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DEPARTAMENTO DE FISIOLOGÍA ANIMAL II

TESIS DOCTORAL

Función inmunitaria y estado redox en el envejecimiento cronológico, prematuro y patológico de ratones

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Rashed Ibrahim Rashed Manassra

Directora

Mónica de la Fuente del Rey

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TESIS DOCTORAL

Rashed I.R. Manassra

Vº Bº de la Directora de Tesis **Vº Bº del Interesado**

Dra. Mónica de la Fuente del Rey **Rashed I.R. Manassra**

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DRA. MÓNICA DE LA FUENTE DEL REY, Catedrática del Departamento de Fisiología Animal II, perteneciente a la Facultad de Ciencias Biológicas de la Universidad Complutense de Madrid,

CERTIFICA

Que **D. RASHED I.R. MANASSRA** ha realizado bajo mi dirección el trabajo correspondiente a su Tesis Doctoral, titulado "FUNCIÓN INMUNITARIA Y ESTADO REDOX EN EL ENVEJECIMIENTO CRONOLÓGICO, PREMATURO Y PATOLÓGICO DE RATONES" y que dicho trabajo reúne los requisitos necesarios para su presentación.

LA DIRECTORA

Fdo. Mónica de la Fuente del Rey

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DOCTORAL THESIS

IMMUNE FUNCTION AND REDOX STATE IN CRONOLOGICAL, PREMATURE AND PATHOLOGICAL AGING OF MICE

A dissertation submitted to the department of Animal Physiology

and the committee on graduate studies of Complutense University of Madrid in partial fulfillment of the requirements for the degree of doctor of philosophy

Presented by

Rashed Ibrahim Rashed Manassra

Under super vision of

Prof. Mónica De la Fuente

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Dedication

I would like to dedicate my dissertation work to my loving parents, whose words of encouragement and push for tenacity ring in my ears.

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To all my dearest friends and relatives I dedicate this work

RESUMEN

"FUNCIÓN INMUNITARIA Y ESTADO REDOX EN EL ENVEJECIMIENTO CRONOLÓGICO, PREMATURO Y PATOLÓGICO DE RATONES"

INTRODUCCIÓN

El proceso de envejecimiento supone un deterioro progresivo y generalizado de la función del organismo, con una menor capacidad para responder a los cambios, y por tanto con una menor posibilidad de restaurar la homeostasis. Esto es debido a que al envejecer ese deterioro funcional se aprecia de forma importante en los sistemas reguladores, el nervioso, el endocrino y el inmunitario, así como en la comunicación que existe entre los mismos. Por ello, con el envejecimiento hay más dificultades para restaurar el equilibrio homeostático y esto explica las mayores posibilidades de enfermar y morir que suceden al avanzar la edad. Dada la heterogeneidad que representa el proceso de envejecimiento, en el que los sistemas del organismo tienen una distinta velocidad de alteración, y también es diferente la velocidad a la que envejece cada persona, se instauró el concepto de "edad biológica" para definir esa velocidad a la que se lleva a cabo el proceso de envejecimiento por parte de cada individuo, y que es más indicativa que la edad cronológica para saber cómo se está efectuando ese proceso. El sistema inmunitario, el encargado de defendernos de las continuas infecciones con las que nos enfrentamos y de los cánceres que constantemente nos aparecen, sufre una serie de alteraciones al avanzar la edad, lo que se denomina inmunosenescencia. Este deterioro explica el aumento de infecciones, cánceres y enfermedades autoinmunes que aparecen al envejecer. La causa de la inmunosenescencia es el estrés oxidativo que experimentan las células inmunitarias al envejecer. Se ha comprobado que el sistema inmunitario puede ser un buen marcador de la velocidad a la que cada individuo envejece y también que el estado funcional de este sistema puede repercutir en el estado de oxidación del individuo, y de esta forma incidir en su velocidad de envejecimiento (De la Fuente y Miquel, 2009).

OBJETIVOS

Un aspecto interesante dentro del marco de la comunicación neuroinmunoendocrina, y no investigado en el contexto del envejecimiento cronológico, es el de las variaciones circadianas en parámetros de función y estrés oxidativo de las células inmunitarias. Ya han sido estudiados los cambios en una serie de funciones inmunitarias y de estado redox de las células peritoneales de ratones ICR/CD1, a lo largo de la edad, y las valoraciones, realizadas hasta el momento, tanto en estudios trasversales

como longitudinales, se han llevado a cabo siempre a las 8-9 de la mañana (inicio del periodo de actividad de los animales en nuestro estabulario). Dado que es conocida la implicación del ritmo circadiano en algunos cambios de la función inmunitaria y que la respuesta circadiana puede modificarse con la edad, en el **Primer objetivo** se ha querido profundizar en los mecanismos de la inmunosenescencia y su repercusión en el envejecimiento del individuo, comprobando como en animales adultos y viejos se modifican una serie de funciones y parámetros de estrés oxidativo en células inmunitarias de la suspensión peritoneal a lo largo del día. También, dado que hay resultados contradictorios en una de las capacidades funcionales de las células inmunitarias como es la secreción de citoquinas, dependiendo del tipo de muestra en la que se lleve a cabo la valoración, se estableció como parte de este objetivo analizar los cambios circadianos en la secreción de citoquinas pro y anti-inflamatorias en sangre periférica de ratones adultos y viejos en respuesta a LPS. De este modo se podrían comparar con las secretadas por las células peritoneales en respuesta a este mitógeno. En paralelo, las variaciones circadianas en los niveles plasmáticos de una hormona muy claramente relacionada con la función inmunitaria y con un establecido ritmo a lo largo del día en sus secreciones, la corticosterona, fueron también planteadas en este primer objetivo.

Primer objetivo: Estudiar el efecto del ritmo circadiano en varias funciones inmunitarias y en parámetros de estrés oxidativo en leucocitos de ratones adultos y viejos.

De los modelos de envejecimiento prematuro establecidos en nuestro grupo de investigación, es el de los animales con inadecuada respuesta al estrés y con ansiedad, los que hemos denominado "Prematurely ageing mice" (PAM), el mejor caracterizado en muchos aspectos, especialmente en los referentes a su prematuro envejecimiento inmunitario y nervioso (neuroquímica cerebral y conducta), el cual se relaciona con su menor esperanza de vida en relación a los no prematuramente envejecidos (NPAM) de la misma edad cronológica. Como ya se ha indicado, la base de la inmunosenescencia es el estrés oxidativo que manifiestan las células inmunitarias. En los PAM ese estrés oxidativo a nivel de las células del peritoneo ha sido ya comprobado. No obstante, los parámetros de estado redox han sido muy poco investigados en órganos inmunitarios como el bazo. Dado que en otros modelos de envejecimiento prematuro se había comprobado que el estrés oxidativo que se aprecia a nivel de las células peritoneales, es también observado en órganos inmunitarios y en otros órganos del individuo, el **Segundo objetivo** fue profundizar en los mecanismos de estrés oxidativo en el modelo de PAM. Para ello se ha llevado a cabo el estudio del estado redox en diferentes órganos (inmunitarios como el bazo y no inmunitarios como el corazón y el hígado) en animales PAM y NPAM de edad

adulta. En los tres órganos se han analizado una serie de defensas antioxidantes y en el hígado, se han evaluado también los de oxidantes y el estado de estrés oxidativo. Como resultados previos analizando otros parámetros de oxidación en PAM y NPAM (datos en vías de publicación) parecen apuntar a que las diferencias entre esos dos grupos se van modificando al avanzar la edad cronológica de los animales, en este segundo objetivo se han analizado los parámetros indicados además de en animales adultos, en maduros y viejos.

Segundo objetivo: Estudiar la situación de estrés oxidativo (concretamente del ciclo del glutation) en varios órganos tales como corazón, hígad y bazo de PAM y NPAM adultos, maduros y viejos.

Como modelo de envejecimiento patológico, se han elegido a ratones triples transgénicos para la enfermedad de Alzheimer (3xTgAD). En estos animales, en comparación con los de la cepa salvaje de la que se obtuvo el transgénico (NTg), se han estudiado los cambios que tienen lugar a nivel de ciertas funciones inmunitarias en bazo y timo de hembras y machos viejos, de 15 meses de edad, momento en el que la patología ya está claramente establecida. Además de comprobarse una serie de alteraciones inmunitarias, se han observado algunas variaciones entre machos y hembras, apuntándose un mayor envejecimiento en los machos que se relaciona con su menor longevidad. Más recientemente se ha observado que en células inmunitarias del peritoneo de esos animales 3xTgAD también se aprecian alteraciones funcionales y de estrés oxidativo respecto a los NTg, las cuales aparecen en algunos casos en edades tan tempranas como los 2 meses, y de forma clara se aprecia la inmunosenescencia prematura a los 4 meses de edad. Por ello, el **Tercer objetivo** fue avanzar en el conocimiento de los cambios inmunológicos y de estrés oxidativo en 3xTgAD.

Para ello, en una primera parte de este objetivo se planteó estudiar una serie de funciones y la secreción de citoquinas en leucocitos de bazo y timo, así como analizar las defensas antioxidantes en bazo, de ratones machos y hembras 3xTgAD y NTg de 4 y 9 meses de edad.

Dado que en estudios previos se ha comprobado que la ovariectomía en las hembras, lo que supone la pérdida de estrógenos, representa un mayor envejecimiento inmunitario y conductual, así como un estado de mayor oxidación, se propuso comprobar en una segunda parte de este objetivo los efectos que tendría la ovariectomía de las hembras 3xTgAD y NTg, llevada a cabo a los 4 meses de edad en los parámetros antes indicados cuando los animales llegan a los 9 meses. Además, puesto que las hembras ovariectomizadas parecen asemejarse a los machos en los aspectos inmunitarios y de

estado redox, en esta parte del objetivo se ha querido también comparar los resultados de las hembras ovariectomizadas con los de los machos de la misma edad.

El ejercicio físico moderado es una adecuada estrategia para mejorar la respuesta inmunitaria, por esa razón, se propuso como tercer subobjetivo estudiar el efecto de un ejercicio moderado en machos y hembras 3xTgAD y NTg así como en las hembras ovariectomizadas.

Tercer objetivo: Avanzar en el conocimiento de la funcionalidad inmunitaria y el estado de estrés oxidativo en ratones 3xTg-AD, machos y hembras, jóvenes y adultos-maduros, así como estudiar en estos animales maduros el efecto de la ovariectomía y del ejercicio físico moderado en varias funciones inmunitarias.

MATERIAL Y MÉTODOS

Se han llevado a cabo 3 grandes diseños experimentales, siendo cada uno de ellos correspondiente a uno de los objetivos planteados.

1. Para estudiar el efecto del ritmo circadiano en varias funciones inmunitarias y en parámetros de estrés oxidativo en leucocitos de ratones adultos y viejos se han utilizado ratones hembras ICR/CD1. En ratones adultos (7±1 meses) y viejos (18±1 meses de edad) se obtuvo suspensión peritoneal, de cada animal, a las 8:00, 13:00 y 18:00 horas. En esas células se estudiaron las siguientes funciones: en macrófagos, la adherencia (a superficies inertes), quimiotaxis (hacia un foco infeccioso generado por péptido formilado), la capacidad fagocítica (de partículas de latex) y la capacidad digestiva (niveles de anión superóxido intracelular); en linfocitos, la capacidad de adherencia, quimiotaxis y proliferación (basal y en presencia de mitógenos como ConA y LPS). También, se analizó la actividad NK (capacidad de lisar células tumorales murinas) y los niveles de citoquinas (IL-2, IL-1beta, TNF-alfa y de IL-10) secretadas en los cultivos de leucocitos peritoneales tras 48 de incubación en presencia de los mitógenos indicados. Los parámetros de estrés oxidativo estudiados fueron: los niveles de glutatión total (TG), y las actividades de las enzimas de su ciclo, la glutatión peroxidasa (GPx) y la glutatión reductasa (GR), como defensas antioxidantes. Como oxidante los niveles de anión superóxido extracelular.

En muestras de sangre obtenidas de los animales adultos y viejos, a las horas indicadas, se analizaron los niveles de citoquinas (IL-10, IL-1beta y TNF-alfa) secretadas

tras 4 horas de incubación en presencia de LPS. Así mismo, se valoró la corticosterona plasmática.

2. Para estudiar la situación de estrés oxidativo en varios órganos de animales prematuramente envejecidos a la edad adulta, madura y en la vejez se utilizaron ratones ICR/DC1 hembras clasificados en PAM (prematurely ageing mice) y NPAM (non-prematurely ageing mice) tras someterles a la prueba del laberinto en T a la edad de 6 meses. Los animales que en las 4 veces que se lleva a cabo la prueba conductual (una vez por semana, durante 4 semenas) recorren el brazo largo de la T en más de 10 s son considerados PAM y si lo hacen en menos de ese tiempo NPAM. Los intermedios se retiraron del experimento. A la edad de 7 meses (adultos), 13 meses (maduros) y 18 meses (viejos) se sacrificaron y se obtuvieron de cada animal: el corazón, el bazo y el hígado. En estos órganos se valoraron las defensas antioxidantes: TG, Gpx y GR. En el hígado, dado su mayor tamño, se ha podido analizar también los niveles de glutation reducido (GSH) (como antioxidante), los de glutation oxidado (GSSG) (como oxidante) y la relación GSSG/GSH (un indicador del estado de estrés oxidativo).

3. Para avanzar en el conocimiento de la funcionalidad inmunitaria y el estado de estrés oxidativo en ratones con envejecimiento patológico como son los triples transgénicos para la enfermedad de Alzheimer (3xTg-AD), se utilizaron ratones de la cepa C57BL/129Sv con 3 transgenes, para la presenilina 1, la proteína precursora amieloide y la proteína tau ($PS1_{M146V,}$ APP_{Swe} and $tau_{P301)}$ (3xTg-AD) y los correspondientes notransgénicos (NTg) machos y hembras, jóvenes (4 meses) y adultos-maduros (9 meses). En leucocitos obtenidos de timo y bazo de esos animales se estudiaron los siguientes parámetros de función inmunitaria: quimiotaxis, actividad NK y proliferación (basal y en respuesta a ConA y LPS, así como el porcentage de proliferación que tiene cada mitógeno). Además se valoraron los niveles de citoquinas (IL-2, IL-10, IL-1beta y TNF-alfa) secretadas en los cultivos de 48 horas de leucocitos de bazo y timo en presencia de esos mitógenos. En homogenados de bazo se analizaron los niveles de TG y las actividades de GPx y GR.

En hembras maduras (9 meses) 3xTg-AD y NTg que habían sido ovariectomizadas a los 4 meses, se estudiaron los mismos parámetros antes indicados, haciendose las comparaciones con 3xTg-AD y NTg hembras sin ovariectomizar y los machos de esa misma edad.

En machos y hembras 3xTg-AD y NTg, a la edad madura (9 meses) se estudió el efecto de un ejercicio voluntario (presencia de una rueda en la caja de estabulación) realizado desde los 6 meses. Los parámetros funcionales antes indicados (con excepción

de las citoquinas) fueron estudiados en los animales que realizaron ejercicio y en los que no lo hicieron al carecer de la rueda (controles). También el efecto de ese ejercicio se valoró en hembras 3xTg-AD y NTg maduras ovariectomizadas, y los valores de los parámetros funcionales se compararon con los de las correspondientes hembras no ovariectomizadas.

RESULTADOS Y CONCLUSIONES

1. Hay cambios circadianos en las funciones inmunitarias y en las defensas antioxidantes de leucocitos peritoneales de ratones adultos, los cuales son similares a los que suceden al avanzar la edad. En la mayoría de los parámetros estudiados no tienen lugar esas variaciones circadianas en células de animales viejos. Los valores obtenidos al analizar las funciones y defensas antioxidantes a las 18:00 h en adultos son similares a los que aparecen en los viejos. Los cambios circadianos en las citoquinas proinflamatorias y antiinflamatorias son diferentes según sean las liberadas por las células inmunitarias peritoneales o por las de sangre. En los animales adultos no hay variaciones circadianas en los niveles plasmáticos de corticosterona, sí los hay en los viejos, y aumentan a lo largo del día.

2. Los órganos (corazón, bazo e hígado) de los animales prematuramente envejecidos (PAM) a la edad adulta, presentan menos defensas antioxidantes (glutatión total, actividad de glutatión peroxidasa y reductasa) que los correspondientes en NPAM. El estrés oxidativo (GSSG y GSSG/GSH) es mayor en el hígado de PAM que de NPAM. Las defensas antioxidantes y el estrés oxidativo disminuyen y aumentan, respectivamente, con la edad en PAM y NPAM, pero, en general, de forma más relevante en los NPAM, por lo que al envejecer disminuyen las diferencias entre ambos grupos (PAM y NPAM).

3. Las funciones de los leucocitos de timo y bazo de 3Tg están deterioradas respecto a las que presentan los NTg, al igual que están más disminuidas las defensas antioxidantes esplénicas. Esto sucede tanto en machos como en hembras, ya a los 4 y a los 9 meses de edad, pero en mayor medida a los 4 meses y en los machos. Las hembras NTg ovariectomizadas muestran un deterioro en sus funciones y defensas antioxidantes, asemejándose los valores a los de los “sham” 3Tg. Sin embargo, la ovariectomia no afectó a los parámetros estudiados en 3Tg. El ejercicio físico voluntario mejoró las funciones y defensas antioxidantes en machos y hembras NTg y en hembras NTg y Tg ovariectomizadas.

SUMMARY

IMMUNE FUNCTION AND REDOX STATE IN CRONOLOGICAL, PREMATURE AND PATHOLOGICAL AGING OF MICE

INTRODUCTION. Ageing is a universal phenomenon that affects nearly all animal species with a progressive and general decline of the organism physiological functions that leads to a lower ability to adaptively react to changes and preserve homeostasis. This is due to the significant functional deterioration shown with ageing particularly in the regulatory systems, nervous, endocrine and immune as well as communication between them. Thus, with aging there is less chance of restoring the homeostatic balance and this explains the greater chance of illness and death that occur with advancing age. Much scientific evidence suggests that the process of ageing is a result of chronic oxidative stress, which affects living organisms, owing to the imbalance between endogenous antioxidant and oxidant compounds. The progressive oxidation leads to the damage of biomolecules and ultimately to a greater degree of oxidation of the organism. Aging is a heterogeneous process, in which body systems have a different rate of change, and also the rate at which you age is different form other people, even of the same chronological age, thus a concept of “biological age " is established to define the rate of aging . The immune system is a homeostatic system, which contributes to an appropriate functional capacity of the organism, it defends the continuous infections that we face and cancers that constantly appear, undergoes a series of changes with advancing age, which is called immunosenescence. This decline explains the increase of infections, cancers and autoimmune diseases that occur with advancing age. Immunosenescence is a consequence of oxidative stress that suffers the immune cells with ageing and may be due to a decreased capacity of the immune system to respond to antigenic stimulation, altered cytokine microenvironment, and impairment of both innate and adaptive immunity. It has been found that the immune system can be a good marker of rate at which each individual ages, and also to the functional of the immune system

can affect the oxidation state of the individual, thus being able to influence the ageing rate of each individual (De la Fuente and Miquel , 2009).

OBJECTIVES. An interesting aspect in the context of neuroimmunoendocrine communication, and not investigated in the context of chronological aging, is the circadian variations in the oxidative stress and functional parameters of immune cells. Some circadian variations have been observed in several immune functions of human beings and experimental animals, at the present few studies on circadian variations in this aspect, especially in mice, have been carried out. Moreover, the circadian rhythm is usually not considered in animal experimental designs. In addition, the age-related changes in the circadian variations of immune functions have not been studied.

The first objective was to study the effect of the circadian rhythm in several immune functions and oxidative stress parameters in peritoneal leucocytes from adult and old mice.

We have proposed a model of premature ageing mice (PAM), which show a premature altered stress-related behavioural response, observed at adult age, in relation to non-prematurely ageing mice (NPAM), of the same gender and chronological. The second objective of the present investigation was to study the oxidative stress situation (concretely in the glutathione cycle) in several organs such as heart, liver and spleen, of adult, mature and old PAM and NPAM.

The ageing process is accompanied with neurodegenerative disorders such as strokes, Parkinson's disease, and Alzheimer's disease (AD) that are associated with oxidative stress. Although in AD there is a systemic disorder, the studies on the peripheral immune system of subjects with AD are scarce. Menopause is mimicked by ovariectomy, we have proposed that ovariectomised rats and mice, with an oestrogen-deprivation, could be a model of premature ageing at immune, oxidative stress and behavior levels. It is well known that physical exercise is an effective means of preventing or delaying chronic disease. Moreover, physical activity strongly modulates the regulatory systems, and concretely shows beneficial effects on the immune system.

The third objective was to advance the knowledge of the immune function and oxidative stress status in 3Tg-AD mice, both male and female, young (4 months) and adult-mature (9 months). To study, in these mature mice the effect of ovariectomy as well as that of moderate physical exercise on several immune functions and redox parameters in both sexes.

MATERIALS AND METHODS. We conducted three large experimental designs corresponding to each one of the objectives.

To study the effect of circadian rhythm in several immune functions and parameters of oxidative stress in leukocytes of adult and old mice, female ICR/CD1 mice were used. In adult mice (7 ± 1 months) and old (18 ± 1 month old) peritoneal suspension of each animal was obtained at 8:00 , 13:00 and 18:00. The functions studied in macrophages were adherence, chemotaxis (towards formyl peptide, a generated infectious agent), the phagocytic capacity (latex particles) and the digestive capacity (intracellular superoxide anion levels); In lymphocytes the functions were: adherence, chemotaxis, natural killer (NK) activity (ability to lyse murine tumor cells) and proliferation (basal and in response to the mitogens Concanavalin A (ConA) and lipopolysaccharide (LPS)). In addition, the release of cytokines (interleukin-2 (IL-2), interleukin-10 (IL10), interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α)) in the supernatant of cultures of peritoneal leucocytes after 48h of incubation in the presence of mitogens were assayed. The oxidative stress parameters studied in peritoneal leucocytes were: Total glutathione (TG) levels and the activities of the glutathione peroxidase (GPx) and glutathione reductase (GR), as antioxidant defense parameters. The extracellular superoxide anion level was analysed as an oxidant parameter.

In blood samples obtained from adult and old animals at the indicated times, the levels of cytokines (IL-10, IL-1beta and TNF-alpha) secreted after 4 hours of incubation in the presence of LPS were analyzed. Also, the plasma corticosterone was evaluated.

To study the situation of oxidative stress in several organs of prematurely ageing animals at adult aged, middle age and old age, female mice ICR/DC1 classified PAM (prematurely aging mice) and NPAM (non- prematurely aging mice) were used after subjecting them to T-maze test at age of 6 months. The animals that performed the behavioral test (once per week for 4 weeks) and walked the long arm of the T maze in 10 s in the 4 times are considered PAM and those which completed that exploration in 10s or less are NPAM). Animals showing an intermediate response were removed from the study.

At the age of 7 months (adult), 13 months (mature) and 18 months (old), animals were sacrificed and the heart, spleen and liver were obtained from each animal. Antioxidant defenses were evaluated in these organs (TG, Gpx and GR). In the liver it has also been able to analyze the levels of reduced glutathione (GSH) (as antioxidant) and the oxidized glutathione (GSSG) (as oxidant) and GSSG / GSH ratio (an indicator of the state of oxidative stress).

To advance the understanding of immune function and the state of oxidative stress in mice with pathological aging such as triple transgenic Alzheimer's disease (3xTg-AD) mice, strain C57BL/129Sv harbouring 3 transgenes (PS1M146V, APPSwe and tauP301L) and corresponding non transgenic (NTg) males and females, young (4 months) and adult - mature (9 months) were used.

In leucocytes obtained from thymus and spleen of the animals the following parameters of immune function were studied: chemotaxis, NK activity and proliferation (basal and in response to Con A and LPS, as well as the percentage of proliferation in response to each mitogen). Moreover, the levels of cytokines (IL-2, IL-10, IL-1beta and TNF- alpha) secreted in 48 hours in cultures of spleen and thymus leukocytes in the presence of these mitogens were evaluated. In spleen TG levels and activities of GPx and GR were analyzed.

In mature females (9 months) 3xTg-AD and NTg that had been ovariectomized at 4 months, the same parameters listed above were studied, by becoming them with non-ovariectomized 3xTg-AD and NTg females and 3xTg-AD and NTg males of the same age.

In 3xTg-AD and NTg male and female, mature age (9 months) the effect of voluntary exercise (presence of a wheel in the cage when the animals were 6 months old) was studied.

The above functional parameters (except cytokines) were studied in animals who exercised and in the controls (those who did not perform the exercise (lacking the wheel in the cage)). Also the effect of this exercise was assessed in ovariectomized 3xTg-AD and NTg mature females, and the values of the functional parameters were compared with those of the corresponding non-ovariectomized females.

RESULTS AND CONCLUSIONS

1. Functions and antioxidant defences of peritoneal leucocytes from adult mice show circadian changes, which are similar to those that occur with ageing. These circadian variations are not shown in old mice. The values of many parameters in adult mice at 18:00h are similar to those in old animals.The circadian changes in pro-inflammatory and anti-inflammatory cytokines in adult and old mice, are different when they are released by peritoneal or blood leucocytes.In adult animals there are no circadian variation in the levels of plasma corticosterone, whereas in old mice, which show higher levels than adult animals, an increase appears in this hormone along the day.

2. The organs (heart, spleen and liver) from adult prematurely ageing mice (PAM) show decreased antioxidant defences (total glutathione levels, glutathione peroxidase and reductase activities) in comparison with the corresponding NPAM. The oxidative stress state (GSSG levels and GSSG/GSH ratio) is higher in the liver of PAM than in that of NPAM. Antioxidant defences and oxidative stress decrease and increase, respectively with ageing in PAM and NPAM. These changes are more pronounced in NPAM and for this reason the differences between both groups decrease with ageing.

3. In 3Tg mice with respect to NTg animals the functions of thymus and spleen leucocytes are impaired, as well as the spleen antioxidant defences being decreased. This occurs in both males and females and at 4 and 9 months of age.

Ovariectomised NTg mice show an impairment of leucocyte functions and antioxidant defences, with values similar to those in sham 3Tg animals. However, ovariectomy does not affect the parameters studied in 3Tg mice. Voluntary physical exercise improves functions and antioxidant defences in male and female NTg mice as well as in ovariectomised NTg and Tg animals.

CONTENTS

1. INTRODUCTION

1.1.The ageing process

Ageing is a universal phenomenon that affects nearly all species, being inevitable in multicellular organisms with sexual reproduction. The process of ageing can be defined, following the 4 rules of Strehler (1977): (1) **universal** in nature (individuals of a species show the same pattern of ageing, although they die for different reasons). (2) **progressive** (the rate of aging is similar at different ages after the adult state). (3) **intrinsic** or endogenous (the determining factors are the internal ones, since even if animals are exposed to optimal environmental conditions throughout life, they still experience the ageing process at the rate characteristic for their species). (4) **deleterious** (aging is obviously detrimental to the individuals since it leads to their death).

Ageing may also be defined as a progressive and general decline of the physiological functions of organism, which leads to a lower ability to adaptively react to changes and preserve homeostasis. Thus, ageing can be characterized by the loss of maintenance of homeostasis as a consequence of the deterioration of the regulatory systems (the nervous, the endocrine and the immune systems). If the principal characteristic of a healthy organism is to maintain the functional balance at all levels, with ageing, this balance fails. An example of this is the lower capacity of elderly persons to endure extreme temperatures, infections or in general the situations in which stress occurs.

Since the adequate function of these systems is the basis of health, with the passing of time, the risk of disease increases and the final result is death. The chances of suffering degenerative diseases increase with ageing, however ageing should not be considered a disease (Helfand and Rogina, 2003; De la Fuente, 2008; De la Fuente and Miquel, 2009).

1.1.1. Longevity

In the concept of longevity or life span, the difference between maximum and mean longevity must be taken into consideration. Maximum life span represents the maximum time that a subject belonging to a determined species can live and is fixed for each species. In human subjects this may reach about 122 years, whereas in some laboratory mouse and rat strains it is only 3 and 4 years, respectively (De la Fuente, 2008).

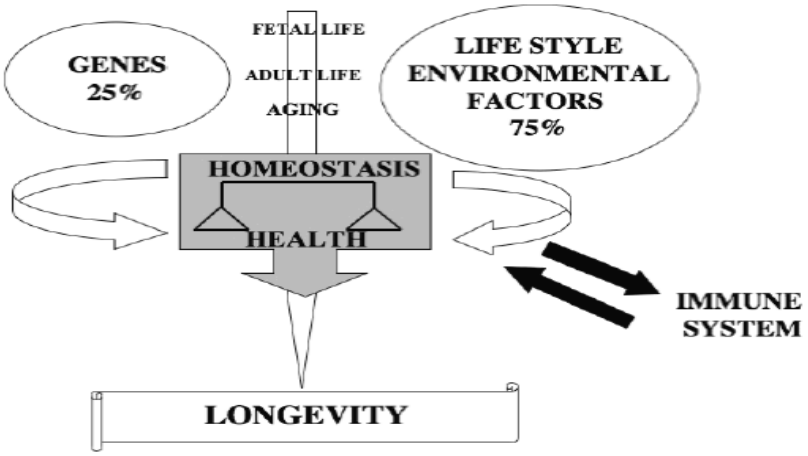


Fig.1 (Original Figure by De la Fuente and Miquel, 2009). How a good and long mean longevity can be reached?. Functional longevity is based on the health maintenance and this depends on preservation of homeostasis (balance at all physiological levels). This health preservation depends on the genes (approximately in a proportion of 25%) and on the style of life and environmental factors (in a 75 %). The regulatory systems deteriorate with ageing that makes the maintenance of homeostasis is difficult. This loss of homeostasis is established at different rate in each subject, and this rate is the result of individual epigenetic mechanisms acting on genes from fetal life throughout the life of the subject.

Mean longevity can be defined as the mean of the time that the members of a population that have been born on the same date, live. This life span varies greatly within populations, this variation is usually attributed to the combined effects of individual genetic and environmental factors and to causes such as infection, accident, starvation, predation and cold. However, even when a population comprises genetically uniform individuals, reared in a constant environment, and protected from extrinsic mortality, the individuals display very different life spans. Thus, the life span of individuals is variable, even when they are of the same genotype and

are raised in a common environment protected from extrinsic hazards (Kirkwood et al., 2005).

Although maximum longevity cannot be increased, the mean lifespan can be extended by environmental and lifestyle factors. Therefore, the preservation of good health not only depends on the genes (25% approximately), but especially on these factors (75 %)(Workshop Report, 2008; De la Fuente and Miquel, 2009) (Fig. 1).

Since human ageing is a serious problem in developed countries because the mean life span is very high (75–83 years), and consequently we spend most of our life ageing (the process of ageing starting at about 18-25 years of age), it is very important to know which life style factors can increase mean longevity and how this can be carried out.

1.1.2. Biological age

Because of the high complexity of a living system (such as the human body) the ageing process may be heterogeneous. Thus, the cells and organs of a body may begin to age at different time points during the lifespan of the organism. Some cells or tissues can function at an optimal level at a given point of time, whereas some others are already in decline. Thus, different parts of the body may follow different patterns of ageing. These differences could be manifested even in similar cell types in a given organ. Besides, the ageing processes in an organism take various directions. There are processes that are enhanced, activated, but at the same time certain levels could be decreasing and down-regulated. All of these processes have heterokinetic characters; i.e. various processes have different kinetic acceleration and speed. It would be, therefore, biased to put all of the emphases on the common features of the advance of senescence. The organs, cell systems, and cell types with alternative functions have special features of ageing, which should be taken into consideration in respect to the problem of ageing (Semsei, 2000).

Ageing, as mentioned above, is associated with a great number of changes at all levels of biological organization, influencing in different ways

the several physiological systems of the members of each species. Thus, the usage of the concept of "biological age" or "functional age", is very useful to assess the level of ageing experienced by each individual and therefore his/her life expectancy (McFarland, 1953). An accurate indication of the aging process cannot be provided by chronological age (Finkel et al., 1995).

Although determining the chronological age is straightforward, the calculation of biological age is difficult. Therefore, it is convenient to select a number of biochemical, physiological and psychological parameters that change with age and can be useful as biomarkers of ageing, revealing the level of senescence suffered by the physiological systems of a particular subject and therefore the relations between biological age, chronological age, health loss and life expectancy (De la Fuente, 2008).

Investigations on biological age showed that the subjects presenting certain parameters with values similar to older subjects had a shorter life expectancy than those found in the majority of the subjects of the same chronological age. These biomarkers include those related to respiratory function, systolic arterial tension and reaction times determined by psychometric tests (Borkan and Norris, 1980).

Different physical parameters of biological age have been investigated since the 1970s, including some physiological and biochemical parameters. Since the immune function is a marker of health and longevity (Wayne et al.,1990) and a positive relation has been shown between a good function of several immune cells and longevity (De la Fuente, 2004), presently several immune parameters are considered essential and very representative of the "true" biological age of a subject and thus, they can be considered appropriate biomarkers of biological age.

1.1.3.Theories of ageing

The study of ageing has expanded rapidly due to the surprising lengthening of the average human life span, worldwide and the increasing percentage of elderly in the population, especially in some developed countries (Wachter and, 1997). Biological, epidemiologic, and demographic data have generated a number of theories that attempt to identify a cause or

process to explain ageing and its inevitable consequence, death. There are more than 300 theories that have been proposed to explain the process of ageing (Medvedev, 1990). However, in recent years, the search for a single cause of ageing, such as a single gene or the decline of a key body system, has been replaced by the view of ageing as an extremely complex, multifactorial process (Kowald and Kirkwood, 1996), which may interact simultaneously and may operate at many levels of functional organization (Franceschi et al., 2000). Similarly, different theories of ageing are not mutually exclusive and may adequately describe some or all features of the normal ageing process, alone or in combination with other theories (Weinert and Timiras, 2003). Currently many theories have been accepted that explain ageing from different points of view, and which complement each other, but most of the theories that do not agree with the data from research on humans and laboratory animals have been abandoned.

1.1.3.1. Genetic theories

"The genetic program theories" suggest that ageing is a consequence of a purposeful program driven by the genes and implies that ageing follows a biological timetable, perhaps a continuation of the one that regulates childhood growth and development. This regulation would depend on changes in gene expression that affect the systems responsible for maintenance, repair and defense responses. In this group are included those theories proposing the existence of specific genes of longevity and theories of existence of biological clocks controlling ageing. All molecular processes in a living system are based on and regulated by genes. Thus, an attractive research strategy has been to discover genes for ageing, termed "gerontogenes" (Rattan, 1995; Johnson, 2002). There is sufficient evidence from studies performed on yeast and other fungi, nematodes, insects, rodents and humans that mutations in various genes can either prolong or shorten the lifespan, and could also be the cause of premature ageing syndromes in human beings (Martin, 2005; Kenyon, 2005; Christensen et al., 2006) . Most of these genes have well defined roles in normal metabolism, in intra- and inter-cellular signaling, and in maintenance and repair functions including stress response. It is the damage-induced changes in the regulation, structure

and/or activity of their gene products, which result in their altered biological role with age (Rattan, 1998).

Several studies indicate that the genome is not a solid structure that preserves its original form, which appears after the birth of the individual (Vijg and Knook, 1989). There are alterations in the genome due to ageing that are the effects of external and internal factors (Piko´ et al., 1988 and Ozawa, 1993). These changes could finally lead to alterations in the gene expression (Sato et al., 1990), caused by a point mutation (Cohen and Levinson, 1988) or a gene rearrangement (Murnane, 1986).

Recent work extends the impact of genomic defects to an age-associated deregulation of the epigenome (reviewed in Oberdoerffer and Sinclair, 2007), suggesting that the accumulation of DNA damage and genomic instability with age may be a critical contributor to the ageing process, though perhaps in a more indirect and complex way than first proposed. The accrual of genomic defects can affect cellular function at many levels. For example, mutations in coding regions of DNA can cause abnormal protein expression or function. Chromosomal translocations and rearrangements can also result in apoptosis, tumor formation or senescence (Campisi, 2005). DNA damage and its repair have also been linked to wide-ranging chromatin alterations that surround the sites of damage and may affect a large number of genomic loci, including coding regions and structural components (Downs et al., 2007).

A very popular theory was based on the idea that the somatic cells with replicative potential possess a "mitotic clock" that fixes their maximum lifespan (Hayflick, 1965).

"The shortening telomere" theory, (telomeres have experimentally been shown to shorten with each successive cell division), have to be considered possible explanations of replicative cellular senescence. When the telomeres reach a critical length, the cell stops replicating at an appreciable rate, and so it dies off, which eventually leads to the death of the entire organism (Campisi, 2000).

1.1.3.2. Epigenetic theories. The free radical and oxidation theory

Many researchers and even those who followed the genetic theories in the past, currently believe that the ageing process, which appears after reproductive maturation, is driven by random events and is not gene-programmed (Hayflick, 2007). Thus, there exists the group of "epigenetic theories", which indicate that ageing is the result of events that are not exclusively guided by a program but are stochastic or random events.

Cells and tissues have vital parts that wear out resulting in aging. Like components of an aging car, parts of the body eventually wear out from repeated use, killing them and then the body (Medvedev, 1990; Viña et al., 2007; Pearl, 1928).

In this group of epigenetic theories, several theories can possibly be included such as:

a) Cross linkage theories, in which an accumulation of cross-linked proteins damages cells and tissues, slowing down bodily processes resulting in ageing.

b) Metabolic theories, in which ageing can be considered "a side effect" of aerobic metabolism: In this group of theories "*wear- and- tear"* or disorganization, rate-of-living, oxidation, damage by free radicals, mitochondrial injury can be included (Medvedev, 1990; Viña et al., 2007).; For example, the Rate of living theory indicates that the greater an organism's rate of oxygen basal metabolism, the shorter its life span (Brys et al., 2007).

c) Physiological theories of ageing in which the neuroendocrine and the immunological theories are included (Medvedev, 1990).

The free-radical epigenetic theory of ageing and the mitochondria theory

The theory of "free-radicals" or of "oxidation" is probably now the most widely accepted to explain how the ageing process occurs. This theory proposes that ageing is the consequence of the accumulation of damage by harmful oxidation in biomolecules caused by the high reactivity of the

oxygen free radicals produced in the cells as a result of the required use of oxygen (Harman, 1956; Gerschman, 1962; Miquel and Fleming, 1986).

The mitochondria is an important cellular location of production of free radicals as a consequence of the use of oxygen in the respiratory process that they carry out. This, and the fact that mitochondrial DNA (mtDNA), which is the first target of oxidation by those free radicals, is very susceptible to oxidation led to the "Mitochondria theory" being proposed. The mitochondrial injury by free radicals and the loss of bioenergetic competence lead to the ageing and death of cells and therefore to that of the organism. This is especially relevant in the postmitotic cells, such as most of the neurons, in which regeneration of the damaged mitochondria cannot take place (Miquel et al., 1980; Miquel et al., 1984; Miquel and Fleming, 1986).

Important findings that support the free radical theory of ageing show that the rate of mitochondrial oxygen radical generation, the oxidative damage to mtDNA, as well as the degree of cell membrane fatty acid unsaturation, are lower in the long-lived species than in the short-lived ones (Barja, 2004).

Cells have different antioxidant mechanisms that protect them against oxygen toxicity, by the prevention of the formation of free radicals or reactive oxygen species (ROS) or by neutralizing them after they are produced. Since ROS, in adequate amounts, are necessary for many normal cell activities (Sies, 1997), the correct functioning of the organism is based on a perfect balance between the levels of ROS and antioxidants. An excess in the production of ROS or a deficiency in the antioxidant levels, result in the loss of this balance, which leads to an oxidative stress. This occurs especially in the differentiated cells, underlying ROS-related diseases and ageing (Miquel et al., 1980; Miquel et al., 1984; Sastre et al., 2000; De la Fuente and Miquel, 2009) (Fig. 2).

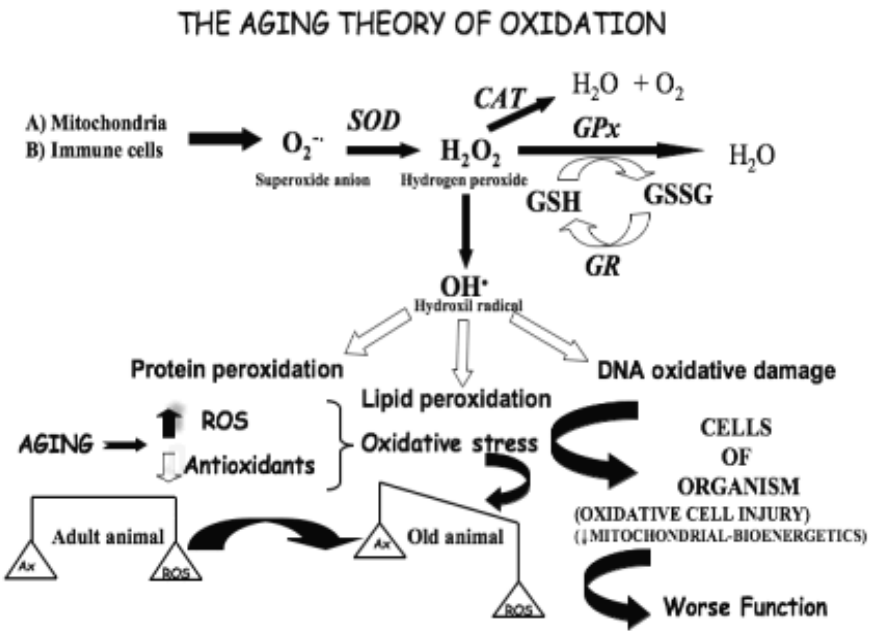


Fig. 2. (Original Figure by De la Fuente and Miquel, 2009). The oxidation theory of ageing. Ageing is the consequence of the accumulation of oxidative damage in biomolecules caused by the high reactivity of the free radicals and reactive oxygen species (ROS) produced in cells, especially in mitochondria, as a result of the necessary use of oxygen. The immune cells also produce important levels of ROS. The first oxygen free radical appearing in cells is the superoxide anion ($O2^{\cdot-}$), which produces hydrogen peroxide (H_2O_2**)** and the hydroxil radical ($OH^{\cdot}$), the most reactive free radical, which carries out the oxidation of biomolecules such as proteins, lipids and DNA. Cells, in order to protect themselves against oxygen toxicity, have developed a variety of antioxidant mechanisms that prevent the formation of ROS or neutralize them after they are produced. Thus, superoxide dismutase (SOD) catalyzes the inactivation of superoxide anion and catalase (CAT) inactivates hydrogen peroxide. The reduced glutathione (GSH) is the most important antioxidant in the organism and neutralizes peroxides using glutathione peroxidase (GPx) and in this action it is transformed to oxidized glutathione (GSSG). The antioxidant enzyme glutathione reductase (GR) is used to catalyze the reduction of glutathione. It should be considered that oxygen is essential for life and that ROS, in certain amounts, are needed for many physiological processes, which are essential for survival. Therefore, the functions of organisms are based on a perfect balance between the levels of ROS and those of antioxidants. However, with ageing a loss of the balance appears, with an excess in the production of ROS or an insufficient availability of antioxidants, which leads to an oxidative stress situation, which results in oxidative cell injury (starting at mitochondrial level, causing a loss of bioenergetic competence, and therefore a worse functioning of cells).

1.2. Oxidative stress

Oxygen is a fundamental component of cellular metabolism. It is essential for efficient energy production in aerobic organisms. However, any situation that results in a consumption of oxygen can lead to the production of free radicals or 'reactive oxygen species' (ROS), which oxidise the different bio-molecules of cells. Thus, in order to protect themselves against

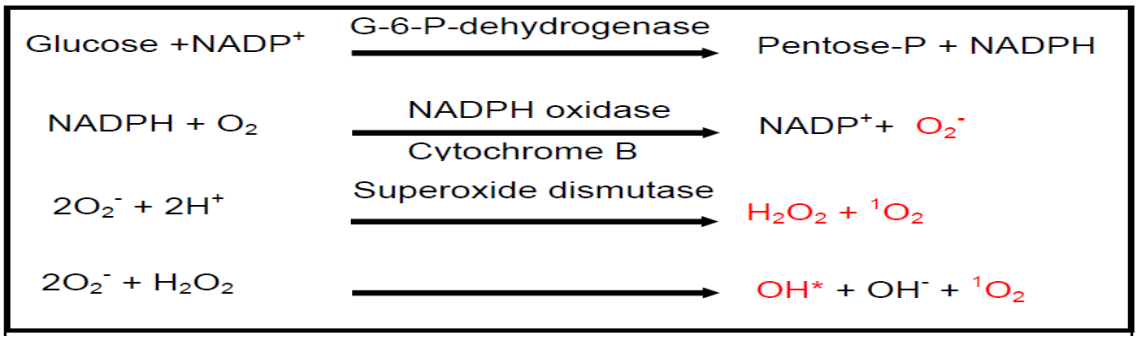
oxygen toxicity, cells have developed a variety of antioxidant mechanisms that prevent the formation of ROS or neutralise them after they are produced. However, these defensive systems are not perfect, and thus when the amount of ROS exceeds the antioxidant protection, an imbalance occurs and an oxidative stress situation appears with resulting cell injury (Sies, 1999; Pacheco and Gonsebatt, 2009) (Gutteridge, 1995). Oxidative stress appears in many diseases such as, cancer, diabetes, atherosclerosis, cardiovascular disease, and neurodegenerative disorders such as stroke, Parkinson's and Alzheimer's diseases. In all these cases there is an association with oxidative stress due to the elevation of ROS or insufficient ROS detoxification (Sies, 1999; Pacheco and Gonsebatt, 2009). The ageing process is also the result of chronic oxidative stress, which affects all living organisms. This progressive oxidation leads to damage of biomolecules, which ultimately causes the age-related decline in physiological functions, including immune function (De la Fuente and Miquel, 2009).

1.2.1. Free radicals and oxidant compounds

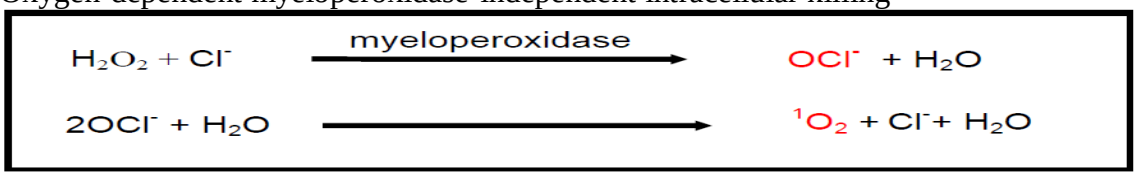
Free radicals are small molecules that contain one or more unpaired electrons. These unpaired electrons alter the chemical reactivity of the molecule, usually making it highly reactive, because they act as capturers of electrons, i.e., as oxidizing agents (Halliwell and Gutteridge, 1988; Gilgun-Sherki et al., 2001). The ROS are molecules, which do not follow the strict definition of free radicals, but produce them. The oxygen-free radicals and ROS represent the most important classes of radical species (Sies, 1997), which along with reactive nitrogen species (RNS) play both deleterious and beneficial roles. Beneficial effects of ROS involve physiological cellular responses to noxia, as for example in defence against infectious agents and in the function of a number of cellular signalling systems. One further beneficial example of ROS, at low concentrations, is the induction of a mitogenic response. In contrast, at high concentrations, ROS can be important mediators of damage by oxidation to cell structures, including lipids, proteins and nucleic acids (Poli et al., 2004).

Under normal physiological conditions, ROS can be produced from both endogenous and exogenous substances such as pollutants in the atmosphere (Cadenas, 1989). Endogenous sources include mitochondria during cellular respiration, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Inoue et al., 2003). Other important sources of cellular ROS and RNS are neutrophils, eosinophils and macrophages. Thus, cells of the immune system, especially activated macrophages, produce both $O_2^{\bullet-}$ and NO during the oxidative burst triggered during inflammatory processes (Carr et al., 2000).

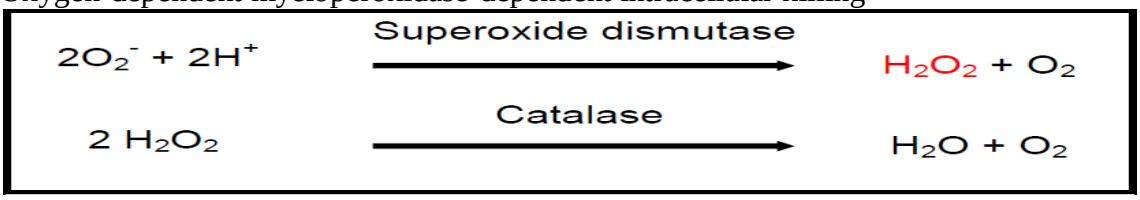
The most abundant free radicals and ROS include nonorganic molecules, such as the superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$), as well as organic molecules such as the alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals (Sies, 1991; Halliwell and Gutteridge, 1999; Halliwell B., 2009). The $O_2^{\bullet-}$, is the primary radical produced from oxygen, and it can interact with other molecules to generate secondary ROS either directly or mainly through enzyme or metal catalysed processes (Fatehi-Hassanabad et al., 2010). Thus, excessive production of $O_2^{\bullet-}$ under stress conditions releases free iron from iron-containing molecules such as haemoglobin, and through the Fenton reaction can generate the $OH^{\bullet}$ radical. This hydroxyl radical, although it has a very short half-life, indiscriminately oxidizes the nearby molecules, generating other radicals such as $ROO^{\bullet}$ and $RO^{\bullet}$. In addition, the superoxide anion generates other ROS such as hydrogen peroxide (H_2O_2), which can also lead to the generation of other ROS as well as RNS through various chemical reactions. Although H_2O_2 has no unpaired electrons, it is highly reactive and relatively stable compared to other ROS, and like other oxidants, also plays an important role contributing to cellular oxidative damage (Fatehi-Hassanabad et al., 2010) (Fig.3).



Oxygen-dependent myeloperoxidase-independent intracellular killing



Oxygen-dependent myeloperoxidase-dependent intracellular killing



Detoxification reactions

Fig.3. Several chemical reactions producing free radicals and ROS.

The free radicals and oxidants may injure cells and tissue directly via oxidative degradation of essential cellular components as well as injure cells indirectly by altering the protease/antiprotease balance that normally exists within the tissue interstitium. It is becoming increasingly apparent that in addition to promoting cytotoxicity, reactive oxygen metabolites may also initiate and/or amplify inflammation via the upregulation of several different genes involved in the inflammatory response, such as those that code proinflammatory cytokines and adhesion molecules. This may occur by the activation of certain transcription factors, such as the nuclear transcription factor κB (NF-κB). NF-κB is a ubiquitous transcription factor and pleiotropic regulator of numerous genes involved in immune and inflammatory response (Conner and Grisham, 1996).

1.2.2. Antioxidant defences

Diverse protective systems must exist to enable adaptation to oxidative environments. Antioxidants are crucial to balance the redox state

in the human body. Under physiological conditions, a homeostatic balance exists between the formation of ROS and their removal by endogenous antioxidant scavenging compounds (Gutteridge and Mitchell, 1999), which can be non-enzymatic and enzymatic antioxidants. Such antioxidant defenses are extremely important as they represent the direct removal of free radicals (prooxidants), thus providing protection for biological sites. The most efficient endogenous enzymatic antioxidants are distributed within the cytoplasm and among various organelles in cells. These antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), work in a complex series of integrated reactions to convert ROS to more stable molecules, which do not cause damage (Mates et al., 1999; Kregel and Zhang, 2007) (Fig.2).

The superoxide anion is depleted undergoing a dismutation reaction by cytosolic and mitochondrial SOD isoenzymes (metal-containing proteins and one of the most effective intracellular enzymatic antioxidants and the first line of defense against oxidation). They convert the $O_2^{\bullet-}$ into H_2O_2 and oxygen (Pollack and Leeuwenburgh, 1999). The CAT, a heme-containing enzyme, is the most reactive enzyme in the majority of the tissues converting toxic H_2O_2 to water. This enzyme which is largely localized in subcellular organelles such as the peroxisomes and mitochondria (Pollack and Leeuwenburgh, 1999; Bai and Cederbaum, 2001). GPx, a selenium-containing enzyme, is found in both cytosol and mitochondria and requires the presence of its substrate, the reduced GSH, for its action. This forms a formidable defense against hydrogen and lipid peroxides. Thus, GPx removes H_2O_2 by coupling its reduction with the oxidation of GSH converting H_2O_2 to H_2O (Pollack and Leeuwenburgh, 1999). The product of the GPx reaction is GSSG, which is a substrate of the flavoenzyme GR, giving GSH (Fig. 4).

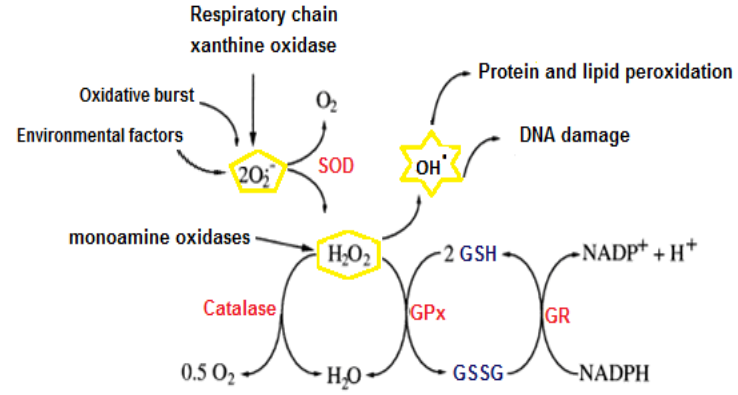


Fig 4. Generation and disposal of superoxide anion and hydrogen peroxide. The superoxide anion generated is converted by SOD to H_2O_2. Monoamine oxidases generate additional H_2O_2. This peroxide is disposed of by catalase and/or GPx.

Antioxidants do not act in isolation but synergistically. Primary antioxidants prevent oxygen radical formation and secondary antioxidants react with ROS, which have already been formed, either to remove or inhibit them (Gutteridge and Mitchell, 1999). However, the endogenous enzymatic defense systems act cooperatively with other endogenous small non-enzymatic compounds, such as GSH and thioredoxin as well as with exogenous antioxidants, such as vitamins E and C, carotenoids, polyphenols and trace metals (i.e. selenium, zinc). Diet is the main source of these antioxidants, but a hormonal product of the pineal gland, such as melatonin and other compounds, can also be involved. Thus, these endogenous and exogenous non-enzymatic antioxidants can also function as direct scavengers of ROS and act interactively (e.g. synergistically) to maintain or re-establish redox homeostasis (McCall and Frei, 1999; Kregel and Zhang, 2007).

In this regard, the principal and most abundant cellular antioxidant is glutathione (GSH), which represents the principal nonprotein thiol source. GSH, which plays a major role in the maintenance of the intracellular redox state, is present in concentrations up to 12 mM in mammalian cells (Dröge, 2002; Cooper, 1997). This antioxidant is very abundant in the cytosol,

nuclei, and mitochondria, and is the major soluble antioxidant in these cell compartments (Masella et al., 2005) (Fig.5).

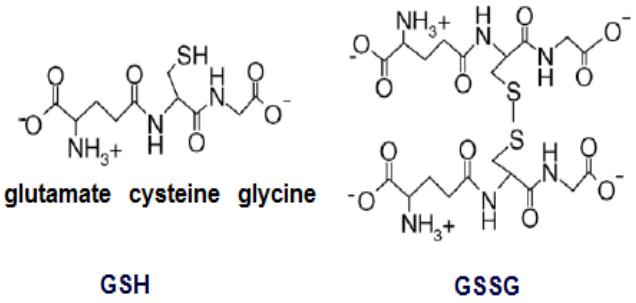


Fig 5. Structures of reduced (GSH) and oxidised (GSSG) glutathione.

The main protective roles of glutathione against oxidative stress (Masella et al., 2005) are: (i) GSH participates in amino acid transport through the plasma membrane; (ii) GSH scavenges hydroxyl radicals and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathionperoxidase; (iii) GSH is able to regenerate antioxidants such as vitamins C (ascorbic acid) and E to their active forms. In fact, glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via the reduction of semidehydroascorbate to ascorbate.

Glutathione is involved in two types of reactions during detoxification of ROS: (i) GSH reacts non-enzymatically with radicals such as the superoxide radical anion, nitric oxide or the hydroxyl radical (Saez et al., 1990; Clancy et al., 1994; Winterbourn and Meto-diewa, 1994; Singh et al., 1996) and (ii) GSH is the electron donor for the reduction of peroxides in the GPx reaction (Chance et al., 1979). The final product of the oxidation of GSH is glutathione disulfide (GSSG). Within cells GSH is regenerated from GSSG by the reaction catalyzed by glutathione reductase (GR). This enzyme transfers electrons from nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) to GSSG, thereby regenerating GSH (Fig. 2 and 4).

The GSSG/GSH ratio is a good measure of the oxidative stress of organisms (Hwang et al., 1992) since too high a concentration of GSSG may cause oxidative damage to many enzymes and compounds.

As mitochondria are the major site of free radical generation, they are highly enriched with antioxidants including GSH and enzymes, such as SOD and GPx, which are present on both sides of their membranes in order to minimise oxidative stress in the organelle (Cadenas and Davies, 2000).

Dietary micronutrients, including vitamin C and E, contribute to the antioxidant defense system. Vitamin C is a critical soluble water antioxidant interacting with GSH and vitamin E maintaining a reduced intracellular environment (Yu, 1994).Vitamin E is the primary antioxidant in cell membranes and often acts as a chain breaking antioxidant attenuating further lipid peroxidation. It is important because of its ability to convert several free radicals such as $O_2^{\bullet -}$, $OH^{\bullet}$ and lipid peroxyl radicals into "repairable" radical forms (Pollack and Leeuwenburgh, 1999; Yu, 1994).

The immune cells are also an important source of oxidant and proinflammatory compounds as a part of their function. The contents of both must be tightly controlled by the antioxidant defenses, therefore, the oxidant–antioxidant balance is essential to these cells. Thus, increased levels of oxidant and pro-inflammatory compounds may damage the biomolecules of the immune cells, as well as the surrounding cells and tissues (De la Fuente, 2008; Knight, 2000). The deficit in antioxidants has been related to impaired immune responses, leading to frequent and severe infections that result in increased mortality (Knight, 2000).

1.3. Immune system and Immunosenescence

The immune system is a remarkably versatile defence system against infection and malignant cells. Its characteristics and its age-related changes will be briefly commented in this section.

1.3.1. General characteristics of the immune system

The defensive activity of the immune system is carried out via different components such as epithelial barriers, immune cells and immune molecules, which act together in a dynamic network (Abbas et al., 2009). One of the main functions of the immune system is self/non-self

discrimination. The foreign substances, which induce immune responses or are the targets of such responses, are called antigens. This ability protects the organism from invading pathogens and eliminates modified or altered cells (e.g. malignant cells). Moreover, since pathogens may replicate intracellularly (viruses and some bacteria and parasites) or extracellularly (most bacteria, fungi and parasites), different components of the immune system have evolved as protection against these. All **cells of the immune system** have their origin in the bone marrow and they include myeloid (neutrophils, basophils, eosinpophils, monocytes/macrophages and dendritic cells) and lymphoid (B lymphocyte, T lymphocyte and Natural Killer (NK) cells). The myeloid progenitor or stem cells in the bone marrow give rise to erythrocytes, platelets, neutrophils, monocytes/macrophages and dendritic cells, whereas the lymphoid stem cells produce NK, T and B cells. In the case of T cell development, the precursor cells must migrate to the thymus where they undergo differentiation into two distinct types of T cells, $CD4^+$ helper cells and $CD8^+$ cytotoxic cells. Helper T cells can be divided into Th1 cells, which help the $CD8^+$ pre-cytotoxic cells to differentiate into cytotoxic T cells, and Th2 cells that help B cells differentiate into plasma cells, which secrete antibodies (Male et al., 2006).

Two types of immunity protect the body, namely the innate (or natural, non-specific) and the adaptive (or acquired, specific). Each of the major subdivisions of the immune system has both cellular and humoral components with which they carry out its protective function. However, this division is somewhat artificial since both are present in the genesis of an immune response. Thus, although these two arms of the immune system have distinct functions, there is interplay between them, with components of the innate immune system that influence the adaptive immune system and vice versa (Male et al., 2006).

Innate immunity, also called natural or native immunity, consists of cellular and biochemical defence mechanisms that are in place even before infection and poised to respond rapidly to it. The principal components of innate immunity are (1) physical and chemical barriers, such as epithelia

and antimicrobial substances produced at epithelial surfaces; (2) phagocytic cells (principally neutrophils and macrophages), which are the first line of defence, as well as NK (natural killer) cells, which are implicated in the resistance to tumours and viral infected cells; (3) blood proteins, including members of the complement system and other mediators of inflammation; and (4) proteins called cytokines that regulate and coordinate many of the activities of innate immunity cells. This immunity provides the early lines of defence against microorganisms. The basic signalling receptors of the innate immune cells in the recognition of pathogens are the Toll-like receptors (TLR), which detect a broad range of molecular patterns that are commonly found on pathogens, called pathogen-associated molecular patterns (PAMPs).

Adaptive immunity develops as a response to infection with the capacity to distinguish among different, even closely related, microbes and molecules, and for this reason it is also called **specific immunity.** The components of adaptive immunity are lymphocytes and their products, which respond not only with a high degree of specificity, also with a striking diversity in its recognition, which allow lymphocytes to recognize billions of unique structures on foreign antigens. Adaptive immunity shows also the remarkable property of "memory", which guarantees a more rapid and robust response to subsequent encounters of a previously experienced antigen. Thus, there are naïve lymphocytes, and very long-lived antigen-experienced lymphocytes (memory lymphocytes), which underpin the possibility of vaccination. There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity. **Humoral immunity** is mediated by molecules in the blood and mucosal secretions, called antibodies, that are produced by **B lymphocytes**. Antibodies recognize microbial antigens, neutralize the infectivity of the microorganisms, and eliminate them by various effector mechanisms. Humoral immunity is the principal defence mechanism against extracellular microorganisms and their toxins. **Cell-mediated immunity,** is mediated by **T lymphocytes**, which are responsible for cellular immunity, coordinating

and controlling the overall immune response, mobilizing other cell types and establishing communication via chemical messengers (cytokines and chemokines). Intracellular microbes, such as viruses and some bacteria, survive and proliferate inside phagocytes and other host cells, where they are inaccessible to circulating antibodies. Defence against such infections is a function of cell-mediated immunity, which promotes the destruction of these microbes to eliminate reservoirs of infection (Abbas et al., 2009). As mentioned previously, there are several kinds of T cells, such as cytotoxic T cells (Tc), that express the surface protein marker CD8+, which directly kill infected cells and tumour cells, and T helper cells (Th), expressing CD4+ as the surface marker, which aid B and other T cells to do their work. The Th cells can be type 1 (Th1) and type 2 (Th2). The Th1 promote cell-mediated reaction providing effective defense against intracellular pathogens. The Th2 activate humoral immunity with antibody production. Other relevant T cells are regulatory T cells (Treg), which suppress the activity of lymphocytes to prevent their overreaction (Male et al., 2006).

Adaptive immune response is divided into three distinct phases 1) the "recognition of foreign agents or antigens"; 2) the "activation" (recruiting a variety of cells and molecules to mount an appropriate response, the proliferation of specific lymphocytes and the production of cytokines being key activities in this stage) and "regulation" of this activation, and 3) the "effective response" to eliminate or neutralize the antigens. In this response an inflammatory situation is generated. Many of the cells involved in this response are then destroyed, but memory cells are produced in the activation stage and thus, although they do not act at that moment, they are maintained in the organism. A later exposure to the same foreign organism induces a memory response, characterized by a more rapid and heightened immune reaction that serves to eliminate the pathogen and prevent disease (De la Fuente, 2004).

1.3.2 Immunosenescence

There are age-related changes in the immune system, which have been observed in humans and in a variety of experimental animal models. These changes are defined as "Immunosenescence" and play a role in the increased susceptibility to infections, malignancies and autoimmune processes, as well as in the decreased response to vaccination and wound healing with age (Paula et al., 2010). Immunosenescence is reflected with alterations at the cellular, molecular, and genetic levels (Tarazona et al, 2002). This is shown by a decreased capacity of the immune system to respond to antigenic stimulation, an altered cytokine microenvironment and an impairment of both innate and adaptive immunity (Hakim et al, 2004).

In view of the key role of an optimal immune function in the preservation of health, it seems logical that one of the theories on the cause(s) of ageing, namely the immunological theory, maintains that the responsibility for the changes that take place in the organism with the passage of time lies in the impairment of the immune system (Baltimore et al., 1969; Walford, 1969).

In spite of the many studies on the age-related changes in the immune system, a full understanding of the mechanisms of immunosenescence has not been achieved. The age-related deterioration of the immune system (Pawelec, 1999), does not necessarily imply a deficit of immune function, since not all immune cell types or all functions of an immune cell show a significant diminishment with age. In fact, several cell types and cell functions are activated, whereas others are not significantly altered with age. Because of this, it is more appropriate to associate immunosenescence with a disregulated functional state. Currently, although there are conflicting observations on this subject, it is accepted that almost every component of the immune system undergoes prominent age-associated restructuring (De la Fuente, 2002,2004; De la Fuente et al., 2002; Pawelec et al 2002). Thus, research using animal models and human subjects suggests that there are several important changes in the innate and adaptive immune responses with

increasing age, which are associated with an increase in morbidity and mortality (Ferguson et al., 1995). In addition, older subjects with fewer features of immunosenescence could have a prolonged and healthy lifespan (Franceschi et al., 1995; Arranz et al., 2010a,b,c; Alonso-Fernandez and De la Fuente, 2011).

1.3.2.1 Age-related changes in the immune organs and cells

Haematopoietic stem cells (HSCs), which give rise to all the cellular components of the immune system (lymphoid and myeloid) and the haematopoietic process in bone marrow, decrease with age (Derhovanessian et al., 2008). There is also a deterioration and a loss of the immune tissues, such as bone marrow, thymus, spleen and ganglions with ageing. Concretely, the thymus has a central role in the age-related changes of the immune system, which has even led to it been called the "biological clock" of ageing (Aw et al., 2007; Gruver et al., 2007). Thymus involution is the first and most striking age-dependent alteration affecting the immune system after puberty. The size of the thymus decreases progressively with age and this starts soon after puberty resulting in a replacement of thymic tissue by fat in the elderly (Aspinall and Andrew, 2001). By 70 years of age, the thymopoietic space, which contains thymocytes and supporting thymic epithelial cells, is replaced by fatty tissue and reduced to approximately 10% of the total (Flores et al., 1999). Thymic output of naïve cells also diminishes by greater than 95% after the age of 70 years (Naylor et al., 2005). The atrophy of this organ occurs, as shown in the murine models, as a consequence of the failure of thymic T cells to undergo differentiation and expression of the T cell receptor (TCR), with a decrease in production of $CD4^+$ and $CD8^+$ cells (Aspinall, 1997). Changes also occur in second messenger pathways, such as defective Fas signalling, as well as in extrathymic factors including hormones, which have been demonstrated to play a role in maintaining thymus function (Zhou et al., 1995).

A characteristic of the aged immune system is the decrease in naïve T cell rates (Mackall et al.,1995), but the total numbers of T cells remain

relatively constant because of the survival and accumulation of memory cells, which proliferate to compensate for the loss of thymic output (Aspinall and Andrew, 2000). The increased survival of memory $CD4^+$ T cells was reported to be due to changes in the microenvironment of the thymus (Timm and Thoman, 1999). Numbers of memory $CD8^+$ T cells might be increased because of chronic viral stimulation (Khan et al., 2002). However, despite prolonged survival, memory $CD4^+$ and $CD8^+$ T cells from aged mice might have poor antigen response (Kapasi et al 2002).

T cell functions and NK activity seem to be the immune responses most affected by ageing (De la Fuente et al., 2004). Additionally, the aged lymphocytes secrete less IL-2 and show a low expression of IL-2 receptors. T cells in the elderly present a decreased proliferation, a weak expression of early activation markers, and diminished intracellular Ca^{+2} levels after TCR stimulation (Hale et al 2006; Miller 1996). The ageing of T cells in humans corresponds to a loss of CD28 expression, which has been well documented (Chamberlain et al., 2000). The consequence of this loss, CD28 being an accessory molecule for TCR signalling, in CD4+ as well as in CD8+ T cells, is the decrease of T-cell signalling, including decreased IL-2 secretion and increased apoptosis (a form of programmed cellular suicide) (Batliwalla et al 2000; Chiu et al., 2006), which should also affect the functional profile of T cells. Immunosenescence is associated with an increased apoptosis in T cells overall. Heightened expression levels of "death receptors" and associated downstream molecules are linked with ageing and are likely explanations for this phenomenon (Gupta, 2000).

Telomere shortening in the $CD28^-$ T cells is more pronounced than in $CD28^+$ T cells indicating that the former have undergone more cell divisions than the latter (Batliwalla et al 2000).

Antigens are presented to T cells via major histocompatibility complex molecules, and it has been shown that aged T cells have a decreased ability to respond to antigens as compared to younger T cells (Schwabet al., 1992). One potential explanation for this is that T cells from

older individuals are less efficient at assembling a signalling complex at the site of antigen presentation (Tamir et al., 2000). Dysregulation of other intracellular signalling molecules such as protein kinase A and C (PKA and PKC), and IκB influences gene expression and consequently T cell activity (Ohkusuet al., 1997). Within 5 minutes of initial activation, T cells from old mice showed diminished intracellular calcium levels compared to young controls, suggesting deficiencies in signalling-associated ion movement (Miller et al., 1987). Other studies showed that elderly people present an increase of activated T cells, which may be associated with a higher frequency of autoimmune phenomena and to an altered regulation of immune function (Gianluigi Mazzoccoli et al., 2011).

The most characteristic function of NK cells is non-MHC restricted killing of target cells, such as tumour and viral infected cells, and thus NK cells are important for maintaining health. Thus, high NK activity is a prerequisite of good health and longevity (Castle, 2000). The numbers of NK cells increase with age, but NK cell cytotoxicity on a per-cell basis is decreased and levels of cytokines and chemokines such as RANTES, MIP1α, and IL-8 produced upon NK cell activation are also reduced (Mocchegiani et al., 2009). The ability of NK cells to exert cytotoxic function is well preserved in our life until old age. Low NK activity in the elderly may be associated with infectious diseases, such as influenza, the most important of all infectious diseases (Ogata et al., 2001). Thus, the decrease of NK activity with age could explain the high frequency of infectious diseases and cancer with ageing. Therefore, low NK activity is a good predictor of morbidity (Levy et al., 1991). The influence of ageing on NK cells can be attributed to various factors, such as decreased responsiveness to the positive modulation with IL-2 (Miller, 1991). The age-associated alterations in NK function may result in part from changes in zinc homeostasis in older individuals, and there is evidence that NK cell function can be improved with zinc supplementation (Mariani et al., 2008).

In contrast to NK cells, the number of natural killer T (NKT) cells, which express a T-cell receptor (Vα14/Vβ8.2 in mice andVα24/Vβ11 in

humans) that is CD1d-restricted and represent a rare class of 'innate immune lymphocytes" (Faunce et al., 2005), decrease in elderly subjects (Jing et al., 2007). However, studies in mice have demonstrated an age-associated increase in number and function of NKT cells (Faunce et al, 2005).

B cell compartment is affected by age (Colonna-Romano et al., 2006). Naïve B cell generation might reduce with age. In humans, a study of bone marrow biopsies from subjects ranging in age from 2 months to 92 years, showed a decrease in the numbers of B-cell precursors (McKenna et al., 2001). Naïve B cell numbers decrease and effector B cells accumulate in old age. This leads to a reduction in the diversity of antibody responses (Allman and Miller ,2005). Defects in isotype switching and somatic mutation, both of which are essential for the production of high-affinity IgG antibodies, result in weak and low-affinity antibody responses in elderly persons (Frasca et al, 2005). In humans, B cells from older adults stimulated by Staphylococcus proteins had similar proliferative ability but diminished plasma cell differentiation capacity as compared to young adults. This inability to differentiate was not due to dysfunctional T cell stimulation as co-culture experiments demonstrated (Ennistet al., 1986). Signal transduction involving the B cell receptor may decline with age (Whisler and Grants, 1993). Alternatively, cytokine production by B cells may be altered by changes in the behaviour of other lymphocytes. Abnormally high constitutive production of IL-12 by macrophages has been experimentally shown to drive abnormally high IL-6 and IL-10 production levels in B cells (Spencer and Daynes, 1997). Plasma cell-produced antibody concentrations may, or may not, decrease with age, but the proportion of functional antibody does decline (Smithet al., 2004).

Until recently it was considered that the most likely cause of B cell failure was a lack of effective T cell help in a T-dependent reaction. Although thymic involution is well known, and there is substantial literature on the functional decline of T cells with age, there are T-independent functions of B cells, such as the polysaccharide responses that are crucial for

anti-bacterial protection, which also appear to be lacking in later life. Additionally, there is emerging evidence to suggest that B cells are important antigen presenting cells in their own right and can be key regulators of T cell development, leading us to speculate that some of the failures of T cell function may yet be blamed on insufficient help from B cells. Although the results are controversial, age-related changes in B cell number and repertoire have been described. Thus, decreased IgM and IgD levels in the elderly suggest a shift from the naïve ($CD27^-$) compartment of the B cell branch towards the memory ($CD27^+$) compartment (Colonna-Romano et al., 2006; Colonna-Romano et al., 2008). In addition, the defects of aged T cells, might account for altered immunoglobulin production (Eaton et al., 2004). In mice, the ability to produce antibodies remains undamaged with age (Dailey et al., 2001) but the antibodies produced show a decreased affinity and avidity for antigens (Doria et al., 1978). This observation is likely to be due to deficient somatic hypermutation, which is responsible for the enhancement of antibody specificity for antigens (Miller and Kelsoe, 1995). Primary antibody responses in aged humans are often weak and short-lived with lower affinity. The decline of the immune response to vaccinations in the aged population is of high clinical relevance.

With respect to phagocytic cells, monocytes, which increase in number with age (Della et al., 2007), show a decrease in function capacity (Boehmer et al., 2004). Moreover, macrophages and neutrophils have a reduced phagocytic capacity in elderly persons (Gomez et al., 2005). Additionally, the up-regulation of MHC class II expression is impaired in old macrophages (van Duin and Shaw, 2007). Nevertheless, the effects of age on macrophage function vary by tissue location (Hanet al, 1995). Peritoneal macrophages isolated from rodents generally display an age-related decrease in levels of functions, in the production of several cytokines and intracellular ROS in the resting state and when they are stimulated in vitro with mitogen, receptor-specific ligand or virus (Kohutet al., 2004; Alvarez et al., 1996). However, stimulated murine alveolar and splenic macrophages generally produce higher levels of cytokines (IL-1β, TNFα,

IL-6, and IL-12) and nitric oxide when compared to younger counterparts (Hanet al., 1995; Kohutet al., 2004 and Tasatet al., 2003). Macrophages from older mice produce greater amounts of PGE2 compared with young mice, and PGE2 is known to suppress T cell effector functions (Beharka et al., 1997).

Furthermore, the innate immune system detects pathogens using pattern-recognition receptors such as the TLRs, which recognise specific molecular patterns present on the surface of pathogens. TLRs are expressed on a variety of cells including macrophages. Interaction between TLRs and a pathogen stimulates the secretion of a wide range of antibacterial peptides that destroy the pathogen and trigger an inflammatory response through cytokine and chemokine secretion. Studies in humans and mice have shown that TLR expression and function decline with age (Aspinall et al., 2007; Renshaw et al., 2002). With ageing, there might be decreased surface expression and altered downstream TLR-mediated signalling, increasing the risk of respiratory tract infections. Macrophages derived from in vitro differentiation of monocytes decrease expression of TLR3 mRNA and intracellular protein in older subjects (Kong et al., 2008). Defects in signal transduction and increased oxidative stress were observed in macrophages from both telomerase-deficient and aged wild-type mice (Sebastian et al.,2009). Thus, there is an age-associated decrease in macrophage function, particularly in the context of TLR activation (Boehmer et al., 2004). Defects in macrophage function in aged humans have also been described in a recent study evaluating delayed type hypersensitivity (DTH) responses to Candida antigens, which are known to diminish with age (Agius et al., 2009).

Macrophages that are not as efficient at presenting antigen or at producing immune cell-stimulatory cytokines would delay an efficient adaptive immune response. Similarly, macrophages with diminished inflammatory cytokine or ROS manufacturing capacity would allow pathogens greater opportunities in the host. Changes such as these may be in part responsible for higher mortality rates due to infections such as influenza and pneumonia in the elderly (Zissel et al., 1999).

Another characteristic of the immunosenescence is the shift from Th1 to Th2 cytokine profiles (Sandmand et al., 2002). Th17 cells are a subgroup of T cells differentiated from naive T cells in the presence of IL-6, that secrete IL-17A, IL-17F, IL-22 and TNF-α, which are increased with ageing (Korn et al.,2007). This explains the age-related increase of IL-17 (Stout-Delgado et al., 2009).

Additionally, to the changes in the T cell subsets and B cell function, cytokine production and function are diminished in older people (Prelog, 2006). Thus, ageing dampens the secretion of IL-7 by bone marrow stromal cells (Tsuboi et al., 2004). IL-7 is an essential survival cytokine in the early stages of thymus development and for developing lymphocytes, and the expression levels of this cytokine are greatly reduced with age (Henson et al., 2004; Gruver et al., 2007). In the elderly macrophages have a reduced ability to secrete tumour necrosis factor (TNF), a key inflammatory cytokine (Wang et al., 1995). Macrophage-derived TNF and interleukin IL-1 are essential for the secretion of other cytokines critical for bone marrow stromal integrity, such as IL-6, IL-11, monocyte colony stimulating factor (M-CSF), granulocyte-monocyte (GM-CSF) and receptor activators of NF-κ B ligand (Rebel et al., 1996).

With ageing, on one hand, there is a decrease in beneficial functions, such as the lymphocyte proliferation or the NK activity. There is also a decline in IL-2 that regulates these two functions, as well as the chemotaxis, phagocytosis and adequate levels of ROS in the phagosomes. On the other hand, there is an increase in the functions that could become noxious, if active in excess, such as those promoting adherence of immune cells to tissue, maybe preventing their arrival to the site where they have to perform their organism-protecting task. Furthermore, there is an increase with age in other potentially harmful immune functions such as the extracellular release of the superoxide anion and pro-inflammatory cytokines like TNF-α. Research carried out by the group of De la Fuente, on the functions of the immune cells in lymphocytes showed that, the ability of lymphocytes to adhere to the vascular endothelia increase with age, while the migration

towards the site of antigen recognition (chemotaxis), the proliferate response to mitogens and the release cytokines such as IL-2, are all decreased with age. In phagocytes the adherence to tissues increase with ageing, while other phagocyte functions decline, such as chemotaxis, ingestion or phagocytosis of foreign particles and destruction of pathogens by means of the intracellular production of free radicals such as the superoxide anion and other ROS located in the phagosome of these cells. In this process of phagocytosis, free radicals can be released to the extracellular space with concomitant damage to the structure of phagocytes and neighboring tissues. The results showed that the intracellular levels of superoxide anion decrease with age, while the extracellular superoxide anion increase (De la Fuente, 2002; De la Fuente et al., 2005; De la Fuente and Miquel, 2009). These age-related changes have been observed in rats, mice and human of both sexes and, in general, in leucocytes of several locations such as peritoneum, thymus, axillary nodes and spleen (De la Fuente et al., 2004).

A variety of mechanisms underlie this age-associated dis-regulation. Thus, a decreased expression and functioning of receptors or signalling rafts (Herreroet al., 2002) and defects in signalling pathways (Eisenbraunet al., 2000) may lead to altered functions in immune cells. For instance, it has been shown that NFkB levels are lower in T cells from aged mice compared to young mice (despite comparable levels of CD3 expression), and that up-regulation of kinases such as protein kinase A (PKA) may lower NFκB expression and consequently the expression of downstream molecules such as interleukin (IL)-2 or IL-2Rα (CD25) (Spauldinget al., 1997). However, an age-related increase of the expression of NFkB, has been observed in peritoneal leukocytes, and is related to the life span of each subject (Arranz et al., 2010). In fact, various transcripts or proteins may be dis-regulated (Fulop, 1994), and declines in function and/or signal-induced relocation of other kinases and accessory molecules within the lymphocytes may instead be impaired with ageing, contributing further to cell dysfunction (Tamiret al., 2000).

Another cause of immunosenescence is a consequence of lifelong persistent antigenic exposure/stress. This can be the result of the continuous challenge represented by the unavoidable exposure to a variety of potential antigens (viruses, bacteria, but also food and self molecules among others), which exert a prolonged attrition on the immune system. The production of memory T cells indicates that the body reacted successfully, to each antigenic stimulus and mounted a response capable of coping with the antigens/stressors. However, these same physiological responses at the same time lead to a progressive accumulation of (expanded) clones of memory cells, which eventually will fill the entire immunological space. Together with thymus involution, and the consequent age-related decrease of new T cells in thymus, the virgin T cells practically disappear. Thus the body is more prone to a variety of infectious, such as bacterial and viral as well as non infectious (atherosclerosis, diabetes, dementia) diseases, where immunity and inflammation play a major role (Claudio et al., 2000).

Several external factors might affect immunosenescence positively or negatively (Grolleau-Julius et al., 2009). Gender differences in life expectancy are partially based on altered immune functions: e.g. androgen hormones are known to contribute to thymus involution. Socio-demographic factors also exert a major impact on susceptibility to age-related diseases; these include residency, institutionalisation, income, level of education, life style and disability in daily living. Unhealthy habits, comorbidities and medications also contribute to declining immune activity. Among these, it is worth mentioning smoking, alcoholism, chronic obstructive pulmonary diseases, hypertension, stroke, heart failure, diabetes mellitus, rheumatic and autoimmune diseases and treatments with chronic oral glucocorticosteroids, as well as severe cognitive impairments and Alzheimer disease (Bulati et al., 2008). In addition, malnutrition is associated with a decrease in immunity and therefore with an increase in susceptibility to many infectious diseases. These effects are exacerbated by ageing (Sliedrecht et al., 2008).

1.3.2.2 Causes of immunosenescence

Recently, it has been accepted that the cause of immunosenescence is the same as that responsible for the senescence of the other cells of the organism, namely the oxidative disorganization linked to the unavoidable use of oxygen to support cellular functions. The immune cells produce free radicals and other oxidant and inflammatory compounds in order to perform many of their defensive functions, and this, together with their membrane characteristics, makes them very vulnerable to oxidative damage. The cells, in order to prevent an excess of the oxidants, need to maintain a balance between the production of oxidants and the antioxidant defenses resulting in oxidative stress. This balance is even more essential to preserve the functional capacity of leucocytes and, consequently, the health of the organism. Thus, oxidative stress is believed to be a major factor of accelerated ageing (Knight, 2000; De la Fuente, 2002,2008; De la Fuente et al., 2005; De la Fuente and Miquel, 2009).

Although some authors have suggested that a cause of immunosenescence is the increased pace of telomere shortening resulting from oxidative DNA damage and the diminished telomerase activity (Gharagozloo et al., 2009), this fact is only a consequence of the age-related oxidative stress affecting cells with mitotic capacity (De la Fuente an Miquel, 2009).

Another feature of immunosenescence is the presence of chronic systemic inflammation, which is referred to as ‘‘inflamm-aging’’ (Franceschi et al., 2007). Thus, ageing has been associated with a chronic proinflammatory state, which is characterized by alterations in cytokine profile, with an increase in proinflammatory cytokine levels, such as IL-1, IL-6 and TNF-α. This might predict morbidity and mortality in the elderly (Ferrucci et al., 2005). In murine models of sepsis using LPS intraperitoneal injection, aged mice were found to have an enhanced inflammatory

response, as measured by cytokine and chemokine production compared with young mice (Gomez et al., 2009).

The research group of De la Fuente have investigated the age-related changes in several oxidant/antioxidant and pro-inflammatory/anti-inflammatory parameters in the immune cells. Results in peritoneal immune cells of mice indicated that ageing leucocytes suffer oxidative and inflammatory stress, resulting in higher levels of the parameters of oxidation and inflammation (extracellular superoxide anion oxidized glutathione, TNF-α, PGE2, xantine oxidase activity, etc) and decreased antioxidant defense (reduced glutathione (GSH), glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase). Therefore, an age-related increase in the oxidative damage to biomolecules such as lipids and DNA, occurs (De la Fuente et al., 2005; De la Fuente and Miquel, 2009). Increased oxidative stress has also been found in the immune cells of prematurely ageing mice (PAM) with respect to those of nonprematurely ageing mice (NPAM), and in the leucocytes of male mice with respect to those of females (Arranz et al., 2007; De la Fuente and Miquel, 2009; Bauer and De la Fuente , 2013; Vida and De la Fuente, 2013).

1.4.The psycho-neuro-endocrine-immune communication

The immune system is a regulatory system, and as mentioned previously, it does not work alone. It is in constant and complex communication with the other homeostatic systems, namely, the nervous and the endocrine systems (Besedovsky and del Rey 2007, 2011), constituting the psychoneuroimmunoendocrine system.

1.4.1. General characteristics

Numerous studies have shown that the three regulatory systems interact with each other in maintaining homeostasis and therefore of health in animals and humans (Besedovsky and Del Rey 1996, Tomaszewska and Przekop, 1997). Immune, endocrine and nervous systems exchange signals for immune regulation and brain functions. An abundance of work confirms

the bidirectional communication between these regulatory systems, which is mediated by their secretions -cytokines, hormones, and neurotransmitters- through the presence of their receptors on the cells of the three systems. Thus, immune, endocrine and neural cells express receptors for cytokines, hormones, neuropeptides and transmitters (Tomaszewska and Przekop, 1997; Bellinger et al., 2001; Besedovsky and Rey, 2007). Moreover, endocrine and neural mediators modulate immune system activity and cytokines act in nervous and endocrine systems (Bellinger et al., 2001). In this context, most of the studies have been focused in how circuits involving immune cell products, the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system can affect immune functions and the course of inflammatory, autoimmune and infectious diseases. In addition, the immune system, which converts recognition of non-cognitive stimuli such as viruses and bacteria into information in the form of cytokines, acting on receptors on the nervous and endocrine systems can alter their functions (Besedovsky and Rey, 2007). Thus, the evidence suggests an immunoregulatory role for the brain and a sensory function for the immune system (Weigent and Blalock, 1995).

In addition, the immune, endocrine and neural cell products coexist in lymphoid, endocrine and neural tissue. In fact, cells of the nervous and endocrine systems are considered a source of cytokines and cells of the immune system produce neurotransmitters and hormones (Fig.6).

In the context of this bidirectional communication, any influence exerted on the immune system will have an effect on the nervous and endocrine systems and vice versa.

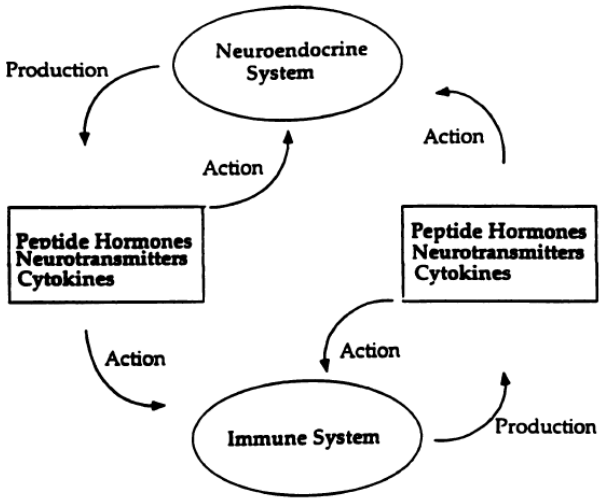


Fig. 6. A molecular communication circuit within and between the immune and neuroendocrine systems involving shared ligands and their receptors (Weigent and Blalock, 1995).

The communication between the regulatory systems can explain a number of facts of everyday life. For instance the situations of emotional stress, depression or anxiety, provoked by the loss of employment or of a close relative, are accompanied by a greater vulnerability to conditions ranging from infectious processes to cancer or autoimmune diseases. This is completely agrees with the notion that the immune system is impaired, and results in worse health and a shorter life span (Guayerbas et al., 2002; Arranz et al 2007; Arranz et al.,2009). By contrast, pleasant emotions help to avoid immune system-related diseases and enjoy better health (Barak, 2006). The changes in the immune system during state of an infection affect and alter the functions of nervous system, which can even lead to psychotic disorders and neural diseases (Merril, 2001).

1.4.2.Changes with ageing in the neuro-endocrine-immune communication

Ageing is associated with a decline of many physiological functions, including those of the regulatory systems, namely the nervous, the endocrine and the immune systems as well as an impairment of the

neuroendocrineimmune network (Fabris,1990; De la Fuente and Medina 2005[a]; Corona et al. 2012).

There is a progressive deterioration in the nervous system and in its function, the hippocampus and the regulation of stress-related disorders in which the hippocampus is involved is highly impaired with ageing. Moreover, the regulation of stress-related disorders in which the hippocampus is involved is clearly impaired with ageing (Garrido 2011; Couillard-Depres et al. 2011).

Several changes accompany healthy ageing in the endocrine system. These include, for example, the increase of several hormones and the decrease of others such as growth hormone/insulin-like factor-1 axis, sexual hormones, dehydroepiandrosterone, and melatonin (Makrantonaki et al. 2010). Moreover, the age related disturbances of the hypothalamic-pituitaryadrenal (HPA) axis seem to be relevant for decreasing stress adaptability in old subjects, this being the cause of their health impairment (Lupien et al. 2009). Thus, there are changes in the innervations of immune organs (such as the decrease of the sympathetic innervation and concentration of noradrenaline (NA) in these organs) and in the expression of receptors of neurotransmitters (as the increase of beta-receptors on the immune cells as a compensatory mechanism). Moreover, the response of immune cells in vitro to neurotransmitters changes with age (Puerto et al. 2005).

Chronic stressful conditions modify immune functions and their interaction with the nervous system, causing detrimental effects on memory, neural plasticity, and neurogenesis (Yirmiya and Goshen 2011). Thus, it has been shown that mice with chronic hyperreactivity to stress and anxiety show a premature immunosenescence and are prematurely aged (Viveros et al. 2007). Likewise, human subjects suffering chronic anxiety or depression show a significant premature immunosenescence (Arranz et al. 2009).

The types and levels of cytokines that contact with the nervous and endocrine cells change with ageing, the age-related increase in the inflammatory cytokine and oxidant compounds, which the immune cells produce (De la Fuente et al., 2005[a]), can modify the nervous function

(Merril, 2001). Also the neuroendocrine modulation of immune response changes with ageing. These changes include the innervations and irrigation of immune organs, the number and affinity of leucocyte receptors for neurotransmitter and hormones, as well as the intracellular signals and the resulting response of the immune cells to them. Thus, some age-related changes in immune function may be due to a disturbance in the communication between the nervous and immune systems (Bellinger et al., 2001; De la Fuente et al., 2001; Puerto et al., 2005). Moreover, the impairment of the immune system with ageing as well as age-related neuroimmunoendocrine network alterations could affect the functions of the other regulatory systems through an increased oxidative and inflammatory stress, resulting in the age-related homeostasis alteration and increase in morbidity and mortality (De la Fuente et al., 2005[b] and De la Fuente, 2008) (Fig. 7). In fact, mice with chronic hyperreactivity to stress and anxiety show a premature immunosenescence and are prematurely ageing (Viveros et al., 2007; De la Fuente and Miquel, 2009). Likewise, human subjects suffering chronic anxiety or depression show a significant premature immunosenescence (Arranz et al.,2007 and Arranz et al., 2009). In addition, the *in vitro* response of immune cells to a wide range of neurotransmitter concentrations changes with the age of the subject (De la Fuente et al., 2001; Puerto et al., 2005). Thus, the age-related deterioration in the communication between those homeostatic systems justifies the loss of homeostatic capacity and the consequent increase of morbidity and mortality that appear with aging (De la Fuente et al., 2005[a]; De la Fuente, 2008).

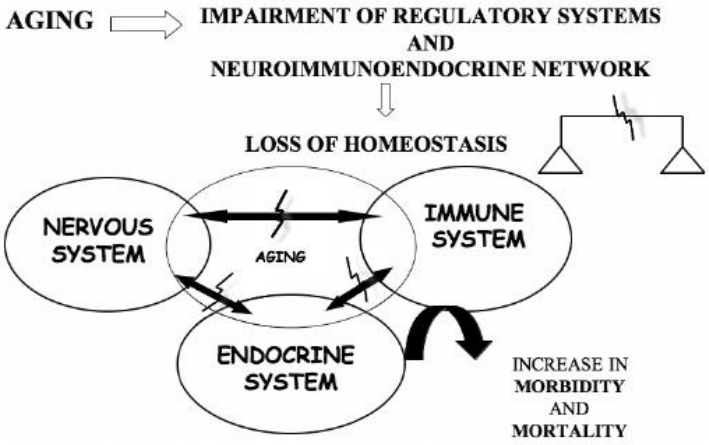


Fig. 7. Regulatory systems and ageing. With ageing there is an impairment of the regulatory systems, namely the nervous, the endocrine and the immune system as well as of the neuro-endocrine-immune communication. These age-related alterations lead to loss of homeostasis and therefore to the age-related increase in morbidity and mortality.

1.5.The immune system: a marker of biological age and predictor of longevity

As previously stated, the process of ageing, with its morphological and physiological modifications, is very heterogeneous in the different organs, tissues and systems of each individual, similarly occurs when comparing subjects of the same chronological age. This fact led to the concept of biological age, which describes the rate of ageing of each subject. As mentioned in the corresponding section, it is very difficult to find markers of biological age. The immune system is a homeostatic system, which contributes to an appropriate functional capacity of the organism, and thus it has been proposed as one of the best markers of health (Wayne et al. 1990; De la Fuente 2004; Alonso and De la Fuente, 2008; De la Fuente, 2014). In fact, various studies have suggested an association between depressed cell-mediated immunity and increased mortality in elderly persons (Wayne et al 1990). An immune risk phenotype (IRP) has been proposed as being related to a higher morbidity and mortality. Thus, parameters such as high CD8 and low CD4 cell numbers and poor lymphocyte proliferative response (Wikby et al., 2005), low interleukine-2

(IL-2) secretion, increased IL-6 levels and a CD4: CD8 ratio <1, define the "Immune Risk Profile" in humans (Pawelec, 2006). Moreover, low NK activity in elderly subjects also proves to be a predictor of morbidity and mortality (Levy et al., 1991). In genetically heterogeneous mice, studies analysing changes in T-cell subsets in peripheral blood showed that the strongest immunological predictor of life span was the proportion of CD4 memory cells, with high levels of these cells associated with a shorter life-span (Miller et al. 1997). The allogenic mice with the longest life spans were the ones, which had the greatest percentage of lymphocytes from the parental strain with the longer lifespan (Warner et al. 1985). All these studies emphasise the relation between an effective immune response and a prolonged life span.

In view of the above mentioned, our research group decided to investigate if some immune functions could be useful as markers of biological age and therefore as predictors of longevity (De la Fuente and Miquel 2009). Since a longitudinal study is impossible to carry out on human subjects throughout the whole ageing process, several functional parameters in leucocytes of peripheral blood in the different decades of the life of human subjects, from their twenties until their eighties, were analysed. As species with a shorter lifespan than to carry out longitudinal studies, mice were chosen, which show a mean longevity of about two years. Although most studies on immune cells in mice involve the sacrifice of the animals (to obtain the spleen, thymus, etc.) using peritoneal leucocytes is possible to extract these cells without necessity of killing them or even using anesthesia, and to study the same functional parameters from adult age until the death of the animals. Thus, functions such as adherence to the vascular endothelia, migration towards the site of antigen recognition (chemotaxis), phagocytosis of foreign particles, anti-tumour capacity of NK cells, proliferation of lymphocytes in response to mitogens and release cytokines, both pro- and anti-inflammatories, have been studied in both species. Surprisingly our results showed that in the members of both species similar age-related changes occur in those immune parameters studied. With ageing there is a decrease of functions such as the lymphoproliferative

response, the IL-2 release, the chemotaxis as well as the NK activity against tumour cells, the latex phagocytosis and the levels of ROS in the phagosomes. In addition, there is an increase of other functions such as adherence of immune cells to tissue, which may prevent their arrival to the site where they have to perform their organism-protecting task (De la Fuente and Miquel 2009). There is also an increase in the release of several pro-inflammatory cytokines, especially those pro-inflammatory, which is accompanied by a decrease in others such as the anti-inflammatory cytokines (Arranz et al. 2010d).

In order to identify the above parameters as markers of biological age, it is necessary to confirm that the levels shown in particular subjects reveal their real health and senescent conditions and consequently, their rate of ageing. This has been achieved in the following two ways:

A) Ascertaining that the individuals with those parameters showing values older than those of most subjects of the same population, sex and chronological age, die before their counterparts. This can be confirmed only in experimental animals and we have used several murine models of premature ageing, especially one of mice with poor response to stress and with anxiety, which will be covert later.

B) Finding that the subjects reaching a very advanced age preserve these parameters at levels similar to those of adults. This can be tested on both humans (centenarians) and experimental animals, such as extremely long-lived mice. Whilst biologically older animals showing the immune competence levels characteristic of chronologically older individuals have been found to die prematurely (Vida and De la Fuente, 2013) centenarians and long-lived mice exhibit a high degree of preservation of several immune functions. This may be related to their ability to reach a very advanced age in a healthy condition (Alonso-Fernandez and De la Fuente 2011). All the above results confirm that the immune system is a good marker of biological age and a predictor of longevity. Moreover, since the evolution of these immune functions is similar in mice and humans, it can be assumed that those humans showing immune parameters at the levels of older subjects have a higher biological age and a shorter longevity.

1.5.1. Models of premature immunosenescence

1.5.1.1. Males versus females

Women live longer than men in different countries and in every era. Several hypotheses were proposed for sex differences in longevity, including more active female immune functioning, the protective effect of oestrogen, compensatory effects of the second X chromosome, reduction in the activity of growth hormone and the insulin-like growth factor 1 signaling cascade, and the influence of oxidative stress on aging and disease (Austad, 2006). Most age-related diseases are delayed in women compared with men. For example, coronary atherosclerosis is postponed in women, also cancer and most other diseases of aging occur earlier in men than in women (Giampaoli , 2000). Women also live more years than men free of each of these diseases (Crimmins et al, 2002). It has been found that slower erosion of human telomeres favours females (Moller et al., 2009) and, even further, the rate of leucocyte telomere shortening predicts mortality from cardio-vascular disease in elderly men (Epel et al., 2009). The higher mean life span of mammalian females than males is today accepted due to the role of the oestrogens, which allow females to live in a less oxidized condition (Viña et al., 2006).

With respect to the mammalian immune system, this works more efficiently in females than in males (De la Fuente et al 2004). The involution of the thymus, occurs more rapidly in males than in females (Richard, 2000), the difference being at the level of the $CD4^{+}CD8^{+}$ immature thymocyte, with less of these being found in males than in females as well as their output of the thymus (Aspinall and Andrew, 2000). These gender-related differences in thymus and immune functions as well as in the fact that males have a higher oxidative and inflammatory state than females, may be causes of the differences seen with longevity between males and females (Baeza et al., 2011; De la Fuente et al., 2004; Guayerbas and De la Fuente, 2003).

1.5.1.2. Menopausal models

Menopause is a complex physiological process, that results from reduced secretion of the ovarian hormones oestrogen and progesterone, which takes place as the finite store of ovarian follicles is depleted (Nelson, 2008). It is known that a chronic deficiency of sex hormones has many implications in a wide variety of non-reproductive functions, the most studied symptoms being, skin ageing and the high risk of osteoporosis and cardiovascular disease (Miquel et al., 2006). Moreover, different psycho-emotional symptoms have also been described that overlap with depressive symptoms and include disturbed sleep, lack of concentration, anxiety, irritability, frustration, mood lability, depression and fatigue (Rasgon et al., 2005; Sarkaki et al., 2008).

It is well known that oestrogens are responsible for the higher immunocompetence of females. Oestrogens appear to play a central role in the immune response and immune-mediated diseases (Cutolo et al., 1995). In fact, women are more susceptible to autoimmune diseases than men (Jansson and Holmdahl, 1998). Several studies have shown the presence of oestrogen receptors on the cells involved in the immune response, namely thymocytes, macrophages and endothelial cells (Cutolo et al., 1995). It is shown that β-oestradiol decreases the production of proinflammatory LPS-induced cytokine such as IL-1 alpha, IL-6, and TNF-α, but does not modified IL-10, IL-12 release (Deshpande, 1997). Thus, in several studies has been observed that oestrogen-deprivation might decrease many immune functions (Keller et al. 2001), whereas hormone replacement therapy in menopause modulates and improves several immune parameters (Brunelli et al., 1996).

To imitate human menopause in rodents, especially in rats and mice, and to obtain a model of long-term ovarian oestrogen deprivation, it is necessary to carry out an ovariectomy. Studies on a model of menopause in rats have shown an impairment in several functions of leucocytes such as chemotaxis, lymphoproliferation, interleukin-2 (IL-2) release and NK cell activity of leucocytes from spleen and axillary nodes, giving values similar

to those in older animals. Thus, in this model of menopause there is an accelerated immunosenescence. In a model of model menopause in mice, these animals also show higher immunosenescence than the age-corresponding controls in their peritoneal leucocytes, as well as behaviour responses typical of older subjects. Thus, ovariectomized rodents showed an older biological age, which did not correspond to their chronological age. This deterioration of homeostatic systems allows us to propose the ovariectomised rodents as a model of premature ageing (De la Fuente et al., 2004, 2011; Baeza et al., 2010b). In addition, the cause of this accelerated ageing seems to be the oxidative stress in these rats and mice (Baeza et al., 2010c), as that, impairment of the immune function and redox state by ovariectomy was partially or completely reversed by administration of natural phyto-oestrogens (Baeza et al., 2010a).

1. 5.1.3. Models of poor response to stress and of anxiety

It is accepted that an inadequate response to stress is one of the conditions leading to an acceleration of ageing, accompanied by an impaired immune system and other physiological systems. Moreover, the changes in cellular trafficking as well as cell-mediated immunity observed in ageing are similarly found following stress or chronic glucocorticoid exposure (Bauer, 2005). In addition, the lifespan of rodent strains appears to be inversely related to the intensity of their behavioural and neuroendocrine responses to stressful stimuli in an exploration test (Dellu et al., 1994), and reduced longevity could be caused by an accelerated age-dependent neurodegeneration (Gilal and Gilal, 1995). In this context, several studies from our laboratory have shown that interindividual differences among members of outbred Swiss or IDR/CD1 and inbred BALC/c mouse populations, both male and female, may be related to their behaviour in a simple T-maze test. Moreover, animals which exhibit immobility or "freezing behaviour" (high levels of anxiety) when placed in a new environment, such as the T-maze, took longer to perform the spontaneous exploration of this maze, show also a worse immune function than those mice that performed the test correctly (De la Fuente et al., 1998; Guayerbas

et al., 2000; 2002a,b,c; Viveros et al., 2001). These animals with premature immunosenescence also have a shorter life span (Guayerbas et al., 2002a,c; Guayerbas and De la Fuente, 2003), and for that they were denominated as prematurely aging mice (PAM), whereas those of the same age, that explored the first arm of the T-maze in 10s or less, and show appropriate immune functions and life span were called non-prematurely aging mice (NPAM) (Viveros et al., 2007). An efficient exploratory behavior relies on an adequate level of CNS functions, which support the ability of the NPAM to adapt quickly to novel stress-causing environments (Haas and Schauenstein, 1997). In fact, PAM are "biologically older," i.e., suffer premature immunosenescence, show a neurochemistry similar to mice of an older chronological age and display higher levels of anxiety and emotionality with respect to NPAM of the same chronological age (Viveros et al., 2007). Based on the fact that ageing results in an alteration of the neuro-immune interactions with resulting cognitive and immune function decline, changes in these functions can be considered biomarkers of physiological age and therefore the present model of premature immunosenescence may be useful in gerontological research. In addition, PAM show a greater oxidative stress in their immune cells as well as in the brain, liver, heart and kidney (Viveros et al., 2007), which show the relation between redox state, immune function and longevity (De la Fuente and Miquel, 2009).

1.5.2. The role of the immune system in oxi-inflamm-ageing

Studies on murine animals showed that, there is a relation between the redox state of the immune cells, their functional capacities and the life span of the subject, when an animal shows a great oxidative stress in its immune cells, that animal showed bad immune functions and shows a decreased longevity. On the other hand, when animals maintain better redox state, their functions will be better and this animal reaches greater longevity (De la Fuente, 2008; De la Fuente and Miquel, 2009).

Body of evidence supports that the ageing is accompanied by inflammation and oxidation condition (Chung et al., 2006; De Martinis et

al., 2006, De la Fuente and Miquel, 2009). In fact, ageing-related diseases such as atherosclerosis, arthritis, dementia, osteoporosis and cardiovascular diseases, among others, are inflammation and oxidation-related. Molecular inflammation hypothesis of the ageing views the redox derangement that occurs during ageing as the major factor for increased risk for age-related inflammation. Accumulated data strongly indicate the activation of redox-sensitive transcription factors and dysregulated gene expression under the age-related oxidative stress, seems to be the major cause of inflammation and ageing. Key players involved in the inflammatory process are the age-related upregulation of NF-κB, IL-1beta, IL-6, TNF-α, cyclooxygenase-2, adhesion molecules, and inducible NO synthase. Furthermore, data are presented on the molecular events involved in age-related NF-κB activation and phosphorylation by IκB kinase/NIK and MAPKs (Chung et al., 2006). Thus, inflammation and oxidation markers might can be considered as the most powerful predictors of frailty and mortality in the elderly (De la Fuente et al., 2005; De Martinis et al., 2006).

De la Fuente et al., have proposed an oxidative inflammatory theory of ageing (De la Fuente et al., 2005; De la Fuente, 2008; De la Fuente and Miquel, 2009). They suggested that the ageing process is highly linked to a chronic oxidative stress, which affects all cells of the organism, particularly those of the regulatory systems; the nervous, endocrine and immune system. Thus regulatory systems would show the greatest oxidative damage and loss the ability to maintain their redox balance, thus their functions that preserve the homeostasis would deteriorate as a consequence, with a resulting increase in morbidity and mortality such as found in old age. The oxidative and inflammatory stress appears to play a fundamental role in the ageing of the immune system and other regulatory systems. The immune system, because of its need to generate continuously oxidative and inflammatory compounds could activate, if it is not well regulated, factors such as the NF-κB, which after reaching certain level of activation stimulates the expression of genes programming the production of higher amounts of those compounds. And if the production of oxidative and inflammatory compounds is not well controlled, a great amount of oxidant and

inflammatory compounds produced by the immune system would activate even more the further production of the same noxious compounds through factors such as NF-κB. These noxious compounds would disorganize with the passage of time not only the immune cells, but also all other cells of the ageing organism and contributing to maintain the chronic oxidative stress of the organism if the are not well controlled, It can be concluded that the immune system can play a role in the uncontrolled oxidation and inflammation process linked to ageing and thus affect the rate of ageing (De la Fuente and Miquel, 2009; Vida et al., 2014) (Fig. 8).

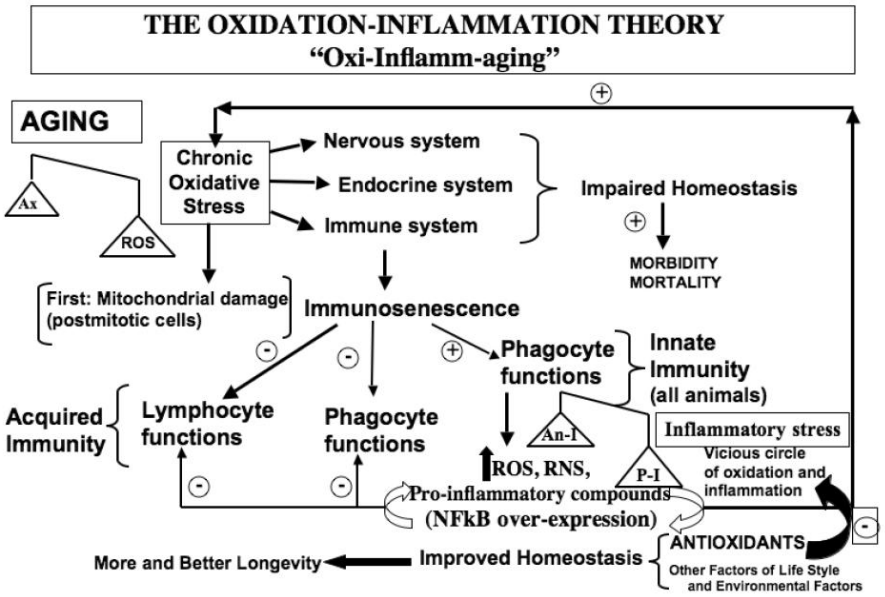


Fig. (8). (Original Figure by De la Fuente and Miquel, 2009). A more developed scheme of the oxidation-inflammation theory than that previously published. Aging is a chronic oxidative stress condition (higher levels of oxidants than of antioxidants) affecting all cells, especially those of the regulatory systems, i.e., the nervous, endocrine and immune system and the communication among them. This explains the impaired homeostasis and the increased morbidity and mortality found in old age. According to the oxidative mitochondrial theory of aging, the differentiated postmitotic cells are the first to suffer damage with age. In animals with a complex immune system the T lymphocytes, and especially the T memory cells (which are the most abundant T cells in aged subjects and are also the most postmitotic cells of the immune system) are those that suffer the effects of the oxidative stress of aging. In the age-associated re-structuring of the immune cells or immunosenescence, there is a decrease of several lymphocyte and phagocyte functions (those related to the acquired immunity), but an increase in other functions, specially those carried out by phagocytic cells generating continuously oxidative and inflammatory compounds (cells and functions responsible for the innate immunity and present in all animals). These compounds produced in order to eliminate foreign agents, could activate the factor of transcription NF-κB, which after reaching certain level of activation stimulates the expression of genes programming the production of higher amounts of those compounds. If this process is not regulated well a vicious circle of oxidation-inflammation could be established, which would increase the oxidative stress and the inflammatory stress, and consequently accelerate aging. Thus we could conclude that in all animal species the immune cells modulate the rate of aging of each subject. In agreement with this concept, the administration of antioxidants has been shown to cut the mentioned vicious circle, improving both the nervous and immune systems, decreasing their

oxidative stress, and consequently improving homeostasis and increasing longevity. Other factors of the environment and life style also have to be considered.

1.6. Biological Rhythms

1.6.1 General characteristics

Chronobiology is a field of biology that studies all the time-dependent variations, known as biological rhythms. These are periodic changes in biological variables of an organism that occur in a specific temporal frame, since the physiology and behaviour of all living creatures are manifested by regular variations and not as continuous processes. Most biological activities fluctuate throughout the day and contribute to a better adaptation to the organism's daily activity. Chronobiology has aimed at studying these biological rhythms, explaining most of the biological mechanisms of a) the endogenous circadian rhythmicity, b) the neurophysiological mechanisms of the photic system that allows its external resetting, and c) the neuroendocrine mechanisms of internal rhythm synchronization (Bourdon and Buguet, 2004).

Biological rhythms can be classified, according to their frequency and period, as: a) ultradian, with a period lower than 20 hours; b) circadian, with a period of about 24 hours and c) seasonal or circannual rhythms (Goulding and Hall, 1993), due to the steady influence of daily and seasonal changes resulting from the Earth´s rotation around its axis and its orbit around the sun. This periodic pattern is most notably manifested by the light-dark cycle and has led to the establishment of endogenous circadian timing systems that provide organisms the ability to anticipate periodical changes in environment such as light and darkness, temperature or food availability and to internal physiological challenges occurring with regular frequency (Moore-Ede and Am, 1986) to adjust the homeostatic system, including sleep and wakefulness, hormonal secretions, immune function and various other bodily functions, to the 24-h cycle (Buijs and Eden, 2003; Collins and Blau, 2006) (Fig. 9).

The suprachiasmatic nucleus (SCN) of the hypothalamus acts as the master circadian pacemaker in mammals. This SCN consists of two small

clusters of about 10,000 heterogeneous neurons. The circadian system is composed of many individual, tissue-specific cellular clocks. To generate coherent physiological and behavioural responses, the phases of this multitude of cellular clocks are coordinated by SCN (Ralph et al., 1990; Okamura, 2003), and coordinate autonomous peripheral clocks located in organs (Reppert et al., 2005). Thus, surgical removal and reimplantation of these nuclei respectively abolish and restore many circadian rhythms in rodents (Scheving et al 1983 and Filipski et al., 2004). The SCN synchronizes other parts of the brain and the periphery through the autonomic nervous system and the neuroendocrine system, via neural efferent and humoral signals, modulating physiological and behavioral variables, including leukocyte physiology (Arjona, 2008).

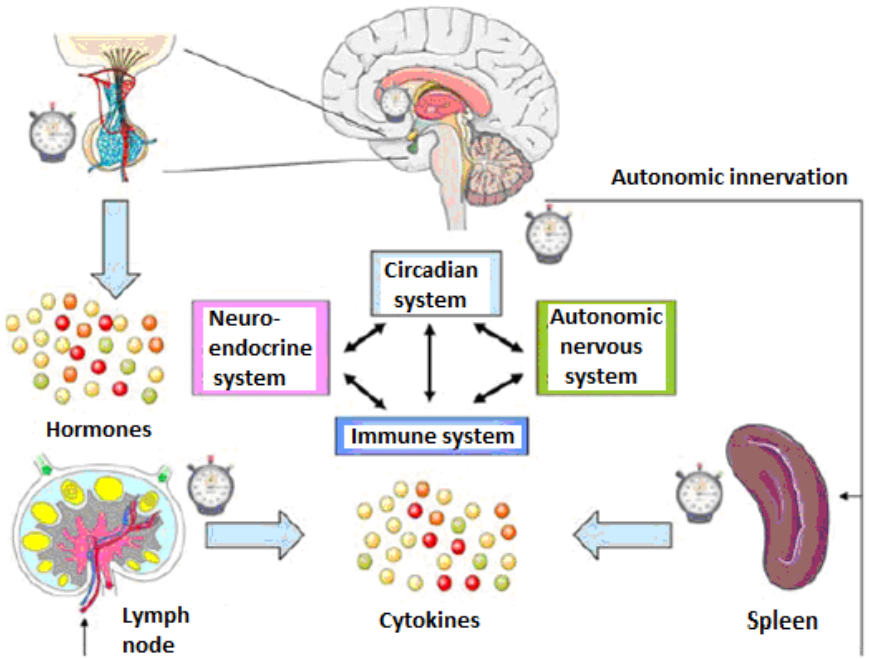


Fig. 9. Temporal interactions among the neuroendocrine system, the autonomic nervous system and the immune system as adaptive mechanisms to the environmental changes.

Circadian rhythms seem to be the result of the expression of the so-called 'clock genes' and their synchronization by environmental and endogenous factors (Yamaguchi et al., 2003). The molecular clock is the result of complex autoregulatory transcription/translation feedback loops, where a set of circadian clock genes, expressed in a circadian manner in

SCN neurons and in overall human cells such as immune and endocrine cells, coding for proteins that regulate not only their own expression, but also that of clock output genes and pathways throughout the genome, completing a circadian cycle in a period of about 24 h (Reppert and Weaver, 2002; Lowrey and Takahashi, 2004; Preitner et al. 2002).

Each cell within the SCN expresses clock genes in a rhythmic and coordinated fashion. Other regions of the brain (Guilding and Piggins, 2007) and many peripheral tissues and even cultured cell lines (Balsalobre et al., 1998) have been shown to express clock genes in a rhythmic and ordered fashion. Subsequent, detailed studies of various clock genes have revealed the circadian-regulated expression in organs as diverse as liver, heart, vasculature, pancreas, kidneys, ovaries, muscle, and adipose tissue (Scheving and Russell, 2007).

The diurnal changes in environmental illumination are conveyed from the retina to the brain to entrain circadian rhythms throughout the body. Light is the major time cue responsible for synchronizing the circadian timing system, although early work also suggested that nonphotic time cues such as meals, caffeine, exercise, and sleep-wake cycle were important synchronizers of the human circadian clock (Aschoff et al., 1971). The main input pathway to the SCN comes from the retina through a monosynaptic retinohypothalamic tract (RHT), which uses glutamate, the main photic signal for the circadian clock (Diego and Ruth, 2010 and Eide et al., 2002), as well as aspartate and pituitary adenylate cyclase-activating polypeptide (PACAP). A multisynaptic pathway, also originating from the retina, innervates the clock from the ventral lateral geniculate nucleus and the thalamic intergeniculate leaflet, using neuropeptide Y (NPY) and γ-amino-butyric acid (GABA) as transmitters (Harrington, 1997). The phase of the central clock is amenable to ambient light signals captured by the visual rod-cone photoreceptors and non-visual melanopsin in the retina. These light signals are transmitted to the SCN through the retinohypothalamic tract, and transduced therein by mitogen-activated protein kinase and other signaling molecules to induce gene expression, which eventually elicits phase-dependent phase shifts of the clock. The central clock controls

peripheral clocks directly and indirectly by virtue of neural, humoral, and other signals in a coordinated manner. Changes in feeding time resets the peripheral clocks in a SCN-independent manner, possibly by food metabolites and body temperature rhythms (Hirota and Fukada, 2004). Output from the SCN to target sites is via both humoral and neuronal connections, and clock output is known to have significant influence on the architecture of the sleep/wake cycle, food intake, attention, learning and memory and other cognitive functions (Kalsbeek, 2006).

The autonomic nervous system and the neuroendocrine system are considered the major conveyers of the circadian information generated in the hypothalamus. The fact that these two systems have been shown to modulate cytokine production, leukocyte trafficking, proliferation, and apoptosis, strongly suggests that circadian mechanisms modulate the immune response through signals ultimately regulated at the hypothalamic level. For example, enhanced prolactin (PRL) and growth hormone (GH) production together with low cortisol levels during the early nocturnal sleep synergistically act to increase Th1 cytokine activity (Dimitrov et al., 2004). Additional supporting evidence for the circadian regulation of the immune response arises from the multiple immunomodulatory properties of melatonin (Carrillo-Vico et al.,2005), a pineal hormone with a pivotal role in the circadian system. Immune mediators such as cytokines might also modulate circadian regulatory mechanisms providing an immune-mediated, circadian-paced feedback loop (Ohdo et al., 2001).

1.6.2 Circadian rhythms in the nervous, endocrine and immune systems

All neurological, endocrine and immunological variables in mammals display biological periodicity, showing an intricate time structure with rhythms and pulsatile variations in multiple frequencies (Haus, 2007). Recordings from single dispersed SCN neurons have demonstrated that the circadian mechanism is not an emergent property of the SCN neuronal network but is expressed in each individual cell. Multisynaptic links of SCN occur through the hypothalamic sub-paraventricular nucleus (PVN) zone

outflow to the adrenocorticotropic and other neuroendocrine axes and to autonomic ganglia that innervate the viscera including all the endocrine and immune systems (Saper et al., 2005). Brain slice cultures of the SCN exhibit circadian rhythms of electrical activity, demonstrating that this structure is able to generate rhythms autonomously (Herzog et al., 1997). However, this property is not due to a "neural oscillator circuit", since even single dissociated SCN neurons show circadian patterns of activity (Welsh et al., 1995). Investigation on the brains of human subjects collected at autopsy showed that populations of arginine, vasopressin and vasoactive intestinal polypeptide (VIP) expressing neurons, located in the shell and core of the suprachiasmatic nucleus, respectively, showed marked circadian rhythms with an asymmetrical, bimodal waveform (Hofman, 2003). Marked diurnal oscillations were observed in the neuropeptide content of the SCN in human subjects. Vasopressin, for example, one of the most abundant peptides in the human SCN, exhibited a diurnal rhythm, with low values at night and peak values during the early morning (Hofman, 2000). Rat SCN neurons in vivo exhibit increased sensitivity to serotonin during the projected night (Mason, 1986). Acetylcholine (ACh) release in the medial prefrontal cortex (mPFC) during the dark phase was significantly greater than during the light phase in rats (Takase et al., 2009).

Neuropeptide Y (NPY) is one of the more abundant peptides in the central nervous system. It acts as a neurohormone and as a neuromodulator, particularly in the hypothalamus, involved in the regulation of several physiological functions such as hormonal release, circadian rhythms, thermoregulation, stress response, anxiety, sleep and others. Both sleep and wakefulness promoting effects of NPY were found in animals, depending on the site of injection as well as on the functional state of the structure. In humans, NPY acts as a physiological antagonist of the corticotropin-releasing hormone (CRH) (Dyzma et al., 2010). Many reports have shown increases in NPY levels both at the beginning and at the end of the light portion of the daily photoperiod in rats (Calzá et al., 1990). Studies on rat and hamster SCN neurons reveal circadian variation in cellular

responsiveness to NPY with many more cells responding during the projected light phase (Mason et al., 1987; Albers et al., 1990). NPY has a modulatory role in the immune system; this neurotransmitter inhibits NO production as well as the expression of inducible nitric-oxide syntheses in microglia, and also strongly impairing the release of IL-1β. The activation of this cytokine on NF-κB was also inhibited by NPY (Ferreira et al., 2010).

Daily oscillations in the levels of enzymes and hormones that affect the timing of cell function, division, and growth have been observed (Mazzoccoli, 2011). Recent studies showed the circadian profiles of a wide group of hormones measured in rats and common marmosets. A significant time effect was observed for adrenocorticotropic hormone (ACTH), corticosterone, prolactin (PRL), thyroid stimulating hormone (TSH), GH, follicle-stimulating hormone, brain-derived neurotrophic factor, ghrelin, insulin, leptin, insulin-like growth factor-1, adiponectin and interleukin-10 (Bertani et al., 2010).

Lymphoid cells express specific receptors for a wide range of substances that show periodicity (Plaut, 1987) such as the hypothalamic-pituitary-adrenal (HPA) axis, and other rhythmic hormones including β-endorphin, melatonin and PRL, which can exert potent effects on lymphocyte behaviour (Hastings,1991). The patterns of neuroendocrine hormone secretion, which regulates aspects of immune function, differ dramatically between day-active and nocturnal species. For example, ACTH and subsequent corticosteroid release is maximal during the day in man but during the dark phase in mice and rats, whereas melatonin is secreted during the dark phase for both groups. This means that these factors may interact in opposite ways depending on the species under investigation. Such differences might give some evidence as to the hormonal mechanisms underlying any particular rhythmicity under investigation (Goulding, 1993).

The HPA axis represents one of the major homeostatic systems of the body. It has been proposed as the primary feed back regulator of the physiological response to stress (Munck et al., 1984). The system can sense

neural, endocrine, metabolic and immune stress signals and respond with hormonal cascade releasing of corticotrophin-releasing hormone (CRH) from the paraventricular nuclei of the hypothalamus. This in turn acts on the pituitary corticotroph cells, resulting in the release of ACTH which stimulates adrenal gland cells to secrete cortisol in man or corticosterone in rodents, which are secreted into the systemic circulation and act on a variety of cells and tissues (Hermus and Sweep, 1990; Khansari et al., 1990). Thus, the levels of circulating GC are regulated systemically by the hypothalamo-pituitary-adrenal axis (Nagalski and Kiersztan, 2010). SCN control of glucocorticoid (GC) secretion is mediated partly via sympathetic input to the adrenal gland. The adrenal peripheral clock is a necessary partner in generating the robust glucocorticoid rhythms that synchronize peripheral clocks (Dickmeis, 2009) and can be enhanced at any time in response to a stressor. These neuroendocrine signals regulate rhythms of peripheral cellular physiology including cell proliferation, apoptosis, secretion of hormones and cytokines, immune cell trafficking, and cytotoxicity (Fu and Lee, 2003). One function of glucocorticoids is the regulation of cell proliferation. Moreover, depending on the tissue, this can involve both negative and positive regulation of a variety of processes, including cell differentiation and cell death (Thomas Dickmeiset al., 2011). Daily fluctuation in plasma cortisol account for most of the circadian periodicity of immune cell distribution and function, since, glucocorticoid hormones exert profound effects on the immune system (Boumpas et al., 1991). In rat liver and human peripheral blood leukocytes, glucocorticoid receptor expressions have shown circadian rhythms with the peak between 04:00 and 08:00 and the lowest point between 23:00 and midnight (Bagdy et al., 1991). The circadian peaks for cortisol occurred between 08:00 and 08:30 hours in human (Gianoulakis et al., 2005). The effects of increased cortisol activity *in vivo* include changes in the production of soluble mediators of immune and inflammatory responses, alterations in the migratory pattern and distribution of leukocytes and the down-regulation of end-stage functions of immune response including granule release, generation of

bactericidal enzymes and oxygen free radicals, phagocytosis, cell cytotoxisity and apoptosis (Bagdy et al., 1991).

The circadian clock of the SCN controls the release of norepinephrine from the dense pineal sympathetic afferents. Norepinephrine has a pivotal role in the nocturnal stimulation of melatonin synthesis in the pineal gland (Simonneaux and Ribelayga, 2003). Melatonin is synthesized at high levels at night and is therefore, called the 'hormone of darkness'. Cells from the SCN also express melatonin receptors (River-Bermudez et al., 2004). This synthesis is under the control of a clock gene in the SCN. The SCN-regulations can also influence the circadian rhythms of some immunological characteristics, which is supported by findings that experimental destruction of the SCN markedly alters corticosterone concentration and consequently lymphocyte counts (Filipski et al., 2003). Melatonin is an important synchronizer of circadian rhythms in the whole body, and also has immune and haematopoietic roles. Many studies show the various effects of melatonin in immune tissues and blood cells, including modulation of the T helper cell (Th) and NK activity, thymus cellularity, leucocyte and lymphocyte counts, IL-2, IL-6, IL-12, and IFN-γ production, etc. (Skwarlo-Sonta et al., 2003). Melatonin alone does not affect lymphocyte proliferation, but does potentiate the corticosteroid inhibition of lymphocyte proliferation (Rogers et al., 1997). Also melatonin has significant immunomodulatory roles in immunocompromised states. In mice, the inhibition of melatonin synthesis causes inhibition of cellular and humoral responses (Maestroni et al., 1986). The administration of exogenous melatonin is also important in both innate and cellular immunity. The chronic administration of melatonin augmented the spontaneous NK cell activity and also the circulating number of NK cells (Angeli et al., 1992). This increased NK cell number was attributed partly to the increased production of cytokines by melatonin stimulated T helper cells. IL-2, IL-6, IL-12 and IFN-γ have all been suggested as the possible cytokines that mediate melatonin-induced increase of NK cell number (Srinivasan et al., 2005).

ACTH secretion demonstrates both ultradian and circadian rhythms. Studies in rats have shown that the diurnal surge of secretion is primarily under the control of CRH (Bagdy et al., 1991). Peripheral blood lymphocytes possess receptors for ACTH and this hormone affects the cytokine and antibody production and activates NK cells and macrophages (Weigent and Blalock, 1987). The circadian peaks ACTH occurred between 08:00 and 08:30 hours in humans (Gianoulakis et al., 2005). CRH shows a diurnal periodicity in synchrony with those of ACTH and glucocorticoid and is responsible for the evening surge in levels of ACTH (Carnes et al 1989). CRH acts as the trigger for stress-induced HPA axis stimulation, and also acts as a modulator of cytokines in peripheral tissues. Receptors of CRH have been found in many tissues such as CNS as well as on the surface of peripheral blood leukocytes. Although it acts as an immunosuppressor terminating in the production of glucocorticoids, its peripheral effects are immunomodulatory. It stimulates lymphocyte proliferation, up-regulates IL-2 receptor expression by T lymphocytes (Singh, 1990), and induces the production of IL-1, IL-2 and IL-6 (Singh and Leu, 1991; Leu and Singh, 1992). CRH induces β-endorphin (Levin et al., 1989), and the immune cells, including monocytes, lymphocytes and polymorphonuclear leukocytes, possess opioid receptors (Bagdy et al., 1990). Thus, β-endorphin and other opioids affect leukocyte functions, inducing several mononuclear cell activities such as mitogen-induced T cell proliferation (Gilmore et al., 1990) the production of IL-1 and IL-2 (Carr, 1991), and enhance NK cell activity (Kay et al., 1990). The circadian peaks for β-endorphin, ACTH, and cortisol occurred between 08:00 and 08:30 hours in humans (Gianoulakis et al., 2005).

PRL is secreted by cells in the anterior pituitary and its plasma concentration rises following the onset of sleep and fall upon awakening (Frantz, 1978). Both T and B lymphocytes express PRL receptors (Russell and Larson, 1985) and this hormone induces expression of high affinity IL-2 receptors (Mukherjee et al., 1990). An inhibitory effect of PRL on NK cell activity has also been shown (Gerli et al., 1986). Suppression of PRL

secretion in laboratory animals resulted in depressed T cell proliferation, cytokine production and macrophage mediated cytotoxicity (Bernton et al., 1988).

There are a number of lines of evidence that suggest that an animal's immune-status can influence circadian timekeeping processes and there is accumulating evidence linking the immune system with circadian regulation. For instance, several light input path-ways, including the SCN, may modulate the immune system (Chacon et al., 2002 and Roberts, 2000). It has been shown that the circadian clock influences the immune system, mice with a loss of function mutation in the *Per2* gene display a loss of interferon-γ (IFN-γ) mRNA cycling in the spleen (Arjona and Sarkar, 2006). Additionally, these animals are also more resistant to lipopolysaccharide-induced endotoxic shock than wild type mice and show decreased levels of pro-inflammatory cytokines in the serum (Sun et al., 2006). Moreover, the human circadian clock genes Per1, Per2, and Per3 were found to be expressed in a circadian manner in human peripheral blood mononuclear cells, with a peak level occurring during the second part of the active phase (Boivin et al., 2003; Kusanagi et al., 2004).

Circadian rhythms are evident in both enumerative and functional immune measures. Peripheral counts of monocytes, B cells, and helper T cells peak nocturnally, while granulocytes, monocytes, macrophages, natural killer (NK) cells, and cytotoxic T lymphocytes (CTL) peak diurnally (Born et al., 1997). Further, several studies have described circadian variations of different immune parameters such as lymphocyte proliferation, antigen presentation, and cytokine gene expression (Demas et al., 2003; Levi et al., 1991 and Maestroni and Conti, 1996). Rhythmic variations have been reported in the activity of a number of vertebrate immune system components, including horse and human neutrophils (Raidalet al., 2000), ring dove heterophils (Rodriguez et al., 1999), rat and hamster NK cells (Arjona et al., 2004; Yellon et al., 2005), and complement proteins in sea bass and gilthead seabream (Esteban et al., 2006). But most studies have

concentrated on numbers of circulating cells and their surface phenotype, with relatively few reports of changes in functional activity.

Peritoneal macrophage functions in mice such as phagocytosis exhibited a circadian variation that peaks during the light period and bottoms during the dark period. Furthermore, the cytokine/chemokine expressions display a circadian rhythm that is regulated by a molecular clock. These diurnal changes of phagocytosis activity in macrophages were induced without exogenous stimulants. The expression of the clock genes including brain and muscle Arnt-like protein-1 (BMAL1) exhibited robust circadian rhythms in macrophages. The expression patterns of the clock genes in macrophages were similar to those in the suprachiasmatic nucleus and other peripheral tissues (Mitsuaki et al., 2007). Phagocytosis of neutrophils showed circadian patterns in mice and rats **(**Hriscu, 2005). The production of superoxide anions by human neutrophils incubated by chemotactic peptide F-Met-Leu-Phe showed a trough during the day (Muniain et al., 1991) The chemotactic response of these cells to F-Met-Leu-Phe also showed diurnal variation with the maximal activity during the day (Muniain et al., 1988).

The activity of NK cells varies depending on the time of day (Arjona et al., 2004). NK cell showed a maximum activity in the dark phase in rats (Fernandes et al., 1979), and it is found to double along the circadian time scale in mice, the highest NK cell activity being observed at the transition from the rest span to the activity span of mice, Furthermore, murine data indeed indicate that the organism was more resistant to tumour cells inoculated in the middle-to-late rest span (light) (Fernandes, 1979). In healthy human beings, both NK cell activity and the circulating count of NK cells in peripheral blood are found to be highest near awakening (Gattiet al., 1988; Leviet al., 1988). Cytotoxicity mediated by NK cells in man showed a circadian rhythm with maximal activity in the morning (Williams et al., 1979).

Cell proliferation as measured by DNA synthesis was found to be highest around 12.00-20.00 h in human bone marrow (Smaaland et al., 1989). The proliferative ability of bonemarrow granulomonocytic precursors was also highest in the second half of the activity span both in man (Smaaland et al., 1989) and in mice (Levi et al., 1988).

In rodents, early studies demonstrated that the number of circulating and splenic lymphocytes varied in a circadian fashion, with peak values being measured during the light (resting) phase (Abo et al., 1979; Kawate at al., 1981).

Several studies have investigated the changes in cytokine levels that occur during the 24 h sleep-wake cycle in humans. Plasma TNF-α levels peak during the dark phase of the cycle, before going to sleep, and the circadian rhythm of TNF release is disrupted by sleep pathologies such as obstructive sleep apnea. Plasma IL-1β levels also have a circadian variation, being highest at the onset of non-REM sleep. The levels of other cytokines (including IL-2, IL-6, IL-10, and IL-12) also change during the 24-h cycle. Thus, the production of IL-2 increases during sleep, independent of migratory changes in T-cell distribution (Pandi-Perumal et al., 2007). Among inflammation factors examined in peritoneal macrophage in mice, the level of monocyte chemoattractant protein-1 (MCP-1/JE) mRNA exhibited most robust circadian oscillation. Expression of other cytokines such as IL-1β , IL-6 and TNFα showed mild diurnal changes (Mitsuaki et al., 2007).

1.6.3 Circadian rhythms with ageing

Ageing is characterized by changes in neuroendocrine functions, such as thyroid, adrenal, or growth/metabolic functions among others (Rehman and Masson, 2001; Wise et al., 2002). Moreover it is characterized by a diminished ability to respond to stress (Weinert and Timiras, 2003). As we age, the circadian clock begins to break down. For example, the molecular rhythmicity in expression or activity of some proteins dampens and our sleep/wake cycles become disrupted. This waning clock may have

numerous consequences on our cognitive processes including learning and memory (Eckel-Mahan and Storm, 2009). A reduced amplitude, shorter free-running periods and desynchronization of circadian rhythms are associated with advanced age in both rodents and humans (Kondratov, 2007). Aged rodents showed, among others, an altered response to the phase-shifting effects of light pulses and changes in the time it takes to re-entrain to a new light- dark cycle. In healthy elderly humans, although circadian rhythmicity persists, a number of 24-hour rhythms are dampened and/or advanced (Rehman and Masson, 2001). Age related changes in the circadian rhythms and sleep quality has been linked with impairment in the function of the suprachiasmatic nucleus (SCN) and melatonin secretion. The precursor of melatonin, serotonin (5-HT) is a neurotransmitter involved in the synchronisation of the circadian clock located in the SCN, which shows decreased levels with age (Celia Garau et al., 2006).

In the context of nervous, endocrine and immune systems communication, Gianluigi Mazzoccoli et al. found in older human beings that the function of these systems shows patterns of circadian rhythmicity and a number of age-related changes in the 24-h hormonal and non-hormonal rhythms. They found circadian rhythms for CD3 (with a phase delay of 3 h), CD8, CD4/CD8 ratio, CD16, CD25 and NK cells, cortisol (with a phase delay of 1 h) and melatonin (Gianluigi Mazzoccoli et al., 2011).

Studies documented the existence of significant disruption of circadian organization of components of the hypothalamic-hypophyseal unit in aged rats. Both the efficacy of input and output pathways from the central nervous system circadian pacemaker (the hypothalamic SCN) and the functioning of the central pacemaker itself, change with advancing age. In addition, some of the decline in overt circadian rhythmicity can be due to the deterioration of the effector systems (Kolker and Turek, 2001).

1.7. Alzheimer ´s Disease (AD)

1.7.1. General characteristics

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common form of senile dementia, characterized by a progressive loss of cognitive function. The prevalence increases with age, occurring in mid to late life in human populations and affects about 10% of individuals older than 65 and about 40% of people older than 80 (Evans et al., 1989). The etiology of AD is largely unknown, but mutations of the genes encoding the β-amyloid precursor protein (APP) (Goate et al., 1991) and presenilin (PS) 1 (Sherrington et al., 1995) and PS2 (Rogaev et al., 1995) have been observed in inherited AD. In addition, there is an association between AD and the apolipoprotein E (APOE) gene, (Corder et al., 1993) α-macroglobulin (Blacker et al., 1998) and endothelial nitric oxide (NO) synthase-3 genes (Dahiyat et al., 1999).

Clinical manifestations of AD are primarily progressive impairments in memory, language, calculation, visiospatial perceptions, judgment, and behaviour (McKhann et al., 1984). Some patients show evidence of psychosis. Activities of daily living become progressively more impaired, and the patients are profoundly demented and often mute, incontinent, and bedridden in the late stage of the disease. Alzheimer's disease is characterized histopathologically by intracellular neurofibrillary tangles (NFT), numerous extracellular senile (neuritic) plaques (SP), and synaptic loss. NFT consists of paired helical filaments and related straight filaments, which are composed of aggregates of the hyperphosphorylated microtubule-associated protein tau (Grundke-Iqbal et al., 1986). The main component of SP is amyloid β-peptide (Aβ), a 40-42-amino-acid peptide derived from proteolytic cleavage of APP by the action of β- and γ-secretases. Aβ is considered to play a causal role in the development and progress of AD (Selkoe, 2001). The cholinergic neurons in the nucleus basalis of Meynert (NBM) are lost early in the course of AD, and the dysfunction of these neurons is believed to be involved in cognitive deficits in this disease (Coyle et al., 1983).

Several lines of evidence suggest an important role for oxidative stress in the pathogenesis and progression of AD (Markesbery, 1997). This damage is characterized by oxidative modification of a number of cellular macromolecular targets, including proteins, lipids, carbohydrates, DNA, and RNA (Butterfield and Lauderback, 2002), in the AD brain, protein oxidation occurs in Aβ-rich regions such as the cortex and hippocampus, but is not observed in the cerebellum, where the Aβ was found to be negligible (Hensley et al., 1995).

1.7.2. Models of AD in mice

Despite considerable progress, a complete description of the molecular pathology of AD has yet to be elucidated. The usual criteria for establishing useful rodent models for Alzheimer's Disease (AD) focus on the reproduction of Alzheimer's like pathology in the rodent brain, accompanied by behavioral deficits related to dementia, for defining the character and the spatial and temporal development of cellular abnormalities in brains with AD, to illustrate the mechanisms that cause brain dysfunction, and for development and testing of potential treatments.

AD is a typical human disease and experimental animal models of AD are designed to reproduce a subset of selected neuropathological, biochemical and behavioural changes accompanying AD (Selkoe, 1991 and Wenk et al., 1992). No single animal model presents the whole range of pathological, neurochemical and behavioural alterations typical of the disease, although they can be useful to perceive and analyse specific aspects of this neurodegenerative disorder. A valid animal model for AD should exhibit (1) progressive AD-like neuropathology and (2) cognitive deficits, and (3) should be verified in several laboratories. Transgenic models should be able to (4) discern pathogenic effects of familial forms (FAD) mutations from those of transgene overexpression. Models derived from microinjection of FAD mutant alleles should (5) encompass more than one Tg line (Janus and Westaway, 2001). During the past decade, nontransgenic Animal models of AD, as well as transgenic mice with familial AD genes,

that mimic some of the neuropathological changes and cognitive impairments in AD have been generated.

Amyloid β-peptide-related animal models: Several studies in mice have demonstrated that acute injection or continuous infusion of amyloid β-peptide (Aβ) in the brain causes brain dysfunction characterised by neurodegeneration and impairment of learning, memory (Pepeu et al., 1996; Nabeshima and Itoh, 1997) and behavior as recognized by using two-choice active avoidance learning in a Y-maze (Flood et al., 1991) and Water-maze (Nitta et al., 1994). More research using complex behavioral tasks is needed to determine if Aβ-induced deficits are similar to those observed in Alzheimer patients.

Animal models based on gene transfer techniques facilitate studying the effects of overexpression of mutant human amyloid precursor protein (APP) and presenilin-1 (PS1) or presenilin-2 (PS2) genes in rodents (Mattson, 2004). Several mutations in early-onset AD are known, so making transgenic AD model mice can be achieved by insertion all or part of the gene that codes for the human amyloid precursor protein (APP) into the murine blastocyst. The transgenic mice typically overexpress APP and sometimes have pre-amyloid deposits (Higgins et al., 1994). Different types of transgenic mice exhibiting senile plaques and Aβ-associated neuropathology have been generated, such as those expressing human APP (Quon et al., 1991), Aβ (LaFerla et al., 1995), the C-terminal fragment of APP (Kammesheidt et al., 1992) and the APP genes carrying familial AD mutations (Games et al., 1992).

The production of β-amyloid plaques accompanied by neurodegenerative changes in a mutant human APP717 transgenic mouse have been produced (Games et al., 1995). These mice express 8-10 times the normal level of amyloid precursor protein, APP, and this high level of APP expression is thought to be a key factor in the successful production of plaques. Transgenic mice overexpressing human APP751, which develop early AD-like histopathology exhibit age-dependent deficits in spatial

learning in a water maze and show age-dependent deficits in spatial learning in a water maze task and in spontaneous alteration behaviour in a Y-maze, Aβ elevation and amyloid plaques (Moran et al., 1995and Hsiao et al., 1996).

Neurofibrillary tangles-related animal models: Development of AD animal models that show neurofibrillary tangles (NFT)-like neuropathology has been attempted using pharmacological or transgenic technology. NFT consist of paired helical filaments and the related straight filaments, which are made by hyperphosphorylated tau protein. Chronic intracerebroventricular infusion of phosphatase 1/2A inhibitor, okadaic acid, causes an induction of paired helical filament-like hyperphosphoprylation of tau, APP expression and Aβ deposition, which were associated with marked memory impairment (Arendt et al., 1994; Arendt et al., 1994).

Presenilin-related animal models: Transgenic mice overexpressing human mutant PS1, increased selectively brain Aβ42 (43) compared with the wild-type. The brain Aβ42(43) levels in the transgenic mice with mutant PS2 gene were also higher than those in wild-type PS2 transgenic mice (Borchelt et al., 1996; Oyama et al., 1998).

The doubly transgenic mice were developed when a PS1 mutation (M146L), which causes a modest increase in Aβ42(43), is introduced into the Tg2576 mice expressing human APP695 containing the double mutation (K670N/M671L) (Holcomb et al., 1998; Holcomb et al., 1999). The doubly transgenic mice develop large numbers of fibrillar Aβ deposits in the cerebral cortex and hippocampus far earlier than singly transgenic Tg2576 mice. Both doubly and singly transgenic Tg2576 mice showed reduced spontaneous alteration performance in a Y-maze before substantial Aβ deposition was apparent, indicating that some behavioural alterations in these mice may be related to an event that precedes plaque formation (Holcomb et al., 1998).

1.7.3.Triple-trangenic mice for AD

The first triple-transgenic 3xTgAD mice harboring *PS1*$_{M146V,}$ *APP*$_{Swe}$ and *tau*$_{P301L}$ transgenes was developed in 2003 in the laboratory of Prof. Frank M. LaFerla, by microinjection transgenes into single-cell embryos from homozygous *PS1*$_{M146V}$ knockin. This animal model mimics both amyloid and tau AD neuropathologies in an age-dependent manner in disease-relevant brain regions and reveals the important role of the intraneuronal accumulation of oligomeric Aβ in the etiopathogenesis of the disease. The 3xTgAD mice progressively develop plaques and tangles. Synaptic dysfunction, including LTP deficits, manifests in an age-related manner (Oddo et al., 2003), and present the characteristic reactive gliosis inflammatory profile as well as cognition impairment (learning and memory deficits) (Kitazawa et al., 2005; Billings et al., 2005) and psychological and behavioral symptoms of dementia (Giménez et al., 2007).

Studies on this model in our laboratory in 3xTgAD and NTg mice of 15 months of age, showed: (1) an impairment of the neuroimmunoendocrine network in the 3xTgAD mice (especially in males) similar to those occur with ageing. (2) The life span of triple-transgenic 3xTgAD mice was shorter than that in age-matched wild-type NTg mice. (3) Several immune functions, studied in leucocytes from spleen and thymus of old female 3xTgAD mice, showed alterations with respect to old NTg mice. (4) The levels of reduced glutathione in the spleen and thymus leukocytes of 3xTgAD mice were similar to those of NTg mice (Giménez-Llort et al., 2008).

Recently, in peritoneal leucocytes from 3xTgAD and NTg mice of 2, 4, 6, 12 and 15 months of age have been studied several functions and redox parameters. The results have shown a premature immunosenescence and oxidative stress situation in 3xTgAD at 4 months of age, when the immunoreactivity against intracellular amyloid-beta (Aβ) fibrils appears. Moreover, several function and redox parameters were already changed before the onset of AD, at the age of 2 months. Furthermore, the changes in

some parameters continue at 15 months of age, when AD pathophysiology is completely established. A short life span was also observed in 3xTgAD. Thus, it has been suggested that peripheral immune cell functions and their oxidative stress status could be good early peripheral markers of the preclinical and prodromal stages and progression of AD (Mate et al. 2014) (Fig. 10).

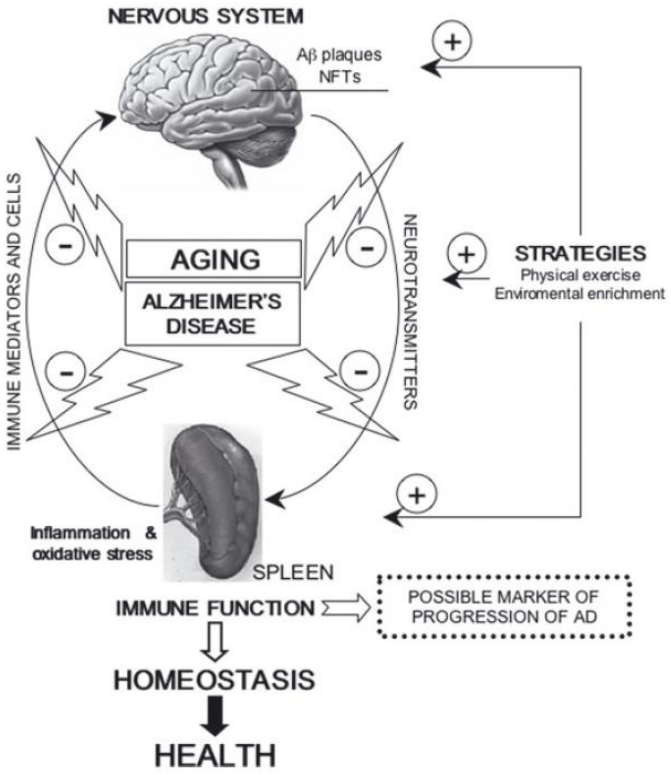


Figure 10. (**Original Figure by Gimenez-Llort et al., 2012**) With age, all regulatory systems (the nervous and immune system) involved in homeostasis show impairment due to an increase of inflammatory and oxidative stress. This age-related loss of homeostasis is exacerbated in AD and supports the hypothesis of a premature immunosenescence as a relevant factor of AD. Moreover, the analysis of immune function could be a marker of the progression of AD. Several strategies, such as physical exercise and environmental enrichment, seem to reestablish the homeostatic systems. Aβ plaques, beta amyloid plaques; NFTs, neurofibrillary tangles; (+), positive effect; (−), negative effect.

1.8. Physical activity

1.8.1. Physical activity and the immune system

There is a body of evidence which shows that exercise training has a number of beneficial physiological effects, e.g., increases insulin sensitivity, improves lipid profile, increases maximal oxygen uptake, maintenance of muscle strength, and increases bone mass (Astrand, 1997). Moreover, the association of physical exercise with health is well known (Kokkinos 2012). Although there are conflicting results, depending on the type, intensity, and frequency of exercise, as well as the immune function studied and state of the subject, it is accepted that physical exercise modulates the immune functions (Sugiura et al., 2000). In general, acute or strenuous physical exercise induces an impairment of immune response, increasing the risk of infections, whereas, regular and moderate training exercise leads to adaptations of the immune cells with an improvement of their functions (Radak et al. 2008; Hoffman and Pedersen, 1994). Thus, moderate training exercise causes an enhancement of total leukocyte (lymphocyte, granulocyte, monocyte and natural killer cell) count, as well as of the helper:suppressor cell ratio, lymphocyte proliferation in response to mitogens, and production and serum levels of immunoglobulins (Roy and Pang,1994). However, intense exercise training tends to produce adverse changes in these same indices, and also may lead to immunosuppression and elevated risk for infectious diseases (Roy and Pang,1994; Nieman, 1998). Nevertheless, the effects of exercise on immune functions depend not only on the intensity but also on the duration of the activity. Thus, it has been reported that the NK cell activity is increased when measured immediately after, or during, both moderate and intense exercise (bicycle exercise) of a few minutes (Pedersen and Goetz, 2000). Many studies show contradictory results with respect to the effects of exercise on immune response. For example, lymphocyte proliferation in response to pokeweed mitogen (PWM) and lipopolysaccharide (LPS), B-cell mitogens, increases or remains unchanged after exercise in humans(Field et al., 1991).

In mice, Ferrandez and De la Fuente (1999) reported that a swimming exercise for 90 min/day over a period of 20 days enhances several functions (chemotaxis capacity, phagocytosis capacity, and superoxide anion production) of peritoneal macrophages compared to sedentary control mice. A treadmill running exercise as well as a voluntary running exercise for 8-12 weeks in mice also enhance phagocytic activities in peritoneal macrophages as well as lymphocyte proliferation in response to concanavalin A (Sugiura et al., 2001). In young mice (4 weeks of age) a forced running exercise (on a flat floor for 60 min a day, during 5 days per week for 12 weeks) results in an increase in the spleen and thymus weight, an increase in phagocytic index of peritoneal macrophages to latex beads and an increase in the proliferation of leukocytes induced by Con A (Sigira et al., 1993).

1.8.2 Physical activity and oxidative stress

Several studies in experimental animals and humans have shown evidence of free radical generation during, and after, exercise (Evans et al., 1991; Packer, 1984). These free radicals may come from several sources: 1) The mitochondria, from which oxygen radicals that have escaped scavenging enzymes present in this organelle may leak into the sarcoplasm. 2) The capillary endothelium, where a hypoxia or reoxygenation process is created during exercise; and 3) An oxidative burst from inflammatory cells mobilized as a result of muscle or tissue damage (Evans, 2000). Nevertheless, in response to repetitive or graded exercise training, a decrease in oxidative stress and a resistance to oxidative damage also appears. This seems to be due to a down regulation of the release of ROS as consequence of an adaptation of antioxidant defenses, which increases their levels and activities (Walsh et al. 2011; De la Fuente et al. 2011). Thus, the increase exercise-related levels of ROS and oxidative damage are initiators of specific adaptive responses, such as the activation of antioxidant enzymes (Jell et al, 2004) and enhanced oxidative damage repair (Radak et al, 1999). Although the effects of training on oxidative stress depend on training characteristics (i.e., intensity, type, volume, duration) (Vincent et al,

2002;Takahashi et al., 2013), several studies have demonstrated that in humans continuous aerobic training, characterized by a constant sub-maximal intensity, reduces ROS production and increases antioxidant defences (Fatouros et al., 2004; Miyazaki et al., 2001). Vitamin C and, especially, vitamin E are shown to decrease the rate of lipid peroxidation induced by exercise (William, 2000). Exercise has a positive effect on antioxidant processes in red blood cells, increasing glutathione reductase activity (Evelo et al., 1992).

However, acute exercise in a person who is not in condition or unaccustomed to exercise induces oxidative damage and results in muscle injury. Thus, significant increases in catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase activity was found higher in erythrocyte of trained runners than in untrained subjects (Toskulkao and Glinsukon, 1996; Ohno et al., 1988).

Exercise could be a preventive tool against neurodegeneration-associated oxidative challenge, since some studies showed that regular physical exercise can attenuate oxidative damage in the brain by decreasing the production of ROS and stimulating the antioxidant systems (Radak et al., 2008; Radak et al., 2010).

Whereas some studies showed that blood amounts of GSSG (indicative of oxidative stress) increased immediately after intensive exercise on a treadmill, the values returning to rest within 1 h, other research reported no change in blood GSH and GSSG during 30 min of treadmill running (Marin et al., 1990), or a decrease in GSH (Gohil et al., 1988). It was suggested that decreased plasma GSH after exercise reflects its consumption by skeletal muscle, which results in a reduced export rate from muscle into plasma (Kretzschmar and Muller, 1993). In contrast, other studies showed that blood GSH increased after progressive-intensity or prolonged exercise (Ji et al., 1993; Sahlin et al., 1991). A significant relationship between weekly training distance and erythrocyte antioxidant

capacity in runners, was observed, with an enhancement of vitamin E, GSH and catalase activity (Robertson et al., 1991).

In mice which performed treadmill training over a period of eight weeks, the GSH content and GSH/GSSG ratio was increased in the soleus and plantaris skeletal muscles as well as an improved cellular redox status was observed (Julio et al., 2010)

1.8.3 Physical activity and immunosenescence

Although in old animals or elderly humans the effects of physical exercise on the immune functions have been scarcely studied, the available data show that the practice of regular and moderate exercise is an important candidate for improving the immune function throughout the ageing process, delaying the onset of immunosenescence (Simpson et al. 2012). It is well known that physical exercise training is important in the prevention of age-associated diseases, such as type II diabetes, atherosclerosis, hypertension and osteoporosis in elderly humans since it influences physiological functions (Bruunsgaard and Pedersen, 2000) including immune system response (Sugiura et al., 2000). Most studies suggest that moderate exercise training exerts little effect on immune function in healthy populations, but it is possible, that moderate exercise training improves the immune functions in other groups, such as the elderly (Bruunsgaard and Pedersen, 2000). Studies showed that the immune response to acute exercise is maintained with age (Pedersen et al., 1999) whereas others argue that it is similar but reduced in elders (Ceddia et al., 1999). Acute exercise in older subjects is known to increase NK cell activity (Crist et al., 1989) and circulating numbers of neutrophils (Cannon et al., 1994). It also changes circulating T cell populations and modulates T cell proliferation (Ceddia et al., 1999). Moreover, in female athletes over 65 years of age, it was found that the NK cell activity was higher than in sedentary controls (Nieman et al., 1993). However, other results demonstrated similar (Shinkai et al., 1995) or slightly greater (Shinkai et al., 1998) NK activity in elderly runners in comparison to the corresponding controls. It was found that the older

adults demonstrated higher cytotoxic T cell response to the influenza vaccine, as assessed by granzyme B, after 10 months of aerobic exercise compared to control subjects (Kohut et al., 2004). It was also reported that the lymphocytes from older recreational male runners showed higher proliferation in response to phytohemagglutinin (PHA) and pokeweed mitogen (PWM) than the controls (Shinkai et al., 1995; Shinkai et al., 1998). Similar findings were reported with respect to PHA-induced lymphocyte proliferation in older competitive female athletes (Nieman et al., 1993). The age-associated decline in neutrophil phagocytic function is also attenuated by exercise in adults aged over 60 (Yan et al., 2001).

In experimental animals, an age-associated decline in macrophage function may be reversed by exercise. Thus, moderate and regular exercise (5 days/week, 45 minutes/day, 16 weeks) in older mice increased the capacity of peritoneal macrophages to kill tumour cells *in vitro* (Lu et al., 1999). In old rats (27 months of age), it was found that 15 weeks of exercise training (running) increased proliferation in response to Con-A, and that 21 months of daily exercise also resulted in a trend towards increased proliferation to PHA in old animals (Nasrullah and Mazzeo, 1992; Utsuyama et al., 1996.). Many other studies are reviewed by De la Fuente et al., (2011).

2. OBJECTIVES

Much scientific evidence suggests that the process of ageing is a result of chronic oxidative stress, which affects living organisms, owing to the imbalance between endogenous antioxidant and oxidant compounds. The progressive oxidation leads to the damage of biomolecules and ultimately to a greater degree of oxidation of the organism. This causes an age-related decline of physiological functions, especially those of the regulatory systems such as the nervous, endocrine and immune systems as well as the communication between them. Several functions of the immune cells have been proposed as markers of the rate of ageing and predictors of longevity. This idea has been suggested using mouse models of premature ageing in which the premature immunosenescence, associated with higher oxidative stress of immune cells, of a subject is accompaied by his/her shorter life span. Thus, mice showing a poor response to stress situations and high anxiety levels, mice without oestrogens (by ovariectomy) and mice with a neurodegenerative illness such as Alzheimer´s disease, are models that, in this context, are being studied in our laboratory. Nevertheless, many aspects of the changes in the redox state and in the immune functions of these models are still unknown. In addition, another relevant aspect of immunosenescence, which has not been previously studied, is the effect of circadian rhythm on the functions and oxidative stress of the immune cells as well as the possible age-related change of this rhythm.

In an attempt to study in more depth the above mentioned aspect of the ageing process, three objectives have been proposed in the present work.

First Objective:

The circadian rhythm seems to be a key component of the neuro-immuno-endocrine system. Although in the immune system, some circadian variations have been observed in several immune functions of human beings and experimental animals, at the present few studies on circadian variations in this aspect, especially in mice, have been carried out. Moreover, the

circadian rhythm is usually not considered in animal experimental designs. In addition, the age-related changes in the circadian variations of immune functions have not been studied.

For this reason the first objective was **to study the effect of the circadian rhythm in several immune functions and oxidative stress parameters in peritoneal leucocytes from adult and old mice**.

Second objective:

We have proposed a model of premature ageing mice (PAM), which show a premature altered stress-related behavioural response, observed at adult age. PAM, in relation to non-prematurely ageing mice (NPAM), of the same gender and chronological age, take longer to explore a T-shaped maze, have high anxiety levels, a premature immunosenescence, higher oxidative stress of peritoneal immune cells, more aged endocrine and nervous systems, and a shorter life span. Although the premature immunosenescence in PAM has been previously studied in immune cells from peritoneum, thymus, spleen and axillary nodes, the oxidative stress state in immune organs as well as in postmitotic and nonpostmitotic organs such as heart and liver, respectivelly, have not been studied yet. Moreover, there is an age-related oxidative stress and the above mentioned differences between PAM and NPAM has been observed principally at the adult age. However, whether these differences are maintened with the chronological age is currently unknown.

Thus, the second objective of the present investigation was **to study the oxidative stress situation (concretely in the glutathione cycle) in several organs such as heart, liver and spleen, of adult, mature and old PAM and NPAM.**

Third objective:

The ageing process is accompanied with neurodegenerative disorders such as strokes, Parkinson's disease, and Alzheimer's disease (AD) that are associated with oxidative stress. Although in AD there is a systemic disorder, the studies on the peripheral immune system of subjects with AD

are scarce. In a model of triple transgenic mice for AD (3xTgAD) our research group has shown, in a preliminary study, an impairment of the neuroimmunoendocrine network similar to that which occurs with ageing in animals of 15 months of age, when AD pathophysiology is completely established. However, in 3xTgAD at 4 months of age, when the immunoreactivity against intracellular amyloid beta (Aβ) fibrils appears, and at 9 months when beta amyloid plaques exist but the tau alterations have not yet occured, the modifications of immune functions in thymus and spleen are unknown.

Menopause is a complex physiological process, characterized by a reduced secretion of the ovarian hormones oestrogen and progesterone. In rodents, menopause is mimicked by ovariectomy. We have proposed that ovariectomized rats and mice, with an oestrogen-deprivation, could be a model of premature ageing at immune, oxidative stress and behavior levels. Since in male rats and mice there is a premature immunosenescence and oxidative stress, compared to females, we have observed that ovariectomy causes immunosenescence and oxi-inflamm-ageing in peritoneal leucocytes of female mice similar to that present in males.

It is well known that physical exercise is an effective means of preventing or delaying chronic disease. Moreover, physical activity strongly modulates the regulatory systems, and concretely shows beneficial effects on the immune system. Although the exercise has shown benefits in the behaviour and of 3xTg-AD mice, the effects of exercise on the immune system has not been studied in these animals.

Thus, the third objective was **to advance the knowledge of the immune function and oxidative stress status in 3TgAD mice, both male and female, young (4 months) and adult-mature (9 months). To study, in these mature mice the effect of ovariectomy as well as that of moderate physical exercise on several immune functions and redox parameters in both sexes**.

3. MATERIALS AND METHODS

3.1. Material

3.1.1. Animals

In the present study two strains of mice have been used. Female ICR/CD1 mice (used in the first and second objectives), were purchased from (Harlan Interfauna Ibérica) (Barcelona, Spain) at the adult age (28±4 weeks). The animals were specifically pathogen free as tested by Harlan and according to the Federation of European Laboratory Science Associations recommendations. In our Animal Facility, placed at the Faculty of Biology of the Complutense University of Madrid (UCM), they were housed at 6±1 per cage and maintained at a constant temperature (22±2 ºC) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France), on a 12/12 h reversed light/dark cycle (lights on at 20:00 h). Mice had access to tap water and standard Sander Mus pellets (A04 diet from Panlab L.S., Barcelona, Spain) *ad libitum*. The diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals.

Male and female triple transgenic Alzheimer's disease (3xTg-AD) mice harbouring $PS1_{M146V,}$ APP_{Swe} and tau_{P301L} transgenes, as well as those belonging to the wild strain from which they produced (C57BL/129Sv) were used in the experiments of the third objective. Genotypes were confirmed by PCR analysis of DNA obtained from tail biopsies. Animals were maintained, in similar conditions to those previously mentioned, under approval from the local animal ethics committee (CEEA, UB). This animal model mimics both amyloid and tau AD neuropathologies in an age-dependent manner in disease-relevant brain regions and reveals the important role of the intraneuronal accumulation of oligomeric Aβ in the etiopathogenesis of the disease (Oddo et al., 2003[a]).

All procedures were in accordance with Spanish legislation on the "Protection of Animals Used for Experimental and Other Scientific Purposes", the guidelines and protocols of the Royal Decree 1201/2005

regarding the care and use of laboratory animals for experimental procedures and the European Communities Council Directive (86/609/EEC) on this subject.

3.1.2. Cell lines

To assess the natural killer activity of immune cells, the murine Yac-1 cell line from a murine Tcell lymphoma induced by the Moloney virus, was used as targets. This line was supplied by the Immunology Service of the Clinical Hospital of Madrid, and maintained until used at -80°C in sterile conditions in aliquots of $2x10^6$ cell/ml of complete medium (RPMI 1640 with 1% gentamicin, 0.1 mg / ml, and supplemented with 10% fetal calf serum) and 10% DMSO as cryo-protectant.

3.1.3. Saline solutions and culture media

Phosphate buffer saline (PBS) (pH 7.4), contains sodium chloride (NaCl) 123,2 mM (PANREAC); disodium hydrogen phosphate (Na2HPO4) 10,84 mM (PANREAC); potassium dihydrogen phosphate (KH2PO4) 3,23 mM (BDH CHEMICALS) and ultrapure water (1L). PBS was sterilised in an autoclave, for 1 hr at 1 atmosphere pressure and 120°C, and stored at 4°C.

Hank´s saline solution (pH 7.4), contains D (+)-glucose (C6H12O6) 5,55 mM (PANREAC); magnesium chloride (MgCl2) 1 mM (PANREAC); sodium chloride (NaCl) 136,89 mM (PANREAC); potassium chloride (KCl) 5,36 mM (PANREAC); calcium chloride (CaCl2) 1,26 mM (PANREAC); magnesium hydrogen phosphate (MgHPO4) 0,80 mM (PANREAC); monopotassium phosphate (KH2PO4) 0,44 mM (BDH CHEMICALS); disodium hydrogen phosphate (Na2HPO4) 0,42 mM (PANREAC); sodium bicarbonate (NaHCO3) 4,16 mM (PANREAC) and 1L ultrapure water. The Hank´s solution was sterilized by filtering through a membrane of 0.22 µm pore diameter (Millipore), and stored at 4°C.

Tampon phosphate 50 mM pH 7.4 (1L), contains disodium hydrogen phosphate (Na2HPO4) (PANREAC) 7.1g and monopotassium phosphate (KH2PO4) (BDH CHEMICALS) 6.81g.

RPMI 1640 medium. An RPMI 1640 medium with phenol red, HEPES (25 mM), L-glutamine (20 mM) (PAA), 1% gentamicin (0.1 mg / ml) (GIBCO BRL) and supplemented with 10% fetal calf serum (GIBCO BRL) (heated in water bath at 56°C for 30 min and sterilised by filtration) was used. Also an RPMI 1640 without phenol red and with L-glutamine (PAA) was utilized. These media were maintained in sterile conditions at 4ºC.

3.1.4. Reagents and commercial kits

- β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH) (SIGMA).

  5,5 '-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma).
- Bovine serum albumin (BSA) (SIGMA).
- Calcium chloride (CaCl2) (Panreac).
- Chemotactic peptide (formyl-Met-Leu-Phe, FMLP) from *E. coli* (Sigma), reconstituted in PBS (10^{-2} M), aliquoted and stored at -20 ° C until use.
- Chloroform (RIEDEL-DE HAËN).
- Colorimetric kit to assess cytotoxic activity (PROMEGA).
- Concanavalin A (ConA) (Sigma) at a concentration of 200 g/ml in sterile PBS. Cumene hydroperoxide (SIGMA).
- D (+)-Glucose (C6H12O6) (Panreac).
- Dihydrogen phosphate (KH2PO4) (BDH Chemicals).
- Dihydrogen phosphate (KH2PO4) for HPLC (Fluka).
- Dimethyl sulfoxide (DMSO) (SIGMA).
- Dioxane (Merck).
- Ethanol (MERCK).
- Disodium phosphate (Na2HPO4) (Panreac).
- Distilled and deionized water
- Ethylenediaminetetraacetic acid (EDTA) (SIGMA)

- Fetal bovine serum (GIBCO BRL).
- Gentamicin (GIBCO BRL).
- Glutathione (GSH) (SIGMA).
- Glutathione reductase (GR) (SIGMA).
- Histopaque density 1,077 g/ml (Sigma Diagnostics).
- Hydrochloric acid 37% (SIGMA).
- Latex particles of 1.091 ± 0.0082 μm in diameter (SIGMA), diluted to 1% in sterile PBS and stored at 4 ° C until use.
- Lipopolysaccharide (LPS) (SIGMA) of *E. coli* (055: B5), aliquots of 100 μg/ml in sterile PBS and stored at -20 ° C.
- Magnesium chloride (MgCl2) (Panreac).
- Magnesium phosphate (MgHPO4) (Panreac).
- Methanol (Merck).
- MILLIPLEX® $_{MAP}$ Mouse Cytokine/Chemokine kit (Immunoassay), detects and quantifies a combination of cytokines in cell cultures (Millipore).
- N-ethyl-maleimide (NEM) (SIGMA).
- Nitroblue tetrazolium (NBT) (Sigma), diluted with Hank's solution at the time of their use in a concentration of 1 mg/ml, protected from light and stored at 4°C.
- Nitrogen gas (AIR LIQUID).
- Potassium chloride (KCl) (Panreac).
- Potassium hydroxide (KOH) (Merck).
- RIA to assess levels of corticosterone (SIEMENS).
- Sodium azide (SIGMA).
- Sodium bicarbonate (NaHCO3) (PANREAC).
- Sodium chloride (NaCl) (Panreac).
- Trichloroacetic acid (TCA) (PANREAC y SIGMA).
- Tritiated thymidine (ICN) with a specific activity of 35 Ci / mmol, diluted to 0.1 mCi / ml in sterile PBS and stored at 4°C.

3.1.5. Laboratory equipment

- Automatic pipettes (Gilson and Boece).
- Boyden chambers, used in tests of chemotaxis, made of acrylic material. These chambers have two compartments (13 mm outer diameter, 9 mm inner diameter and 5 mm in height).
- Chronometers.
- Culture flasks (IWAKI GLASS).
- Dissecting materials: scissors, tweezers and scalpels.
- Filters (MILLIPORE) of 25 mm diameter and 0.22 μm pore diameter for sterilization of media.
- Glass materials: tubes, Pasteur pipettes, slides, coverslips, Petri dishes, test tubes, beakers, etc.
- Manual cell counter (REXEL).
- Needles.
- Nitrocellulose filters (MILLIPORE) (transparent, 13 mm in diameter and 3 μm pore diameter, for testing chemotaxis**.**
- Optical glass and quartz cuvettes of 1 cm pathlength (STARNA).
- Plastic materials: disposable syringes, tubes, eppendorfs, gloves, pipette tips, parafilm, etc.
- Plates M.I.F. (KARTELL) of 8 wells (1.5 cm diameter) for testing of phagocytosis.
- Plates of 96-well: flat bottom (COSTAR) and U-shaped bottom (NUNC).

* Sterilization of material takes place in an autoclave at 120 ° C and 1 atmosphere pressure for 1 hour.

3.1.6. Instruments

- Autoclave (SELECT).
- Precision balances (SAUTER y SARTORIUS).
- Sonicating bath (UPSTATE).
- Centrifuge 32R (HETTICH ZENTRIFUGEN).
- Centrifuge 5702R (EPPENDORF).
- Crushed ice dispenser (SCOTSMAN).

- Filtration pump (MILLIPORE).
- Fluorometer (TECAN).
- Freezers -20 º C (LIEBHERR) and -80 ° C (HERAEUS)
- Incubator thermostat, controlled CO_2 atmosphere (KOWELL and HERAEUS).
- Luminometer (Luminex 100TM, UPSTATE).
- Optical microscopes phase contrast (NIKON).
- pH meter with electrode (CRISON) and micropH meter (HANNA INSTRUMENTS).
- Plate filtration system (MILLIPORE).
- Plate shakers (BUNSEN).
- Refrigerated centrifuge (2.0 RS HERAEUS).
- Refrigerators (FAGOR y LIEBHERR).
- ***Shaking bath*** with thermostat (PRECISION SCIENTIFIC).
- Sonicator (BANDELIN SONOPULS).
- Spectrophotometer (GENESIS ESPECTRONICS 5).
- T-shaped maze (PANLAB).
- Ultrapure water distiller (MILLIPORE).
- Universal vacuum system UVS400A (THERMO ELECTRON CORPORATION).
- Vortex (PACIS).
- Water distiller (MILLIPORE).
- β counter (PERKIN-ELMER).

3.2. Experimental designs

The experimental design of each objective of the present work will be described in continuation.

3.2.1. Circadian rhythms

ICR/CD1 female adult (7±1 months of age) and old (18±1 months) mice, in groups of 10, were used. To identify each animal, they were labeled in yellow with picric acid. Peritoneal leucocyte samples were obtained and the tests were performed with fresh samples.

3.2.1.1.Collection of peritoneal leucocytes and function and redox parameters studied

Peritoneal suspensions were obtained from each animal at 8:00, 13:00 and 18:00 h. The use of peritoneal cell samples has the main advantage of not having to sacrifice the animals and being a non-invasive technique. Without using anesthesia, mice were held by cervical skin, the abdomen was cleansed with 70% ethanol, and 3 ml of sterile Hank´s, previously tempered at 37°C, adjusted to pH 7.4, was injected intraperitoneally. After abdominal massage, approximately 80% of the injected volume was recovered from the hole previously made by the needle employed for the injection of Hank´s. Then, the number of macrophages and lymphocytes from the peritoneal suspensions were identified by their morphology and quantified in Neubauer chambers using optical microscopy (40x), and then leucocytes were adjusted by dilution with Hank's solution to 5×10^5 macrophages/ml. The peritoneal samples to assess functions of immune cells were obtained during Winter, whereas those used to analyse oxidative stress parameters were obtained during Summer.

The functions studied in macrophages were: adherence, chemotaxis, phagocytosis and digestion capacity (intracellular superoxide anion levels). In lymphocytes the functions were: adherence, chemotaxis, natural killer activity (NK) and proliferation (basal and in response to the mitogens Concanavalin A (ConA) and lipopolysaccharide (LPS)). In addition, the release of cytokines (interleukin-2 (IL-2), interleukin-10 (IL10), interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α)) in the supernatant of cultures of peritoneal leucocytes after 48h of incubation in the presence of ConA (1μg/ ml) or LPS (1 μg/ml) were assayed.

The oxidative stress parameters studied in peritoneal leucocytes adjusted to 10^6 cells/ml, were: Total glutathione (TG) levels and the activities of the glutathione peroxidase (GPx) and glutathione reductase (GR), as antioxidant defense parameters. The extracellular superoxide anion level was analysed as an oxidant parameter.

3.2.1.2. Collection of peripheral blood and parameters studied

Blood was obtained in Summer from another group of mice with the same characteristics as those mentioned above, and under the same conditions, at 8:00, 13:00 and 18:00 h.

Blood collection was carried out from the peri-orbital sinus of each mouse by Dr. Fernández-Malavé (Department of Immunology. Faculty of Medicine of UCM), an expert in this technique, using a microhematocrit tube with anticoagulant (heparin). The mouse has a large peri-orbital venous sinus that fills the bony orbit of the eye. The microhematocrit tube was inserted through the conjunctiva and into the orbital sinus by quickly rotating it. The eye was not damaged because the tube passed under the eye. After filling the tube this was withdrawn and bleeding usually ceased by the orbit pressure alone. In some cases haemorrhage was controlled by direct pressure with a Kleenex over the eyelid.

Aliquots of blood samples were incubated 4h in the presence of LPS (25μg/ml). After centrifuging, the concentrations of interleukin-2 (IL-2), interleukin-10 (IL10), interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α), were determined in the supernatants.

Further aliquots were centrifuged and the plasma was used to measure the concentrations of corticosterone.

3.2.2. Animal Model of poor response to stress and anxiety (PAM and NPAM)

A prematurely aging mice (PAM) model, spontaneously developed from female ICR-CD1 mice, was used. These mice, which take longer to explore a T-shaped maze, showed in previous studies carried out by our group a premature immunosenescence, high anxiety levels and similar

neurochemical parameters to those of ageing mice, as well as a lower life span (Guayerbas et al., 2002a,c; Guayerbas and De la Fuente, 2003). At 6 months of age the spontaneous exploratory behavior of each mouse was tested in a T-shaped maze (Guayerbas and De la Fuente, 2003) to determine if it was PAM or NPAM. Six groups of female ICR/CD-1: adult (7 months of age), mature (13 months of age) and old (18 months of age) PAM and NPAM were used.

3.2.2.1. T-shaped maze test

The T-shape maze essentially consists of three arms made of wood, their internal face being covered with black methacrylate. The inside dimensions of each arm was 10 cm wide, 25 cm long, and 10 cm high. The floor was made of cylindrical aluminum rods 3mm thick placed perpendicularly to the walls.

The test was performed holding the mouse from the tip of the tail and placing it inside the ‘‘vertical’’ arm of the maze with its head facing the end of the wall. The performance is evaluated by determining with a chronometer the time elapsed until the animal crosses with both hindlegs the intersection of the three arms. This test was performed four times, one each week, in order to sort out the PAM (prematurely aging mice, which required, four times, over 10s to complete the exploration) from the NPAM (which completed that exploration in 10s or less). Animals showing an intermediate response were removed from the study. Adult PAM (7 months of age), mature PAM (13 months), old PAM (18 months), and their NPAM counterparts were used. This test was always performed between 9:00 and 11:00 h, to minimize circadian variations, under red light.

3.2.2.2. Collection of organs and parameters studied

Adult, mature and old PAM and NPAM (n=10 per age/type) were sacrificed by decapitation. Spleens, hearts and livers were removed aseptically and frozen at -80ºC until processed. Total glutathione levels, glutathione peroxidase and glutathione reductase activities were analysed in spleens, hearts and livers. In livers, glutathione reduced (GSH) and

glutathione oxidized (GSSG) levels were also evaluated, as well as the GSSG/GSH ratios.

3.2.3 Triple transgenic-AD (Tg) mice

The fresh spleen and thymus of triple-transgenic (Tg) male and female mice and those of their NTg counterparts, were received from the Institute of Neuroscience and Medical Psychology Unit, Department of Psychiatry and Forensic Medicine, Autonomous University of Barcelona, E-08193 Bellaterra, Barcelona, as well as from the Institute of Biomedical Research of Barcelona (IIBB), CSIC, IDIBAPS, E-08036 Barcelona, Spain. These organs corresponded to animals of the three different experiments: 1) Differences in several functions of leucocytes from spleen and thymus as well as in spleen antioxidant defenses, between animals (males and females NTg and Tg) of 4 and 9 months of age. 2) Effects of ovariectomy (carried out at 4 months of age) on several functions of spleen and thymus leucocytes as well as in spleen antioxidant defenses from NTg and Tg of 9 months of age. 3) Effects of physical exercise on several functions of spleen and thymus leucocytes as well as in spleen antioxidant defenses from male and female NTg and Tg mice, as well as female ovariectomiced (NTg and Tg) at 9 months of age.

Animals were killed by decapitation. Spleen and thymus were immediately removed in sterile conditions and maintained at 4ºC in Hank's balanced saline solution until the collection of leukocytes, which was performed 24h later (see below). In the case of spleen, half of each organ was frozen at -80ºC until the antioxidant defence analysis was carried out.

3.2.3.1. Isolation of leucocytes from spleen and thymus

Spleen and thymus were freed of fat, minced with scissors and gently pressed in PBS through a mesh screen (Sigma, St Louis, and USA). The cell suspensions of spleen were centrifuged in a gradient of Ficoll-Hypaque (Sigma) with a density of 1.070 g/ml, and cells from the interface were collected. Then, both spleen and thymus leucocytes were washed in phosphate puffer saline (PBS), and the number of leucocytes was

determined and adjusted to 1×10^6 cells/ml. Cellular viability, routinely measured before and after each experiment by the trypan-blue exclusion test, was higher than 95% in all experiments. All incubations were performed at 37°C in a humidified atmosphere of 5% CO2.

3.2.3.2. Effects of age on functions of spleen and thymus leucocytes and on spleen antioxidant defences

The spleen and thymus of young (4 months old) and adult (9 months old) triple-transgenic (Tg) male and female mice and those of their NTg counterparts, were used.

Parameters studied

The following functions were studied in leucocytes (collected as previously mentioned) from these organs: chemotaxis capacity, NK activity, lymphoproliferation in basal conditions as well as in response to mitogens (ConA and LPS). We also analysed the levels of some cytokines (interleukin-2 (IL-2), interleukin-10 (IL10), interleukin-1beta (IL-1β) and tumour necrosis factor alpha (TNF-α) in supernatants of cultures (48 h) of spleen leucocytes.

In the homogenized spleen, the following antioxidant defences were analysed: total glutathione (TG) levels as well as the activity of the glutathione peroxidase (GPx) and glutathione reductase (GR).

3.2.3.3. Effect of ovariectomy on functions of spleen and thymus leucocytes and on spleen antioxidant defences

Female Tg and NTg mice (n=8-9 per group) were submitted to ovariectomy or sham operation at 4 months of age. At 9 months of age, the animals were sacrificed by decapitation and spleens and thymus were collected as previously described. Spleens and thymus from male Tg and NTg of the same age were also used.

Surgical procedure: ovariectomy

For the surgical procedure animals were anaesthetised with a mixture of ketamine 80 mg/kg (Ketolar 50 mg/ml^R, Pfizer) and xylazine 5 mg/kg

(Rompun^{R}, Bayer), which was intraperitoneally injected. Ovaries were extirpated bilaterally after ligation of the oviductes. The dorsal part of the lumbar region was shaved and then cleaned with ethanol. One small incision (1 cm) was made through the skin and the muscle wall on each side of the backbone, in the dorsal aspect. The ovaries were then located and a braided silk sterile suture (Lorca Marín, Murcia, Spain) was performed around the craneal area of the uterine horns, which were sectioned thereafter, and the ovaries removed. The wound was closed in two layers, i.e.: muscle and skin using sterile sutures (Lorca Marín, Murcia, Spain). Non ovariectomized mice were submitted to a sham operation. Sham animals were also anaesthetised, the skin and muscle layers were opened and the uterus and ovaries manipulated but not excised. The abdominal cavity was closed in the same way as in the ovariectomized mice. After recovery from the surgery, the mice were housed individually for some hours to allow recovery and then re-grouped in their home cages in groups of 4-5 per cage.

Parameters studied

The following functions were studied in leucocytes (collected as previously mentioned) from these organs: chemotaxis capacity, NK activity, lymphoproliferation in basal conditions as well as in response to mitogens (ConA and LPS). We also analysed the levels of some cytokines (interleukin-2 (IL-2), interleukin-10 (IL10), interleukin-1beta (IL-1β) and tumour necrosis factor alpha (TNF-α) in supernatants of cultures (48 h) of spleen leucocytes.

In homogenized spleen, the following antioxidant defences were analysed: total glutathione (TG) levels as well as the activity of the glutathione peroxidase (GPx) and glutathione reductase (GR).

3.2.3.4. Effect of moderate physical exercise on functions of spleen and thymus leucocytes and on spleen antioxidant defences from males and females Tg and NTg as well as from ovariectomized females

Spleen and thymus of male and female Tg and NTg (9 months of age) submitted to moderate exercise as well as those of ovariectomized and non-

ovariectomized Tg and NTg female mice (9 moths of age), after carrying out physical exercise, were used.

Exercise protocol

At the age of 6 months, 8 animals of each group (males and females, Tg and NTg) were submitted to a chronic voluntary physical exercise (with one running wheel in the cage). Another four groups of 8 mice, were housed in standard cages without access to the wheel. After 3 months, when the animals were 9 months of age, these male and female NTg and Tg mice were sacrificed following the previously mentioned protocol and the spleens and thymus were collected.

Female Tg and NTg mice, submitted to ovariectomy or sham operation at 4 months of age, were submitted to a similar exercise protocol to that previously described. At 9 months of age these animals and the corresponding controls were sacrificed and the spleens and thymus were collected.

Parameters studied

The following functions were studied in leucocytes (collected as previously mentioned) from spleen and thymus: chemotaxis capacity, NK activity, lymphoproliferation in basal conditions as well as in response to mitogens (ConA and LPS). We also analysed the levels of some cytokines (interleukin-2 (IL-2), interleukin-10 (IL10), interleukin-1beta (IL-1β) and tumour necrosis factor alpha (TNF-α) in supernatants of cultures (48 h) of spleen leucocytes.

In homogenized spleen, the following antioxidant defences were analysed: total glutathione (TG) levels as well as the activity of the glutathione peroxidase (GPx) and glutathione reductase (GR).

3.3. Methods

3.3.1. Function and redox parameters studied in peritoneal suspensions (corresponding to the first objective)

The function and redox parameters studied in peritoneal suspensions of ICR/CD1 female adult (7±1 months of age) and old (18±1 months), collected as above mentioned, were the following.

3.3.1.1. Function parameters

Adherence capacity

The adherence capacity of peritoneal macrophages and lymphocytes was measured following the method described by De la Fuente et al. (1991). Briefly, aliquots containing 200 µl of peritoneal suspension (5×10^5 macrophages/ml Hank's medium) or (5×10^6 lymphocytes/ml Hank's medium) were placed in eppendorf tubes and incubated for 10 min at 37°C. After gently shaking, the number of macrophages and lymphocytes in the suspension was determined. The Adherence Index (AI) was calculated according to the following equation: AI = ([cells/ml initial sample (time 0)]-[cells/ml supernatant after 10 min of incubation]/ [cells/ml initial sample (time 0)]) × 100%.

Chemotaxis capacity

The chemotaxis of peritoneal macrophages and lymphocytes were assessed according to a previous described method (De la Fuente et al., 1991), using chambers with two compartments separated by a filter (3µm pore diameter, Millipore Iberica, Madrid, Spain). Aliquots of 300 µl macrophage (5×10^5 cells/ml Hank's medium) suspensions were deposited in the upper chamber compartment, and the lower compartment was filled with FMLP (formyl-met-leu-phe) at 10^{-8} M (Sigma, St. Louis, MO) as chemoattractant agent. Chambers were incubated for 3 h at 37 °C in a humidified atmosphere of 5% CO_2. Then, filters were fixed and stained, and the Chemotaxis Index (CI), which represents the number of macrophages or

lymphocytes counted in four scans of 5 mm on the lower face of the filter using an optical microscope (×100 magnification), was determined.

Phagocytic capacity

The phagocytic capacity of inert particles (latex beads, 1.09 μm diameter diluted to 1% in PBS; Sigma St Louis, MO) was assayed according to the method described by De la Fuente et al. (De la Fuente, 1985). Aliquots of 200 μl peritoneal macrophage suspension (5×10^5 cells/ml Hank's medium) were incubated in migratory inhibitory factor (MIF) plates for 30 min at 37 °C in a humidified atmosphere of 5% CO_2. The adhered monolayer obtained was washed with pre-warmed PBS, and then 200 μl Hank's solution and 20 μl latex bead suspension, were added. After 30 min of incubation under the same conditions, plates were washed, fixed, and stained, and the number of particles ingested by 100 macrophages, which presents the phagocytic index (PI), as well as the phagocytic efficacy (P.E) (the number of ingesting macrophages per 100 macrophages), were determined by counting in an optical microscope.

Digestion capacity. Intracellular superoxide anion levels

Superoxide anion levels were evaluated assessing the reduction of nitroblue tetrazolium (NBT) in peritoneal leucocytes following the method described by Hedley Currie (1978) and modified by De la Fuente et al. (1991, 2008). Briefly, aliquots of 250 μl peritoneal suspension (5×10^5 macrophages/ml Hank's medium) were mixed with 250 μl NBT (1 mg/ml in Hank's solution, Sigma), and then 50 μl of Hank's medium and 50 μl of the latex bead suspension (1%) were added to non-stimulated and stimulated samples, respectively. After 60 min of incubation at 37°C, the samples were centrifuged (the supernatant was used to determine the extracellular superoxide anion levels). The intracellular reduced NBT was extracted with dioxin (SIGMA) and after centrifuging, the absorbance of supernatant was measured as described previously. The levels of superoxide measured are the result of anion generation and neutralization by antioxidant defenses

present in the cells. The results were expressed as nanomols per 10^6 cells using a pattern curve made with NBT reducing agent.

Natural killer activity assay

Natural killer (NK) activity of the peritoneal leucocyte suspension was evaluated using an enzymatic colorimetric assay for cytolytic measurements of target cells (Cytotox 96 TM Promega, Madison, WI, USA; Cytotox 96 TM Promega, Boerinher Ingelheim, Germany) based on the determination of lactate dehydrogenase (LDH) activity using a tetrazolium salt (Ferrandez et al., 1999). Yac-1 cells (a murine tumoural line) were used as target cells and seeded in 96-well U-bottom culture plates (Nunclon, Denmark) at 10^4 cells/well in RPMI-1640 without phenol red. Effector cells (leukocytes from peritoneum) were added at 10^5 cells/well, obtaining an effector/target rate of 10/1 in the NK assay. Each sample was measured in triplicate. The plates were centrifuged at 250×g for 4 min to facilitate cell-to-cell contacts and then they were incubated for 4 h. After incubation, LDH activity was measured in 50 µl/well of the supernatants by addition of the enzyme substrate and absorbance recording at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. The results were expressed as percentages of lysis of target cells, which were determined with the following equation: % lysis = ((E-ES-TS) / (M-ES-TS)) × 100, where E is the mean of absorbances in the presence of effector cells; ES the mean of absorbances of effector cells incubated alone; TS the mean of absorbances in target cells incubated with medium alone; and M is the mean of maximum absorbances after incubating target cells with lysis solution.

Lymphoproliferation assay

The proliferation of peritoneal lymphocytes in response to the mitogens concanavalin A (ConA) and lipopolysaccharide (LPS) was assayed following a method previously described (Guayerbas et al., 2002). Aliquots of 200 µl of peritoneal cell suspensions (1×10^6 cells/ml complete medium containing RPMI-1640 [Gibco] supplemented with 1% gentamicin

[1 mg/ml, Gibco] and 10% fetal calf serum [PAA]) were dispensed in 96-well flat-bottom culture plates (Nunclon, Denmark). 20 µl of LPS (Sigma, 1 µg/ml) or ConA (1µg/ml) were added to the stimulated wells, and 20 µl of complete medium to the control wells. Each sample was assayed in triplicate. The cultures were incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO2. After 48 h incubation 100 µl of supernatant were collected for cytokine measurements, and 0.5 µCi [^{3}H] thymidine (ICN, Costa Mesa, USA) was added to each well. After 24 h cells were harvested in a semiautomatic microharvester, and thymidine uptake was measured in a beta counter (LKB, Uppsala, Sweden). The results are expressed in counts per minute (cpm).

Cytokine levels in cultures of peritoneal leukocytes

The concentrations of interleukin-2 (IL-2), interleukin-10 (IL10), interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) were determined in the supernatant of cultures of peritoneal leukocytes after 48h of incubation in the presence of ConA (1µg/ ml) or LPS (1 µg/ml). An immunoassay method specific for mice (Millipore's MILLIPLEX® $_{MAP}$ Mouse Cytokine/Chemokine kit), which can be used to detect and quantify a combination of cytokines in cell cultures, was curried out. Each assay was run in duplicate. The minimum detectable concentration of IL-2, IL10, IL-1β and TNF-α was 3.2 pg/ml. The results were expressed as pg/ml.

3.3.1.2. Redox parameters in peritoneal leucocytes

Extracellular superoxide anion

The method used was the above mentioned for the intracellular superoxide anion. For extracellular superoxide anion measurement, the supernatants of the samples centrifuged after 60 minutes of incubation (aliquots of peritoneal suspension mixed with NBT and Hank's medium or latex bead suspension in non-stimulated and stimulated samples, respectively) were used. The results were expressed as nanomols per 10^6 cells using a pattern curve made with NBT reducing agent.

Total glutathione (TG) levels

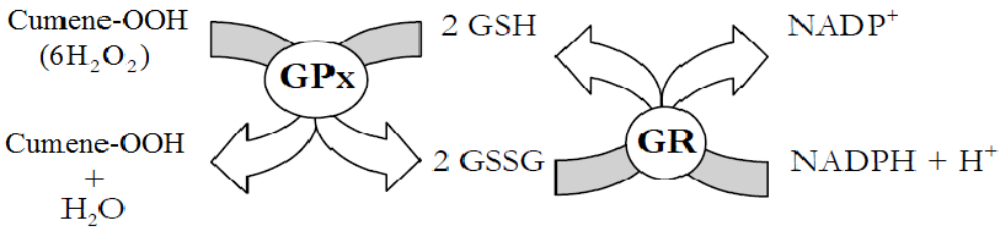
Total glutathione (TG) was spectrophotometrically evaluated according to the method of Tietze (Tietze, 1969) with some modifications (De la Fuente et al., 2004). Briefly, 1 ml of peritoneal leukocyte suspension (10^6 cells/ml Hank's medium) was centrifuged at 1200g for 10 min at 4°C and the pellet was resuspended in 100 µl of medium containing 5% trichloroacetic acid (TCA, Panreac, Spain) in 0.01 N HCl (Panreac). Then, samples were sonicated for 10 s, three times with a 20s break between each cycle, and 400 µl of medium TCA-HCl were added, and centrifuged at 3200g for 5 min at 4°C. Aliquots of supernatant were measured in the spectrophotometer using the following reaction mixture: 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 6 mM, Sigma), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, 0.3 mM, Sigma) and glutathione reductase (10U/ml, Sigma). The reaction was monitored for 240 s. The results were expressed as nmol TG/10^6 cells.

nmol TG/10^6 cells = [(ΔDO/min)/0.0003]×(10^6/X)

- ΔDO/min: ΔDO/min reaction with sample-ΔDO/min reaction without sample.
- (ΔDO/min)/0.0003: pmol TGSH in the cuvet.
- X = number of cells in the assessed sample (70 µl) = (1.4 x 10^5 cells)

Glutathione peroxidase (GPx) activity

The GPx is an enzyme which plays a fundamental role in the glutathione system allowing its antioxidant function to protect cells from potential damage by free radicals. GPx catalyzes the reduction of hydrogen peroxide (H_2O_2) and hydroperoxide (R-O-O-H) to water and the corresponding stable alcohol, thereby effectively removing toxic peroxides from living cells and inhibiting the formation of free radicals formed by peroxide decomposition.



The GPx enzyme was measured according to the original technique described by Lawrence and Burk (1976), with some modifications (Alvarado et al., 2006). The peritoneal cell suspension was adjusted to 10^6 cells/ml and aliquots of 500 µl were used to carry out the enzymatic assay. Cells were centrifuged at 1200g for 10 min at 4°C, and the pellets were resuspended in 50 mM phosphate buffer. Afterwards, the samples were sonicated (3 cycles of 10 s sonication and a 20 s break between each) and centrifuged at 3200g for 20 min at 4°C. Total activity was determined using cumene hydroperoxide (cumene-OOH, Sigma), which carried out the oxidation of glutathione. This is regenerated by the addition of β-nicotinamide adenine di-nucleotide phosphate, in its reduced form (β-NADPH, Sigma), in the presence of GR (Sigma).Aliquots of the supernatants were measured in the spectrophotometer using the following reaction solutions that were prepared in dark bottles and pumped to remove O_2 at the moment of measurement of the enzyme activity.

1) Reactive solution: EDTA 1 mM, Sodium Azide 4 mM, NADPH 0,2 mM, GSH 4 mM, GR 1 U/ml in tampon phosphate (50 mM).
2) Cumene Hidroperoxide (Cumene-OOH) solution (0,71 mM) in tampon phosphate (50 mM).

It is necessary to consider that the reaction between cumene hydroperoxide and GSH can occur even in the absence of enzyme GPx. Therefore, to avoid a possible overestimation of the enzyme activity, this was carried out in the absence of a reaction sample (called non-catalyzed reaction), which allowed the quantification of the spontaneous reaction.

The measurements were done in optic glass microcuvettes containing the following reactions:

1) Catalyzed reaction: 650 µl reactive solution, 25 µl sample and 25 µl cumene hydroperoxide solution.

- The blank of catalyzed reaction contains (675 µl of reactive solution and 25 µl sample).

2) Non-catalyzed reaction: 650 µl reactive solution, 25 µl tampon phosphate and 25 µl of cumene hydroperoxide solution.

- The blank of none catalyzed reaction contains (675 µl reactive solution and 25 µl tampon phosphate).

Before adding cumene hydroperoxide, both reactions were incubated for 5 min at 37°C. The reaction was initiated upon the addition of cumene hydroperoxide in the cells. The decrease in absorbance at 340 nm from that moment every 40 s for a total of 5 min was monitored. The results were expressed as miliunits (mU) of enzymatic activity per 10^6 cells.

The activity of GPx was calculated as following

$$\text{mU.I. GPx}/10^6 \text{ cells} = [(\Delta DO/min) \times F / Є] \times (10^6/X)$$

- DO/min: ΔDO/min catalyzed reaction - ΔDO/min non catalyzed reaction
- F: dilution factor of the sample in the cuvette (700/25=28).
- Є: molar absorption coefficient of the NADPH at 340 nm ($6{,}22 \times 10^{-3}$ nM^{-1} cm^{-1}).
- X = number of cells in the assessed sample (25 µl) = (2.5×10^5 cells).

Glutathione reductase (GR)

The glutathione reductase (GR) enzyme plays an important role in the maintenance of reduced glutathione (GSH) levels, being coupled to the activity of GPx.

The GR activity was assessed according to the technique described by Massey and Williams (1965), with some modifications. The peritoneal suspension was adjusted to 10^6 cells/ml and aliquots of 500 µl were used to carry out the enzymatic assay. Cells were centrifuged at 1200g for 10 min at 4°C, the pellets were resuspended in 50mM phosphate buffer and 6.3mM EDTA (Sigma). Afterwards, the samples were sonicated (3 cycles) and

centrifuged at 3200g for 20 min at 4 °C. The total activity was determined spectrophotometrically following the oxidation of NADPH at 340 nm.

$$GSSG + NADPH + H^{+} \xrightarrow{GR} 2\,GSH + NADP^{+}$$

The measurements were done in optic glass microcuvettes containing the following:

1. Catalyzed reaction: 580 µl tampon phosphate-EDTA solution 6.3 mM, 50 µl sample, 35 µl GSSG 80 mM and 35 µl NADPH.
   - The blank of catalyzed reaction contained: 650 µl tampon phosphate-EDTA solution 6.3 mM and 50 µl sample.
2. Non-catalyzed reaction: 620 µl tampon phosphate-EDTA solution 6.3 mM, 35 µl GSSG and 35 µl NADPH.
   - The blank of non-catalyzed reaction contained: 700 µl tampon phosphate-EDTA solution 6.3 mM.

The results were expressed as miliunits (mU) of enzymatic activity per 10^6 cells.

mU.I. GR/10^6 cells = [(ΔDO/min)×F /Є]× (10^6/X)

- DO/min: ΔDO/min catalyzed reaction - ΔDO/min non catalyzed reaction
- F: dilution factor of the sample in the cuvet (700/50 =14).
- Є: molar absorption coefficient of the NADPH at 340 nm (6,22x10^{-3} nM^{-1} cm^{-1}).
- X = number of cells in the assessed sample (50 µl) = (0.5 x 10^6 cells)

3.3.2. Cytokine and hormone levels in peripheral blood

Samples of blood from the peri-orbital sinus collected as was above mentioned, were used.

3.3.2.1. Cytokine levels in cultures of peripheral blood

Aliquots of 250 µl blood were incubated with 250 µl RPMI 1640 with phenol red, HEPES (25 mM) and without L-glutamine (PAA) supplemented with 10% fetal calf serum (GIBCO BRL) and 5 µl gentamicin (GIBCO BRL) for 4 h in the presence of 5 µl LPS (25 µg/ml). After centrifuging, the concentrations of interleukin-2 (IL-2), interleukin-10

(IL10), interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α), were determined in the supernatants. The same immunoassay method specific for mice (Millipore's MILLIPLEX® MAP Mouse Cytokine/Chemokine kit) as mentioned above, was used. The results were expressed as pg/ml.

3.3.2.2. Plasma corticosterone levels

Concentrations of corticosterone were measured in the blood plasma mentioned previously, using a commercial Radioimmunoassay (RIA) method (Siemens). Briefly, the assay consisted of a RIA solid phase, in which 125I-labeled murine corticosterone competes for a fixed time (2 h incubation at room temperature) with the corticosterone in the sample for antibody sites, immobilized on the walls of polypropylene tubes. A gamma counter was used to measure the levels of corticosterone, expressed in pg/ml after using a calibration curve made with known concentrations of this hormone.

3.3.3. Function and redox parameters studied in organs

3.3.3.1. Function parameters in leucocytes from spleen and thymus

The following function parameters in leucocytes collected from spleen and thymus were studied.

Chemotaxis capacity

Chemotaxis of spleen and thymus lymphocyte suspensions was evaluated according to the previously mentioned technique described by De la Fuente et al. (1991) with slight modifications (De la Fuente et al., 1993). The leucocyte suspension (0.3 ml, $1x10^6$ cells/ml) was deposited in the upper compartment of the Boyden chambers and then, a similar procedure to that mentioned in the 3.3.1. section was carried out.

Natural killer activity assay

Natural killer (NK) activity of spleen and thymus lymphocyte suspensions was evaluated using the previously mentioned enzymatic colorimetric assay (Ferrandez et al., 1999).

Lymphoproliferation assay

The proliferation of lymphocytes from spleen and thymus in response to both mitogens, Concanavalin A (ConA) and lipopolysaccharide (LPS), was assayed following the method described by De la Fuente et al. (1993). The leucocyte suspensions were adjusted to $1x10^6$ cells/ml and then the method described previously to peritoneal cells was carried out (3.3.1. section).

Cytokine levels in cultures of spleen leukocytes

The concentrations of interleukin-2 (IL-2), interleukin-10 (IL10), interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) were determined using the supernatants of cultures of spleen and thymus leukocytes after 48h of incubation in the presence of ConA (1µg/ ml) or LPS (1 µg/ml). The same immunoassay method specific for mice (Millipore's MILLIPLEX® $_{MAP}$ Mouse Cytokine/Chemokine kit) previously mentioned was used. The assay was run in duplicate.

3.3.3.2. Redox parameters in organs

Total glutathione (TG) levels

Total glutathione were spectrophotometrically evaluated in the liver, spleen and heart according to the method published by Tietze (1969), previously described, with some modifications in peritoneal suspension (De la Fuente et al., 2004) and in organs (Baeza et al. 2010). Samples of liver, spleen or heart were homogenized with 5% trichloroacetic acid (TCA, Panreac) and 0.01 N HCl (Panreac), in a cold room (4°C), the samples being maintained in ice throughout the process. Homogenates used to measure total glutathione were resuspended in a medium containing 5% trichloroacetic acid (TCA, Panreac) and 0.01 N HCl (Panreac). Thereafter,

all samples were centrifuged at 3200g for 5 min at 4°C. Aliquots of the supernatants were measured in the spectrophotometer using the following reaction mixture: 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 6 mM, Sigma), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, 0.3 mM, Sigma), and glutathione reductase (10U/ml, Sigma). The reaction was monitored for 240 s, and the measurements were done in optic glass microcuvets containing the following reactions:

1) Blanc:

500 µl NADPH, 70 µl DTNB, 70 µl sample and 70 µl of TCA solution.

2) Reaction with sample to measure total GSH:

500 µl NADPH, 70 µl DTNB, 70 µl sample and 70 µl GR.

3) Reaction without sample:

500 µl NADPH, 70 µl DTNB, 70 µl of TCA solution and 70 µl GR.

Standard curves were prepared using a glutathione solution (Sigma), 16 mg de GSH prepared in 250 ml of 5% TCA 0,01 N HCl, and measured under the same conditions of samples.

Results were expressed as nmol/mg tissue:

nmol GSH/mg tissue=[(ΔDO/min)+0.0021/0,3951]×(1/X)

-ΔDO/min: ΔDO/min reaction with sample-ΔDO/min reaction without sample.

-X= Volume of the sample in the cuvet (0.07 ml) ×sample concentration (no. of mg tissue/1 ml).

Oxidized glutathione (GSSG) levels

The oxidized form of glutathione (GSSG) was evaluated according the same method used for TG in the liver of all mice groups, by monitoring the change in absorbance at 412 nm. Samples were homogenized with NEM solution (medium containing phosphate buffer 50 mM (pH 7.4), EDTA 1 mM (Sigma) and NEM (N-etilmaleimide, Sigma) 12.5 mM) in a cold room (4°C), and samples were maintained in ice, samples then were left for 15 min at the ambient temperature, and then 25 µl of KOH (10%) were added and left 5 min more at the ambient temperature. Afterword the samples were transferred to the ice and 560 µl of TCA-HCl were added to each sample,

and then all samples were centrifuged at 3200g for 5 min at 4°C. (Another tube containing 1 ml NEM solution were processed as the same as the samples for currying out a control test). Then aliquots of the supernatants were measured in the spectrophotometer using the following reaction mixture: 5,50-dithiobis(2-nitrobenzoic acid) (DTNB, 6 mM, Sigma), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, 0.3 mM, Sigma), and glutathione reductase (10U/ml, Sigma). The reaction was monitored for 140 s.

The measurements were done in optic glass microcuvets containing the following reactions:

1) Blanc:

500 µl sample, 70 µl NADPH, 70 µl DTNB, 70 µl NEM solution.

2) Reaction with sample:

500 µl sample, 70 µl NADPH, 70 µl DTNB, 70 µl GR.

3) Reaction without sample:

500 µl NEM solution (that processed as the samples), 70 µl NADPH, 70 µl DTNB,70 µl GR.

Results were expressed as nmol/mg:

nmol GSSG/mg tissue=[(ΔDO/min)+0.0021/0,3951]×(1/X)

- ΔDO/min: ΔDO/min reaction with sample-ΔDO/min reaction without sample.
- X= Volume of the sample in the cuvet (0.5 ml) × sample concentration (no. of mg tissue/1 ml)

Reduced Glutathione (GSH) levels

The GSH (reduced form) was obtained by subtracting the GSSG values from the total glutathione values, and then the GSSG/GSH ratio was calculated.

Glutathione peroxidase (GPx) activity

GPx activity was spectrophotometrically evaluated in the liver, spleen or heart according to the Lawrence and Burk technique previously described (Lawrence and Burck., 1976) with some modifications (Alvarado et al., 2006), by monitoring the decrease of the absorbance at 340 nm, every 40 S

during 5 min, Using cumene hydroperoxide (cumene-OOH, Sigma) as substrate, that carried out the oxidation of glutathione, which is regenerated by the addition of β-nicotinamide adenine di-nucleotide phosphate, in its reduced form (β-NADPH, Sigma), in the presence of GR (Sigma).

The reaction was followed spectrophotometrically by the decrease of the absorbance at 340 nm.

Samples of liver, spleen or heart were homogenized with tampon phosphate (50 mM), in a cold room (4°C), and samples were maintained in ice during all the process. Thereafter, all samples were centrifuged at 3200g for 20 min at 4°C. Aliquots of the supernatants were measured in the spectrophotometer using the same previous reactions and reaction solutions.

The activity of GPx was calculated as the following and results were expressed as mU of enzymatic activity per mg of tissue (mU.I. GPx/mg tissue).

mU.I. GPx/mg tissue=[(ΔDO/min)×F /Є]×(1/X)

- DO/min: ΔDO/min catalyzed reaction -ΔDO/min non catalyzed reaction
- Є: molar absorption coefficient of the NADPH at 340 nm ($6{,}22x10^{-3}$ nM^{-1} cm^{-1}).
- F: dillution factor of the sample in the cuvet (700/25=28).
- X= Volume of the sample in the cuvet (0.025 ml) ×sample concentration (no. of mg tissue/1 ml)

Glutathione reductase (GR) activity

GR activity was spectrophotometrically assessed in liver, spleen and heart according to the previously mentioned technique described by by Massey and Williams (Massey and Williams, 1965). Tissue samples of liver, spleen or heart were homogenized with tampon phosphate (50 mM) with EDTA (6.3 mM) that is always pumped to remove O_2, in a cold room (4°C). Thereafter, all samples were centrifuged at 3200g for 20 min at 4°C. Aliquots of the supernatants were measured in the spectrophotometer using the NADPH (6 mM) and GSSG (80 mM) reagents (they were prepared in dark bottles).

The measurements were done in optic glass microcuvets containing the same previous reactions. The activity of GR was calculated as the following and results were expressed as miliunits (mU) of enzymatic activity per mg of tissue (mU GR/mg tissue).mU.I. GR/mg tissue=[(ΔDO/min)×F /Є]×(1/X)

- DO/min: ΔDO/min catalyzed reaction -ΔDO/min non catalyzed reaction
- Є: molar absorption coefficient of the NADPH at 340 nm ($6,22x10^{-3}$ nM^{-1} cm^{-1}).
- F: dillution factor of the sample in the cuvet (700/50=14).
- X:Volume of the sample in the cuvet (0.05 ml) ×sample concentration (no. of mg tissue/1 ml).

4. RESULTS

The results will be shown following the order of the objectives proposed. Thus, there will be 3 sections corresponding to the circadian changes in adult and chronological old mice, the oxidative stress situation in PAM and NPAM (model of premature aging), and the results corresponding to the triple transgenic mice for Alzheimer´s disease.

4.1 Circadian changes

In this section of the results, the changes in several parameters throughout the day in adult and old mice are shown. The samples analyzed were obtained from each animal at 8:00 h, 13:00h and 18:00 h, and peritoneal cell suspensions, peripheral blood and plasma were used.

4.1.1. Circadian changes in peritoneal cell suspension parameters

In peritoneal cell suspensions from adult and old mice obtained at 8:00, 13:00 and 18:00 h were studied several functions of macrophages and lymphocytes, secretion of cytokines by peritoneal leucocytes in culture, as well as oxidative stress parameters (antioxidant defences and extracellular superoxide anion).

4.1.1.1. Circadian changes in peritoneal cell functions

The results on several function parameters of peritoneal leucocytes, both macrophages and lymphocytes, are shown in this subsection.

Circadian changes in macrophage functions

The results obtained in the different function studied in macrophages are shown in Figures 11-14 and in Table 1.

Adherence capacity of peritoneal macrophages

The circadian variations in adherence capacity (Adherence Index: A.I.) of peritoneal macrophages are shown in Fig.11. The results showed that in both short (10 min) (Fig. 11A) and long (30 min) time of incubation (Fig. 11B) a progressive and significant increase of macrophage adherence capacity in adult mice at 18:00 h with respect to the values at 8:00 h, ($p<0.01$, $p<0.05$ respectively) was observed. No statistically significant differences were found in adherence capacity of macrophages from old mice along the day with10 min of incubation. Nevertheless, a significant increase ($p<0.05$) it was found at 13:00 h and at 18:00 h in the case of 30 min of incubation. Comparing both ages, the macrophage presented a higher adherence index in old than in young animals with statistically significant differences at 8:00 h ($p<0.01$) and at 13:00 h ($p<0.05$) when cells were incubated for 10 min (Fig.11A), and at 8h and at 18h ($p <0.05$) when cells were incubated for 30 min (Fig.11B).

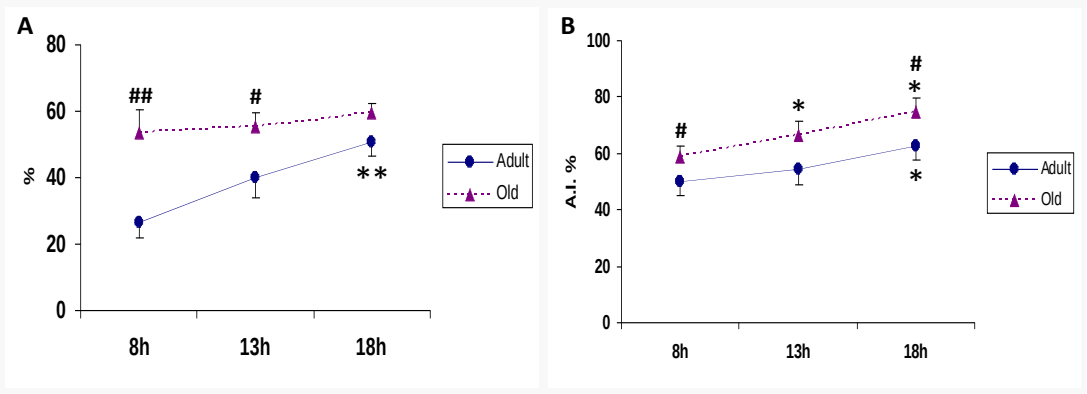


Fig.11. Adherence Index (percentage of macrophage adherence to eppendorf tubes) of peritoneal macrophages, with a short incubation time (10 min) (A) and with a long incubation time (30 min) (B), from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$with respect to the values at 8h. #$p < 0.05$; ##$p < 0.01$ with respect to the corresponding values in adult animals.

Chemotaxis capacity of peritoneal macrophages

The circadian variations in chemotaxis capacity (chemotaxis index: C.I.) of peritoneal macrophages in adult as well as in old mice are shown in (Fig.12). The results showed a progressive and significant decrease in chemotactic capacity of peritoneal macrophages along the day. In both macrophages from adult and old mice C.I. decreased at 13:00 h and 18:00 h with respect to the values at 8:00 h. Macrophages from old animals showed lower chemotaxis capacity than those from adults at both 8:00 h and 13:00 h with statistically significant differences ($p<0.05$ and $p<0.01$, respectively). No statistically significant differences in chemotactic capacity were found at 18:00 h between adult and old animals.

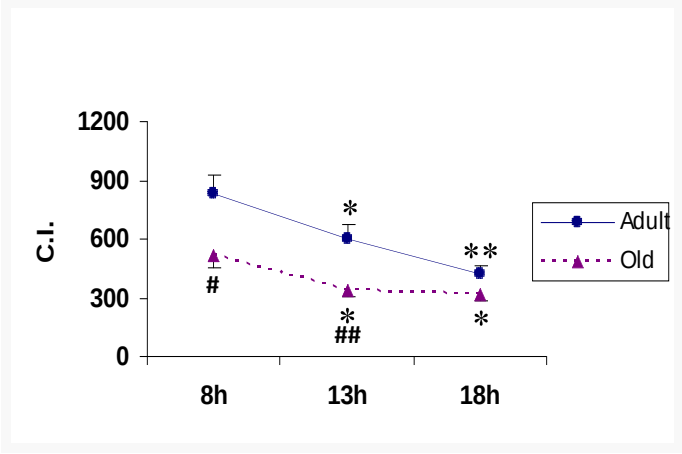


Fig. 12. Chemotaxis Index (C.I.) (number of cells on the filter) of peritoneal macrophages, from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals, and each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$with respect to the values at 8h. #$p < 0.05$; ##$p < 0.01$ with respect to the corresponding values in adult animals.

Phagocytic capacity of peritoneal macrophages

The phagocytosis index (P.I) and Phagocytes efficiency (P.E) of peritoneal macrophages from adult and old mice are shown in Fig.13. Figures 13A and 13B show these indexes obtained in macrophages incubated in the presence of only B lymphocytes (adherent cells). A significant decrease was observed in PI (Fig. 13A) of adult peritoneal macrophages at 18h with respect to the values at 8:00 h and 13:00 h ($p<0.01$

and p<0.05, respectively). Macrophages from old animals showed lower phagocytic capacity at both 13:00 h and 18:00h than at 8:00h (p<0.05). These phagocytosis indexes were higher in adult mice than in old at 8:00 h and 13:00 h (p<0.05) but not at 18:00 h.

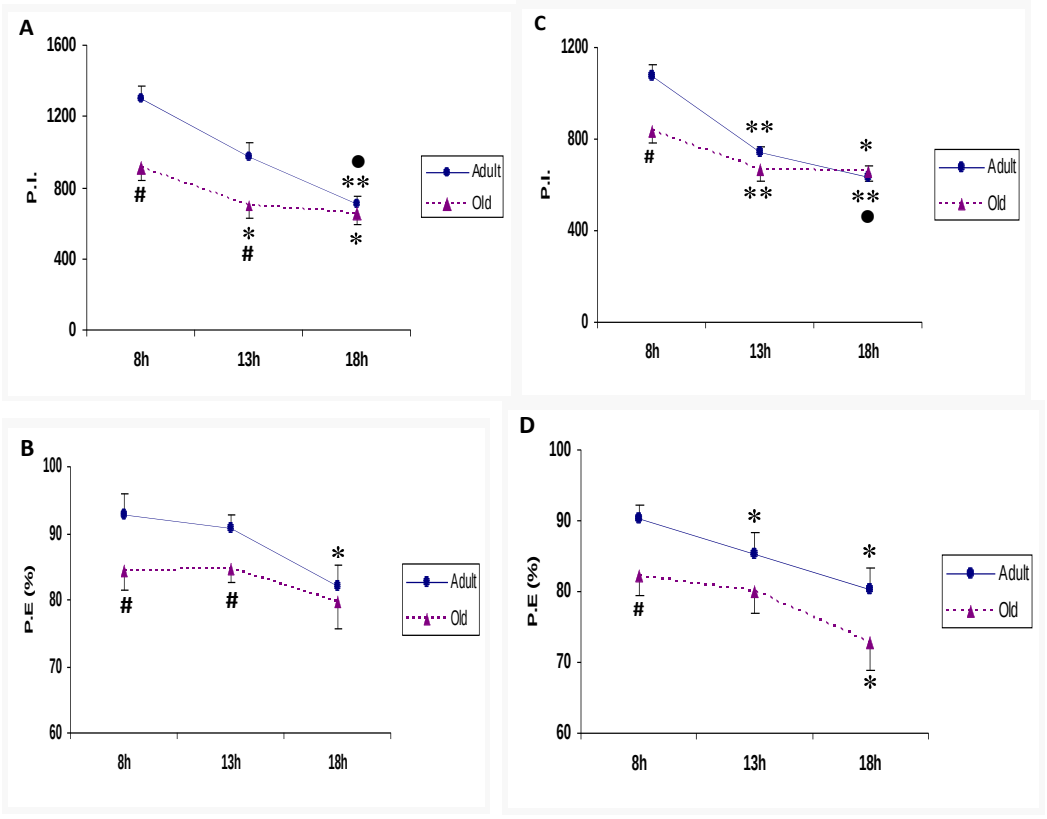


Fig 13. Phagocytosis index (P.I) of peritoneal macrophages (number of latex particles ingested by 100 peritoneal macrophages) when peritoneal macrophages were present with B lymphocytes (A), and when peritoneal macrophages were present with the total leucocyte population (C). Phagocytic efficiency (P.E) of peritoneal macrophages (percentage of macrophages ingesting at least one latex particles) when peritoneal macrophages were present with B lymphocytes (B) and when they were present with total leucocyte population (D). Cells were from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals and being the mean of duplicate assays.*p < 0.05; **p < 0.01with respect to the values at 8h. • p< 0.05 with respect to the values at 13h. #p< 0.05 with respect to the values of adult animals.

In the phagocytosis efficiency (P.E) of those macrophages (Fig.13B), similarly to the PI, significant decreases were observed for those of adult at 18h with respect to the values at 8h (p<0.01), whereas macrophages from old animals did not show circadian variation. These indexes in adult mice are higher than those in old animals at 8:00 h and 13:00 h (p<0.05).

Phagocytosis indexes of macrophage from total population of peritoneal leucocytes (macrophages, B lymphocytes and T lymphocytes) represented in (Fig.13C) showed in adult animals a decrease in the course of the day ($p<0.01$ at 13:00 h and 18:00 h with respect to the values at 8h, and $p<0.05$ at 18:00 h with respect to the values at 13h). Phagocytosis index from old animals decreased at 13:00 h and almost maintain the same trend at 18h, ($p<0.01$ and $p<0.05$ at 13h and 18:00 h respectively with respect to the values at 8h). A significant difference between adult and old was observed only at 8:00 h ($p<0.05$), with higher values in adult than in old, and later no significant differences were observed in the course of the day.

Phagocytosis efficiency (P.E) of macrophage from total peritoneal leucocytes (Fig.13D) showed in adults, a significant decrease at 13:00 h and 18:00 h with respect to the values at 8:00 h ($p<0.01$). Macrophages from old animals showed a significant decrease in phagocytosis efficiency at 18h. A significant difference between adult and old was observed only at 8:00 h, with higher ($p<0.05$) values in adult than in old.

Digestive capacity of peritoneal leucocytes: Intracellular superoxide anion levels

To determine whether the respiratory burst response of ICR-CD1 mice peritoneal macrophages capacity varied throughout the day, intracellular superoxide anion ($O_2^{-}\cdot$) levels were measured in peritoneal cell suspensions under basal and latex beads stimulated conditions (a marker of the digestion capacity of the ingested material by these cells).

The results (Fig. 14), showed a significant increase in the intracellular superoxide anion levels measured in both nonstimulated control (Fig. 14A) and stimulated cells (Fig. 14B) along the day in peritoneal cells from adult and old animals ($p<0.05$ at 13:00 h and $p<0.001$ at 18:00 h with respect to the values of adult at 8:00 h, and $p<0.001$ at 13:00 h and $p<0.01$ with respect to the values of the old at 8:00 h in the case of nonstimulated conditions) and ($p<0.01$ at 13:00 h and at 18:00 h with respect to the values

of adult at 8:00 h, and p <0.05 at 13:00 h and p<0.01 with respect to the values of the old at 8:00 h in the case of stimulated conditions).

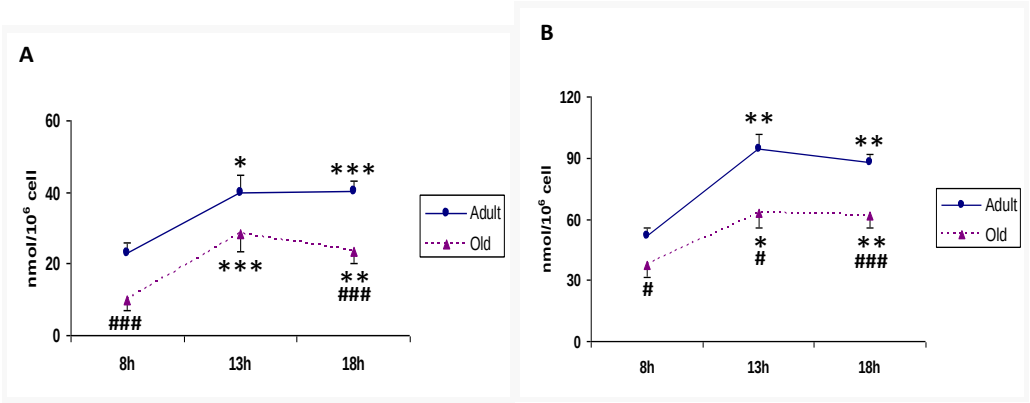


Fig. 14. Intracellular superoxide anion levels (nmols/10^6 leukocytes) of peritoneal cells nonstimulated (A) and Stimulated (B) from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals and being the mean of duplicate assays.*p < 0.05; **p < 0.01; ***p< 0.001 with respect to the values at 8h. #p < 0.05; ###p< 0.001 with respect to the values of adult animals.

Adult mice showed higher levels of intracellular superoxide anion (O_2^{-} ·) production measured in both nonstimulated control (Fig. 14A) and stimulated cells (Fig. 14B) in the course of the day in comparison to those in old animals (p<0.001 at 8:00 h and 18:00 h, for nonstimulated and at 18:00 h in stimulated, as well as p<0.05 at 8h and 13:00 h for intracellular stimulated). No statistically significant differences were observed at 13:00 h between adult and old animals in the case of nonstimulated control conditions.

Table 1. Circadian variations in several function parameters in peritoneal macrophages from adult and old mice.

	Adult			**Old**		
	8h	**13h**	**18h**	**8h**	**13h**	**18h**
Adherence Index (A.I) of macrophages at 10 min of incubation						
	27±6	40±15	51±8**	53±17$^{\#\#}$	55±8$^{\#}$	59±9

Adherence Index of macrophages at 30 min of incubation						
	50±7	55±13	63±11*	59±5$^{\#}$	67±5*	75±7*$^{\#}$
Chemotaxis Index (C.I) of macrophages						
	835±213	603±178*	423±107**	516±141$^{\#}$	338±78*$^{\#\#}$	312±80*
Phagocytosis index (P.I) of adherent population						
	1302±102	973±201	708±59**$^{\bullet}$	911±104$^{\#}$	696±113*$^{\#}$	652±118*
Phagocytosis index (P.I) of total population						
	1078±111	739±77**	637±86**$^{\bullet}$	835±102$^{\#}$	665±72**	661±82*
Phagocytosis efficiency (P.E) (%) of adherent population						
	93±3	91±3	82±7*	84±6$^{\#}$	85±2$^{\#}$	80±5
Phagocytosis efficiency (P.E) (%) of total population						
	90±4	85±6*	80±7*	82±6$^{\#}$	80±4	73±5*
Intracellular superoxide anion(O_2^{-}•) levels non-stimulated (nmols/10^6 leucocytes)						
	23±2	40±13*	40±3***	10±3$^{\#\#\#}$	29±8***	23±5**$^{\#\#\#}$
Intracellular superoxide anion(O_2^{-}•) levels stimulated (nmols/10^6 leucocytes)						
	52±11	95±22**	88±7**	38±7$^{\#}$	63±15*$^{\#}$	62±7**$^{\#\#\#}$

Each value is the mean ± SD of six values corresponding to six animals, each value being the mean of duplicate assays.*p < 0.05; **p < 0.01; ***p< 0.001 with respect to the values at 8h. • p< 0.05; •• p< 0.01; ••• p< 0.001 with respect to the values at 13h. #p < 0.05; ##p < 0.01; ###p< 0.001 with respect to the values of adult animals.

Circadian changes in lymphocyte functions

The results obtained in the different function studied in lymphocytes are shown in Figures 15-19 and in Table 2.

Adherence capacity of peritoneal lymphocytes

The circadian variations in adherence capacity of peritoneal lymphocytes are shown in Fig.15. The results showed that the adherence

indexes increased along the day, but in adults only there were statistically significant differences with respect to the values at 8:00h ($p<0.05$) at 13h when cells were incubated for 10 min (Fig.15A). In old mice there were a significant increases of lymphocyte adherence capacity at 13:00 h and 18:00 h ($p< 0.01$) when cells incubated for 10 min, and at 13h ($p<0.05$) and 18:00 h ($p<0.001$) when cells incubated for 30 min with respect to the values at 8:00 h (Fig. 15A and 15B, respectively). Comparing the values between adult and old animals, these showed higher adherence indexes than those in adults, with statistically significant differences at 8:00 h ($p<0.05$), at 13h ($p<0.01$) and at 18:00 h ($p<0.001$) when cells were incubated for 10 min (Fig.15A), and at 13:00 h and at 18:00 h ($p<0.05$) when cells were incubated for 30 min (Fig.15B).

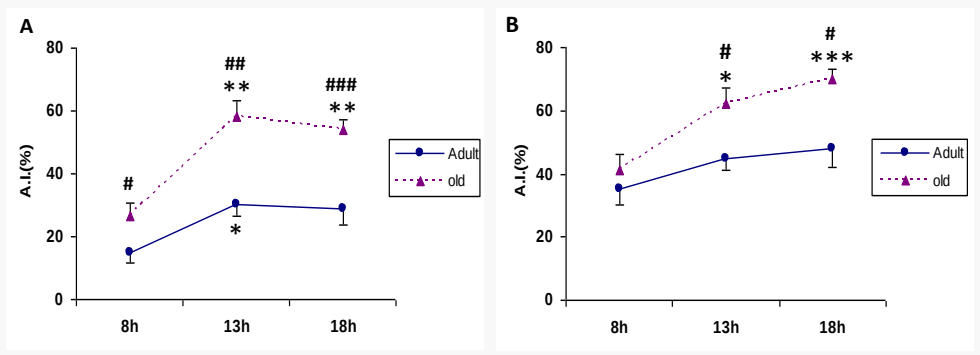


Fig. 15. Adherence Index (percentage of lymphocyte adherent to eppendorf tubes) of peritoneal lymphocytes at short time (10 min) of incubation (A) and at long time (30 min) of incubation (B) from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ with respect to the values at 8h. #$p < 0.05$; ##$p < 0.01$; ###$p< 0.001$ with respect to the values of adult animals.

Chemotaxis capacity of peritoneal lymphocytes

The circadian variations in chemotaxis index of peritoneal lymphocytes in adult as well as in old mice are shown in (Fig.16). The results showed a progressive and significant decrease in chemotactic capacity of lymphocytes along the day in adult as well as in old mice with statistically significant differences ($p<0.05$) at 13:00 h and 18:00h in adults, and at 13:00 h ($p<0.01$) and at 18:00 h ($p<0.05$) in old mice, with respect to

the corresponding values at 8:00 h. Lymphocytes showed lower ($p<0.05$) chemotaxis indexes in old animals than those from adults at both 8:00 h and 13:00 h. No statistically significant differences in chemotactic capacity were found at 18:00 h between adult and old animals.

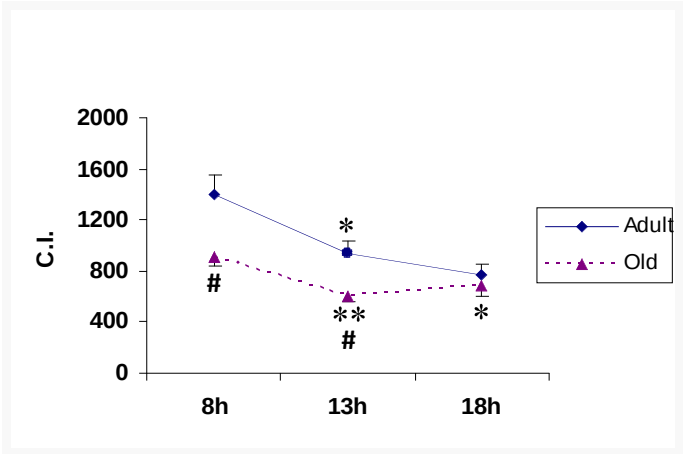


Fig. 16. Chemotaxis Index (C.I.) (number of cells on the filter) of peritoneal lymphocytes, from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals and being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$with respect to the values at 8h. #$p < 0.05$; ##$p < 0.01$ with respect to the values of adult animals.

Proliferation of peritoneal lymphocytes: Basal and in response to the mitogens: ConA and LPS

The results of the proliferation of peritoneal lymphocytes are shown in Figure 17. In response to ConA stimulation (Fig. 17A) cells from adult mice showed a significant increase at 13:00 h ($p<0.01$) followed by significant decrease at 18:00 h ($p<0.01$) with respect to the values at both 8:00 h and 13:00 h. Nevertheless, a significant increase in the course of the day was observed in old animals ($p<0.01$ at 13h and $p<0.05$ at 18:00 h with respect to the values at 8:00 h). No statistically significant differences were observed in circadian variation of lymphoproliferation in response to LPS (Fig. 17C) neither in adult nor in old animals in the course of the day. Peritoneal lymphocytes from old animals showed lower lymphoproliferation than those from adults in response to ConA at 8:00 h and 13:00 h ($p<0.001$)

but not at 18:00 h (Fig. 17A) and in response to LPS stimulation at 8h ($p<0.05$) but not at 13h and 18h (Fig. 17C).

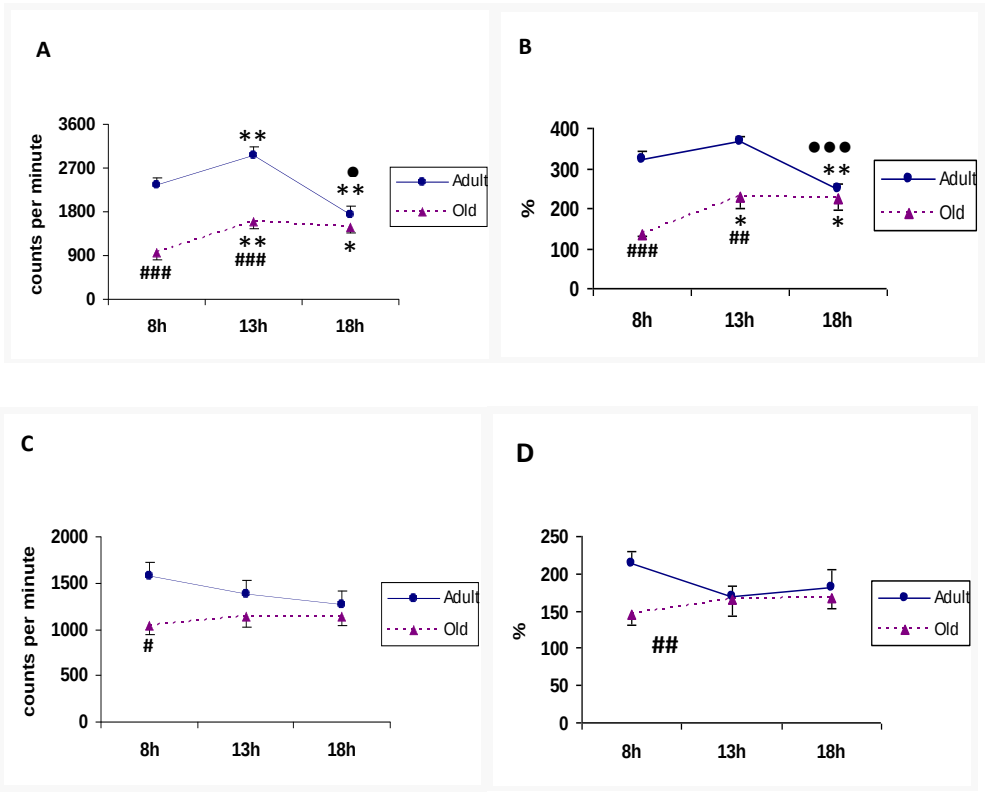


Fig 17. Lymphoproliferation (counts per minute) in response to ConA (A), in response to LPS (C), and percentage of lymphoproliferation (%) in response to ConA (B) and in response to LPS (D) in female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of triplicate assays.*p < 0.05; **p < 0.01 with respect to the values at 8h. •• p< 0.01 with respect to the values at 13h. #p < 0.05; ###p< 0.001 with respect to the values of adult animals.

The stimulation index (%) of lymphocyte proliferation in response to ConA (Fig.17B) and LPS (Fig.17D) showed almost the same trend of the corresponding values of counts per minute above commented.

Natural killer (NK) activity

The circadian variations in natural killer (NK) activity from peritoneal cells are shown in (Fig. 18). The results showed a decrease of NK activity in adults in the course of the day more than in old animals with statistically differences ($p<0.05$ at 13:00 h and $p<0.01$ at 18:00 h for adults and $p<0.05$ at 18:00 h for old animals with respect to the values at 8:00 h). The values

of NK activity in adult mice were higher than those in old animals, with statistically differences at 8:00 h ($p<0.05$) but not at 13:00 h and 18:00 h.

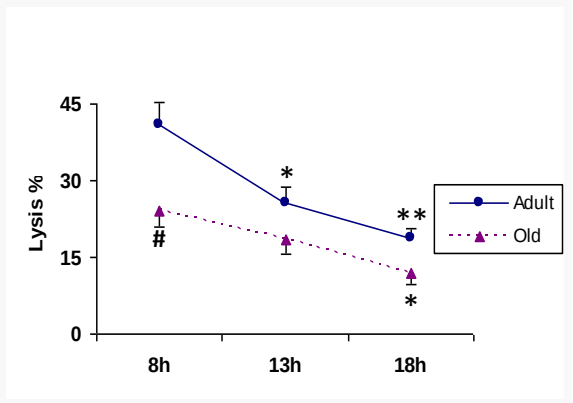


Fig. 18. Natural killer (NK) activity (percentage of lysis of mice tumoural cells) in female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$ with respect to the values at 8h. #$p < 0.05$ with respect to the values of adult animals.

Levels of Interleukin-2 (IL-2) released in peritoneal leucocyte cultures

The results corresponding to the levels of IL-2 released in peritoneal cell cultures for 48 h in response to ConA are shown in Fig. 19. Both adult and old animals demonstrated circadian variations in the interleukin-2 (IL-2) release levels in response to ConA in the course of the day, with decreased levels at 13:00 h ($p<0.05$) in adults and at 18:00 h ($p<0.05$) in old with respect to the corresponding values at 8h. Peritoneal cells from old animals showed lower IL-2 levels in response to ConA than those from adults with significant difference at 8:00 h ($p<0.05$).

4.1.1.2. Circadian changes in the levels of anti-inflammatory and pro-inflammatory cytokines released by peritoneal leukocytes

The results of the circadian changes in anti-inflammatory (IL-10) and pro-inflammatory (IL-1β and TNF-α) cytokines released by peritoneal

leucocytes from adult and old mice are shown in Fig. 20 and 21 as well as in Table 3.

Anti-inflammatory cytokine: Interleukin-10 (IL-10)

The results of the release of IL-10 by peritoneal cell cultures (48h) in response to ConA and LPS from adult and old mice are shown in Fig. 20. Cells from adult mice showed a decrease at 13:00h with respect to 8:00h with both mitogens, ConA ($p<0.01$) (Fig. 20A) and LPS ($p<0.05$) (Fig. 20B). However, in old animals an increase of IL-10 levels was shown in response to both mitogens ($p<0.05$ at 18:00 h with ConA and at 13:00h and 18:00 h with LPS with respect to the values at 8:00h). Comparing the levels of IL-10 in adult and old mice, at 13:00 h and at 18:00 h they are similar. Nevertheless, at 8:00 h, the release of IL-10 by peritoneal leucocytes is smaller ($p<0.05$) in those from old animals. Peritoneal leucocytes produced higher levels of IL-10 in both adults and old animals in response to LPS than in response to ConA.

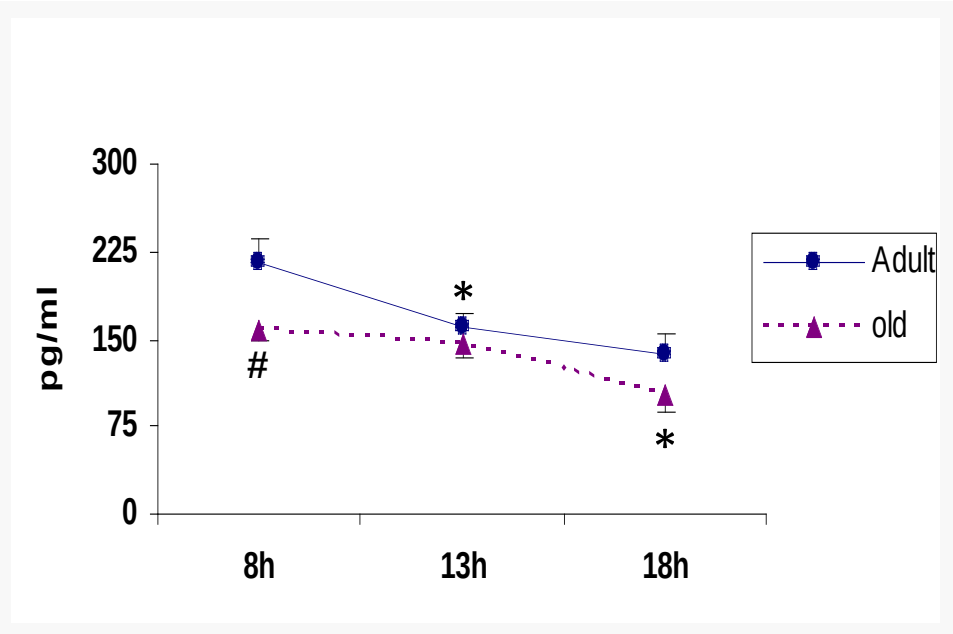


Fig 19. Interleukin-2 (IL-2) release levels (pg/ml) in the peritoneal cell incubated 48 h in presence of ConA. Cells were from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays. *$p < 0.05$ with respect to the values at 8h. #$p < 0.05$ with respect to the corresponding values in adult animals.

Table 2. Circadian variations of several function parameters in peritoneal lymphocytes from adult and old mice.

	Adult			Old		
	8h	**13h**	**18h**	**8h**	**13h**	**18h**
Adherence Index of Lymphocytes at 10 min of incubation						
	15±6	30±11*	29±12	27±9$^{\#}$	58±13**	54±7**$^{\#\#\#}$
Adherence Index of Lymphocytes at 30 min of incubation						
	35±10	45±9	48±15	41±12	62±13*$^{\#}$	70±8***$^{\#}$
Chemotaxis Index (C.I) of lymphocytes						
	1399±377	932±247*	773±217*	902±156$^{\#}$	607±129**$^{\#}$	684±185
Proliferation in response to LPS (cpm)						
	1574±355	1377±366	1263±388	1047±271$^{\#}$	1141±271	1145±264
Proliferation in response to ConA (cpm)						
	2365±265	2954±440**	1750±136**$^{\bullet\bullet}$	969±72$^{\#\#\#}$	1586±334**$^{\#\#\#}$	1480±270*
Proliferation index (%) in response to LPS						
	213±41	170±32	181±62	145±31$^{\#\#}$	165±55	168±33
Proliferation index (%) in response to ConA						
	323±50	366±30	249±30**$^{\bullet\bullet\bullet}$	135±13$^{\#\#\#}$	228±67*$^{\#\#}$	223±66*
NK activity (% lysis)						
	41±11	26±7*	19±4**	24±8$^{\#}$	19±6	12±4*
IL-2 (pg/ml)in peritoneal cell cultures stimulated with ConA (48h)						
	214±56	159±28*	138±42	159±27$^{\#}$	145±30	101±28*

Each value is the mean ± SD of six values corresponding to six animals, each value being the mean of duplicate or triplicate assays.*p < 0.05; **p < 0.01; ***p< 0.001 with respect to the values at 8h. • p< 0.05; •• p< 0.01; ••• p< 0.001 with respect to the values at 13h. #p < 0.05; ##p < 0.01; ###p< 0.001 with respect to the values of adult animals.

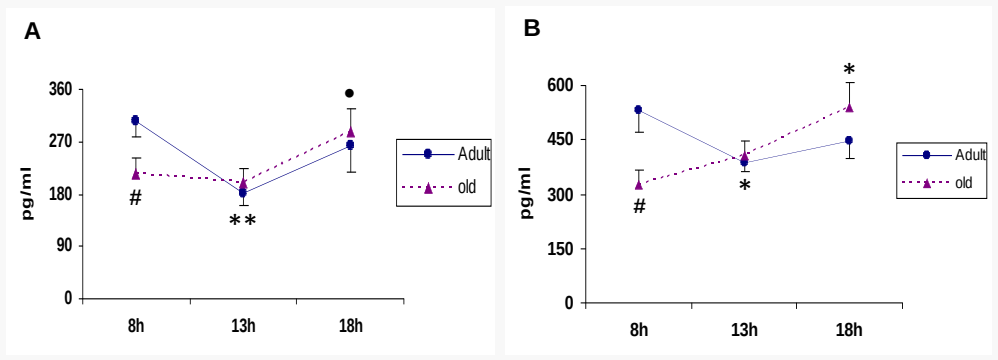


Fig 20. Interleukin-10 (IL-10) release levels (pg/ml) in peritoneal cells incubated 48 h in presence of ConA (A) and LPS (B). Cells were obtained from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals, and each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$ with respect to the values at 8h. • $p < 0.05$ with respect to the values at 13h. #$p < 0.05$ with respect to the corresponding values of adult animals.

Pro-inflammatory cytokines: Interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α)

The results obtained with respect to the release of pro-inflammatory cytokines in peritoneal cell cultures (48h) in response to ConA and LPS stimulation from adult and old mice were shown in Fig. 21.

In the Interleukin-1beta (IL-1β) release (Fig.21 A and B), cells of adult mice showed decreased levels in response to ConA at 13:00 h ($p<0.01$), whereas these levels significantly increased at 18h ($p<0.05$ and $p<0.01$ with respect to the values at 8h and 13:00 h respectively). Old animals showed a significant increase in those levels in the course of the day ($p<0.05$ at 13:00 h with respect to the values at 8:00 h, and $p<0.01$ at 18:00 h with respect to both values at 8:00 h and 13:00 h). Peritoneal cells from old animals showed lower ($p<0.01$) levels in response to ConA than those from adults at 8:00 h, whereas the levels were higher ($p<0.05$) at 13:00 h. No significant differences was observed at 18:00 h comparing adult and old mice (Fig.21 A).

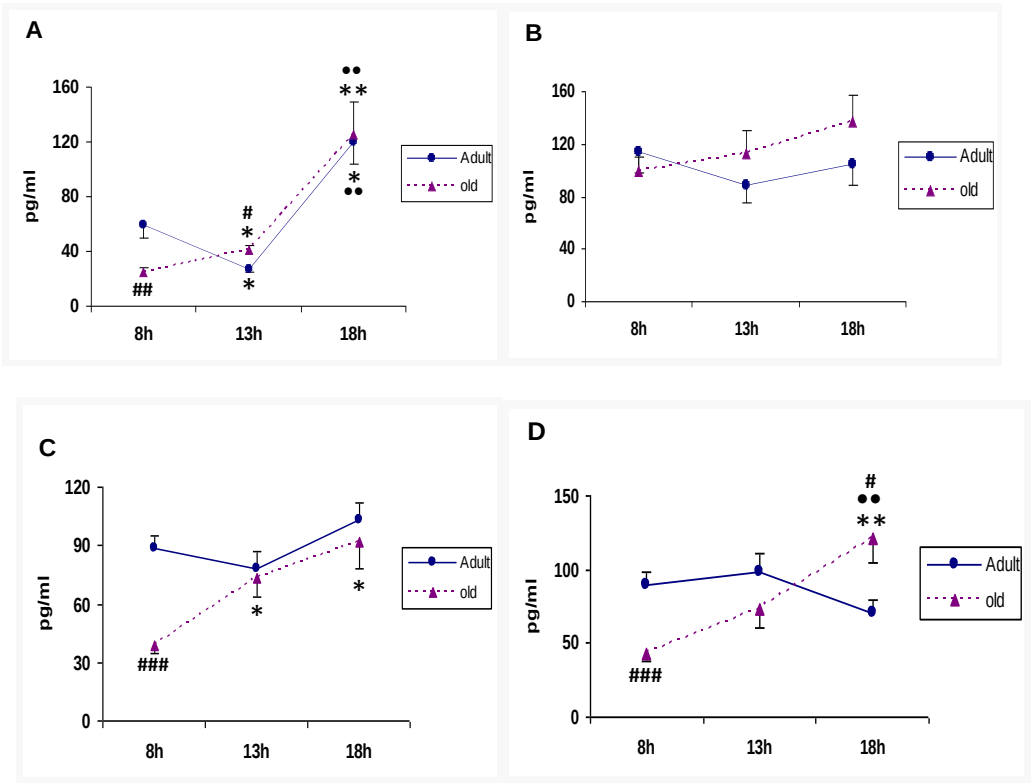


Fig. 21. Interleukin-1beta (IL-1β) release levels (pg/ml) by the peritoneal cells in response to ConA (A) and LPS (B) and tumor necrosis factor-alpha (TNF-α) release in response to ConA (C) and LPS (D), from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals and each value being the mean of duplicate assays.*p < 0.05; **p < 0.01 with respect to the values at 8h. ••p< 0.01 with respect to the values at 13h. #p < 0.05; ##p < 0.01; ###p < 0.001 with respect to the corresponding values of adult animals.

In response to LPS (Fig. 21B), no significant differences were observed in the course of the day or comparing adult and old animals.

The levels of tumor necrosis factor-alpha (TNF-α) in response to ConA (Fig. 21C) in adult animals did not show circadian variations, whereas in old animals significant increases of TNF-α levels in the course of the day ($p<0.05$ at both 13:00 h and 18:00 h with respect to the values at 8:00 h) were observed. Peritoneal leucocytes from old animals showed lower levels of this cytokine than those from adults at 8:00 h ($p<0.001$), then no significant differences were observed in the course of the day.

In response to LPS (Fig. 21D) adult animals did not show a circadian variation, whereas in old animals a significant increase in TNF-α levels at 18:00 h ($p<0.01$ with respect to both values at 8:00 h and 13:00 h) was shown. Old animals

showed lower TNF-α release than adult mice at 8:00 h ($p<0.001$) and higher at 18:00 h ($p<0.05$).

Table 3. Circadian variation of anti-inflammatory and pro-inflammatory cytokine levels released in peritoneal leukocyte cultures from adult and old mice.

	Adult			Old		
	8h	**13h**	**18h**	**8h**	**13h**	**18h**
Anti-inflammatory cytokine (*IL-10*) *(pg/ml) in peritoneal cell cultures (48h) stimulated with*						
ConA	304±61	182±51**	262±105	215±68$^{\#}$	199±61	287±98$^{\bullet}$
LPS	531±145	387±62*	447±116	325±105$^{\#}$	408±91	538±169**$^{\bullet}$**
Pro-inflammatory cytokine (*IL-1β) (pg/ml) in peritoneal cell cultures (48h) stimulated with*						
ConA	59±21	27±8*	120±40*$^{\bullet\bullet}$	25±7$^{\#\#}$	41±9*$^{\#}$	126±56**$^{\bullet\bullet}$
LPS	115±41	89±35	105±40	100±28	112±43	137±49
Pro-inflammatory cytokine (*TNF-α) (pg/ml) in peritoneal cell cultures (48h) stimulated with*						
ConA	89±14	78±21	103±21	38±10$^{\#\#\#}$	73±23*	92±34*
LPS	89±22	99±30	70±22	42±12$^{\#\#\#}$	73±28	121±39**$^{\bullet\bullet\#}$

Each value is the mean ± SD of six values corresponding to six animals, each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ with respect to the values at 8h. • $p< 0.05$; •• $p< 0.01$; ••• $p< 0.001$ with respect to the values at 13h. #$p < 0.05$; ##$p < 0.01$; ###$p< 0.001$ with respect to the values of adult animals.

4.1.1.3. Circadian changes in oxidant compounds and antioxidant defences in peritoneal leucocytes

The results corresponding to the circadian changes in oxidative stress parameters, studied in peritoneal leucocytes from adult and old mice are shown in Fig. 22 and Fig. 23 and in Table 4.

Oxidants: Extracellular superoxide anion (O_2^{-}.) levels

The extracellular superoxide anion levels release by peritoneal leucocytes from adult and old mice, are shown in Fig. 22A and Fig 22B, in non-stimulated and stimulated situations, respectively. Adults showed higher levels of extracellular superoxide anion at 13:00 h with respect to the values at 8h, under both nonstimulated ($p<0.05$) and stimulated ($p<0.001$) conditions. A significant decrease was observed in these levels in cells from old mice at 18:00 h under both nonstimulated ($p<0.05$ with respect to the values at 8:00 h and 13:00 h) and stimulated conditions ($p<0.05$ with respect to the values at 13:00 h).

Comparing the results between adult and old mice, in the latest higher levels of extracellular superoxide anion were observed in both nonstimulated ($p<0.001$ and $p<0.01$ at 8:00 h and 13:00 h, respectively) and stimulated cells ($p<0.05$ at 8:00 h and 13:00 h).

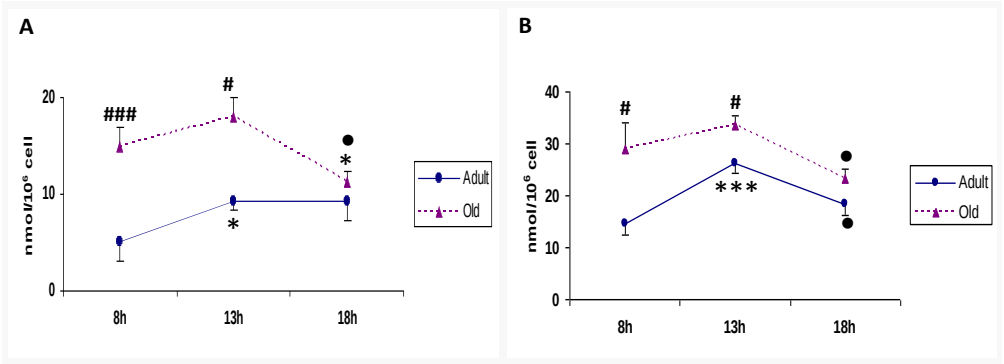


Fig. 22. Extracellular superoxide anion levels (nmols/10^6 leukocytes) of peritoneal cells in nonstimulated (A) and stimulated (B) conditions, from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals and each value being the mean of duplicate assays.*$p < 0.05$; ***$p< 0.001$ with respect to the values at 8h. • $p< 0.05$ with respect to the values at 13h. #$p < 0.05$; ##$p < 0.01$; ###$p< 0.001$ with respect to the values of adult animals.

Antioxidant defences: Total glutathione (TG) and the enzymes of its cycle; glutathione peroxidase (GPx) and glutathione reductase (GR)

With respect to the TG levels (Fig. 23A), cells from adult animals showed a significant decrease at 18:00 h ($p<0.01$ with respect to the values

at 8h), while in old animals at 18:00 h ($p<0.05$ with respect to the values at 13:00 h). Peritoneal leucocytes from old animals produced lower levels of TG than those from adults at 8:00 h ($p<0.001$) and at 13:00 h and 18:00 h ($p<0.05$).

In the antioxidant enzymes studied, with respect to GPx (Fig. 23B), cells from adult animals showed a significant decrease in this activity only at 13:00 h ($p<0.01$ with respect to the values at 8:00 h), whereas leucocytes from old animals did not demonstrate any circadian variation. Peritoneal leucocytes from old animals showed lower activity of GPx than those from adults, with statistically significant differences at 8:00 h ($p<0.05$).

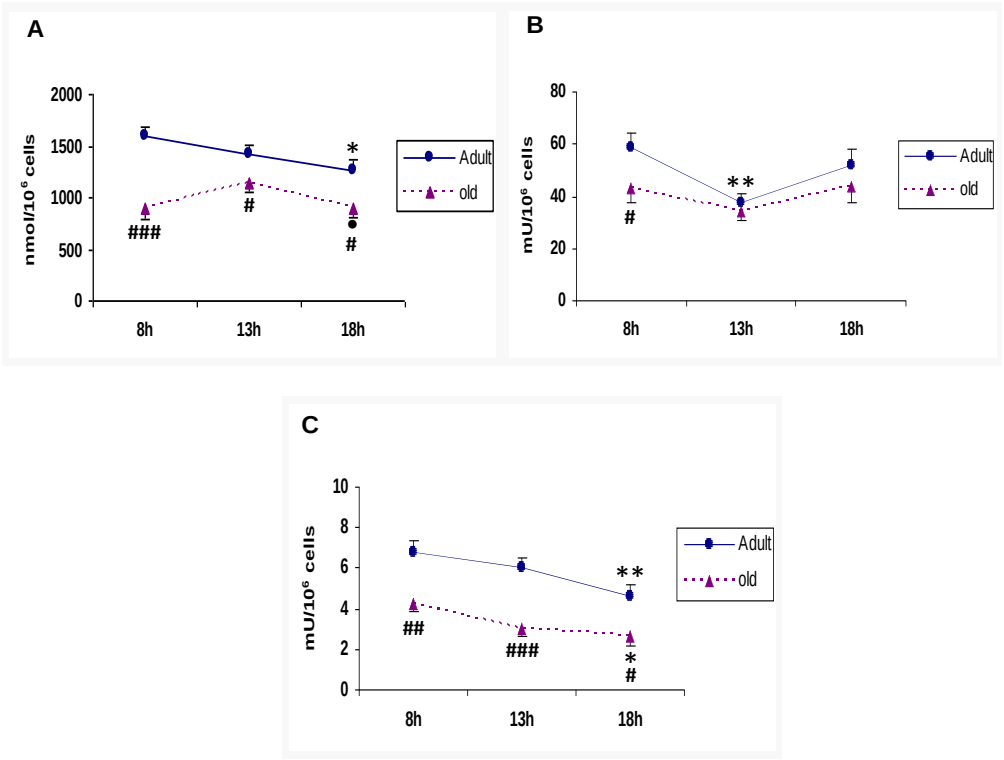


Fig.23: Total glutathione (GSH) (nmole/ 10^6cells) levels (A) and the activities of the enzymes involved in its oxidation reduction reactions, glutathione peroxidase (GPx) (mU/10^6 cells) (B) and glutathione reductase (GR) (mU/10^6 cells) (C) in peritoneal leucocytes from female adult (6 months) and old (18 months) ICR-CD1 mice. Each value is the mean ± standard error of six values corresponding to six animals and each value being the mean of duplicate assays.*$p < 0.05$; **$p< 0.01$ with respect to the values at 8h. • $p< 0.05$ with respect to the values at 13h. #$p < 0.05$; ##$p < 0.01$; ###$p< 0.001$ with respect to the values of adult animals.

The activity of GR (Fig. 23C), decreased in leucocytes from adult mice at 18:00 h ($p<0.01$) with respect to the corresponding value at 8:00h. Similarly occurred in old animals, which suffered a decreased GR activity at 18:00 h with significant differences ($p<0.05$) with respect to the values at 8:00 h. Leucocytes from old animals showed lower activities of this enzyme than those from adult mice at 8:00 h, 13:00 h and 18:00 h ($p<0.01$, $p<0.001$ and $p<0.05$, respectively).

Table 4. Circadian variations in extracellular superoxide anion ($O_2^{-}\bullet$) and total glutathione (TG) levels, and in the activities of the enzymes of glutathione cycle: glutathione peroxidase and glutathione reductase, in peritoneal leucocytes from adult and old mice.

	Adult			Old		
	8h	**13h**	**18h**	**8h**	**13h**	**18h**
$O_2^{-}\bullet$ extracellular non-stimulated (nmols/10^6 leukocytes)						
	5±3	9±2*	9±3	$15±4^{\#\#\#}$	$18±4^{\#\#}$	$11±3^{*\bullet}$
$O_2^{-}\bullet$ extracellular stimulated (nmols/10^6 leukocytes)						
	15±3	26±3***	$18±5^{\bullet}$	$29±11^{\#}$	$34±5^{\#}$	$23±4^{\bullet}$
Total glutathione (TG) (nmol/10^6 cells)						
	1596±354	1425±221	1267±235*	$899±287^{\#\#\#}$	$1143±201^{\#}$	$901±286^{\bullet\#}$
Glutathione peroxidase (GPx) (mU/ 10^6 cells)						
	59±13	38±8**	52±15	$43±12^{\#}$	35±8	44±14
Glutathione reductase (GR) (mU/ 10^6 cells)						
	6.8±1.5	6±1.3	4.7±1.2**	$4.3±1.1^{\#\#}$	$3±0.9^{\#\#\#}$	$2.6±0.6^{*\#\#}$

Each value is the mean ± SD of six values corresponding to six animals, each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ with respect to the values at 8h. • $p< 0.05$; •• $p< 0.01$; ••• $p< 0.001$ with respect to the values at 13h. #$p < 0.05$; ##$p < 0.01$; ###$p< 0.001$ with respect to the values of adult animals.

4.1.2. Circadian changes in parameters measured in blood

4.1.2.1. Circadian changes in anti-inflammatory and pro-inflammatory cytokines released by cells of blood in response to LPS

The results corresponding to IL-10, IL-1β and TNF-α level in plasma of blood, incubated in presence of LPS, from adult and old ICR-CDI female mice at 8:00h, 13:00h and 18:00h are shown in Fig. 24 and Fig. 25 and in Table 5.

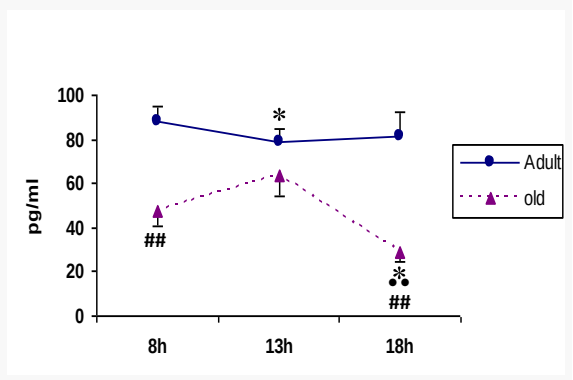


Fig. 24: Interleukin-10 (IL-10) levels (pg/ml) in plasma of blood incubated in presence of LPS, collected from female adult (6 months) and old (18 months) ICR-CD1 mice at 8:00, 13:00 and 18:00 h. Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays.*p < 0.05 with respect to the corresponding value at 8h. •• p<0.01 with respect to the corresponding value at 13h. ##p < 0.01 with respect to the corresponding value of adult animals.

In the IL-10 levels (Fig. 24), adult animals demonstrated a decrease of this cytokine at 13:00 h ($p<0.05$) with respect to the values at 8:00h, whereas old animals showed this decrease at 18:00 h with respect to the values at 8h ($p<0.05$) and 13h ($p<0.01$). Old animals showed lower levels of IL-10 than those from adults, with significant differences at 8h and at 18h ($p<0.01$).

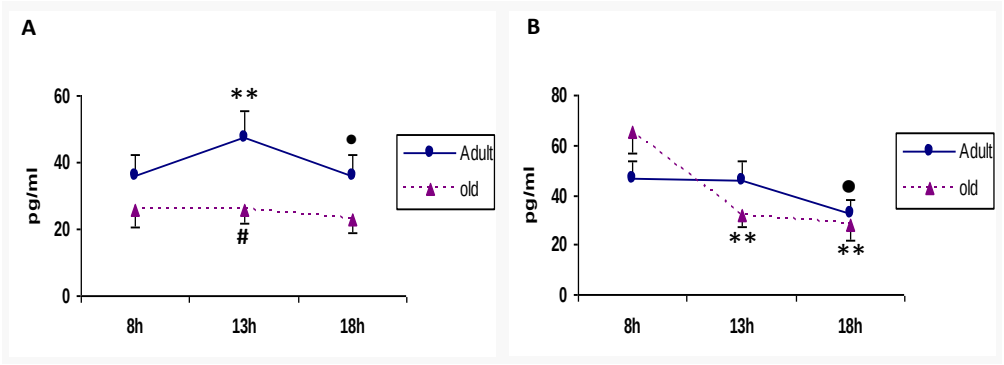


Fig. 25: IL-1β (A) and TNF-α (B) levels (pg/ml) in plasma of blood, incubated in presence of LPS, collected from female adult (6 months) and old (18 months) ICR-CD1 mice at 8:00, 13:00 and 18:00 h. Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays.**$p < 0.01$ with respect to the corresponding value at 8h. • $p< 0.05$ with respect to the corresponding value at 13h. #$p < 0.05$ with respect to the corresponding value of adult animals.

Adult animals showed an increase in IL-1β levels (Fig. 25A) at 13:00 h ($p<0.01$), whereas old animals did not show circadian variations in this cytokine. Comparing the levels of IL-1β between adult and old mice, these animals showed lower levels than those from adults with significant differences at 13h ($p<0.05$).

In TNF-α levels (Fig. 25B), adult animals showed a decrease at 18:00 h ($p<0.05$) with respect to the values at 13:00 h. and old mice at 13:00 h and 18:00 h with respect to the values at 8h ($p<0.01$). No differences were observed between adult and old at each time studied.

Table 5: IL-10, IL-1β and TNF-α levels (pg/ml) in plasma of blood, incubated 4 h in presence of LPS, from adult and old ICR-CD1 female mice.

	Adult			Old		
LPS stimulation	**8h**	**13h**	**18h**	**8h**	**13h**	**18h**
IL-10 (pg/ml)						
	88±7	79±6*	82±11	48±7$^{\#\#}$	63±9	29±4*$^{\bullet\bullet\#\#}$
IL-1β (pg/ml)						
	36±6	47±8**	36±6$^{\bullet}$	26±5	26±4$^{\#}$	23±4
TNF-α (pg/ml)						
	47±7	46±8	32±6$^{\bullet}$	65±9	32±5**	28±6**

Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays.*p < 0.05; **p < 0.01 with respect to the corresponding value at 8h. • p< 0.05 ;•• p< 0.01 with respect to the corresponding value at 13h. #p < 0.05; ##p < 0.01 with respect to the corresponding value of adult animals.

4.1.2.2 Plasma corticosterone levels

The results corresponding to plasma corticosterone levels in adult and old ICR-CDI female mice are shown in figure 26.

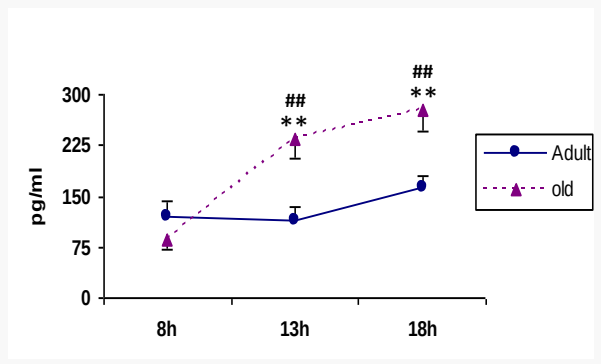


Fig. 26: Corticosterone levels (pg/ml) in the plasma from female adult (6 months) and old (18 months) ICR-CD1 mice at 8:00, 13:00 and 18:00 h. Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays.**p < 0.01 with respect to the corresponding value at 8h. ##p < 0.01 with respect to the corresponding value of adult animals.

Adult animals did not show circadian variation in the levels of plasma corticosterone, whereas old animals showed an increase at 13:00 h and at 18:00 h ($p<0.01$) with respect to the values at 8:00 h. Old mice showed higher ($p<0.01$) levels of corticosterone in plasma than adult animals at 13h and at 18h (Fig.26).

Table 6: Corticosterone levels ***(pg/ml)*** in the plasma from adult and old ICR-CD1 female mice.

	Adult			**Old**		
Plasma	**8h**	**13h**	**18h**	**8h**	**13h**	**18h**
Corticosterone (pg/ml)						
	121±22	115±21	162±19	87±14	235±31**$^{\#\#}$	278±32**$^{\#\#}$

Each value is the mean ± standard error of six values corresponding to six animals, each value being the mean of duplicate assays.**$p < 0.01$ with respect to the corresponding value at 8h. ##$p < 0.01$ with respect to the corresponding value of adult animals.

4.2. Redox parameters in heart, spleen and liver of adult, mature and old prematurely ageing mice (PAM) and non-prematurely ageing mice (NPAM).

The results corresponding to total glutathione (TG) levels and the antioxidant enzyme activities involved in its oxidation-reduction reactions, glutathione peroxidase (GPx) and glutathione reductase (GR) in heart, spleen and liver of adult, mature and old, non-prematurely ageing mice (NPAM) and prematurely ageing mice (PAM) are shown in figures 27, 28 and 29 and in table 7. The values obtained in reduced glutathione (GSH), oxidized glutathione (GSSG) and GSSG/GSH ratio in liver are shown in figure 30 and in table 8.

4.2.1. Antioxidant defenses in heart of adult, mature and old prematurely ageing mice (PAM) and non-prematurely ageing mice (NPAM)

In the heart (Fig. 27) the results showed that TG levels, GR and GPx activities decrease significantly with ageing in both PAM and NPAM. Mature and old PAM and NPAM showed lower levels of TG and lower activity of GPx and GR than the corresponding adults, with exception of the GR activity in mature PAM. In old PAM and NPAM there were lower ($p<0.001$) values of TG and GR than those in mature animals but higher ($p<0.001$) in GPx activity.

Comparing PAM and NPAM, the results showed that TG levels and GPx activities were lower in adult and mature PAM than in the corresponding values of NPAM ($p<0.05$ and $p<0.001$, respectively). Adult PAM showed lower GR activity ($p<0.01$) than their NPAM counterparts, whereas, no significant differences were observed in mature mice. No differences were observed between old PAM and NPAM in any of the parameters studied.

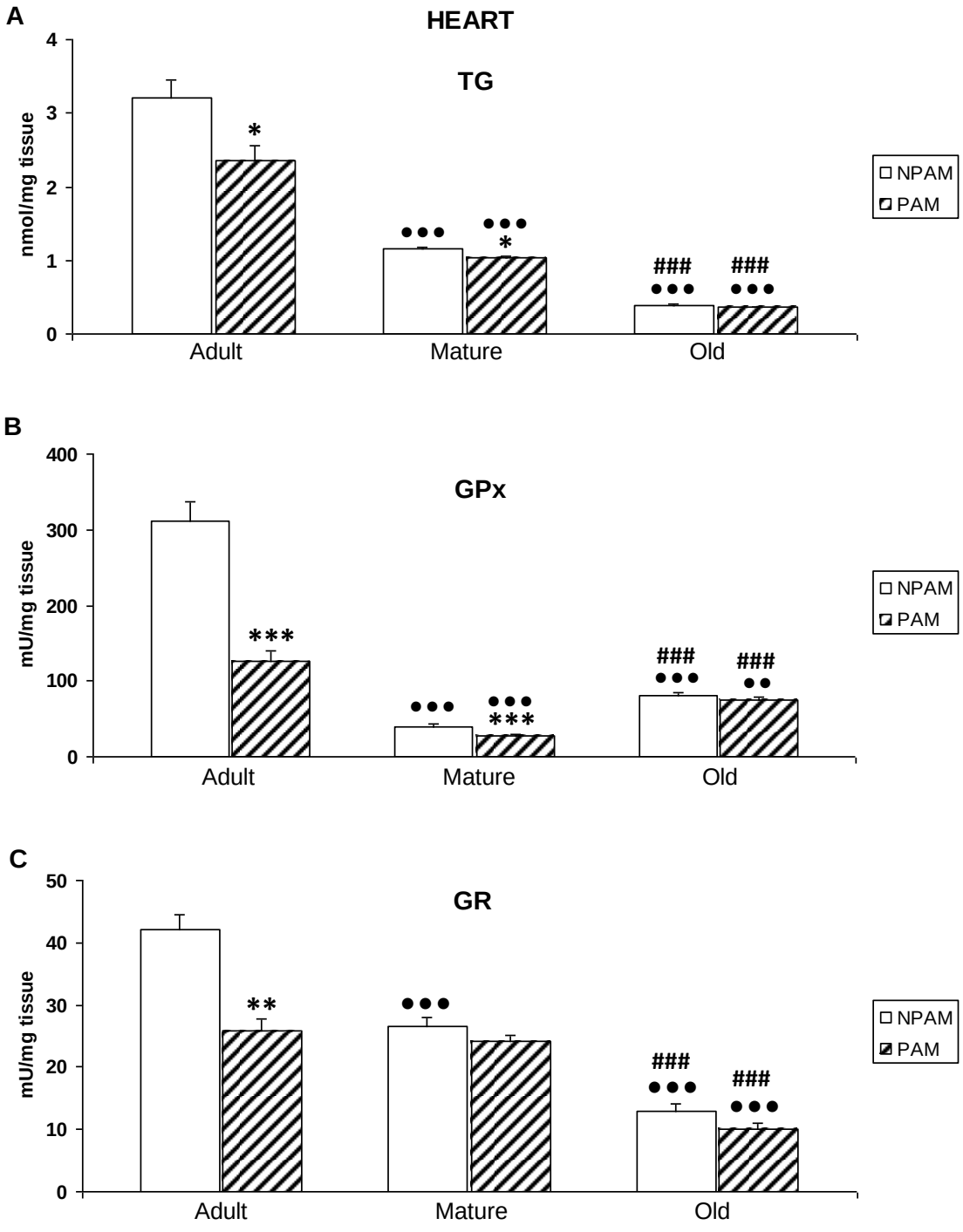


Figure.27. Total glutathione (TG) levels (A) and glutathione peroxidase (GPx) (B) and glutathione reductase (GR) (C) activities in heart of adult, mature and old NPAM and PAM. Each column represents the mean±S.E. of 8-10 values corresponding to 8-10 animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value in NPAM. • p< 0.05; •• p< 0.01; ••• p< 0.001 with respect to the corresponding values in adult. #p < 0.05; ###p< 0.001 with respect to the corresponding values in mature.

Animals were killed by decapitation. Spleen and thymus were immediately removed in sterile conditions and maintained at 4ºC in Hank's balanced saline solution until the collection of leukocytes, which was performed 24h later (see below). In the case of spleen, half of each organ was frozen at -80ºC until the antioxidant defence analysis was carried out.

4.2.2. Antioxidant defenses in spleen of adult, mature and old prematurely ageing mice (PAM) and non-prematurely ageing mice (NPAM)

In the spleen (Fig. 28), the results showed that, in general, TG levels, GPx and GR decreased with ageing in both PAM and NPAM. Thus, mature PAM and NPAM showed lower levels of TG and of GPx activities than those in adults. Old PAM and NPAM showed in all the parameters studied lower levels than the corresponding in adults.

TG levels as well as GPx and GR activities were lower in adult and mature PAM than in NPAM counterparts. These differences between PAM and NPAM disappeared in the old animals, which showed similar levels of TG and activity of GR, although old PAM showed lower activity of GPx than NPAM.

4.2.3. Antioxidant defenses in liver of adult, mature and old prematurely ageing mice (PAM) and non-prematurely ageing mice (NPAM)

In the liver (Fig. 29), the results showed that, TG levels, GPx and GR activities decreased significantly in NPAM and PAM with ageing. Thus, mature and old NPAM and PAM showed lower values of those parameters than those in the corresponding adult mice, with the exception of the GR activity in mature PAM (with similar activity of this enzyme to that in adult mice).

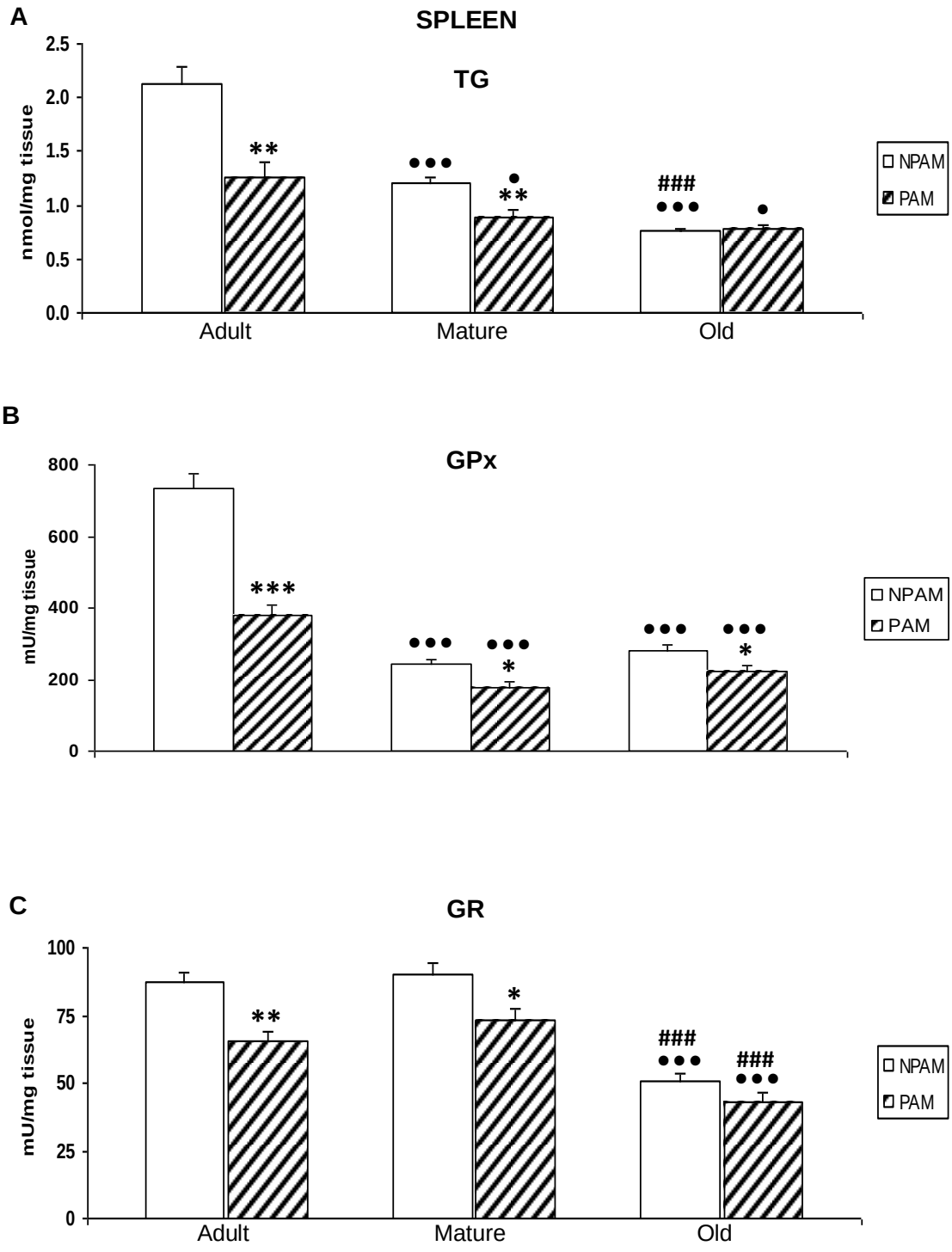


Figure.28. Total glutathione (A), glutathione peroxidase (GPx) (B) and glutathione reductase (GR) (C) in spleen of adult, mature and old NPAM and PAM. Each column represents the mean±S.E. of 8-10 values corresponding to 8-10 animals, each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ with respect to control NPAM. •$p< 0.05$; ••$p< 0.01$; •••$p< 0.001$ with respect to the corresponding values in adult. #$p < 0.05$; ###$p< 0.001$ with respect to the corresponding values in mature.

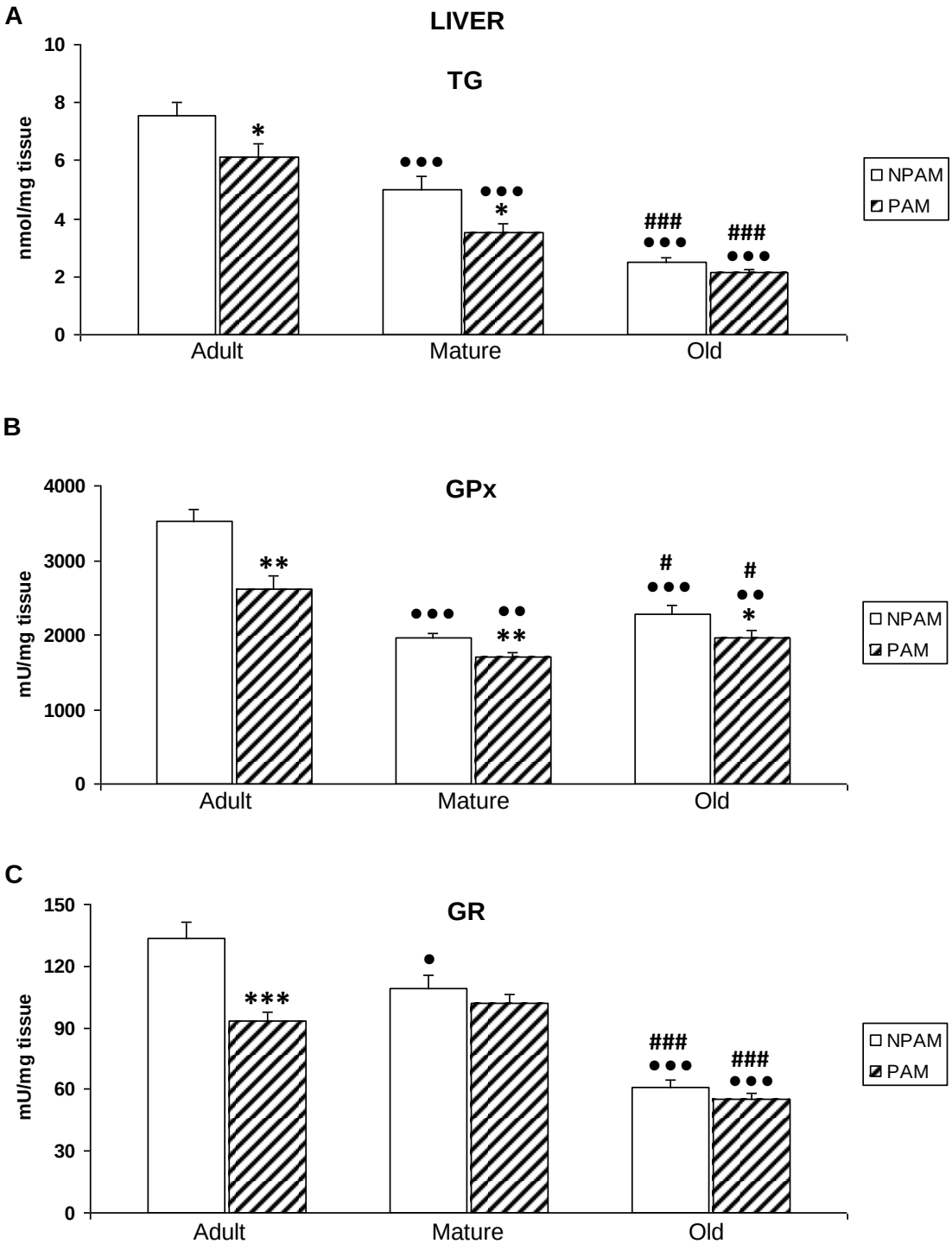


Figure. 29. Total glutathione (A), glutathione peroxidase (GPx) (B) and glutathione reductase (GR) (C) in liver of adult, mature and old NPAM and PAM. Each column represents the mean±S.E. of 8-10 values corresponding to 8-10 animals, each value being the mean of duplicate assays.*p < 0.05; **p < 0.01; ***p< 0.001 with respect to control NPAM. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding values in adult. #p < 0.05; ###p< 0.001 with respect to the corresponding values in mature.

Table 7. Total glutathione (TG) levels, glutathione peroxidase (GPx) and glutathione reductase (GR) activities in liver, heart and spleen from adult, mature and old NPAM and PAM.

Antioxidant defense	Age	Mice	Heart	Spleen	Liver
TG (nmol/mg tissue)	Adult	NPAM	3.2±0.68	2.1±0.48	7.6±1.2
		PAM	2.4±0.53*	1.3±0.40**	6.1±1.3*
	Mature	NPAM	1.15±0.10•••	1.2±0.17•••	5.0±1.2•••
		PAM	1.04±0.08*•••	0.89±0.17**•	3.5±0.8*•••
	Old	NPAM	0.39±0.05•••###	0.75±0.06•••	2.5±0.5•••###
		PAM	0.36±0.04•••###	0.79±0.08•	2.1±0.3•••###
GPx (mU/mg tissue)	Adult	NPAM	312±67	735±112	3517±491
		PAM	127±33***	379±87***	2616±494**
	Mature	NPAM	40±8•••	241±39•••	1970±166•••
		PAM	27±5***•••	178±17*•••	1708±182**••
	Old	NPAM	80±16•••###	279±58•••	2280±365•••#
		PAM	74±15•••###	225±43*•••	1966±283*•••#
GR (mU/mg tissue)	Adult	NPAM	42±6.8	88±10	134±24
		PAM	26±5.49**	66±8**	93±11***
	Mature	NPAM	26.6±4•••	90±12	109±16•
		PAM	24±3	73±11*	102±11
	Old	NPAM	13±4.3•••###	51±11•••###	61±10•••###
		PAM	10±3.2•••###	43±9•••###	55±9•••###

Each value represents the mean ± S.D. of 8-10 values corresponding to 8-10 animals, each value being the mean of duplicate assays.*p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value in NPAM. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value in adult. #p < 0.05; ##p< 0.01; ###p< 0.001 with respect to the corresponding value in mature. NPAM: non-prematurely aging mice; PAM: prematurely aging mice.

Comparing the values between NPAM and PAM, these PAM showed lower levels than NPAM in chronological adult animals in TG levels and in GPx and GR activities ($p<0.05$, $p<0.01$ and $p<0.001$, respectively). In mature PAM the values of TG and GPx were lower than those in NPAM counterparts. These differences disappeared in the old animals, which showed similar values of TG levels and GR activity between PAM and NPAM. However, GPx activity was lower in old PAM than in old NPAM.

4.2.4. Oxidative stress in liver of adult, mature and old prematurely ageing mice (PAM) and non-prematurely ageing mice (NPAM)

The results corresponding to reduced glutathione (GSH) and oxidized glutathione (GSSG) levels as well as the GSSG/GSH ratio in liver of adult, mature and old NPAM and PAM are shown in Fig 30 and Table 8. In PAM and NPAM, the GSH levels decreased with ageing, whereas the GSSG levels and the GSSG/GSH ratio increase with ageing.

With respect to GSH levels (Fig. 30A), mature PAM and NPAM showed lower levels ($p<0.001$) than those in adults but higher ($p<0.001$) than those in old animals. IN adult and mature PAM and NPAM, PAM showed lower levels than those in the corresponding NPAM. However, in old animals these values are similar in PAM and NPAM.

The GSSG levels (Fig.30B) were higher in mature PAM and NPAM than in the corresponding adult animals. Nevertheless, in old PAM and NPAM, although the GSSG levels were higher than those in adult animals, they were similar to those in mature animals in the case of PAM and lower than those in the case of mature NPAM. PAM showed higher values of GSSG levels with respect NPAM at all age studied.

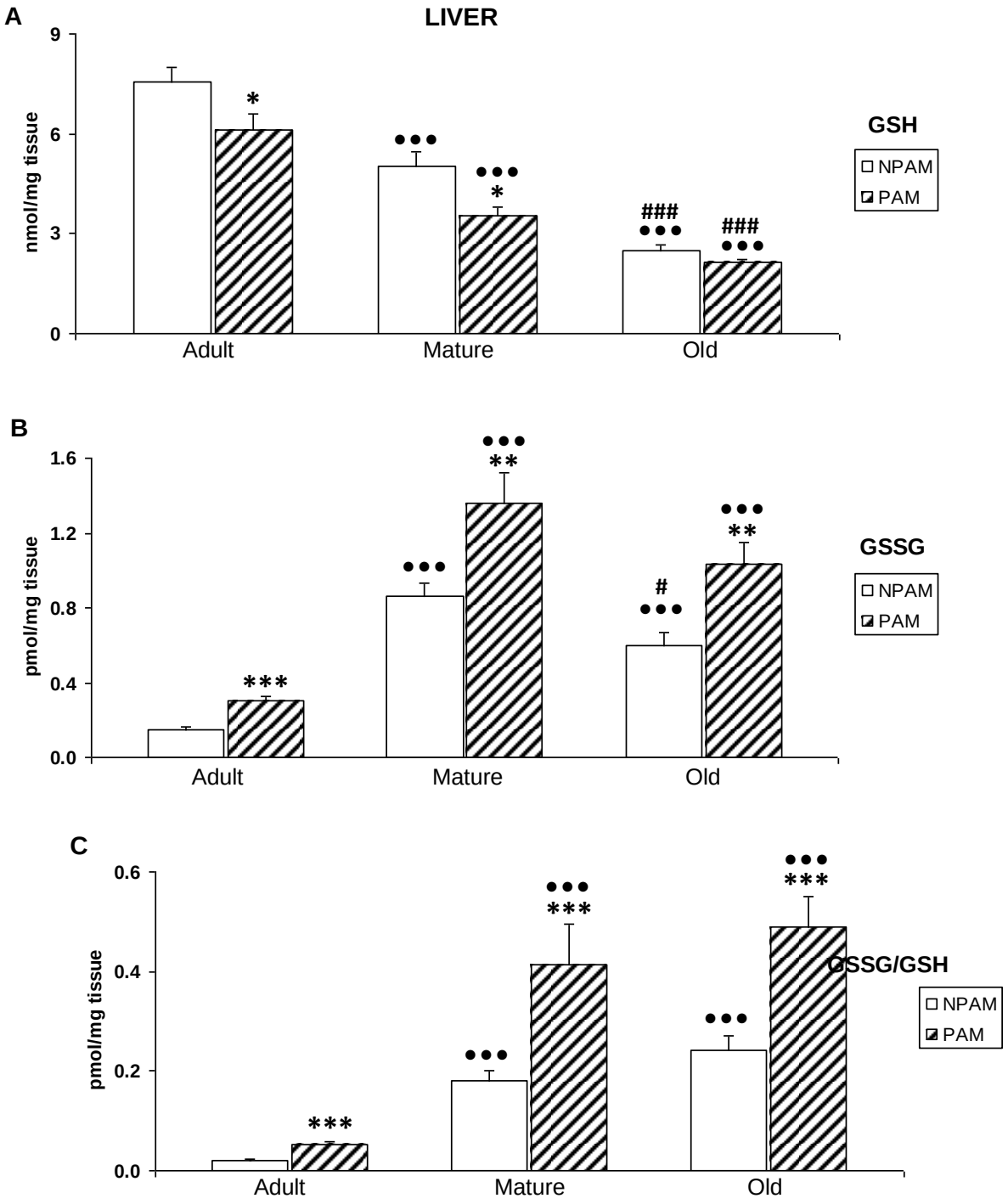


Figure. 30. Reduced glutathione (GSH) (A), oxidized glutathione (GSSG) (B) and GSSG/GSH ratio (C) in liver of adult, mature and old NPAM and PAM. Each column represents the mean ± S.E of 8-10 values corresponding to 8-10 animals, each value being the mean of duplicate assays.*p < 0.05; **p < 0.01; ***p< 0.001 with respect to control NPAM. • p< 0.05; •• p< 0.01; ••• p< 0.001 with respect to the corresponding values in adult. #p < 0.05; ###p< 0.001 with respect to the corresponding values in mature.

The GSSG/GSH ratios (Fig.30C) were increased with ageing in PAM and NPAM. In mature and old PAM and NPAM the values of these ratios were higher (p<0.001) than in the corresponding adult animals. PAM and

NPAM showed the same GSSG/GSH ratio in mature mice than in old animals. PAM, at the three ages studied, showed higher ($p<0.001$) values of the GSSG/GSH ratio than NPAM.

Table 8. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels and GSSG/GSH ratio in liver from adult, mature and old NPAM and PAM.

	Age	Mice	liver
GSH (nmol/mg tissue)	Adult	NPAM	7.57±1.24
		PAM	6.12±1.30*
	Mature	NPAM	5.02±1.20•••
		PAM	3.52±0.80*•••
	Old	NPAM	2.489±0.53•••###
		PAM	2.119±0.33•••###
GSSG (pmol/mg tissue)	Adult	NPAM	0.15±0.04
		PAM	0.31±0.06***
	Mature	NPAM	0.86±0.20•••
		PAM	1.36±0.46**•••
	Old	NPAM	0.60±0.23•••#
		PAM	1.03±0.38•••**
GSSG/GSH $x10^3$	Adult	NPAM	0.02±0.009
		PAM	0.05±0.02***
	Mature	NPAM	0.18±0.06•••
		PAM	0.41±0.23***•••
	Old	NPAM	0.24±0.08•••
		PAM	0.49±0.20***•••

Each value represents the mean ± S.D. of 8-10 values corresponding to 8-10 animals, each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ with respect to the corresponding value in NPAM. •$p< 0.05$; ••$p< 0.01$; •••$p< 0.001$ with respect to the corresponding value in adult. #$p < 0.05$; ##$p< 0.01$; ###$p< 0.001$ with respect to the corresponding value in mature.

4.3. Functional parameters and redox state in young and adult triple transgenic Alzheimer's disease (3xTg-AD) mice.

In this section of the results, the functional and redox state of several parameters in young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice are shown. The samples analyzed were obtained from spleen and thymus.

4.3.1. Functional parameters and antioxidant defense in young and adult transgenic Alzheimer's disease (3xTg-AD) mice.

Several immune functions were studied in leukocytes from spleen and thymus, namely, chemotaxis, natural killer cell activity (NK), lymphoproliferation both in basal conditions and in response to Con A and LPS mitogens, as well as levels of IL-2, IL-10, IL-1β and TNF-α cytokines in the culture supernantants from Con A and LPS. In addition, levels of glutathione (the endogenous antioxidant) and activities of GPx and GR in spleen are also analyzed.

4.3.1.1. Functional parameters of lymphocytes from young and adult female and male transgenic Alzheimer's disease (3xTg-AD) mice.

The results corresponding to chemotaxis, natural killer cell activity (NK), lymphoproliferation both in basal conditions and in response to Con A and LPS mitogens spleen and thymus of youngs and adults, females and males 3xTg-AD mice are shown in figures 31- 38.

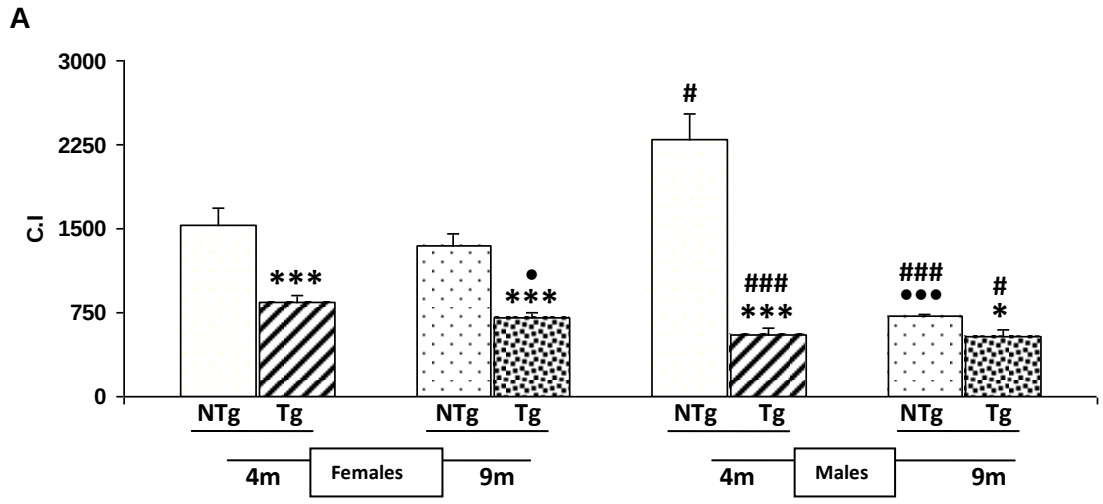


Fig. 31. Chemotaxis index in spleen leukocytes from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *p < 0.05; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;###p< 0.001 with respect to the corresponding value of females.

In spleen leukocytes (Fig. 31), the results showed that young and adult females 3xTg-AD mice showed lower chemotactic index than their NTg counterparts ($p<0.001$). Adult female NTg showed similar chemotactic index as in young, whereas Adult female 3xTg-AD showed lower chemotactic index than in young 3xTg-AD ($p<0.05$). Adult and young 3xTg-AD male mice showed lower chemotactic index from spleen lymphocytes than their NTg counterparts with higher differences in young than in adults ($p<0.001$, $p<0.05$ respectively). Adult males NTg showed lower chemotactic index than in young animals, whereas no differences were observed in adult and young 3xTg-AD. Adult NTg males showed slightly higher chemotactic index than in females ($p<0.05$), on the contrary, adult NTg and 3xTg-AD as well as young 3xTg-AD females showed higher chemotactic index than in males ($p<0.001$, $p<0.05$ and $p<0.001$ respectively) with lower differences between adult female and male 3xTg-AD.

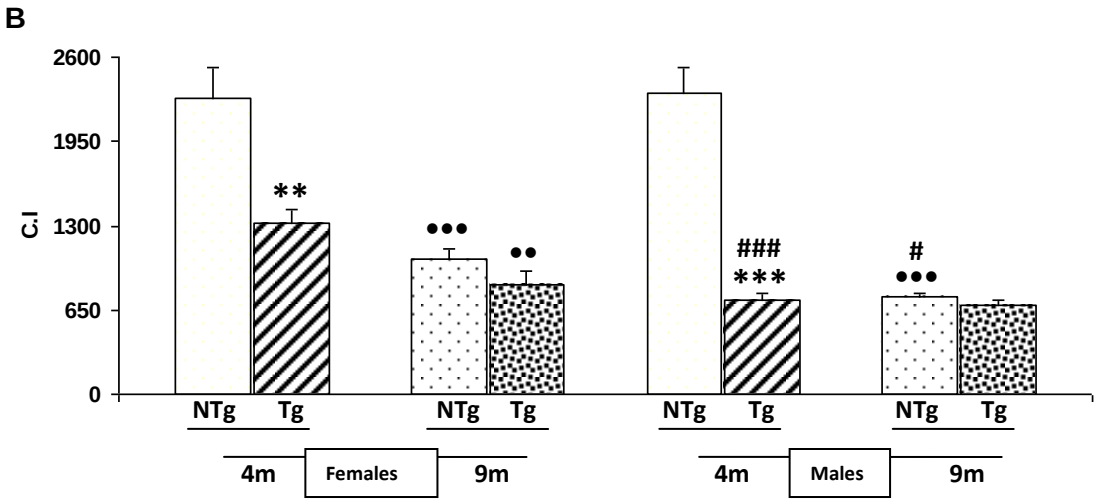


Fig. 32. Chemotaxis index in thymus leukocytes from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05; ###p< 0.001 with respect to the corresponding value of females.

In thymus leukocytes (Fig. 32), the results showed that young females and males 3xTg-AD mice showed lower chemotactic index than in their NTg counterparts ($p<0.01$ and $p<0.001$ respectively). Adult females and males 3xTg-AD showed similar chemotactic index with respect to NTg counterparts. Adult NTg and 3xTg-AD females as well as adult NTg males showed lower chemotactic index than in young mice ($p<0.001$, $p<0.01$ and $p<0.001$ respectively), whereas no significant differences were observed between young and adult 3xTg-AD. Young males and females NTg as well as adult males and females 3xTg-AD showed similar chemotactic index. Young 3xTg-AD and adult NTg males showed lower chemotactic index than in females ($p<0.001$ and $p<0.05$ respectively).

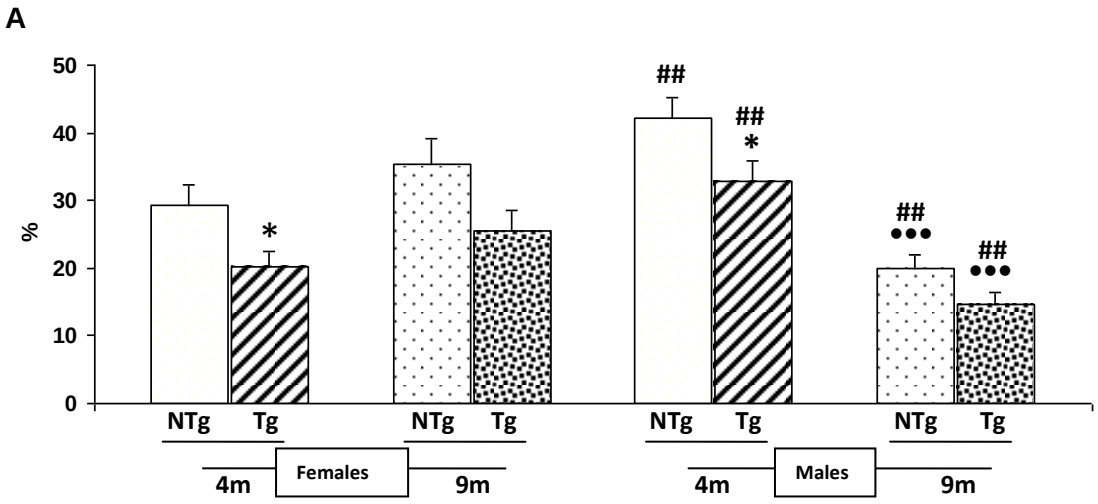


Fig. 33. NK activity in spleen leukocytes from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *p < 0.05 with respect to the corresponding value of control mice. •••p< 0.001 with respect to the corresponding value of young. ##p<0.01 with respect to the corresponding value of females.

In spleen lymphocytes (Fig. 33), the results showed that young 3xTg-AD female and male mice showed lower NK activity than their NTg counterparts ($p<0.05$). 3xTg-AD adult females and males showed similar NK activity as in their NTg counterparts. No significant differences were observed between adult and young NTg and 3xTg-AD female mice, but have been observed in males with significant differences ($p<0.001$). Young NTg and 3xTg-AD males showed higher NK activity from spleen lymphocytes than females ($p<0.01$), on the contrary, adult NTg and 3xTg-AD males showed lower NK activity than in females ($p<0.01$).

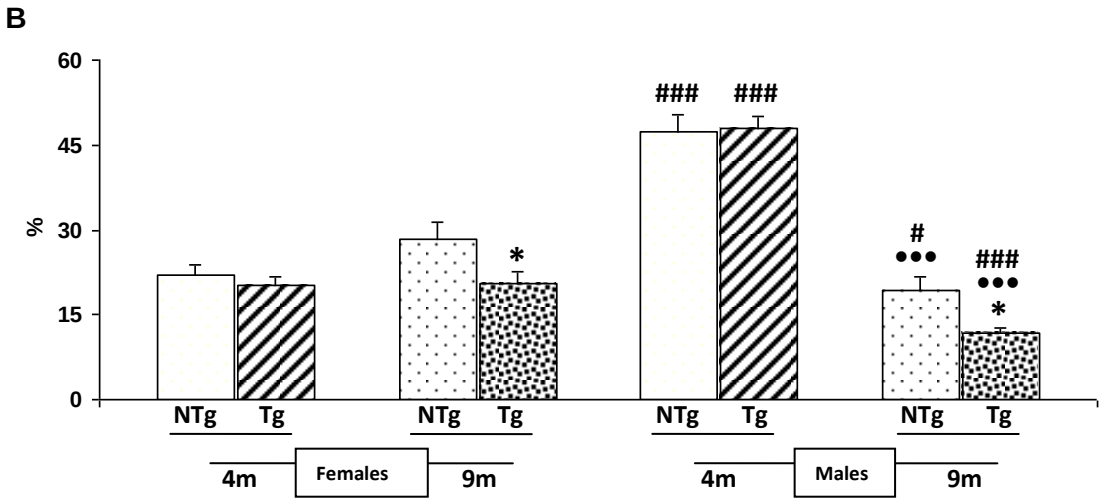


Fig. 34. NK activity in thymus leukocytes from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *p < 0.05 with respect to the corresponding value of control mice. •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;###p< 0.001 with respect to the corresponding value of females.

In thymus (Fig. 34), the results showed that lymphocytes from young 3xTg-AD female and male mice showed similar levels of NK activity as their NTg counterparts. Adult 3xTg-AD females and males showed lower NK activity than in their NTg counterparts ($p<0.05$). No differences were observed between adult and young NTg and 3xTg-AD female mice, but have been observed in males with significant differences ($p<0.001$). Young NTg and 3xTg-AD males showed higher NK activity from thymus lymphocytes than females ($p<0.001$), but in adults, NTg and 3xTg-AD males showed lower NK activity than in females ($p<0.05$ and $p<0.001$ respectively).

Table 9. Chemotaxis capacity (Chemotaxis index) and NK activity (% of tumor lysis) of spleen and thymus leukocytes from female and male young (4 months) and adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice.

Function	Gender	Age (months)	Mice	Spleen	Thymus
Chemotaxis index (C.I)	***Females***	**4**	NTg	1538±472	2281±762
			3xTg-AD	848±160***	1316±359**
		9	NTg	1352±290	1040±242•••
			3xTg-AD	703±117***•	850±291••
	Males	**4**	NTg	2295±705#	2321±644
			3xTg-AD	578±148***###	723±190***###
		9	NTg	715±60•••###	746±89•••#
			3xTg-AD	542±165*#	687±110
NK (%)	***Females***	**4**	NTg	29±10	22±7
			3xTg-AD	20±7*	20±5
		9	NTg	35±12	28±8
			3xTg-AD	26±8	21±5*
	Males	**4**	NTg	42±9##	47±10###
			3xTg-AD	32±9*##	48±7###
		9	NTg	20±6•••##	19±6•••#
			3xTg-AD	15±5•••##	12±3*•••###

Each value is the mean ± S.D of 8-10 values (young 10, adult 8), each value being the mean of duplicate assays.*p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding values of control NTg mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding values in young mice. #p < 0.05; ##p< 0.01; ###p< 0.001 with respect to the corresponding values in females.

Proliferation (counts/minute) of spleen and thymus lymphocytes from young (4 moths) and adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice in basal conditions, in response to ConA and LPS as well as the proliferation stimulation (%) are shown in Figures 35, 36, 37 and 38.

In relation to proliferation in spleen lymphocytes in basal conditions (Fig. 35A), the results showed that 3xTg-AD young and adult female as well as adult male mice showed values of lymphoproliferation similar to those found in NTg mice, whereas young 3xTg-AD males showed lower values ($p<0.01$) than in NTg counterparts.

Adult NTg females showed higher values ($p<0.01$) of lymphoproliferation than in young, whereas adult NTg males showed lower values ($p<0.001$) of lymphoproliferation than in young. 3xTg-AD adult females and males showed lymphoproliferation similar to those found in youngs. NTg young males showed higher values of lymphoproliferation than in females, whereas 3xTg-AD young males showed values similar to those found in females, but NTg and 3xTg-AD adult males showed lower values ($p<0.05$ and $p<0.01$ respectively) of lymphoproliferation than in females.

In relation to proliferation in spleen lymphocytes in response to ConA (Fig. 35B), the results showed that 3xTg-AD young and adult female as well as male mice showed lower lymphoproliferation than in NTg mice ($p<0.05$ in youngs and $p<0.001$ in adults) with higher differences in adults than in young mice. NTg and 3xTg-AD Adult females showed lower values ($p<0.01$ and $p<0.001$ respectively) of lymphoproliferation than in young, as well as NTg and 3xTg-AD Adult males showed lower values ($p<0.001$) of lymphoproliferation than in young mice.

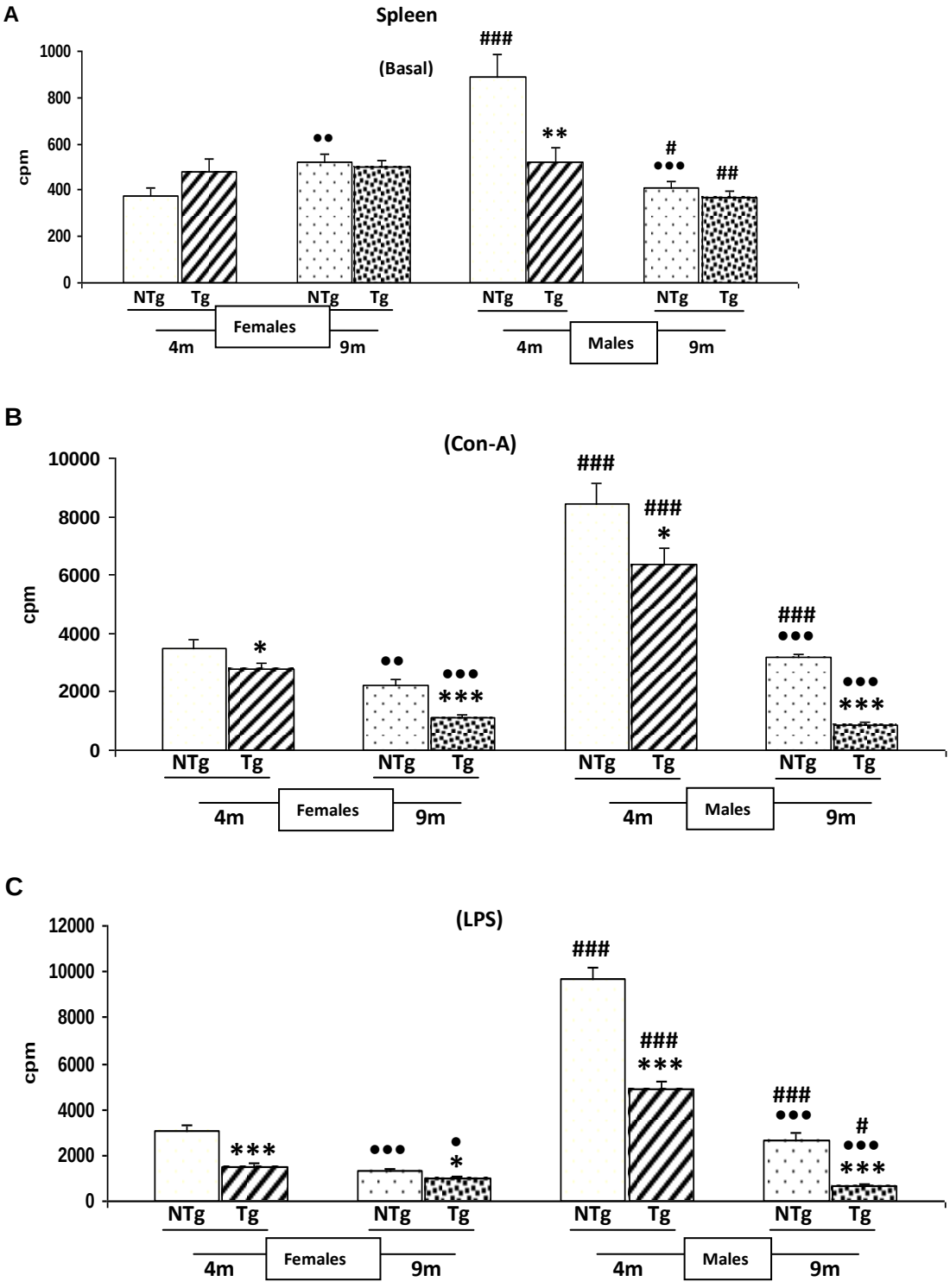


Fig. 35. Lymphoproliferation (counts/minute) of spleen lymphocytes in basal conditions (A) as well as in response to ConA (B) and LPS (C) from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females.

NTg and 3xTg-AD young as well as NTg adult males showed higher values ($p<0.001$) of lymphoproliferation than in females, whereas 3xTg-AD adult males showed values similar to those found in females. In relation to proliferation in spleen lymphocytes in response to LPS (Fig. 35C), the results showed that 3xTg-AD young and adult female as well as young and adult male mice showed lower lymphoproliferation than in NTg mice ($p<0.001$, $p<0.05$ and $p<0.001$ respectively) with higher differences found in adult males than in females. NTg and 3xTg-AD Adult females showed lower values ($p<0.001$ and $p<0.05$ respectively) of lymphoproliferation than in young, as well as NTg and 3xTg-AD Adult males showed lower values ($p<0.001$) of lymphoproliferation than in young mice. NTg and 3xTg-AD young mice showed higher values ($p<0.001$) of lymphoproliferation than in females, as well as NTg and 3xTg-AD adult males showed higher values ($p<0.001$ and $p<0.05$ respectively) of lymphoproliferation than in females.

In relation to proliferation index (%) in spleen lymphocytes in response to ConA (Fig. 36A), the results showed that 3xTg-AD young and adult female as well as adult male mice showed lower lymphoproliferation index than in NTg mice ($p<0.001$, $p<0.05$ and $p<0.001$ respectively). NTg and 3xTg-AD Adult females as well as 3xTg-AD Adult males showed lower values ($p<0.001$) of lymphoproliferation index than in young.

3xTg-AD young and NTg adult males showed higher values ($p<0.001$ and $p<0.01$ respectively) of lymphoproliferation index than in females, whereas NTg young and 3xTg-AD adult males showed values similar to those found in females.

In relation to proliferation index (%) in spleen lymphocytes in response to LPS (Fig. 36B), the results showed that 3xTg-AD young females and adult male mice showed lower lymphoproliferation index than in NTg mice ($p<0.001$). no differences were observed between 3xTg-AD adult females and NTg young males with respect to NTg mice. NTg and 3xTg-AD Adult females as well as NTg and 3xTg-AD Adult males showed lower values ($p<0.001$, $p<0.01$ and $p<0.001$ respectively) of lymphoproliferation index than in young mice. NTg and 3xTg-AD young males as well as NTg adult males showed higher values ($p<0.05$, $p<0.001$ and $p<0.01$ respectively) of lymphoproliferation index than in females, whereas 3xTg-AD adult males showed values similar to those found in females.

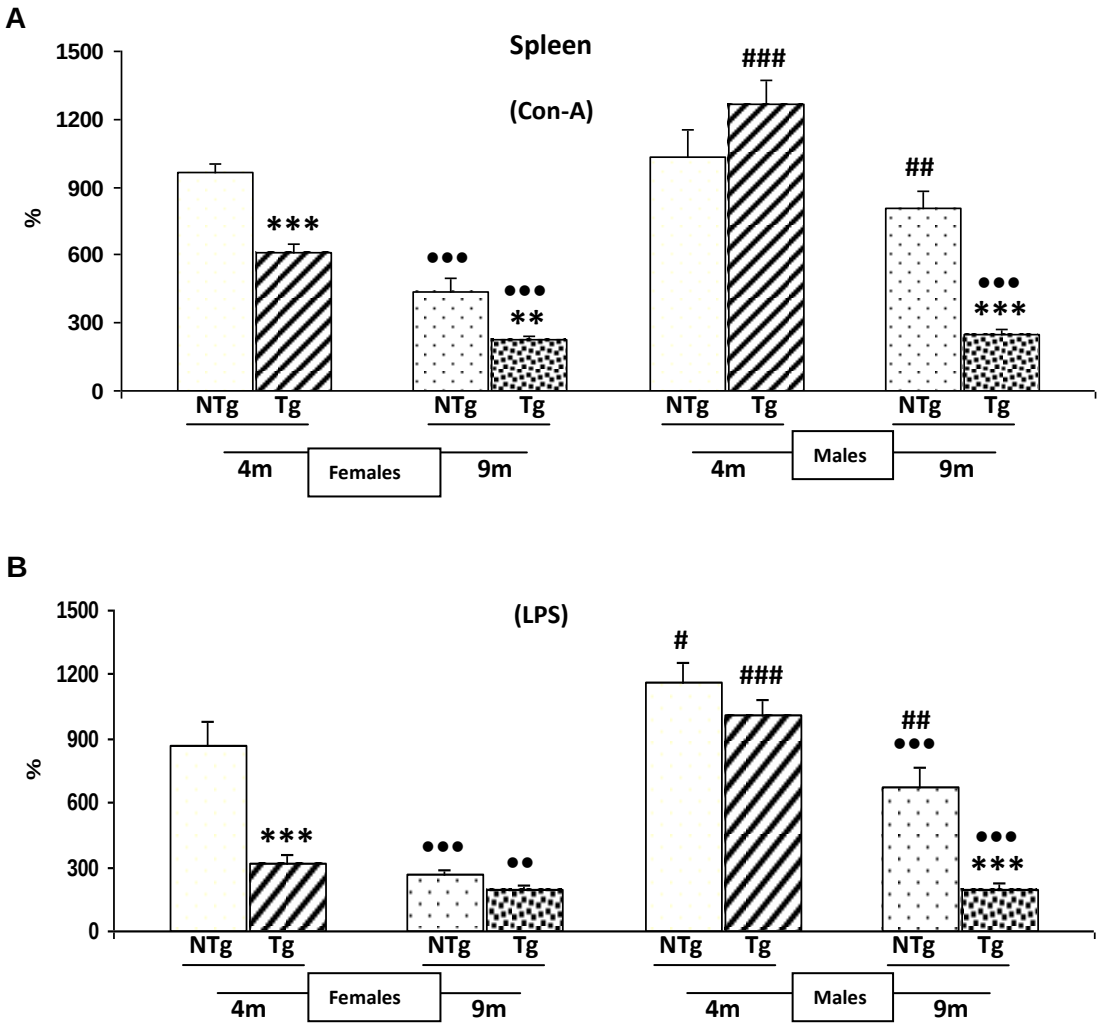


Fig. 36. Lymphoproliferation stimulation (%) of spleen lymphocytes in response to ConA (A) and LPS (B) from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females.

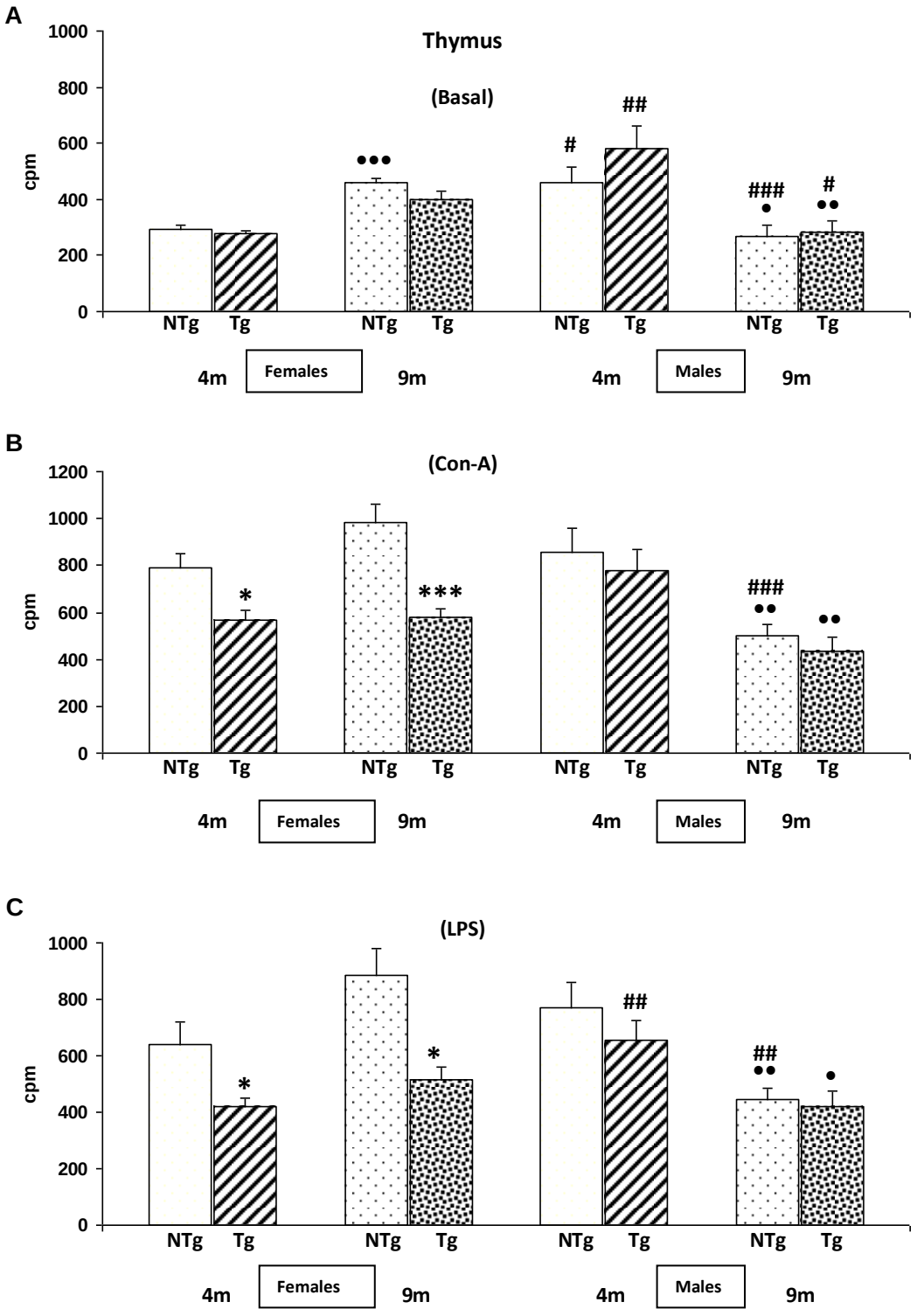


Fig. 37. Lymphoproliferation (counts/minute) of thymus lymphocytes in basal conditions (A) as well as in response to ConA (B) and LPS (C) from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females.

In relation to proliferation in thymus lymphocytes in basal conditions (Fig. 37A), the results showed that 3xTg-AD young and adult female as well as male mice showed values of lymphoproliferation similar to those found in NTg mice. Adult NTg females showed higher values ($p<0.001$) of lymphoproliferation than in young, whereas no differences were observed between 3xTg-AD adult and young females. NTg and 3xTg-AD Adult males showed lower values ($p<0.05$ and $p<0.01$ respectively) of lymphoproliferation than in young. NTg and 3xTg-AD young males showed higher values of lymphoproliferation than in females ($p<0.05$ and $p<0.01$ respectively), whereas NTg and 3xTg-AD adult males showed lower values than those found in females ($p<0.001$ and $p<0.05$ respectively).

In relation to proliferation in thymus lymphocytes in response to ConA (Fig. 37B), the results showed that 3xTg-AD young and adult female mice showed lower lymphoproliferation than in NTg mice ($p<0.05$ and $p<0.001$ respectively) with higher differences in adult than in young mice. 3xTg-AD young and adult males showed lymphoproliferation similar to those found in NTg mice. NTg and 3xTg-AD Adult females showed values of lymphoproliferation similar to those found in young mice, but NTg and 3xTg-AD Adult males showed lower values ($p<0.01$) of lymphoproliferation than in young mice. NTg and 3xTg-AD young as well as 3xTg-AD adult males showed values of lymphoproliferation similar to those found in females, whereas NTg adult males showed lower lymphoproliferation than in female.

In relation to proliferation in thymus lymphocytes in response to LPS (Fig. 37C), the results showed that 3xTg-AD young and adult female mice showed lower lymphoproliferation than in NTg mice ($p<0.05$ and $p<0.01$ respectively). 3xTg-AD young and adult males showed lymphoproliferation similar to those found in NTg mice.

NTg and 3xTg-AD Adult females showed values of lymphoproliferation similar to those found in young mice, but NTg and

3xTg-AD Adult males showed lower values ($p<0.01$ and $p<0.05$) of lymphoproliferation than in young mice.

NTg young and 3xTg-AD adult males showed values of lymphoproliferation similar to those found in females, whereas 3xTg-AD young and NTg adult males showed lower lymphoproliferation than in female ($p<0.01$).

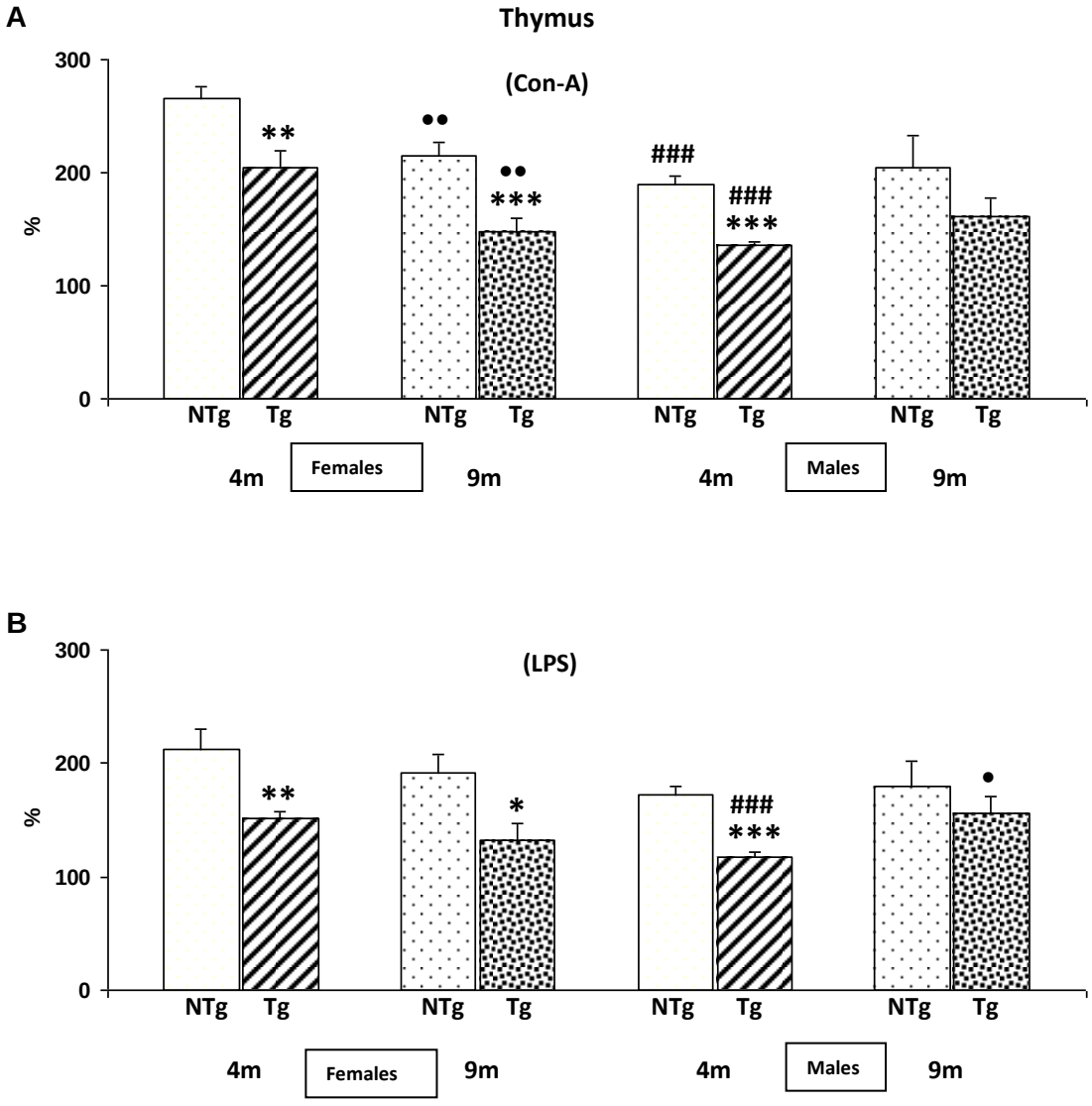


Fig. 38. Lymphoproliferation stimulation (%) of thymus lymphocytes in response to ConA (A) and LPS (B) from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ with respect to the corresponding value of control mice. •$p< 0.05$; ••$p< 0.01$; •••$p< 0.001$ with respect to the corresponding value of young. #$p< 0.05$;##$p<0.01$;###$p< 0.001$ with respect to the corresponding value of females.

In relation to proliferation index (%) in thymus lymphocytes in response to ConA (Fig. 38A), the results showed that 3xTg-AD young and adult female ($p<0.01$, $p<0.001$ respectively) as well as young male ($p<0.001$) mice showed lower lymphoproliferation index than in NTg mice. 3xTg-AD adult mice showed values of lymphoproliferation index similar to those found in NTg mice. NTg and 3xTg-AD Adult females showed lower values ($p<0.01$ and $p<0.001$ respectively) of lymphoproliferation index than in young, whereas NTg and 3xTg-AD Adult males showed values similar to those found in young mice. 3xTg-AD and NTg young males showed lower values ($p<0.001$) of lymphoproliferation index than in females, whereas NTg and 3xTg-AD adult males showed values similar to those found in females.

In relation to proliferation index (%) in thymus lymphocytes in response to LPS (Fig. 38B), the results showed that 3xTg-AD young and adult female ($p<0.01$, $p<0.05$ respectively) as well as young male ($p<0.001$) mice showed lower lymphoproliferation index than in NTg mice. 3xTg-AD adult mice showed values of lymphoproliferation index similar to those found in NTg mice. NTg and 3xTg-AD Adult females as well as 3xTg-AD adult males showed values of lymphoproliferation index similar to those found in young mice. 3xTg-AD young males showed lower values ($p<0.001$) of lymphoproliferation index than in females, whereas NTg young as well as NTg and 3xTg-AD adult males showed values similar to those found in females.

Table 10. Proliferation of spleen and thymus lymphocytes in (3xTg-AD) mice.

Lymphoproliferation							
Spleen			**(cpm)**			**(%)**	
Gender	**Age** (months)	**Mice**	**Basal**	**Con-A**	**LPS**	**Con-A**	**LPS**
Females	4	NTg	374±114	3509±904	3031±933	963±136	871±333
		Tg	479±185	2765±631*	1491±582***	608±130***	320±101***
	9	NTg	521±93••	2222±562••	1331±242•••	441±152•••	264±71•••
		Tg	502±69	1119±232***•••	995±317*•	225±47**•••	198±56••
Males	4	NTg	889±308###	8450±2236###	9721±1442###	1030±383	1168±272#
		Tg	520±204**	6340±2010*###	4882±947***###	1266±332###	1008±228###
	9	NTg	413±79••#	3177±302•••###	2676±746•••###	804±221##	675±243•••##
		Tg	370±83##	878±218***•••	687±217***•••#	246±71***•••	193±76***•••
Thymus			**(cpm)**			**(%)**	
Gender	**Age** (months)	**Mice**	**Basal**	**Con-A**	**LPS**	**Con-A**	**LPS**
Females	4	NTg	294±47	789±206	638±262	266±34	213±60
		Tg	276±36	566±136*	422±96*	205±44**	152±20**
	9	NTg	457±55•••	982±217	883±277	214±37••	191±44
		Tg	398±87•••	576±103***	515±134**	148±30***••	132±40*
Males	4	NTg	457±176#	855±320	770±283	190±21###	172±24
		Tg	582±250##	777±293	655±220##	136±11***###	117±15***###
	9	NTg	269±104•###	501±136••###	446±106••##	204±80	180±62
		Tg	282±112••#	437±164••	419±155•	161±44	157±42•

Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females.

4.3.1.2. Cytokines in cultures of lymphocytes from young and adult female and male transgenic Alzheimer Disease (3xTg-AD) mice

Levels of IL-2, IL-10, IL-1β and TNF-α in cultures of spleen lymphocytes in response to ConA and LPS from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice are shown in Figures 39, 40 and 41.

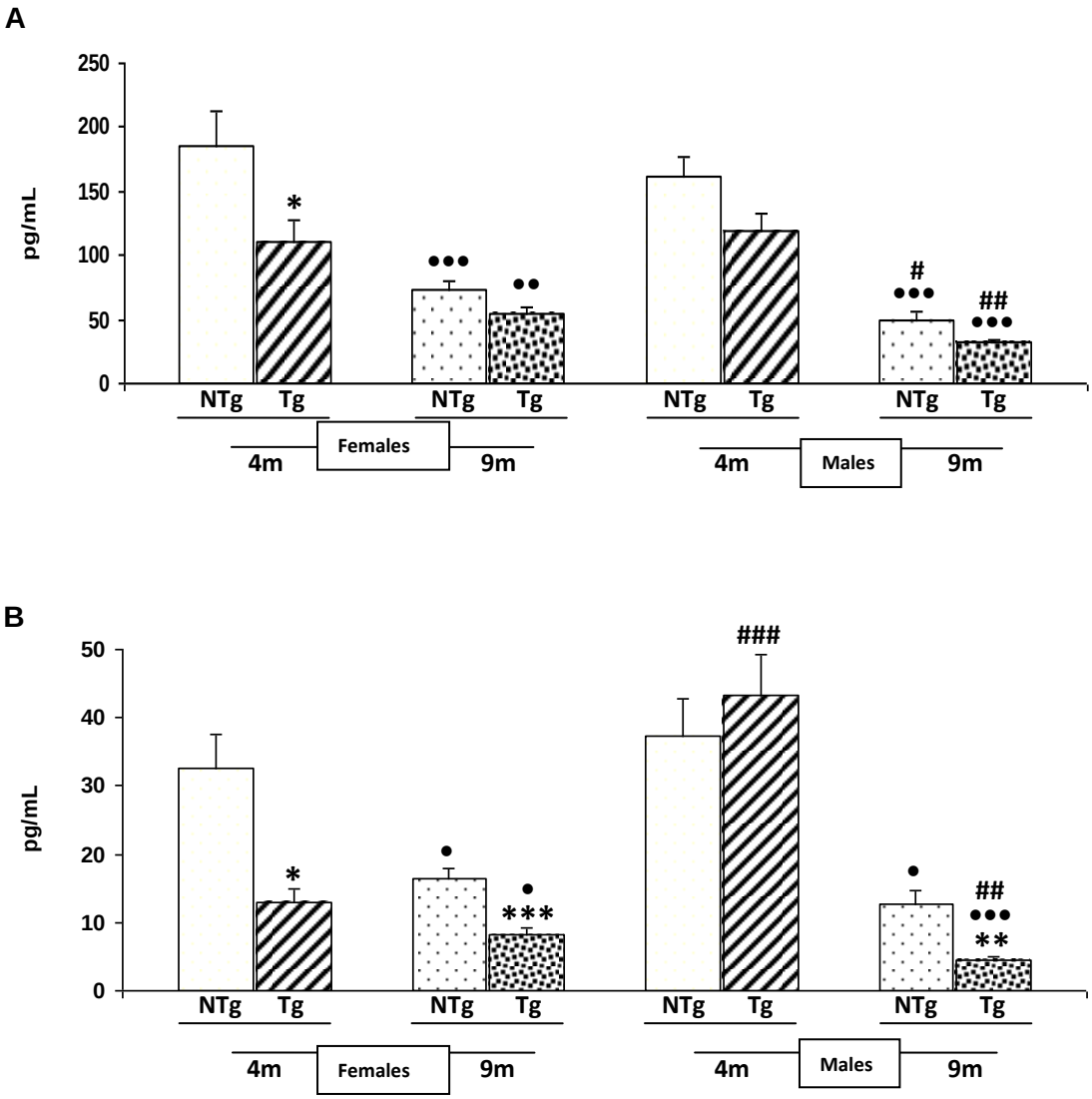


Fig. 39: levels of IL-2 in cultures of spleen lymphocytes in response to ConA (A) and LPS (B) from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8 values corresponding to 8 mice, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females. NTg: non transgenic. Tg: transgenic. m: months.

In relation to the levels of IL-2 in cultures of spleen lymphocytes in response to ConA (Fig. 39A) and LPS (Fig. 39B), the results showed that 3xTg-AD young female mice showed lower levels of IL-2 than in NTg mice ($p<0.05$), whereas no statistical differences were observed in young male mice. Transgenic adult female and male showed lower values of IL-2 than in NTg mice ($p<0.001$ and $p<0.01$ respectively).NTg and 3xTg-AD Adult females showed lower values ($p<0.05$) of IL-2 levels than in young mice, as well as NTg and 3xTg-AD Adult males showed lower values ($p<0.05$ and $p<0.001$ respectively) of IL-2 levels than in young mice. 3xTg-AD young and adult males showed higher values ($p<0.001$) of IL-2 levels than in females, whereas 3xTg-AD adult males showed lower values ($p<0.01$) to those found in females.

With respect to the levels of IL-10 in cultures of spleen lymphocytes in response to ConA (Fig. 40A) and LPS (Fig. 40B), the results showed that 3xTg-AD young female and male as well as adult female mice showed lower levels of IL-10 than in NTg mice ($p<0.001$, $p<0.01$ and $p<0.001$ respectively), whereas no statistical differences were observed in adult male mice. NTg and 3xTg-AD Adult females showed higher values ($p<0.01$ and $p<0.001$ respectively) of IL-10 levels than in young mice, but NTg and 3xTg-AD Adult males showed lower values ($p<0.001$ and $p<0.05$ respectively) of IL-10 levels than in young mice. NTg and 3xTg-AD young males showed higher levels of IL-10 ($p<0.001$) than in females, whereas NTg adult males showed lower values ($p<0.01$) to those found in females. 3xTg-AD adult males showed values similar to those found in females.

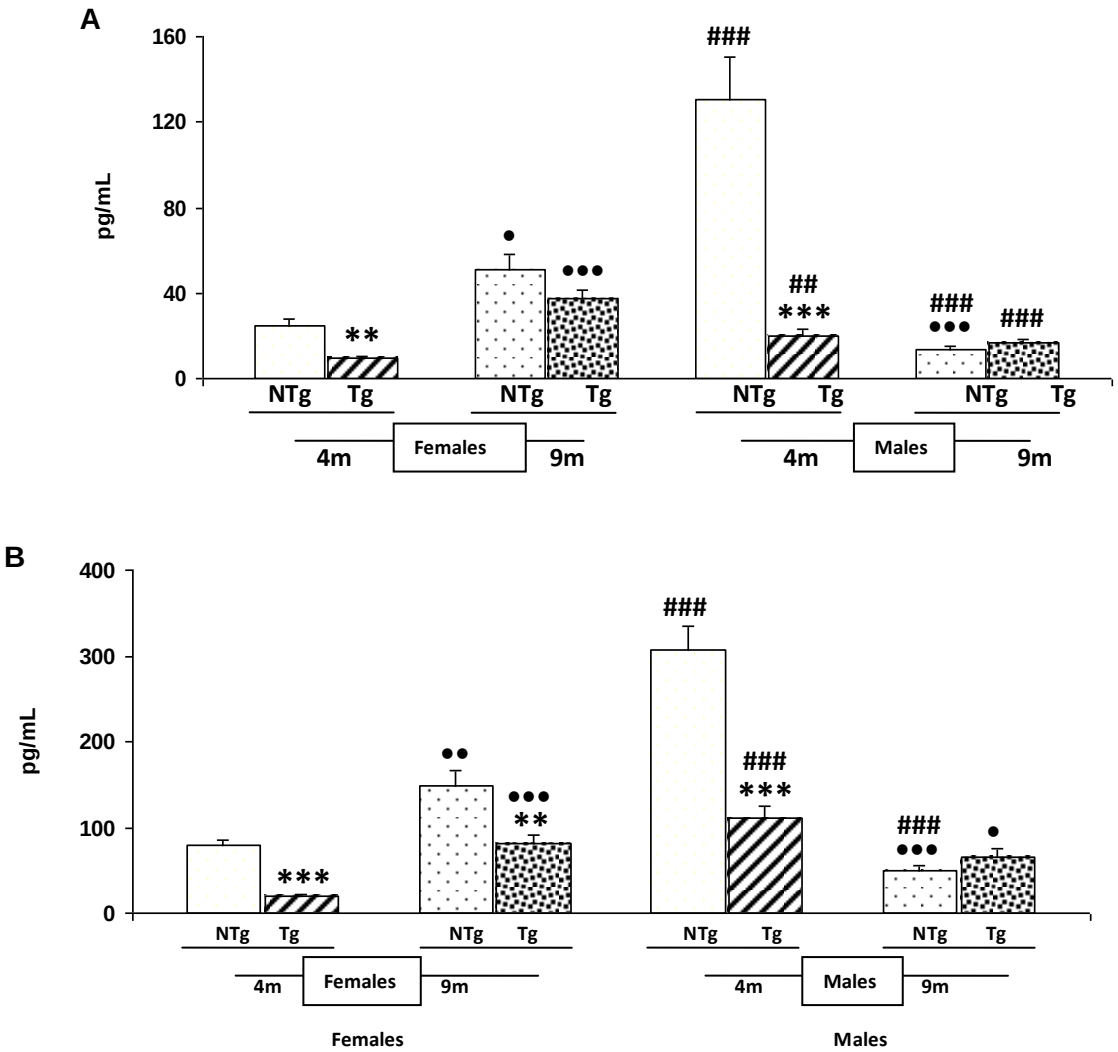


Fig. 40: levels of IL-10 in cultures of spleen lymphocytes in response to ConA (A) and LPS (B) from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8 values corresponding to 8 mice, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females. NTg: non transgenic. Tg: transgenic. m: months.

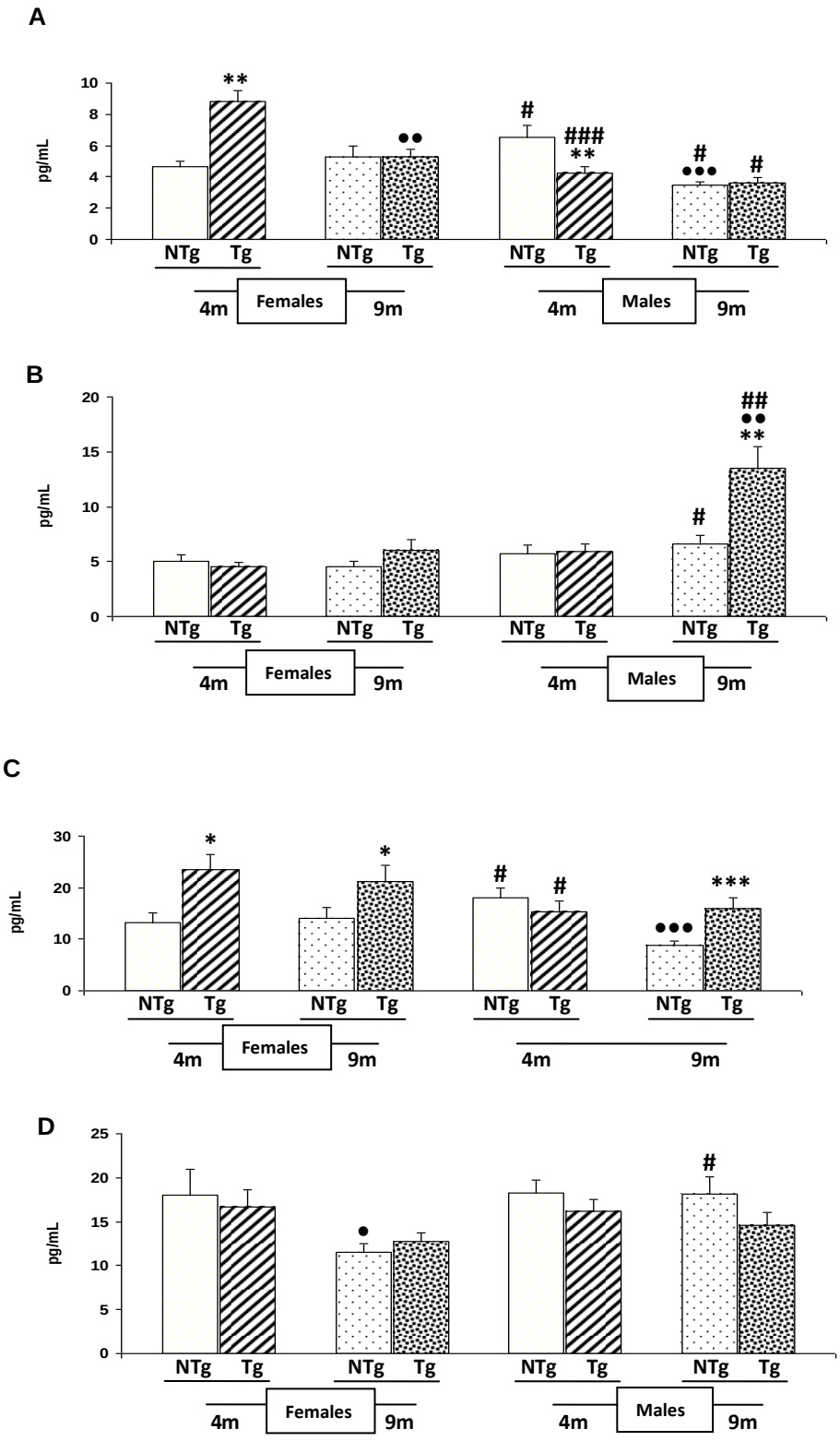


Fig. 41: levels of IL-1β in response to ConA (A) and LPS (B) and levels of TNF-α in response to ConA (C) and LPS (D) in cultures of spleen lymphocytes from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 8 values corresponding to 8 mice, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females. NTg: non transgenic. Tg: transgenic. m: months.

In relation to the levels of IL-1β in cultures of spleen lymphocytes in response to ConA (Fig. 41A), the results showed that 3xTg-AD young female showed higher levels of IL-1β ($p<0.01$) than in NTg mice in response to ConA, whereas young males showed lower levels of IL-1β than in NTg mice. No statistical differences were observed in adult male and female mice. 3xTg-AD adult females and NTg Adult males showed lower levels ($p<0.01$ and $p<0.001$ respectively) of IL-1β than in young mice in response to ConA. NTg young males showed higher levels of IL-1β ($p<0.05$) than in females, whereas 3xTg-AD young males ($p<0.001$) as well as NTg and 3xTg-AD adult males ($p<0.05$) showed lower levels to those found in females in response to ConA.

In relation to the levels of IL-1β in cultures of spleen lymphocytes in response to LPS (Fig. 41B), the results showed that 3xTg-AD young females and males as well as adult females showed similar levels of IL-1β as in NTg mice, whereas 3xTg-AD adult males showed higher levels ($p<0.01$) of IL-1β than in NTg mice. No statistical differences were observed in adult females compared with young mice. 3xTg-AD adult males showed higher levels ($p<0.01$) of IL-1β than in young mice. Young males showed similar levels of IL-1β as in females, whereas NTg and 3xTg-AD adult males showed higher levels ($p<0.05$ and $p<0.01$ respectively) to those found in females.

In relation to the levels of TNF-α in cultures of spleen lymphocytes in response to LPS (Fig. 41D), the results showed that 3xTg-AD young and adult females as well as males showed similar levels of TNF-α as in NTg mice. NTg adult females showed lower levels of TNF-α ($p<0.05$) than in young mice. No statistical differences were observed in adult NTg females and adult males compared with young mice. Only, NTg adult males showed higher levels ($p<0.05$) of TNF-α than in female mice.

In relation to the levels of TNF-α in cultures of spleen lymphocytes in response to ConA (Fig. 41C), the results showed that 3xTg-AD young and

adult females as well as adult males showed higher levels of TNF-α ($p<0.05$ in females and $p<0.001$ in males) than in NTg mice, whereas young males showed similar levels of TNF-α as in NTg mice. NTg adult males showed lower levels of TNF-α ($p<0.001$) than in young mice. No statistical differences were observed in adult females and 3xTg-AD adult males compared with young mice. NTg young males showed higher levels ($p<0.05$) of TNF-α than in female mice, on the contrary, 3xTg-AD young males showed lower levels ($p<0.05$) of TNF-α than in females. Adult males showed similar levels of TNF-α as in female mice.

Table 11: Levels of IL-2, IL-10, IL-1β and TNF-α in cultures of spleen lymphocytes in response to ConA and LPS from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and triple transgenic Alzheimer Disease (3xTg-AD) mice.

Spleen			**Con-A**			
Gender	**Age** (months)	**Mice**	**IL-2** (pg/ml)	**IL-10** (pg/ml)	**IL-1β** (pg/ml)	**TNF-α** (pg/ml)
Females	4	NTg	185±68	25±8	5±1	13±4
		Tg	111±38*	9±3**	9±2**	24±8*
	9	NTg	73±20$^{\bullet\bullet\bullet}$	51±21$^{\bullet}$	5±2	14±5
		Tg	54±17$^{\bullet\bullet}$	38±12$^{\bullet\bullet\bullet}$	5±1$^{\bullet\bullet}$	21±7*
Males	4	NTg	162±42	131±56$^{\#\#\#}$	7±2$^{\#}$	18±5
		Tg	120±37	20±7$^{**\#\#}$	4±1$^{**\#\#\#}$	15±6$^{\#}$
	9	NTg	49±22$^{\bullet\bullet\bullet\#}$	14±5$^{\bullet\bullet\bullet\#\#\#}$	4±1$^{\bullet\bullet\bullet\#}$	9±3$^{\bullet\bullet\bullet\#}$
		Tg	32±9$^{\bullet\bullet\bullet\#\#}$	16±6$^{\#\#\#}$	4±1$^{\#}$	16±6***
Spleen			**LPS**			
Gender	**Age** (months)	**Mice**	**IL-2** (pg/ml)	**IL-10** (pg/ml)	**IL-1β** (pg/ml)	**TNF-α** (pg/ml)
Females	4	NTg	33±13	80±15	5±1	18±6
		Tg	13±4*	20±5***	5±1	17±5
	9	NTg	17±4$^{\bullet}$	148±50$^{\bullet\bullet}$	5±1	12±3$^{\bullet}$
		Tg	8±3$^{***\bullet}$	81±25$^{**\bullet\bullet\bullet}$	6±3	13±3
Males	4	NTg	37±15	306±78	6±2	18±4
		Tg	43±16$^{\#\#\#}$	112±40***	6±2	16±4
	9	NTg	13±5$^{\bullet}$	50±15$^{\bullet\bullet\bullet}$	7±2$^{\#}$	18±6$^{\#}$
		Tg	4±2$^{**\bullet\bullet\bullet\#\#}$	65±27$^{\bullet}$	14±5$^{**\bullet\bullet\#\#}$	15±4

Each value is the mean ± SD of 8 values corresponding to 8 mice, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females. NTg: non transgenic. Tg: transgenic.

Cytokines in cultures of lymphocytes from thymus in young and adult female and male transgenic Alzheimer Disease (3xTg-AD) mice

Levels of IL-2 and IL-10 in cultures of thymus lymphocytes in response to ConA from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice are shown in Figure 42.

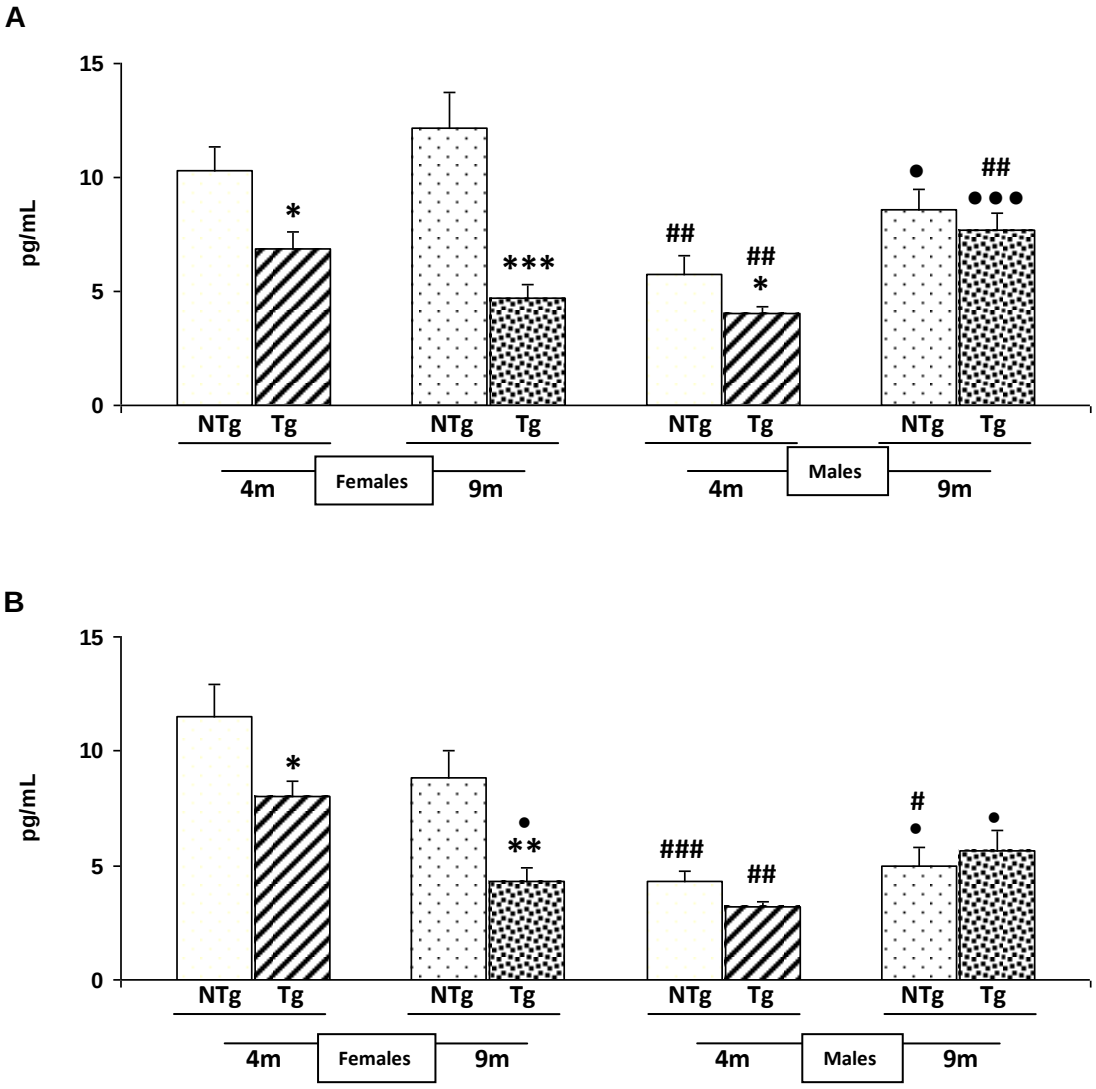


Fig. 42: levels of IL-2 (A) and IL-10 (B) in response to ConA in cultures of thymus lymphocytes from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice. Each value is the mean ± standard error of 6 values corresponding to 6 animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females. NTg: non transgenic. Tg: transgenic. m: months.

In relation to the levels of IL-2 in cultures of thymus lymphocytes in response to ConA (Fig. 42A), the results showed that 3xTg-AD young and adult females as well as adult males showed lower levels ($p<0.05$ in young females and males and $p<0.001$ in adult females) than in NTg mice, whereas adult males showed similar levels of IL-2 as in NTg mice. NTg and 3xTg-AD adult females showed similar levels of IL-2 to those found in young mice. Whereas, NTg and 3xTg-AD adult males showed higher levels of this cytokine ($p<0.05$ and $p<0.001$ respectively) compared to those found in young mice. NTg and 3xTg-AD adult males showed lower levels of this cytokine ($p<0.01$) compared to those found in female mice. Whereas, 3xTg-AD adult males showed higher levels of IL-2 ($p<0.01$) compared to those found in female mice.

In relation to the levels of IL-10 in cultures of thymus lymphocytes in response to ConA (Fig. 42B), the results showed that 3xTg-AD young and adult females showed lower levels ($p<0.05$ and $p<0.01$ respectively) of this cytokine than in NTg mice, whereas 3xTg-AD young and adult males showed similar levels of this cytokine to those found in NTg mice. 3xTg-AD adult females showed lower levels of IL-10 ($p<0.05$) than those found in NTg mice. Whereas, NTg and 3xTg-AD adult males showed higher levels of this cytokine ($p<0.05$) compared to those found in young mice.

NTg and 3xTg-AD young and NTg adult males showed lower levels of this cytokine ($p<0.001$, $p<0.001$ and $p<0.05$ respectively) compared to those found in female mice.

In cultures of lymphocytes from thymus, results showed that the levels of IL-2, IL-10, IL-1β and TNF-α in response to LPS in male and female mice, as well as the levels of IL-1β and TNF-α in response to ConA in young male and female mice were below the detectable limit. With respect to the levels of IL-1β in cultures of thymus lymphocytes, no significant differences were observed between 3xTg-AD and NTg adult mice. Similarly, no differences were observed between males and females Table

12.With respect to the levels of TNF-α in cultures of thymus lymphocytes, no significant differences were observed between 3xTg-AD and NTg adult female mice. Whereas 3xTg-AD males showed higher levels ($p<0.05$) of this cytokine than those found in NTg. Moreover, the NTg and 3xTg-AD males showed higher levels of this cytokine than those found in females Table 12.

Table 12. Levels of IL-2, IL-10, IL-1β and TNF-α in cultures of thymus lymphocytes in response to ConA and LPS from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and triple transgenic Alzheimer Disease (3xTg-AD) mice.

Thymus			**Con-A**			
Gender	**Age** (months)	**Mice**	**IL-2** (pg/ml)	**IL-10** (pg/ml)	**IL-1β** (pg/ml)	**TNF-α** (pg/ml)
Females	4	NTg	10±2	12±3	ND	ND
		Tg	7±2*****	8±3*****	ND	ND
	9	NTg	12±4	9±3	3.2±0.5	3.8±1
		Tg	5±1*******	4±1.5******$^{\bullet}$	4±0.9	6±2
Males	4	NTg	6±2$^{\#\#}$	4±1$^{\#\#\#}$	ND	ND
		Tg	4±1$^{\#\#}$	3±0.5$^{\#\#}$	ND	ND
	9	NTg	9±2$^{\bullet}$	5±2$^{\bullet\#}$	4±0.8	7±2$^{\#}$
		Tg	8±2*****$^{\bullet\bullet\bullet\#\#}$	6±2$^{\bullet}$	6±2	12±4*****$^{\#}$
Thymus			**LPS**			
Gender	**Age** (months)	**Mice**	**IL-2** (pg/ml)	**IL-10** (pg/ml)	**IL-1β** (pg/ml)	**TNF-α** (pg/ml)
Males and Females	**Youngs and adults**		**ND**			

Each value is the mean ± SD of 6 values corresponding to 6 animals, each value being the mean of duplicate assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ with respect to the corresponding value of control mice. •$p< 0.05$; ••$p< 0.01$ with respect to the corresponding value of young. #$p< 0.05$;##$p<0.01$;###$p< 0.001$ with respect to the corresponding value of females. NTg: non transgenic. Tg: transgenic. ND: non-detectable.

4.3.1.3. Antioxidant defense

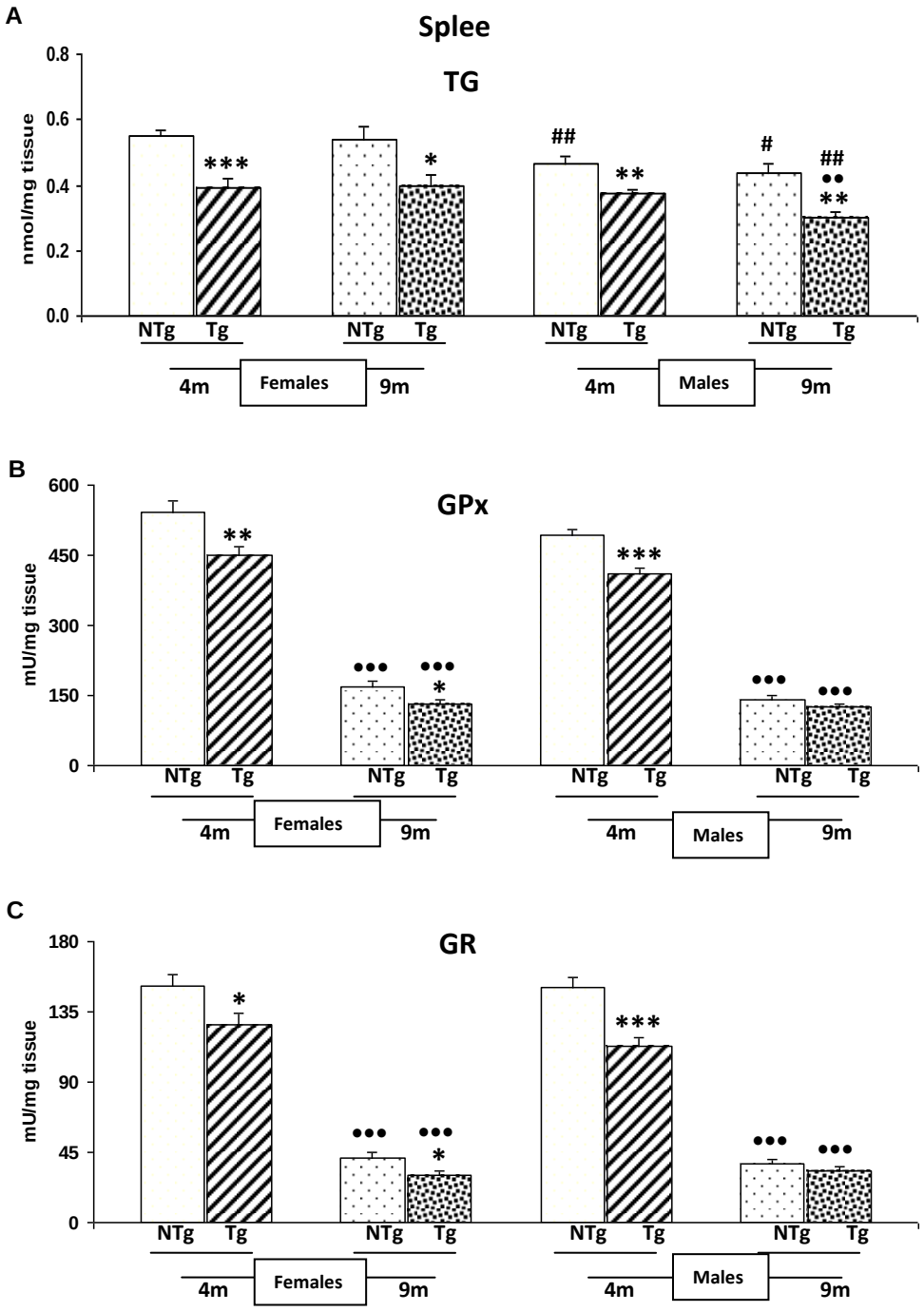


Fig.43. Total glutathione (TG) levels (A) and glutathione peroxidase (GPx) and (B) and glutathione reductase (GR) (C) activities from spleen of young (4 months) and adult (9 months) control NTg and 3xTg-AD female and male mice. Each column represents the mean±S.E. of 8-10 values (young 10, adult 8), each value being the mean of duplicate assays.*$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ with respect to the corresponding values of control NTg. ••$p< 0.01$; •••$p< 0.001$ with respect to the corresponding values in young mice. #$p < 0.05$; ##$p< 0.01$ with respect to the corresponding values in females.

In relation to antioxidant defense in spleen (Fig. 43), the results showed that 3xTg-AD young and adult female showed lower levels of total glutathione (TG) than those found in NTg mice ($p<0.001$ and $p<0.05$ respectively) (Fig. 43A), 3xTg-AD young and adult males showed lower levels ($p<0.01$) than in NTg counterparts. NTg and transgenic adult females as well as NTg adult males showed TG levels similar to those observed in young mice, whereas 3xTg-AD adult males showed lower levels ($p<0.01$) than in young mice. NTg young and adult as well as 3xTg-AD adult males showed lower TG levels than in females ($p<0.01$, $p<0.05$ and $p<0.01$ respectively), but 3xTg-AD young males showed levels similar to those found in females.

In relation to glutathione peroxidase (GPx) activity (Fig. 43B), the results showed that 3xTg-AD young and adult female as well as 3xTg-AD adult males showed lower glutathione peroxidase (GPx) activity than those found in NTg mice ($p<0.01$, $p<0.05$ and $p<0.001$ respectively) with lower differences in adult females, 3xTg-AD adult males showed GPx activiy similar to those found in NTg counterparts. NTg and transgenic adult females as well as males showed lower GPx activity than those found in young mice ($p<0.001$).

NTg and 3xTg-AD young as well as adult males showed GPx activiy similar to those found in females. In relation to glutathione reductase (GR) activity (Fig. 43C), the results showed that 3xTg-AD young and adult female as well as 3xTg-AD adult males showed lower glutathione reductase (GR) activity than those found in NTg mice ($p<0.05$, $p<0.05$ and $p<0.001$ respectively), 3xTg-AD adult males showed GR activiy similar to those found in NTg counterparts. NTg and transgenic adult females as well as males showed lower GR activity than those found in young mice ($p<0.001$). NTg and 3xTg-AD young as well as adult males showed GR activity similar to those found in females.

Table 13A. Antioxidant defense in spleen of young (4 months) and mature (9 months) control NTg and 3xTg-AD female and male mice.

Antioxidant defense	Gender	Age (months)	Mice	Spleen
TG (nmol/mg tissue)	***Females***	**4**	NTg	0.55±0.05
			3xTg-AD	0.39±0.08***
		9	NTg	0.54±0.11
			3xTg-AD	0.40±0.08*
	Males	**4**	NTg	0.47±0.06$^{\#\#}$
			3xTg-AD	0.37±0.05**
		9	NTg	0.43±0.09$^{\#}$
			3xTg-AD	0.30±0.05**$^{\bullet\bullet\#\#}$
GPx (mU/mg tissue)	***Females***	**4**	NTg	542±73
			3xTg-AD	451±56**
		9	NTg	168±37$^{\bullet\bullet\bullet}$
			3xTg-AD	131±21*$^{\bullet\bullet\bullet}$
	Males	**4**	NTg	492±40
			3xTg-AD	409±34***
		9	NTg	141±27$^{\bullet\bullet\bullet}$
			3xTg-AD	124±19$^{\bullet\bullet\bullet}$

Each value is the mean±S.D of 8-10 values Each value is the mean±S.D of 8-10 values (young 10, adult 8), each value being the mean of duplicate assays.*p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding values of control NTg. ••p< 0.01; •••p< 0.001 with respect to the corresponding values in young mice. #p < 0.05; ##p< 0.01 with respect to the corresponding values in females.

Table 13B. Antioxidant defense in spleen of young (4 months) and mature (9 months) control NTg and 3xTg-AD female and male mice.

GR (mU/mg tissue)	***Females***	**4**	NTg	152±21
			3xTg-AD	127±22*****
		9	NTg	41±11**•••**
			3xTg-AD	30±8***•••**
	Males	**4**	NTg	150±20
			3xTg-AD	113±14*******
		9	NTg	38±7**•••**
			3xTg-AD	33±9**•••**

4.3.2. Effects of ovariectomy in leucocytes functional parameters and redox state.

In this section of the results, the functional and redox state of several parameters in adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice are shown. The samples analyzed were obtained from spleen and thymus.

4.3.2.1 Functional parameters

The results corresponding to the effect of overiectomy on chemotaxis, antitumoral NK cell activity, proliferation (cpm) of spleen lymphocytes, proliferation stimulation (%) of lymphocytes in both spleen and thymus of adult triple transgenic Alzheimer's disease (3xTg-AD) female mice are shown in figures 44-49 and in tables 14 and 15.

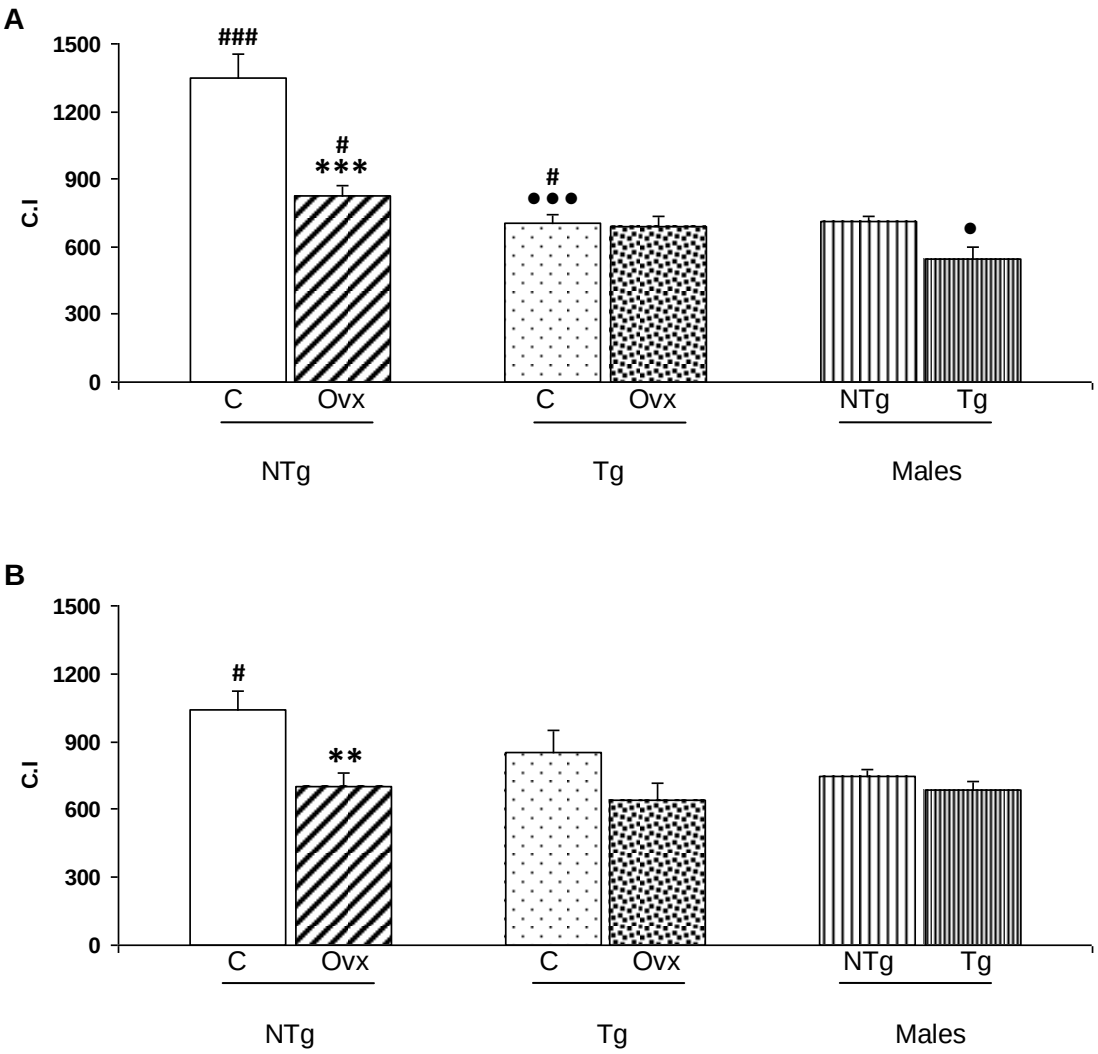


Fig. 44. Effects of ovariectomy (Ovx) on leukocyte chemotaxis in spleen (A) and thymus (B) from adult non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *$p < 0.05$;**$p < 0.01$; ***$p < 0.001$ with respect to the corresponding value of control. •••$p< 0.001$ with respect to the corresponding value of non-transgenic. #$p< 0.05$;###$p<0.001$with respect to the corresponding value of males.

Results showed that ovariectomy decreases the chemotaxis index of spleen and thymus leukocytes ($p<0.001$ and $p<0.01$ respectively) in NTg mice (Fig. 44), but has no effect in 3xTg-AD mice which already have low chemotaxis index with respect to NTg mice.

Spleen leukocytes from NTg males showed lower chemotaxis index than in both control (non-ovariectomized) ($p<0.001$) and ovariectomized ($p<0.05$) females, whereas Spleen leukocytes from 3xTg-AD males showed lower chemotaxis index

than in Tg-control females ($p<0.05$) but showed similar values with respect to Tg-Ovx females.

Thymus leukocytes from NTg males showed lower chemotaxis index than in control (non-ovariectomized) ($p<0.05$) and but similar values with respect to NTg-Ovx females, whereas thymus leukocytes from 3xTg-AD males showed similar values with respect to females.

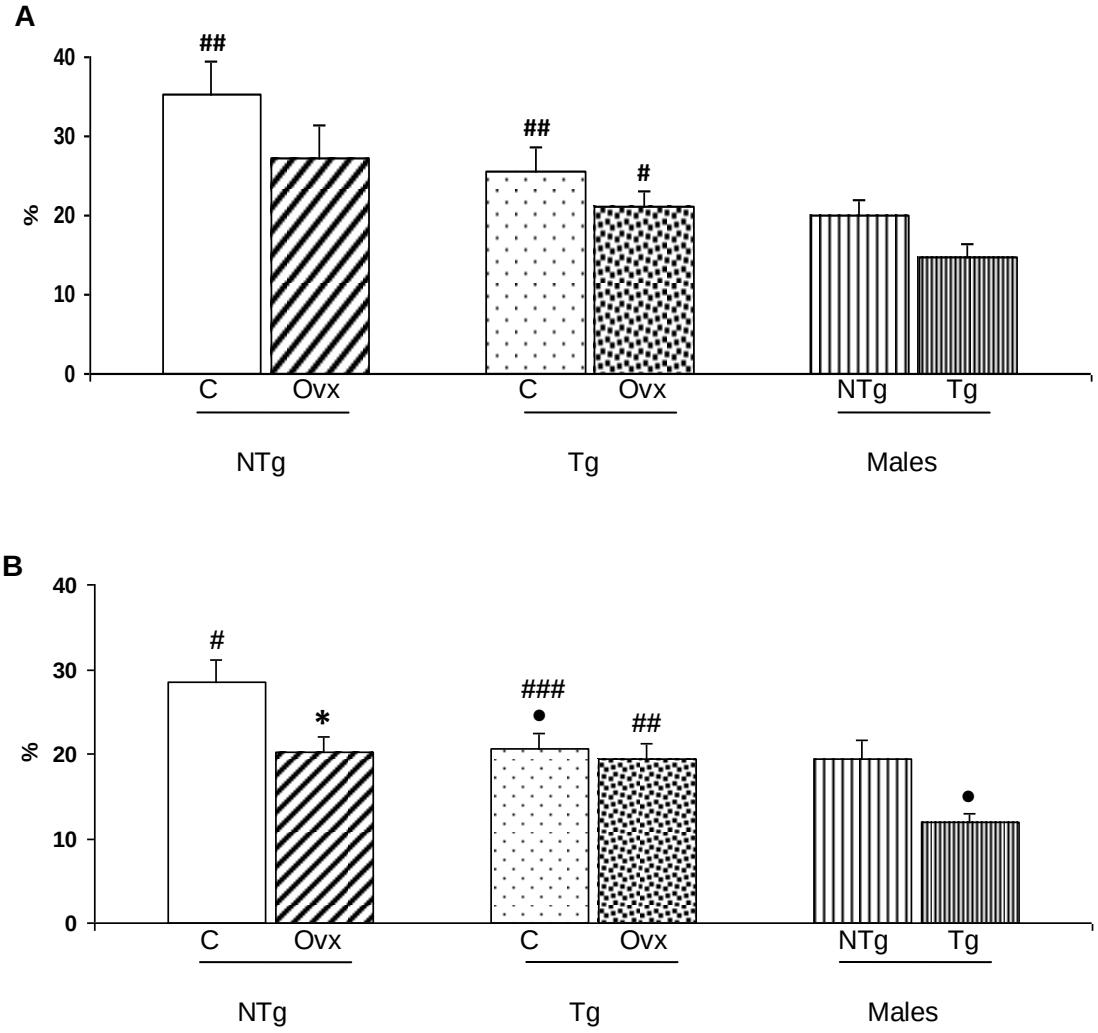


Fig. 45. Effects of ovariectomy (Ovx) on antitumoral NK activity (percentage of lysis of mice tumoral cells) in spleen (A) and thymus (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *$p < 0.05$ with respect to the corresponding value of control. •$p< 0.05$ with respect to the corresponding value of non-transgenic. #$p< 0.05$;##$p<0.01$with respect to the corresponding value of males.

The results obtained from spleen (Fig. 45A), results showed that ovariectomy has no effect on the NK activity in NTg as well as in 3xTg-AD mice. NTg males showed lower NK activity than in control (non-ovariectomized) females ($p<0.01$), but showed similar values with respect to NTg-Ovx females. 3xTg-AD males showed lower NK activity than in both Tg-control and Tg-Ovx females ($p<0.01$ and $p<0.05$ respectively).

The results obtained in thymus (Fig. 45B), results showed that ovariectomy decreases the NK activity in NTg mice ($p<0.05$), but has no effect in 3xTg-AD mice. NTg males showed lower NK activity than in control (non-ovariectomized) females ($p<0.05$), but showed similar values with respect to NTg-Ovx females. 3xTg-AD males showed lower NK activity than in both Tg-control and Tg-Ovx females ($p<0.001$ and $p<0.01$ respectively).

Table 14. Effects of ovariectomy on NK activity (% of tumor lysis) and chemotaxis capacity (Chemotaxis index) of spleen and thymus leukocytes from adult non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice.

	NK (%)		**Chemotaxis Index (C.I)**	
	Spleen	**Thymus**	**Spleen**	**Thymus**
FNTg	35±12	28±8$^{\#}$	1352±290$^{\#\#\#}$	1040±242$^{\#}$
FOvxNTg	27±12	20±6*****	830±115*******$^{\#}$	700±180******
FTg	26±8$^{\#\#}$	21±5$^{\bullet\#\#\#}$	703±117$^{\bullet\bullet\bullet\#}$	850±291
FOvxTg	21±6$^{\#}$	20±5$^{\#\#}$	686±143	643±198
MNTg	20±6	19±6	715±60	746±89
MTg	15±5	12±3$^{\bullet}$	542±165$^{\bullet}$	684±110

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. **$p < 0.01$; ***$p < 0.001$ with respect to the corresponding value in the corresponding control. •$p< 0.05$; ••$p< 0.01$; •••$p< 0.001$ with respect to the corresponding value of non-transgenic. #$p< 0.05$;##$p<0.01$;###$p< 0.001$ with respect to the corresponding value of males. F:female, M: male, Ovx : ovariectomized female.

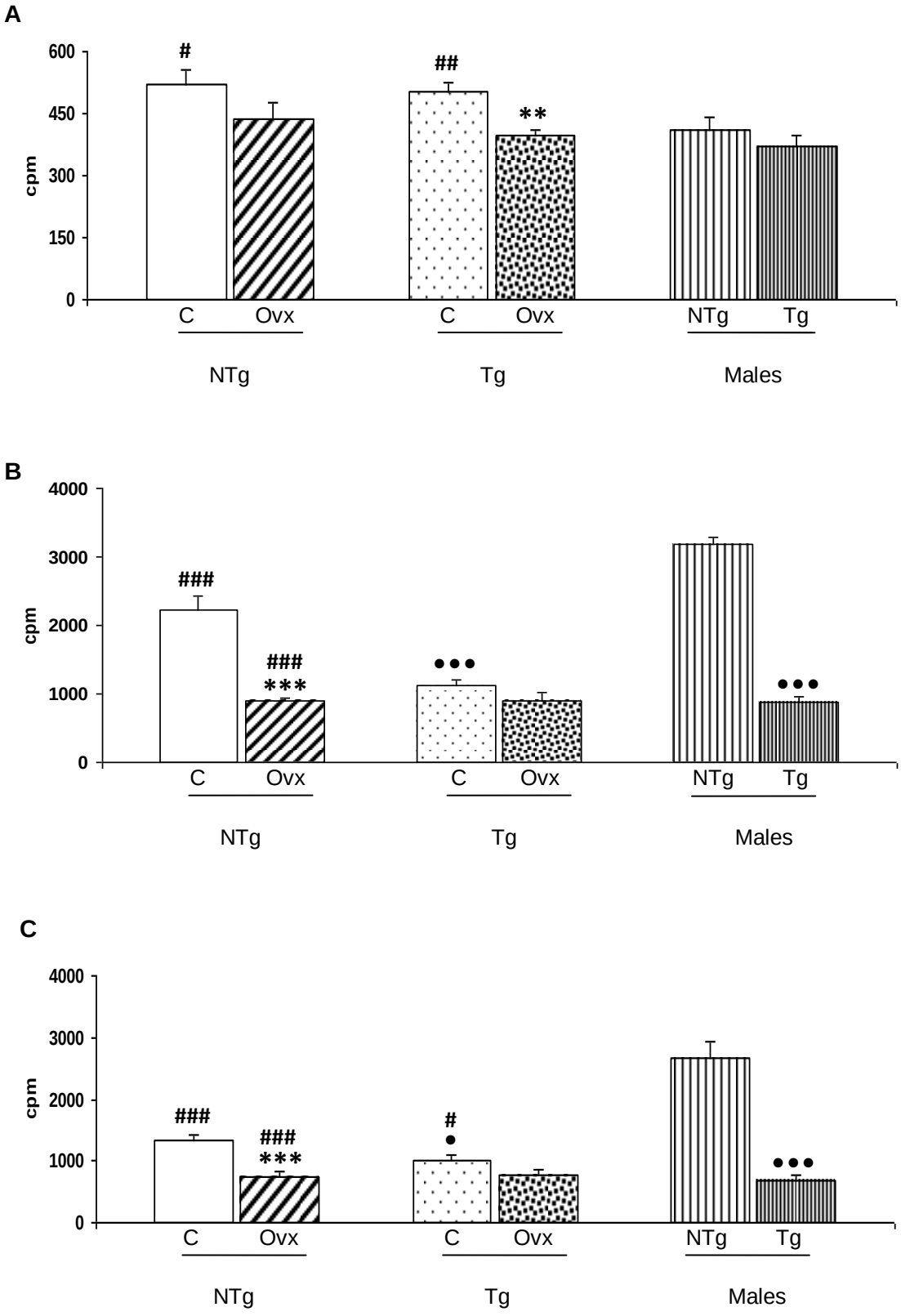


Fig. 46. Effects of ovariectomy (Ovx) on proliferation (cpm) of spleen lymphocytes in basal conditions (A), in response to ConA (B) and in response to LPS (C) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals,

each value being the mean of duplicate assays. **p < 0.01; ***p < 0.001 with respect to the corresponding value of control. •p< 0.05; •••p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of males.

With respect to proliferation in spleen lymphocytes in basal conditions (Fig. 46A), the results showed that the ovariectomy has no effect in NTg mice, but decreases the lymphoproliferation in 3xTg-AD ($p<0.01$). NTg males showed lower proliferation than in control females ($p<0.05$), but showed similar values with respect to NTg-Ovx females, similarly, 3xTg-AD males showed lower proliferation than in Tg-control ($p<0.01$) but similar to Tg-Ovx females.

In relation to proliferation in spleen lymphocytes in response to ConA (Fig. 46B) and LPS (Fig. 46C), the results showed that the ovariectomy decreases the proliferation in NTg mice ($p<0.001$) in response to ConA as well as LPS, but has no effect in 3xTg-AD mice. NTg males showed higher proliferation in response to ConA and LPS than in NTg females ($p<0.001$), but 3xTg-AD males showed similar values of proliferation as in transgenic control as well as ovariectomized (Ovx) females.

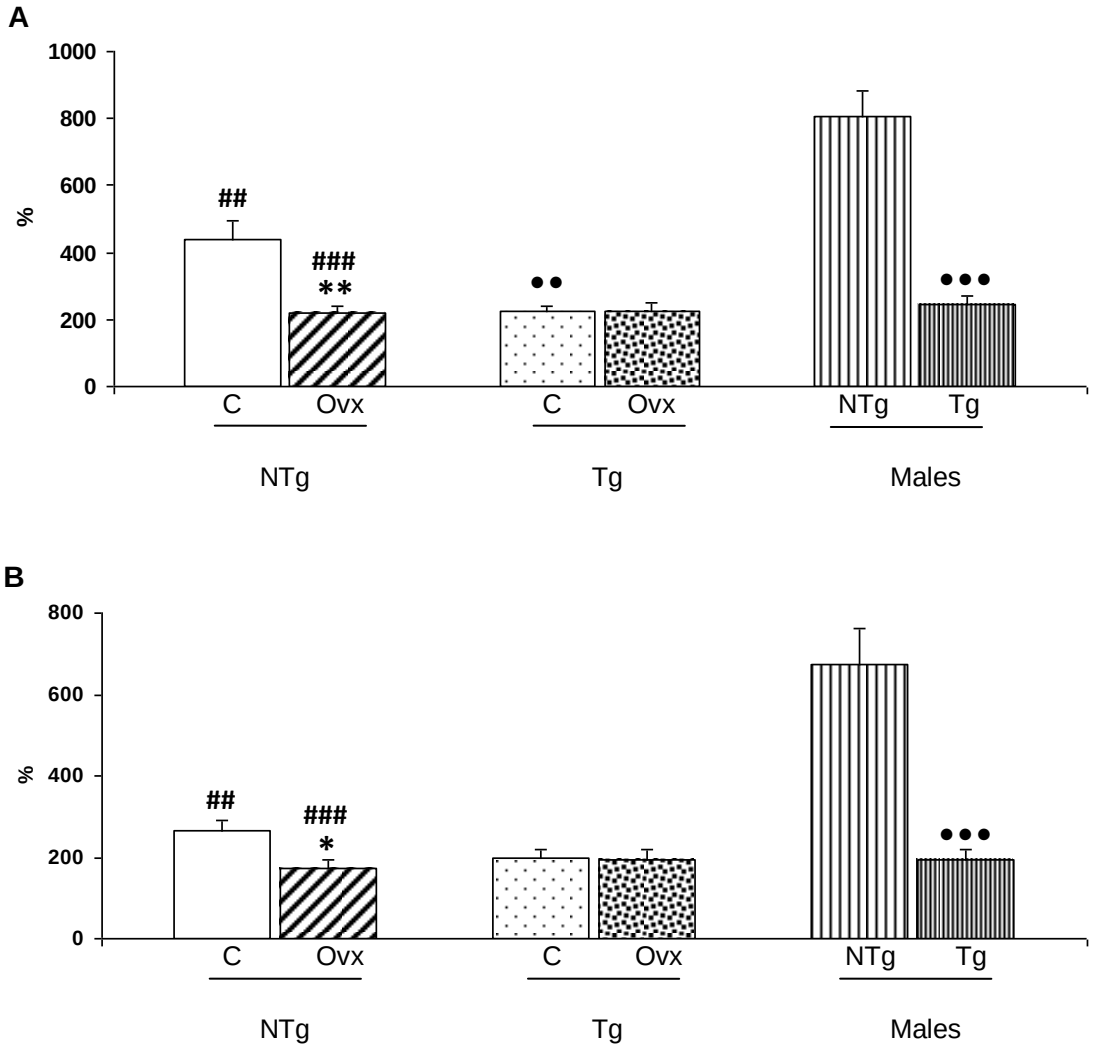


Fig. 47. Effects of ovariectomy (Ovx) on proliferation (%) of spleen lymphocytes in response to ConA (A) and LPS (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control. ••p< 0.01 with respect to the corresponding value of non-transgenic. ##p<0.01;###p< 0.001 with respect to the corresponding value of males.

With respect to proliferation index (%) in spleen lymphocytes in response to ConA (Fig. 47A) and LPS (Fig. 47B), the results showed that the ovariectomy decreases the proliferation index in response to ConA ($p<0.01$) as well as in response to LPS ($p<0.05$) in NTg mice, but has no effect in 3xTg-AD mice. NTg males showed higher proliferation index in response to ConA and LPS than in NTg control as well as ovariectomized (Ovx) females ($p<0.01$ and $p<0.001$

respectively), but 3xTg-AD males showed similar values of proliferation index as in transgenic control and Ovx females.

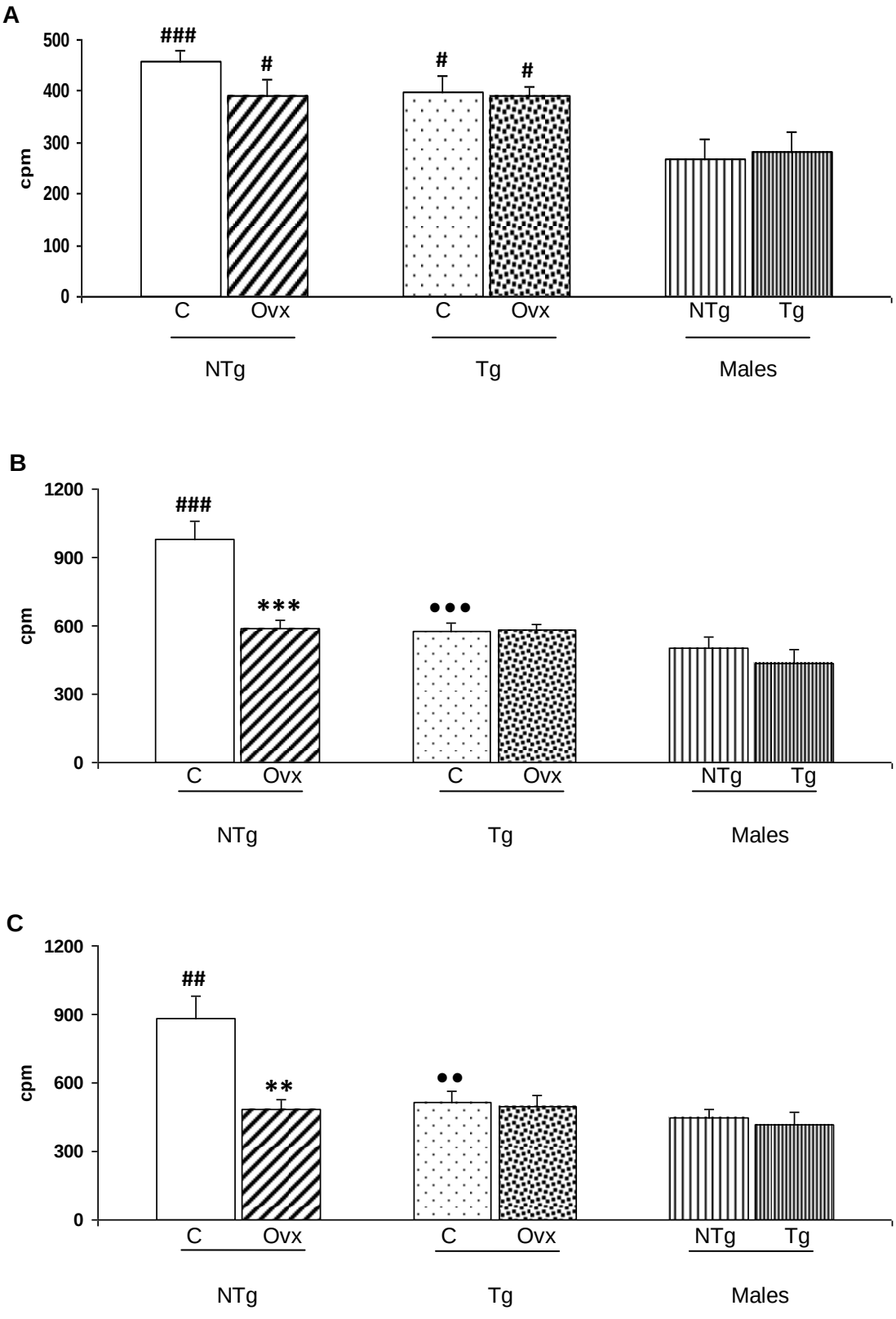


Fig. 48. Effects of ovariectomy (Ovx) on proliferation (cpm) of thymus lymphocytes in basal conditions (A), in response to ConA (B) and in response to LPS (C) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. **p < 0.01; ***p < 0.001 with respect to the corresponding value of control. ••p< 0.01; •••p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of males.

With respect to proliferation in thymus lymphocytes in basal conditions (Fig. 48A), the results showed that the ovariectomy has no effect in both NTg and 3xTg-AD mice. NTg males showed lower proliferation than in NTg-control ($p<0.001$) and NTg-Ovx ($p<0.05$) females. 3xTg-AD males showed lower proliferation than in 3xTg-AD-control and 3xTg-AD-Ovx ($p<0.05$) females.

In relation to proliferation in spleen lymphocytes in response to ConA (Fig. 48B) and LPS (Fig. 48C), the results showed that the ovariectomy decreases the proliferation in NTg mice in response to ConA ($p<0.001$) and LPS ($p<0.01$), but has no effect in 3xTg-AD mice. NTg males showed lower proliferation in response to ConA ($p<0.001$) and LPS ($p<0.01$) than in NTg-control females, but similar values of proliferation as in as in NTg- Ovx females. 3xTg-AD males showed similar values of proliferation as in transgenic control as well as Ovx females.

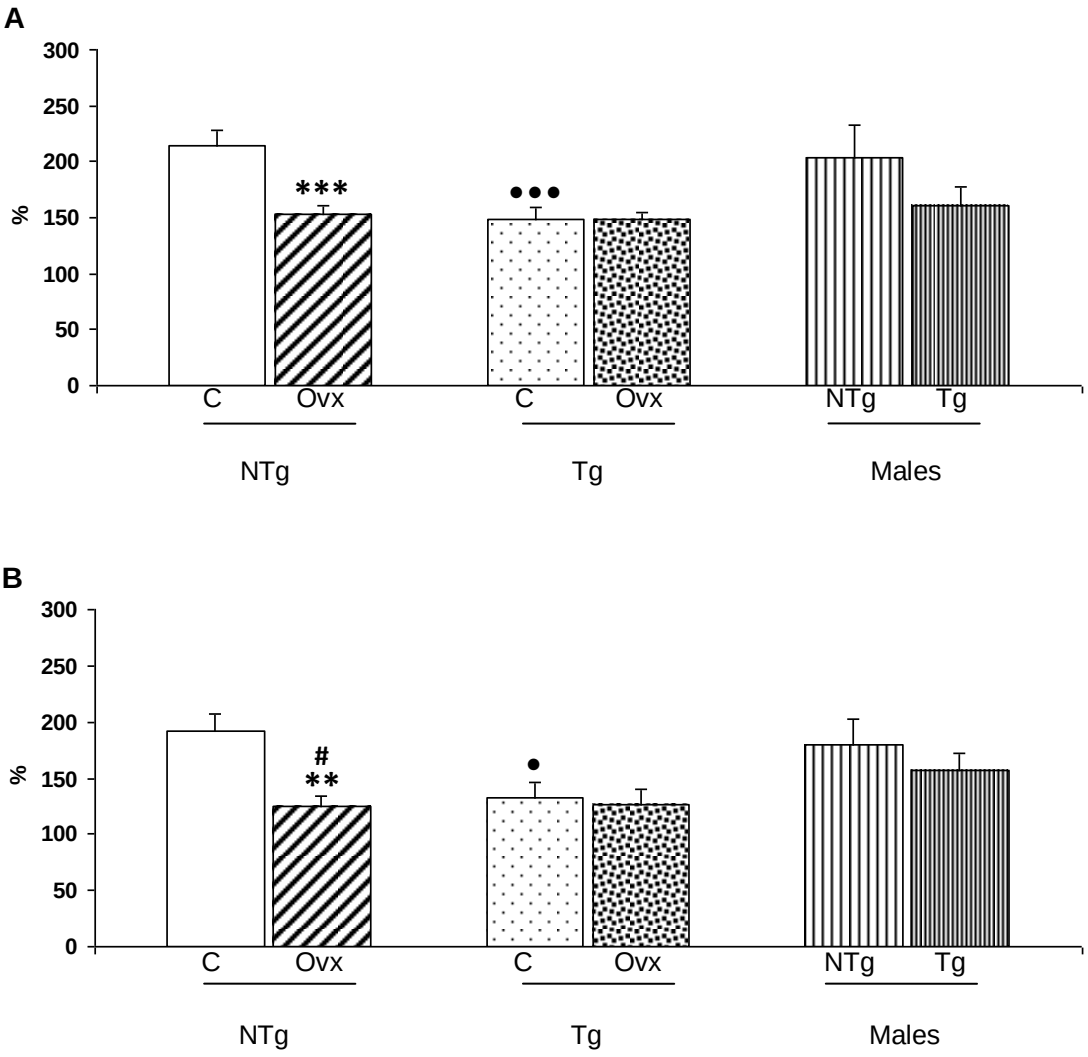


Fig. 49. Effects of ovariectomy (Ovx) on proliferation stimulation (%) of thymus lymphocytes in response to ConA (A) and LPS (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. **$p < 0.01$; ***$p < 0.001$ with respect to the corresponding value of control. •$p< 0.05$; •••$p< 0.001$ with respect to the corresponding value of non-transgenic. #$p< 0.05$ with respect to the corresponding value of males.

With respect to proliferation index (%) in spleen lymphocytes in response to ConA (Fig. 49A) and LPS (Fig. 49B), the results showed that the ovariectomy decreases the proliferation index in response to ConA ($p<0.001$) as well as in response to LPS ($p<0.01$) in NTg mice, but has no effect in 3xTg-AD mice. NTg

and 3xTg-AD males showed similar proliferation index in response to ConA and LPS as in females.

Table 15. Effects of ovariectomy on proliferation of spleen and thymus lymphocytes from Adult non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice in basal conditions, as well as in response to ConA and LPS.

Lymphoproliferation					
	(cpm)			**(%)**	
Spleen	**Basal**	**Con-A**	**LPS**	**Con-A**	**LPS**
FNTg	521±93$^{\#}$	2222±562$^{\#\#\#}$	1331±242$^{\#\#\#}$	441±152$^{\#\#}$	264±71$^{\#\#}$
FOvxNTg	435±115	904±107$^{***\#\#\#}$	732±220$^{***\#\#\#}$	218±55$^{**\#\#\#}$	174±57$^{*\#\#\#}$
FTg	502±69$^{\#\#}$	1119±232$^{\bullet\bullet\bullet}$	995±317$^{\bullet\#}$	225±47$^{\bullet\bullet}$	198±56
FOvxTg	395±46**	904±333	765±286	226±66	194±65
MNTg	413±79	3177±302	2676±746	804±221	676±243
MTg	370±83	878±218$^{\bullet\bullet\bullet}$	687±217$^{\bullet\bullet\bullet}$	246±71$^{\bullet\bullet\bullet}$	194±76$^{\bullet\bullet\bullet}$
	(cpm)			**(%)**	
Thymus	**Basal**	**Con-A**	**LPS**	**Con-A**	**LPS**
FNTg	457±55$^{\#\#\#}$	982±217$^{\#\#\#}$	883±277$^{\#\#}$	215±37	191±44
FOvxNTg	391±90$^{\#}$	589±98***	483±124**	153±19***	125±25$^{**\#}$
FTg	398±90$^{\#}$	576±103$^{\bullet\bullet\bullet}$	515±134$^{\bullet\bullet}$	148±30$^{\bullet\bullet\bullet}$	132±40$^{\bullet}$
FOvxTg	392±44$^{\#}$	579±81	496±141	148±17	127±37
MNTg	269±104	501±136	446±106	204±80	180±62
MTg	282±112	437±164	419±155	161±44	157±42

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays.*p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of males. %: percentage obtained giving 100 to the cpm in basal condition.

Levels of Interleukins released in spleen leucocyte cultures

The results corresponding to the effect of overiectomy on the levels of IL-2, IL-10, IL-1β and TNF-α released in cultures of lymphocytes from spleen in response to Con-A and LPS from adult 3xTg-AD and NTg mice are shown in Figures 50, 51 and 52 and in table 16.

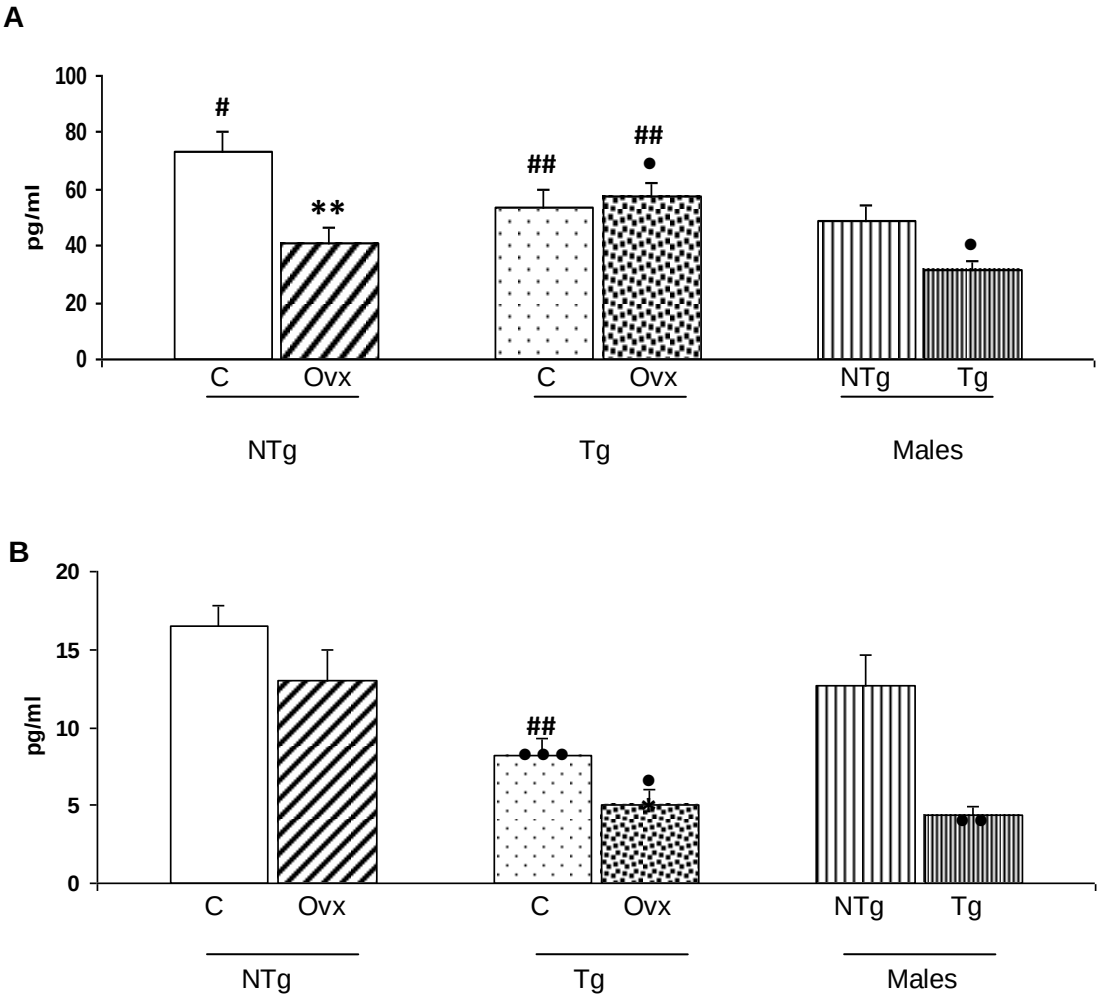


Fig. 50: Effects of ovariectomy (Ovx) on levels of IL-2 in cultures of spleen lymphocytes in response to Con-A (A) and LPS (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. **p < 0.01; ***p < 0.001 with respect to the corresponding value of control. •p< 0.05; •••p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05 with respect to the corresponding value of males.

The results showed that the ovariectomy decreases ($p<0.01$) the levels of IL-2 in cultures of spleen lymphocytes in response to Con-A (Fig. 50A), but has no effects on the levels of this cytokine in 3xTg-AD mice. NTg females showed higher ($p<0.05$) levels of IL-2 in cultures of spleen lymphocytes in response to Con-A than in males. Whereas, NTg-Ovx showed similar levels of this cytokine to those found in males.Control and overiectomized 3xTg-AD females showed higher levels of IL-2 in cultures of spleen lymphocytes in response to Con-A than in males. In relation to the levels of IL-2 in cultures of spleen lymphocytes in response to LPS (Fig. 50B), the results showed that the ovariectomy decreases ($p<0.05$) the levels of this cytokine in 3xTg-AD mice. 3xTg-AD control females showed higher ($p<0.01$) levels of this cytokine than in males.

The results showed that the ovariectomy decreases the levels of IL-10 ($p<0.01$) in response to LPS and Con-A in cultures of spleen lymphocytes (Fig. 51A and B), but has no effects on the levels of this cytokine in 3xTg-AD mice. NTg females showed higher levels of IL-10 ($p<0.01$) in cultures of spleen lymphocytes in response to LPS than in males. Whereas, NTg-Ovx showed similar levels of this cytokine to those found in males. Control and ovx 3xTg-AD females showed NTg-Ovx showed similar levels of this cytokine in response to LPS found in males. .

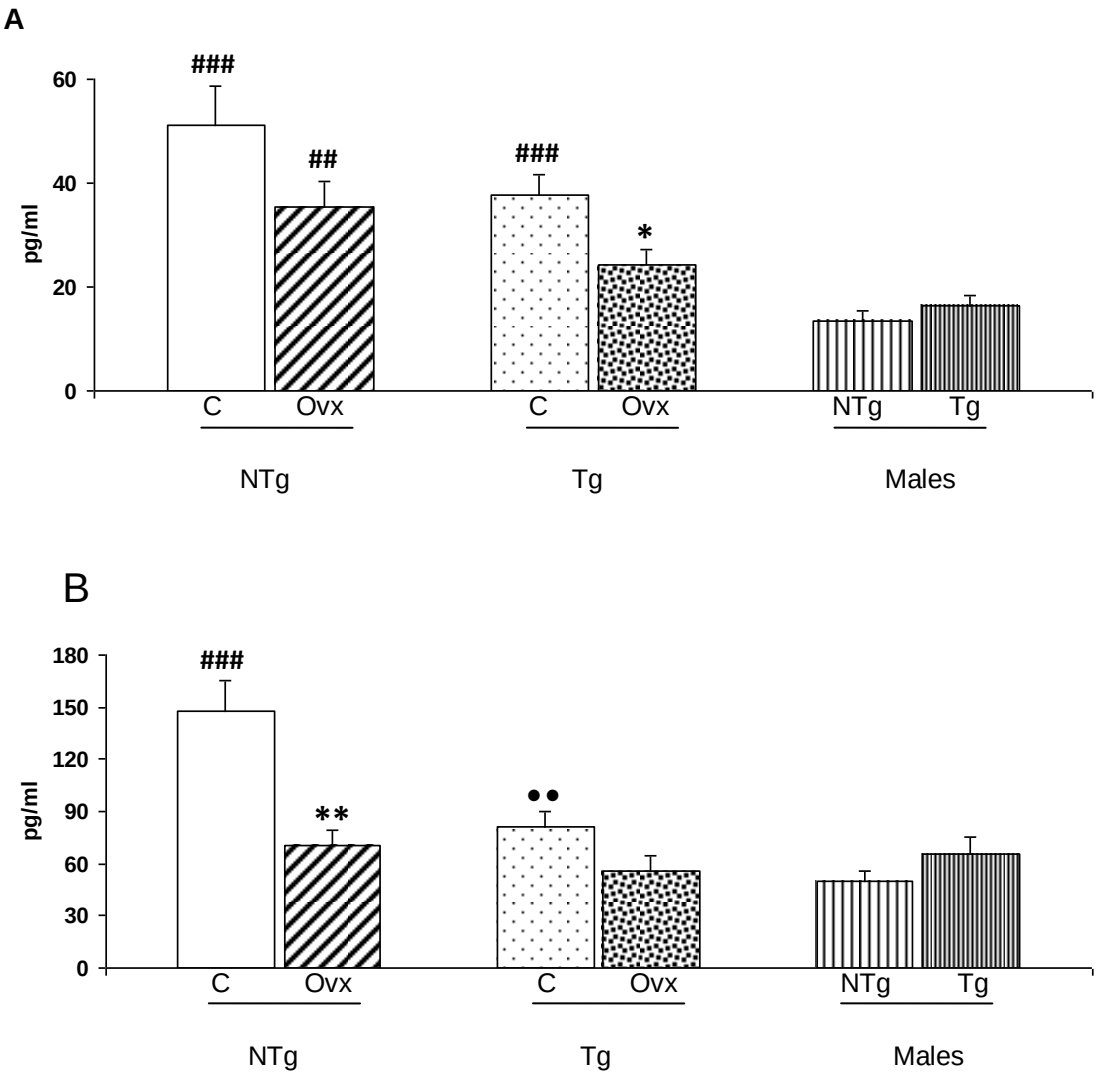


Fig. 51: Effects of ovariectomy (Ovx) on levels of IL-10 in cultures of spleen lymphocytes in response to ConA (A) and LPS (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. **p < 0.01; ***p < 0.001 with respect to the corresponding value of control. •p< 0.05; •••p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05 with respect to the corresponding value of males.

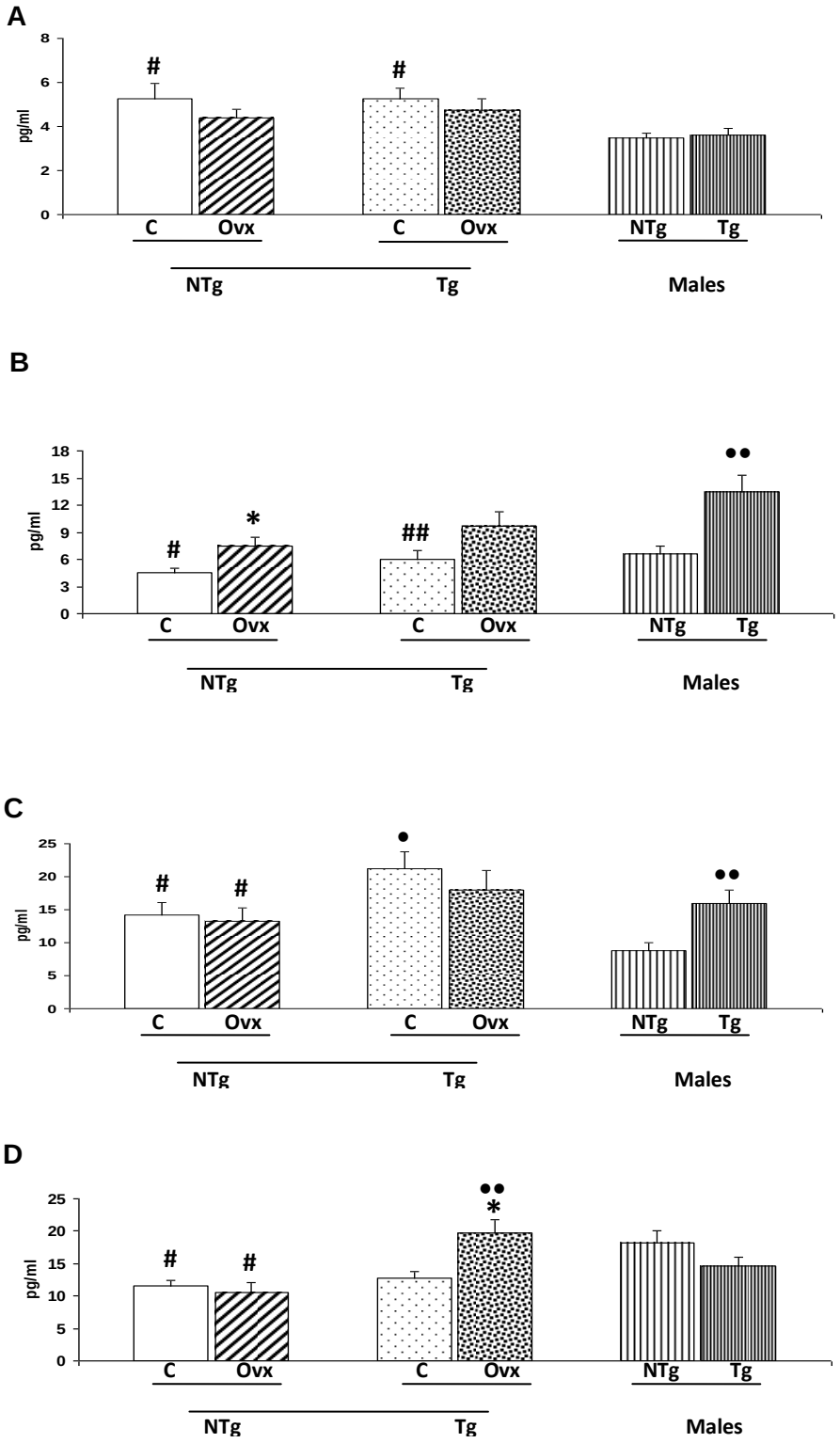


Fig. 52: Effects of ovariectomy (Ovx) on levels of IL-1β in response to ConA (A) and LPS (B) and levels of TNF-α in response to ConA (C) and LPS (D) in cultures of spleen lymphocytes from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. **p < 0.01; ***p < 0.001 with respect to the corresponding value

of control. •p< 0.05; •••p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05 with respect to the corresponding value of males.

Table 16: Levels of IL-2, IL-10, IL-1β and TNF-α in cultures of spleen lymphocytes in response to ConA and LPS from young (4 moths) and adult (9 months) female and male non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) mice.

Spleen	***Con-A***			
	IL-2 (pg/ml)	**IL-10** (pg/ml)	**IL-1β** (pg/ml)	**TNF-α** (pg/ml)
FNTg	73±20$^{\#}$	51±21$^{\#\#\#}$	5±2$^{\#}$	14±5$^{\#}$
FOvxNTg	41±15**	35±14$^{\#\#}$	4±1	13±4$^{\#}$
FTg	54±17$^{\#\#}$	38±12$^{\#\#\#}$	5±1$^{\#}$	21±7$^{\bullet}$
FOvxTg	57±14$^{\bullet\#\#}$	24±9*	5±1	18±6
MNTg	49±16	14±5	3.5±0.5	9±3
MTg	32±9$^{\bullet}$	16±6	3.6±0.9	16±6$^{\bullet\bullet}$
Spleen	***LPS***			
	IL-2 (pg/ml)	**IL-10** (pg/ml)	**IL-1β** (pg/ml)	**TNF-α** (pg/ml)
FNTg	17±4	148±50$^{\#\#\#}$	4.5±1.4$^{\#}$	13±3$^{\#}$
FOvxNTg	13±5	70±24**	7±3*	11±4$^{\#}$
FTg	8±3$^{\bullet\bullet\bullet\#\#}$	81±26$^{\bullet\bullet}$	6±3$^{\#\#}$	13±3
FOvxTg	5±2$^{*\bullet}$	56±23	10±4	20±6$^{*\bullet\bullet}$
MNTg	13±5	50±15	7±2	18±6
MTg	4±2$^{\bullet\bullet}$	65±27	14±5$^{\bullet\bullet}$	15±4

Each value is the mean ± standard error of 8-10 values corresponding to 10 young and 8 adult animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001 with respect to the corresponding value of control mice. •p< 0.05; ••p< 0.01; •••p< 0.001 with respect to the corresponding value of young. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding value of females.

With respect to the levels of IL-1β, the results showed that the ovariectomy increases (p<0.05) the levels of IL-1β in cultures of spleen lymphocytes in response to LPS only in NTg females (Fig. 52B), but has no effects on the levels of this cytokine in response to Con-A. Ovariectomized mice showed similar levels of

this cytokine to those found in males in response to Con-A. Control NTg and 3xTg-AD females showed lower ($p<0.05$ and $p<0.01$ respectively) levels of IL-1β in cultures of spleen lymphocytes in response to LPS than in males. Whereas, no statistical differences were observed in the levels of this cytokine in response to LPS between ovariectomized mice and males.

With respect to the levels of TNF-α, the results showed that the ovariectomy increases ($p<0.05$) the levels of this cytokine in cultures of spleen lymphocytes in response to LPS only in 3xTg females (Fig. 52D), but has no effects on the levels of this cytokine in response to Con-A. Ovariectomized 3xTg mice showed similar levels of this cytokine to those found in males in response to both Con-A and LPS.

Antioxidant defences: Total glutathione (TG) and the enzymes of its cycle; glutathione peroxidase (GPx) and glutathione reductase (GR)

The results corresponding to the effect of overiectomy on total glutathione (TG) levels and the antioxidant enzyme activities involved in its oxidation-reduction reactions, glutathione peroxidase (GPx) and glutathione reductase (GR) in spleen from adult NTg and 3xTg-AD mice are shown in Figures 53 and in table 17.

The results showed that the ovariectomy significantly decreases the total glutathione (TG) levels in both NTg and 3xTg-AD mice ($p<0.001$). NTg males showed lower TG levels ($p<0.05$) than in NTg control female mice, but higher levels ($p<0.001$) than in NTg-Ovx females. Similarly, 3xTg-AD males showed lower TG levels ($p<0.01$) than in 3xTg-AD control female mice, but higher levels ($p<0.001$) than in 3xTg-AD-Ovx females.

The results showed that the ovariectomy significantly decreases the GPx and GR activities ($p<0.05$ and $p<0.01$ respectively) in NTg mice, but has no effect in 3xTg-AD mice, which already have lower activities with respect to NTg mice. NTg and 3xTg-AD males showed GPx activity similar to those found in female mice, NTg males showed GR activity similar to those found in NTg control female mice, but higher than in NTg-Ovx females, whereas 3xTg-AD males showed GR activity similar to those found in female mice.

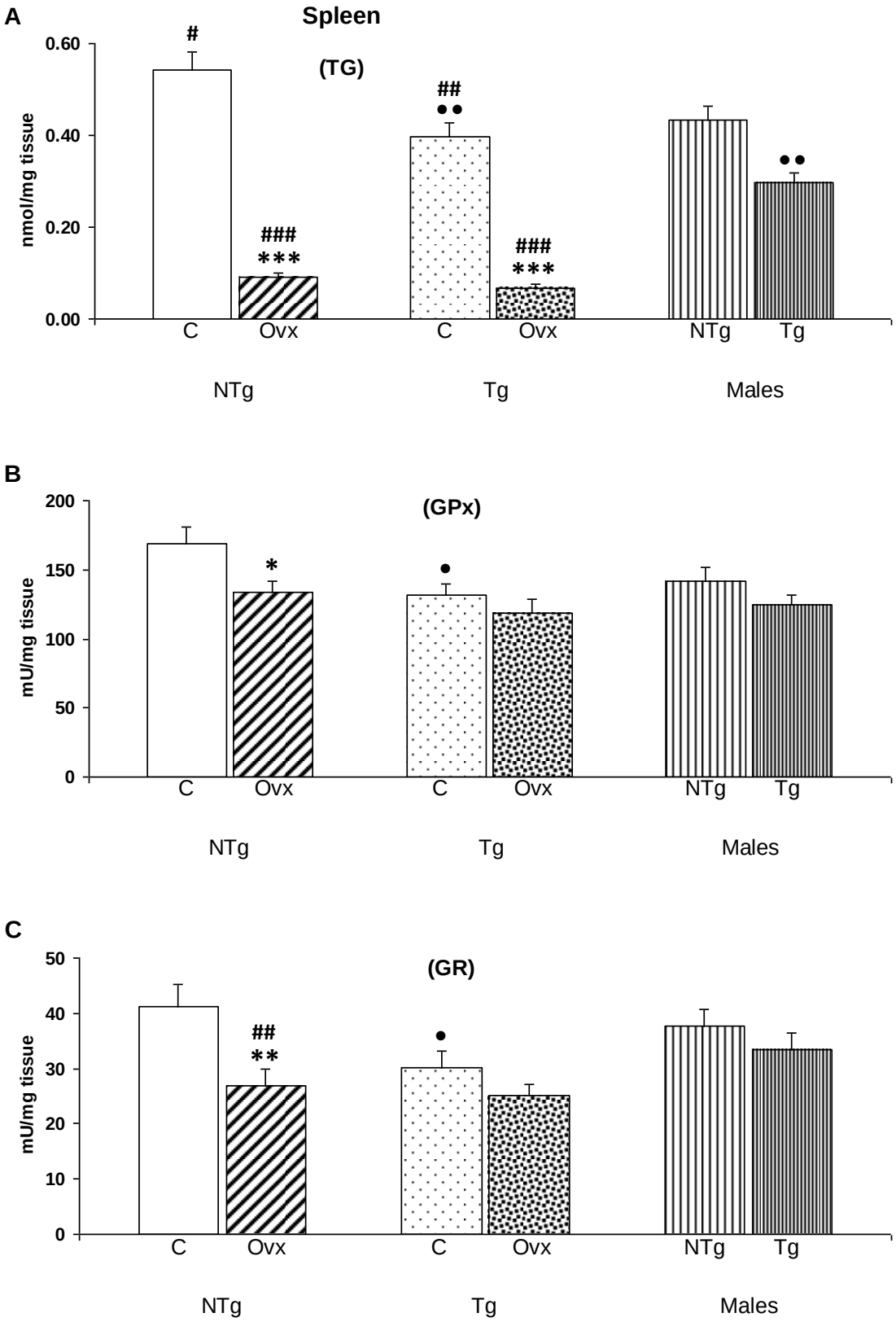


Fig.53. Total glutathione (TG) levels (A), glutathione peroxidase (GPx) (B) and glutathione reductase (GR) (C) activities in spleen from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being

the mean of duplicate assays. *p < 0.05;**p < 0.01; ***p < 0.001 with respect to the corresponding values of control mice. •p< 0.05; ••p< 0.01 with respect to the corresponding values of non-transgenic. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding values of males.

Table 17. Effects of ovariectomy on antioxidant defense in spleen from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female mice.

	Antioxidant defense		
Spleen	**TG**	**GPx**	**GR**
FNTg	0.54±0.11$^{\#}$	168±37	41±11
FOvxNTg	0.09±0.02$^{***\#\#\#}$	133±22*	27±7$^{**\#\#}$
FTg	0.40±0.08$^{\bullet\bullet\#\#}$	131±21$^{\bullet}$	30±8$^{\bullet}$
FOvxTg	0.07±0.01$^{***\#\#\#}$	119±27	25±7
MNTg	0.43±0.09	141±27	38±7
MTg	0.30±0.05$^{\bullet\bullet}$	124±19	33±9

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05;**p < 0.01; ***p < 0.001 with respect to the corresponding values of control mice. •p< 0.05; ••p< 0.01 with respect to the corresponding values of non-transgenic. #p< 0.05;##p<0.01;###p< 0.001 with respect to the corresponding values of males. F: female. Ovx: ovariectomized. M: males.

4.3.3. Effects of physical exercise in adult triple transgenic Alzheimer's disease (3xTg-AD) female and male mice.

The results corresponding to the effect of physical exercise on chemotaxis, antitumoral NK cell activity, proliferation (cpm) of spleen lymphocytes, proliferation stimulation (%) of lymphocytes in both spleen and thymus of adult triple transgenic Alzheimer's disease (3xTg-AD) female and male mice are shown in figures 54- 59 and in tables 18 and 19.

4.3.3.1. Effects of physical exercise on leukocytes functional parameters and redox state in adult 3xTg-AD female and male mice.

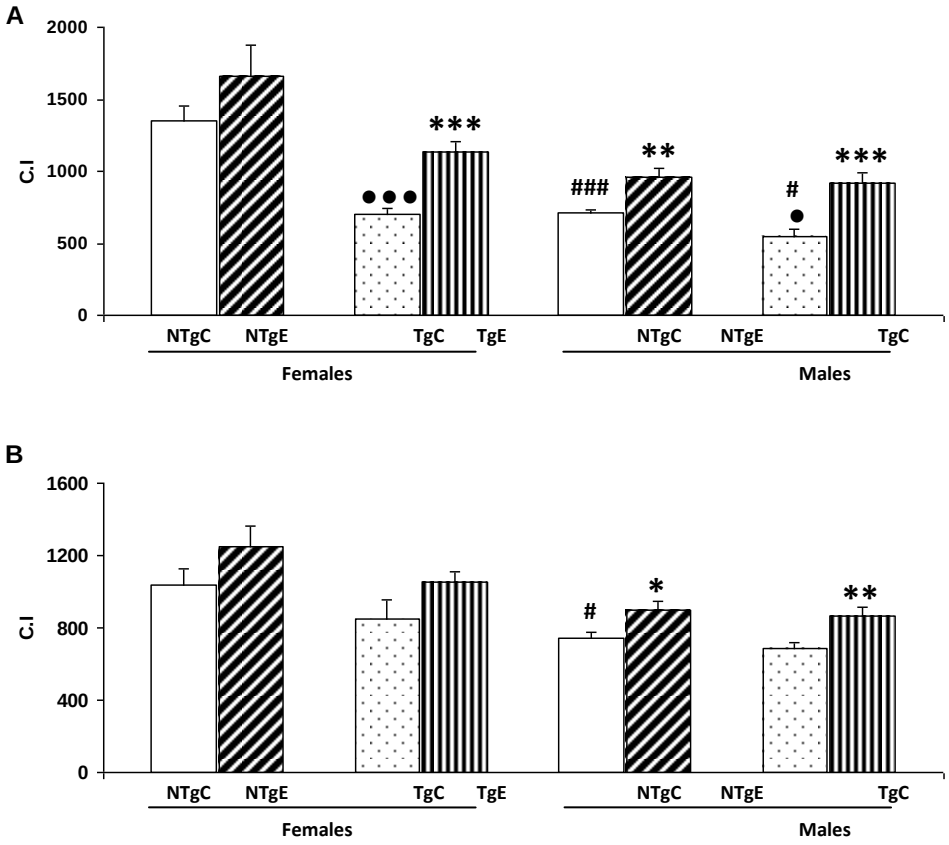


Fig. 54. Effects of physical exercise in chemotaxis of spleen (A) and thymus (B) leukocytes from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ with respect to the corresponding value of control mice. • $p< 0.05$; ••• $p< 0.001$ with respect to the corresponding value of non-transgenic. #$p< 0.05$; ##$p< 0.01$; ###$p< 0.001$ with respect to the corresponding value of females.

In the spleen (Fig. 54A), the results showed that the physical exercise enhances the chemotaxis capacity (chemotaxis index: C.I.) in 3xTg females ($p< 0.001$), whereas it has no effect in NTg. The physical exercise enhances the chemotaxis capacity in both NTg and 3xTg males ($p< 0.01$ and $p< 0.001$ respectively). In the Thymus (Fig. 54B), the results showed that the physical exercise has no effect on the chemotaxis capacity in both NTg and 3xTg females, whereas it enhances the chemotaxis capacity in both NTg and 3xTg males ($p< 0.05$ and $p< 0.01$ respectively).

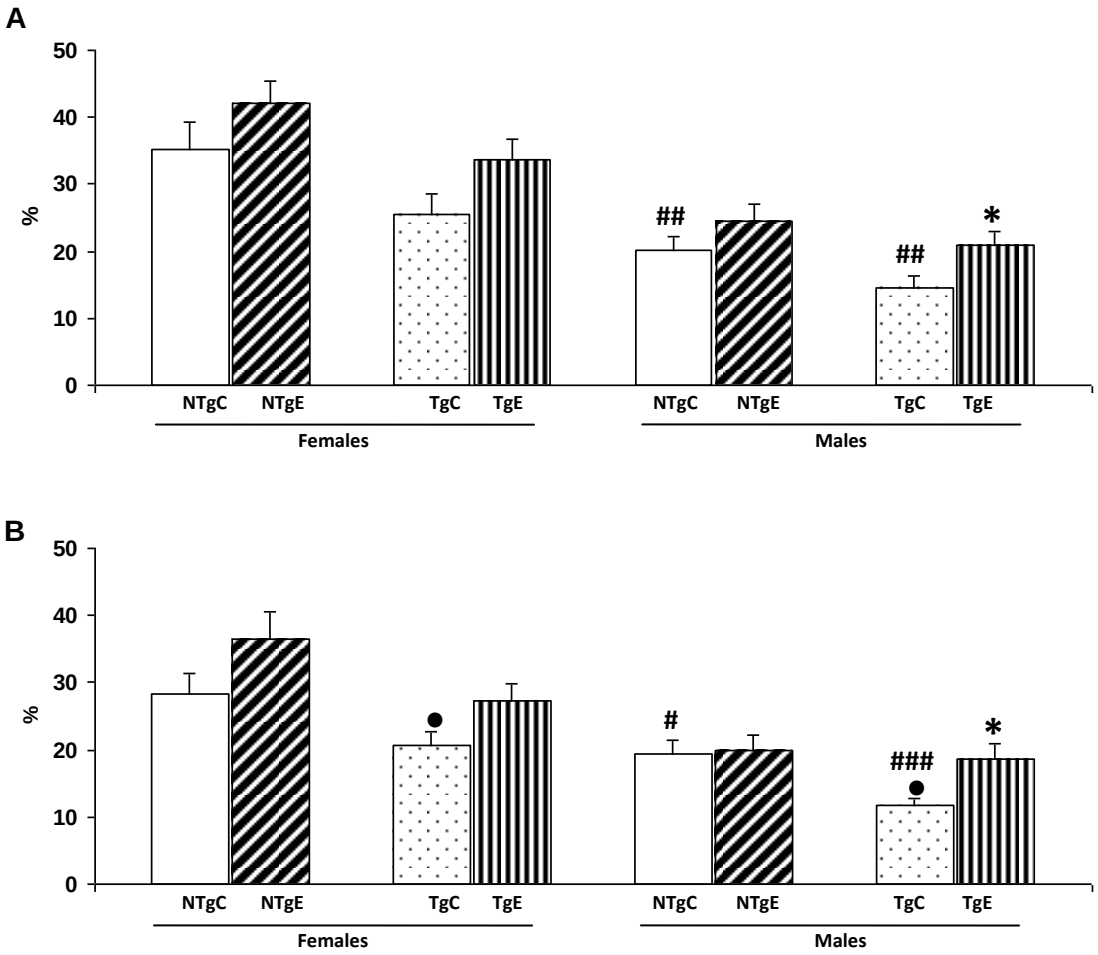


Fig. 55. Effects of physical exercise on antitumoral NK activity of spleen (A) and thymus (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *$p < 0.05$ with respect to the corresponding value of control mice. • $p< 0.05$ with respect to the corresponding value of non-transgenic. #$p< 0.05$; ##$p< 0.01$; ###$p< 0.001$ with respect to the corresponding value of females.

In the spleen and thymus (Fig. 55), the results showed that the physical exercise has no noticeable effect on the antitumoral NK activity in both NTg and 3xTg females, whereas it enhances the NK activity in 3xTg males ($p< 0.05$).

Table 18. Chemotaxis capacity (Chemotaxis index) and NK activity (% of tumor lysis) of spleen and thymus leukocytes from adult non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice.

		Spleen		Thymus	
Function	**Mice**	**Females**	**Males**	**Females**	**Males**
Chemotaxis index (C.I)	***NTg***	1352±290	715±60$^{\#\#\#}$	1040±242	746±89$^{\#}$
	NTgE	1660±613	955±195****	1252±310	899±145*
	Tg	703±117$^{\bullet\bullet\bullet}$	542±105$^{\bullet\#}$	850±291	684±110
	TgE	1132±215*****	919±195*****	1049±167	867±141****
NK (%)	***NTg***	35±12	20±6$^{\#\#}$	28±8	19±6$^{\#}$
	NTgE	42±10	25±7	37±11	20±7
	Tg	26±8	15±5$^{\#\#}$	21±5$^{\bullet}$	12±3$^{\bullet\#\#\#}$
	TgE	34±8	21±6*	27±7	19±6*

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value of control mice. • p< 0.05; ••• p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05; ##p< 0.01; ###p< 0.001 with respect to the corresponding value of females.

The results of the proliferation of spleen lymphocytes are shown in Figure 56. In basal conditions (Fig. 56A), physical exercise shows no effect on this function in NTg and 3xTg female mice, whereas, it enhances the same function in NTg males (p< 0.05). In response to ConA stimulation (Fig. 56B), physical exercise shows no effect on proliferation of spleen lymphocytes in NTg female mice, whereas, it enhances the same function in 3xTg-AD females (p< 0.05). In males, results showed that physical exercise enhances the proliferation in 3xTg-AD mice. In response to LPS stimulation (Fig. 56C), physical exercise shows neither effect on proliferation of spleen lymphocytes in females nor in males.

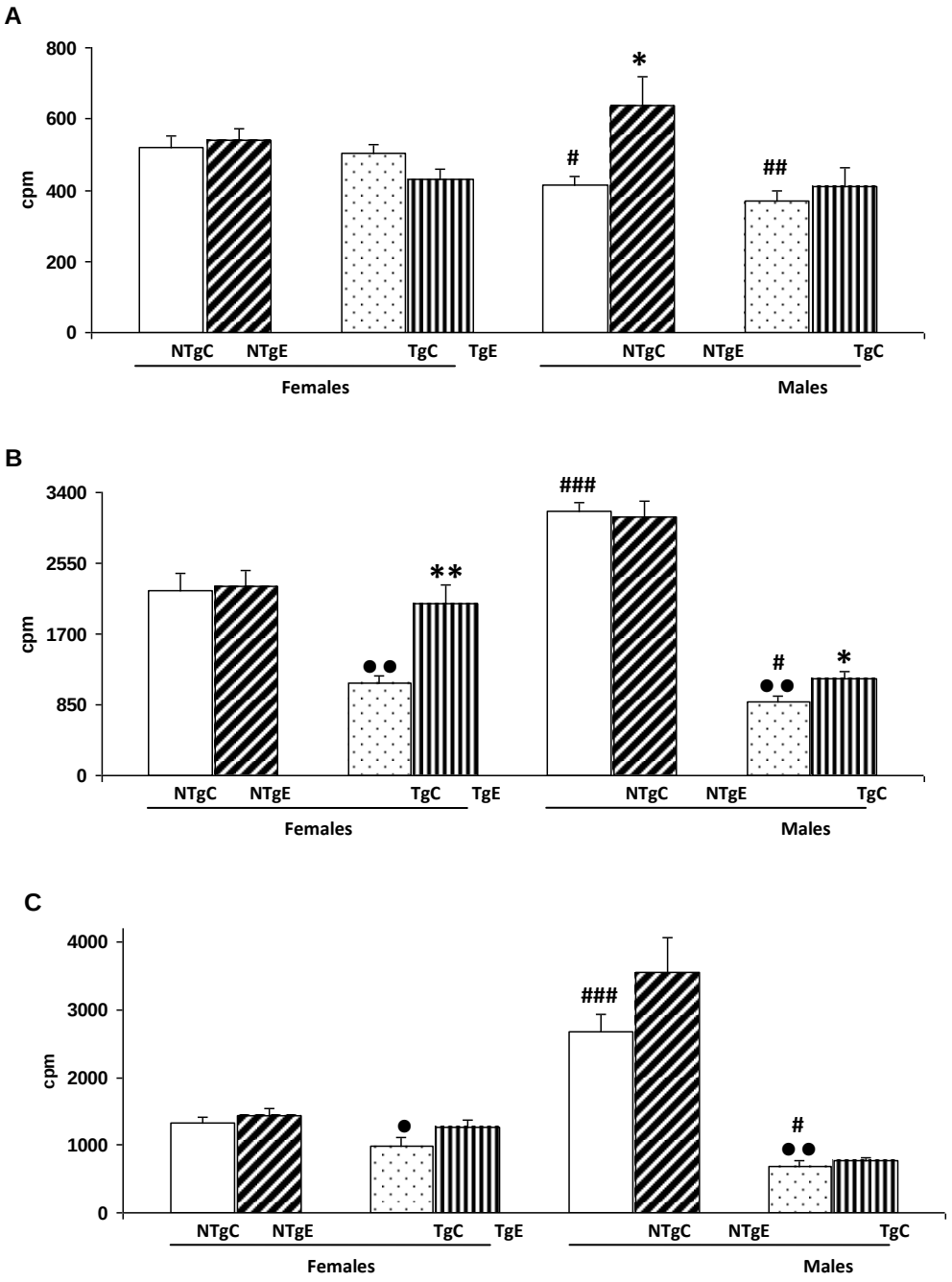


Fig. 56. Effects of physical exercise in proliferation (cpm) of spleen lymphocytes in basal conditions (A), as well as in response to ConA (B) and LPS (C) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *$p < 0.05$ with respect to the corresponding value of control. *$p < 0.05$; **$p < 0.01$ with respect to the corresponding value of control mice. • $p< 0.05$; ••• $p< 0.001$ with respect to the corresponding value of non-transgenic. #$p< 0.05$; ##$p< 0.01$; ###$p< 0.001$ with respect to the corresponding value of females.

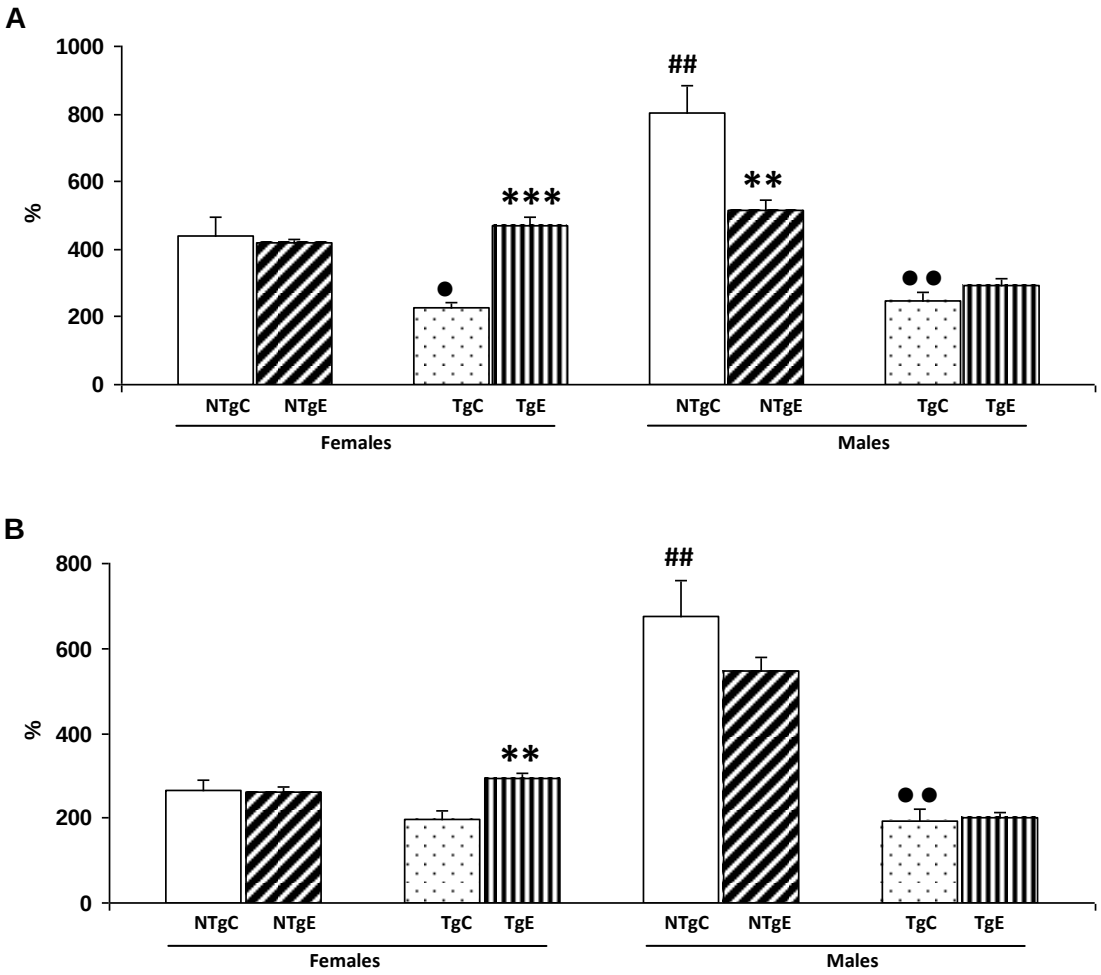
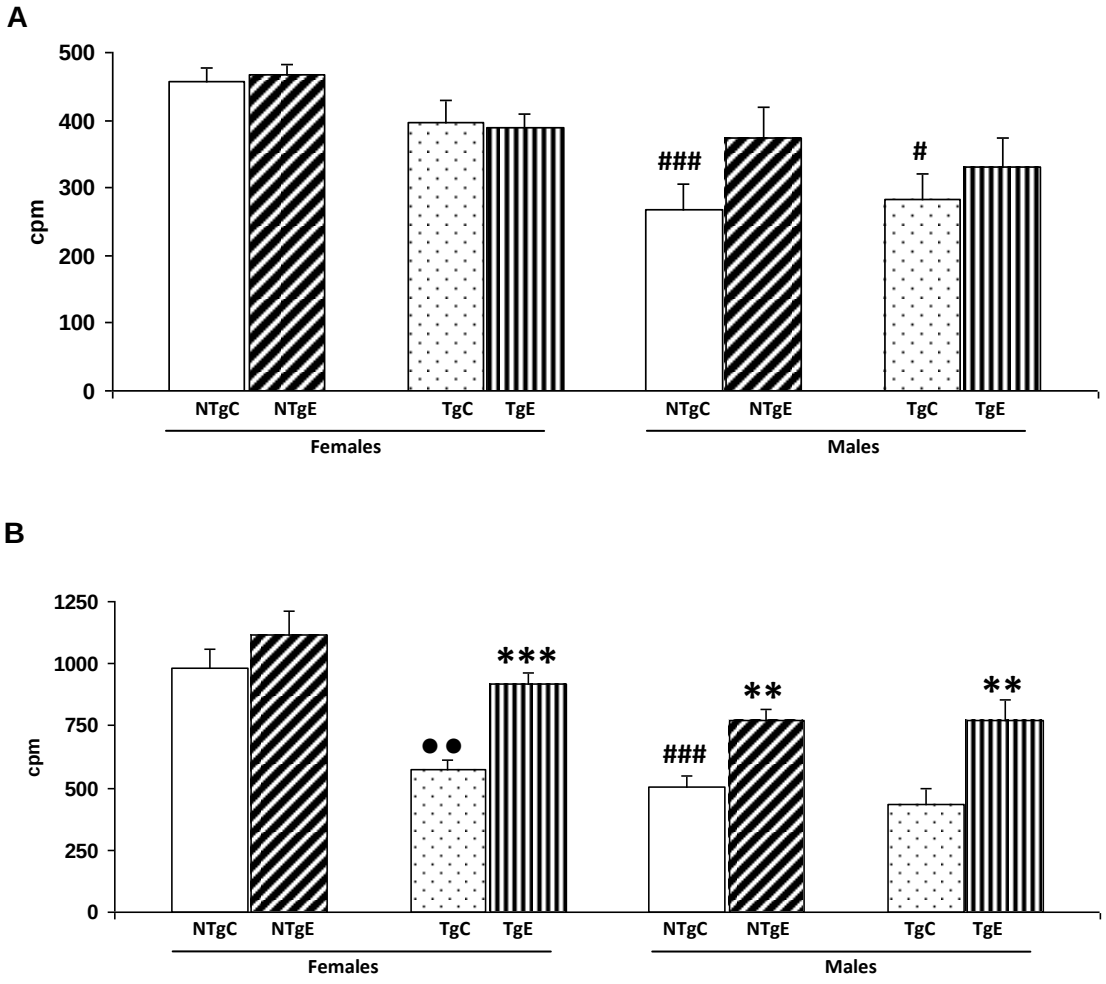


Fig. 57. Effects of physical exercise in proliferation stimulation (%) of spleen lymphocytes in response to ConA (A) and LPS (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice. Each value is the mean ± standard error of eight values corresponding to eight animals. Each value being the mean of duplicate assays. **p < 0.01; ***p< 0.001 with respect to the corresponding value of control mice. •• p< 0.01; ••• p< 0.001 with respect to the corresponding value of non-transgenic. ##p< 0.01 with respect to the corresponding value of females.

With respect to the stimulation index (%) of spleen lymphocyte proliferation in response to ConA (Fig.57A), results showed that physical exercise stimulates this function in 3xTg-AD females (p< 0.001). but it decreases this function in NTg males (p< 0.01).Physical exercise shows no effect on stimulation index (%) of spleen lymphocyte neither in NTg females nor in 3xTg males.

The results of stimulation index (%) of lymphocyte proliferation in response to LPS (Fig.57B) showed that physical exercise stimulates this function in 3xTg-AD females ($p < 0.01$), whereas, it shows neither effect on stimulation index (%) of spleen lymphocyte in NTg females nor in males.



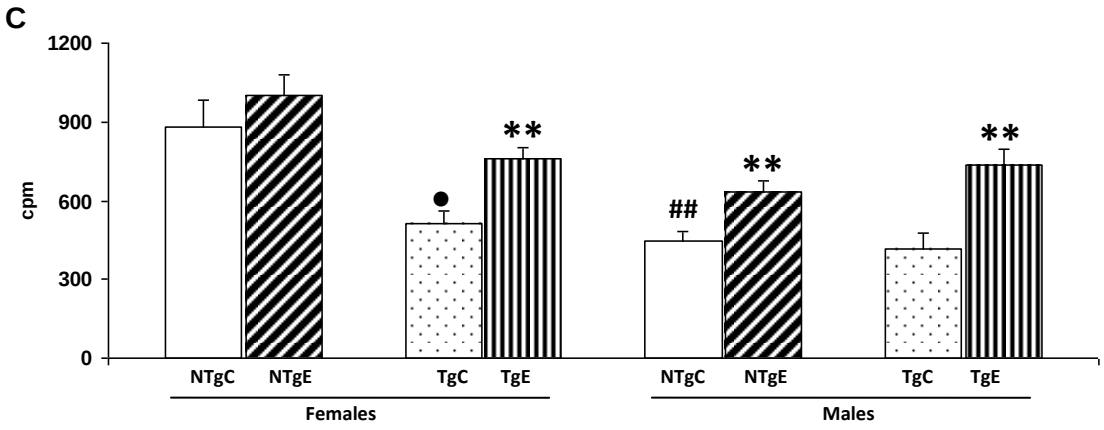


Fig. 58. Effects of physical exercise in proliferation (cpm) of thymus lymphocytes in basal conditions (A), in response to ConA (B) and in response to LPS (C) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. **p < 0.01; ***p< 0.001 with respect to the corresponding value of control mice. •• p< 0.01; ••• p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05; ##p< 0.01; ###p< 0.001 with respect to the corresponding value of females.

The results of the proliferation of thymus lymphocytes are shown in Figure 58. In basal conditions (Fig. 58A), physical exercise shows neither effect on this function in female nor in male mice, whereas, it enhances the same function in NTg and 3xTg-AD males in response to ConA stimulation (p< 0.01) (Fig. 58B) as well as in response to LPS (p< 0.01) (Fig. 58B). Similarly, physical exercise enhances lymphocyte proliferation in 3xTg female mice in response to both ConA (p< 0.001) and LPS (p< 0.01) stimulation.

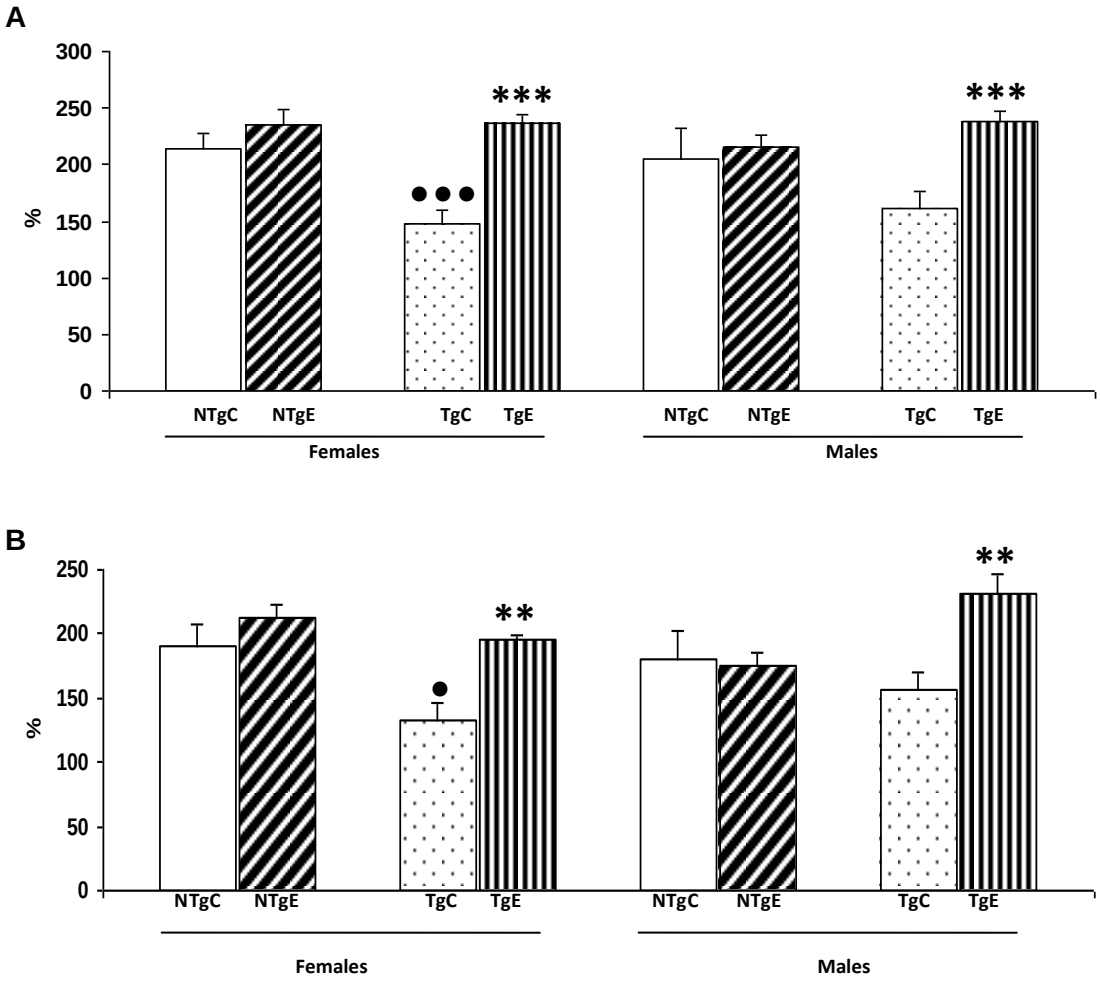


Fig. 59. Effects of physical exercise on proliferation stimulation (%) of thymus lymphocytes in response to ConA (A) and LPS (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) female and male mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. **p < 0.01; ***p< 0.001 with respect to the corresponding value of control mice. • p< 0.05; ••• p< 0.001 with respect to the corresponding value of non-transgenic.

With respect to the stimulation index (%) of thymus lymphocyte proliferation in response to ConA (Fig.59A), results showed that physical exercise stimulates lymphocyte proliferation in both 3xTg-AD females (p< 0.001) as well as in 3xTg-AD males (p< 0.001). Similarly, results showed that physical exercise stimulates lymphocyte proliferation in response to LPS (Fig.59B), in both 3xTg-AD females (p< 0.01) as well as in 3xTg-AD males (p< 0.001). Moreover, results showed that physical exercise has neither effect on stimulation index of thymus lymphocyte proliferation in NTg females nor en NTg males.

Table 19. proliferation of spleen and thymus lymphocytes from adult non-transgenic (NTg) and transgenic Alzheimer's Disease (3xTg-AD) female and male mice.

Lymphoproliferation						
Spleen		**(cpm)**			**(%)**	
Gender	**Mice**	**Basal**	**Con-A**	**LPS**	**Con-A**	**LPS**
Females	***NTg***	521±93	2222±562	1331±242	441±152	264±71
	NTgE	541±76	2275±547	1441±311	417±38	262±29
	Tg	502±69	1119±232$^{\bullet\bullet\bullet}$	995±317$^{\bullet}$	225±47$^{\bullet\bullet}$	198±56
	TgE	431±76	2059±633**	1262±304	470±70***	292±42**
Males	***NTg***	413±79$^{\#}$	3177±302$^{\#\#\#}$	2676±740$^{\#\#\#}$	804±78$^{\#\#}$	676±243$^{\#\#}$
	NTgE	638±222*	3111±544	3562±1442	514±91**	549±91
	Tg	370±83$^{\#\#}$	878±218$^{\bullet\bullet\bullet\#}$	687±212$^{\bullet\bullet\bullet\#}$	246±71$^{\bullet\bullet\bullet}$	194±76$^{\bullet\bullet\bullet}$
	TgE	411±152	1155±272*	777±132	295±53	201±41
Thymus		**(cpm)**			**(%)**	
Gender	***Mice***	**Basal**	**Con-A**	**LPS**	**Con-A**	**LPS**
Females	***NTg***	457±55	982±217	883±277	215±36	191±44
	NTgE	468±42	1114±280	1002±223	235±38	212±30
	Tg	398±87	576±103$^{\bullet\bullet\bullet}$	515±134$^{\bullet\bullet}$	148±30$^{\bullet\bullet\bullet}$	132±40$^{\bullet}$
	TgE	389±60	917±124***	762±119**	237±21***	196±8**
Males	***NTg***	269±104$^{\#\#\#}$	501±136$^{\#\#\#}$	446±106$^{\#\#}$	204±80	180±62
	NTgE	375±127	769±135**	632±118**	215±34	176±27
	Tg	282±112$^{\#}$	437±164	419±155	161±44	157±43
	TgE	331±119	772±241**	733±180**	238±25***	232±41**

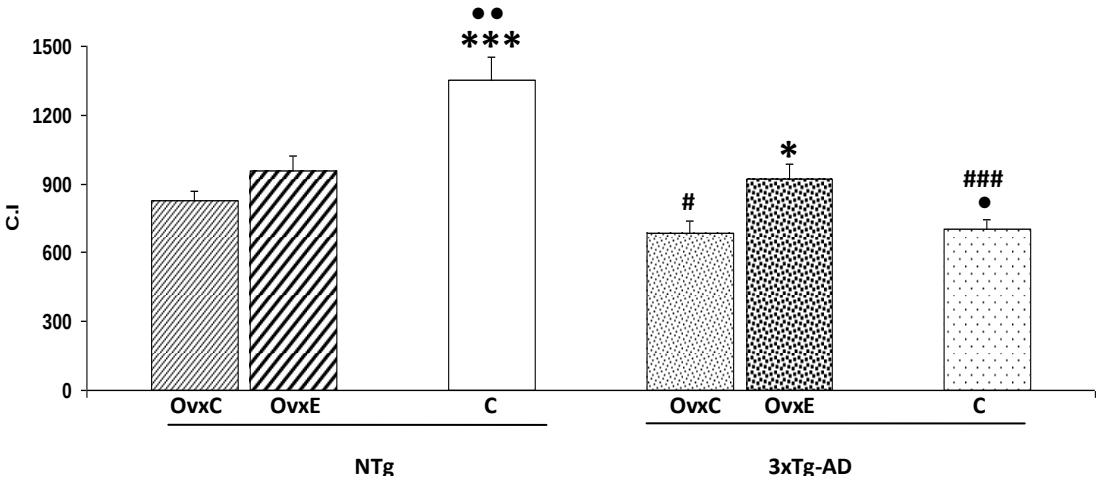
Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value of control mice. • p< 0.05; ••p< 0.01; ••• p< 0.001 with respect to the corresponding value of non-transgenic. #p< 0.05; ##p< 0.01; ###p< 0.001 with respect to the corresponding value of females.

4.3.3.2 Effect of physical exercise in adult ovariectoized triple transgenic Alzheimer's disease (ovx-3xTg-AD) mice and none-transgenic mice.

The results corresponding to the effect of physical exercise on chemotaxis, antitumoral NK cell activity, proliferation (cpm) of spleen lymphocytes, proliferation stimulation (%) of lymphocytes in both spleen and thymus of adult (9 months) ovariectomized triple transgenic Alzheimer's disease (3xTg-AD) mice are shown in figures 60-65 and in tables 20 and 21.

Effects of physical exercise on leukocytes functional parameters in adult overiectomized 3xTg-AD mice

In spleen (Fig.60A) the results showed that the physical exercise enhances the chemotaxis capacity (chemotaxis index: C.I.) in ovariectomized (Ovx) 3xTg-AD mice ($p< 0.05$), whereas it has no effect in Ovx-NTg mice. In thymus (Fig 60B) the results showed that the physical exercise enhances the chemotaxis capacity in ovariectomized Ovx NTg mice ($p< 0.05$).



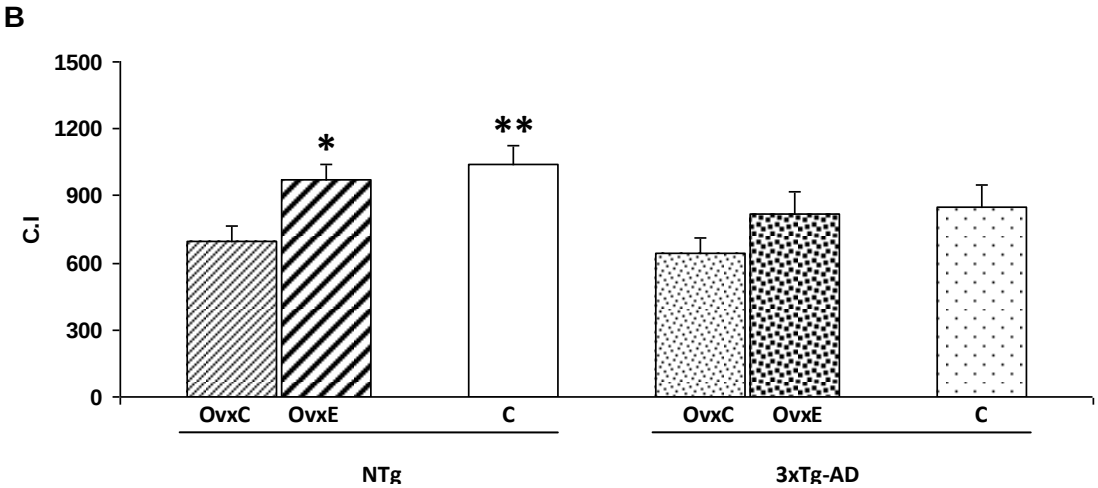
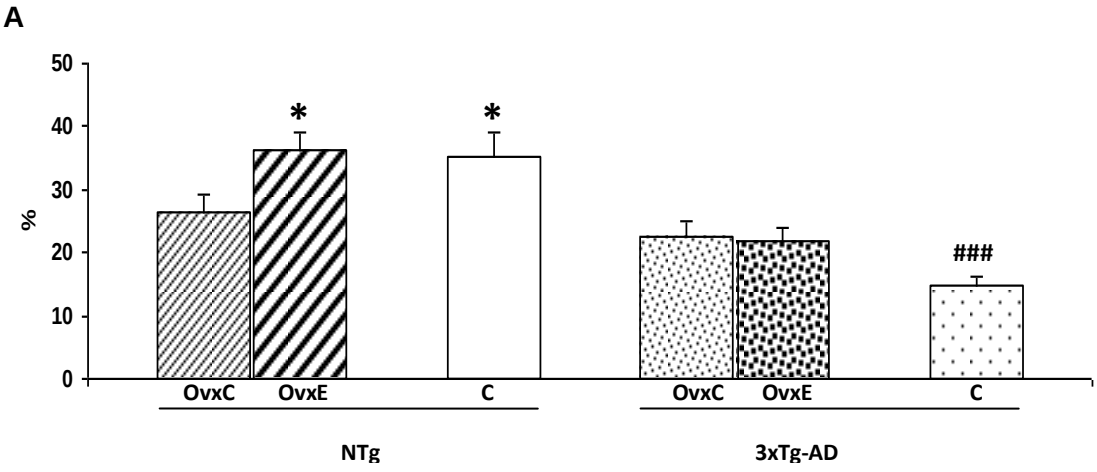


Fig. 60. Effects of physical exercise on chemotaxis in spleen (A) and thymus (B) leukocytes from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01 with respect to the corresponding value of ovariectomized control mice. • p< 0.05; •• p< 0.01 with respect to the corresponding value of ovariectomized mice with exercise. #p< 0.05; ###p< 0.001 with respect to the corresponding value of non-transgenic mice.

In the spleen and thymus (Fig. 61A and B), the results showed that the physical exercise enhances the antitumoral NK activity in Ovx-NTg (p< 0.05) mice, whereas it has no effect on the same functio in Ovx-3xTg mice.



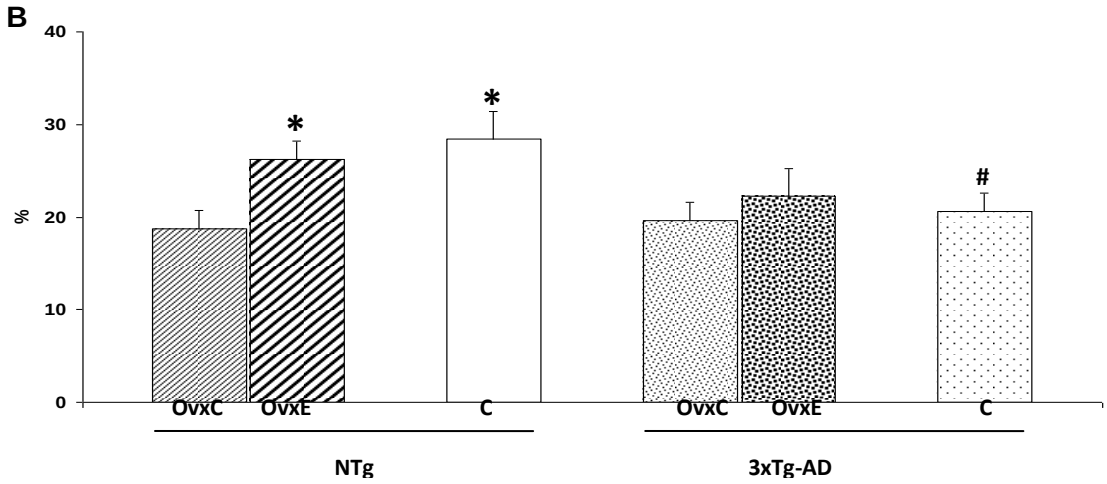


Fig.61. Effects of physical exercise on antitumoral NK activity in spleen (A) and thymus (B) leukocytes from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05 with respect to the corresponding value of ovariectomized control mice. #p< 0.05; ###p< 0.001 with respect to the corresponding value of non-transgenic mice.

Table 20. Effects of physical exercise on NK activity (% of tumor lysis) and chemotaxis capacity (Chemotaxis index) of spleen and thymus leukocytes from adult non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice.

	Chemotaxis Index (C.I)		**NK (%)**	
Mice	**Spleen**	**Thymus**	**Spleen**	**Thymus**
NTgOvx	830±115	700±180	26±9	19±5
NTgOvxE	954±190	969±213*	35±8*	26±5*
NTg	1352±29***••	1040±242**	35±12*	28±8*
TgOvx	686±147#	643±198	22±8	20±5
TgOvxE	919±190*	820±281	21±8	22±8
Tg	703±117•###	850±291	15±5###	21±5#

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value of ovariectomized control mice. • p< 0.05; •• p< 0.01 with respect to the corresponding value of ovariectomized mice with exercise. #p< 0.05; ###p< 0.001 with respect to the corresponding value of non-transgenic mice.

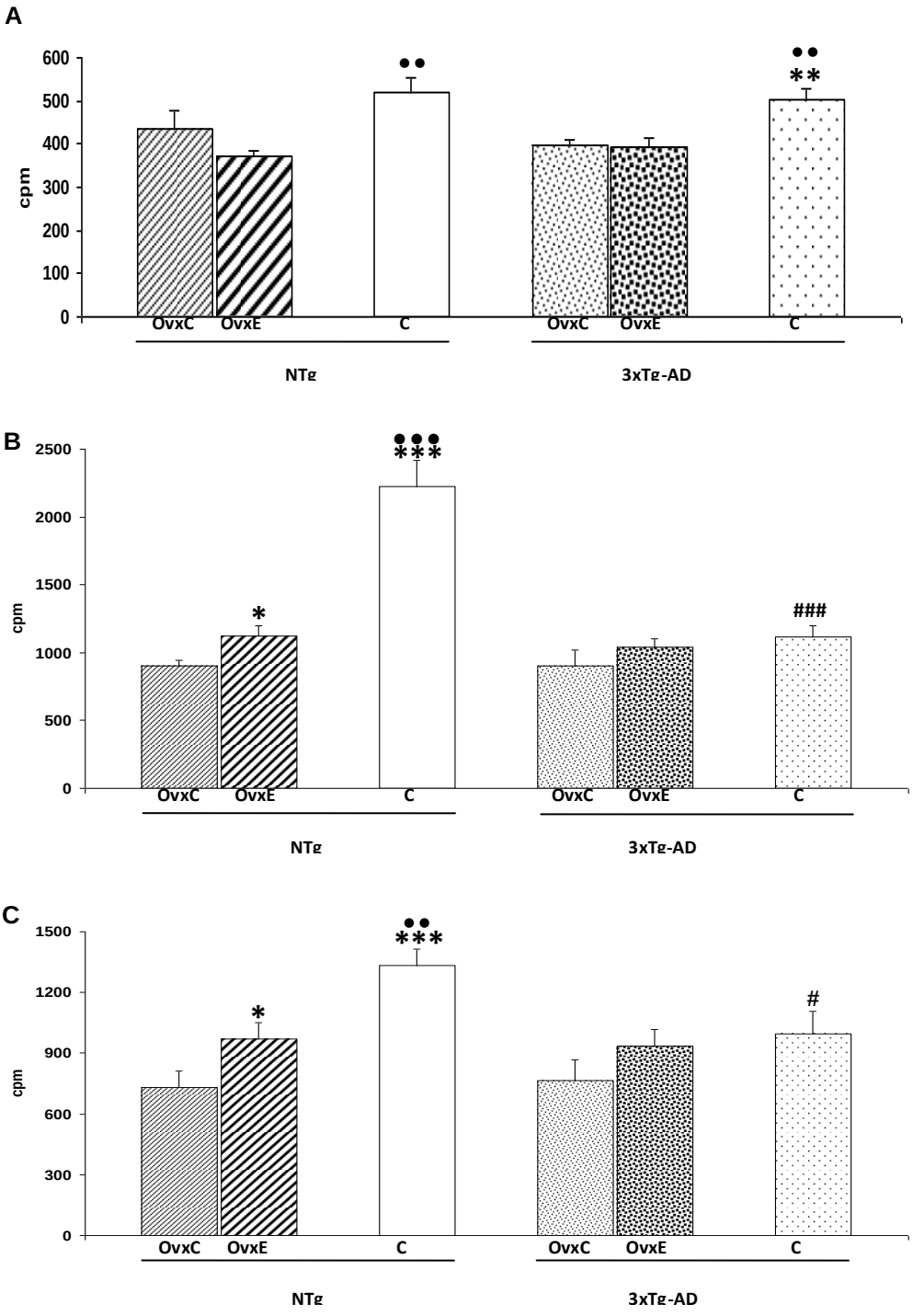
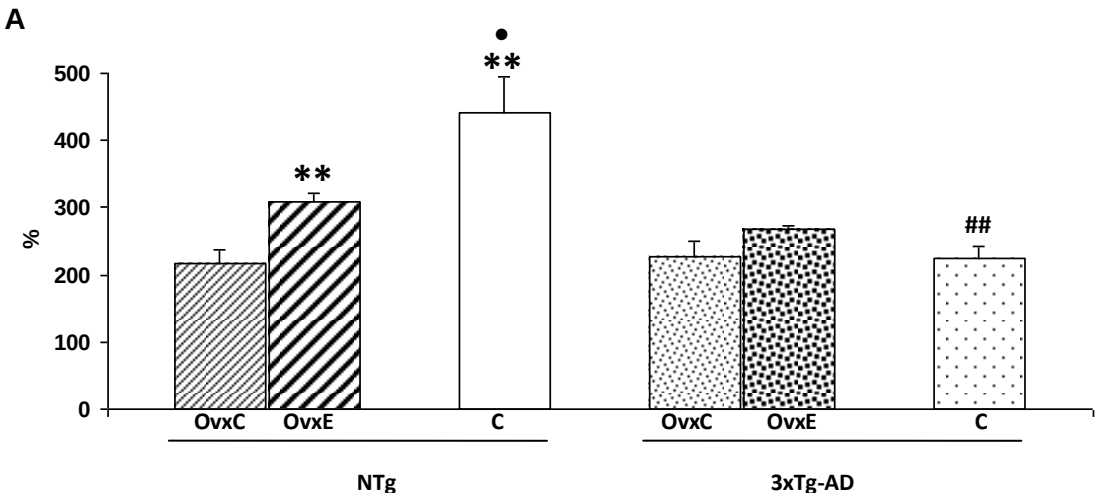


Fig. 62. Effects of physical exercise in proliferation (cpm) of spleen lymphocytes in basal conditions (A) as well as in response to ConA (B) and LPS (C) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value of ovariectomized control mice. •• p< 0.01; ••• p< 0.001 with respect to the corresponding value of ovariectomized mice with exercise. #p< 0.05; ###p< 0.001 with respect to the corresponding value of non-transgenic mice.

The results of the proliferation of spleen lymphocytes are shown in Figure 62. In basal conditions (Fig. 62A), physical exercise shows neither effect on this function in Ovx-NTg nor in Ovx-3xTg-AD females, whereas, it enhances the same function in Ovx-NTg females both in response to ConA and LPS stimulation ($p < 0.05$) (Fig. 62B and C). Physical exercise shows no effect on proliferation of spleen lymphocytes in Ovx-3xTg-AD in response to both ConA and LPS stimulation (Fig. 62B and C).

With respect to the stimulation index (%) of spleen lymphocyte proliferation in response to ConA as well as in response to LPS (Fig.63A and B), results showed that physical exercise stimulates this function in Ovx-NTg mice ($p < 0.01$), and has no effect on stimulation index of spleen lymphocyte proliferation in Ovx-3xTg-AD mice.



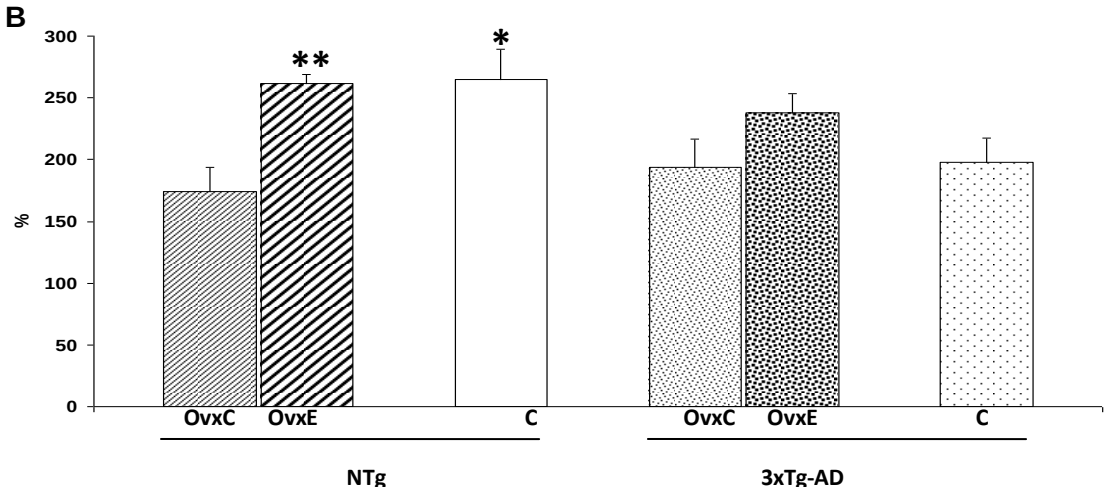


Fig. 63. Effects of physical exercise on proliferation stimulation (%) of spleen lymphocytes in response to ConA (A) and LPS (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01 with respect to the corresponding value of ovariectomized control mice. • p< 0.05 with respect to the corresponding value of ovariectomized mice with exercise. ##p< 0.01 with respect to the corresponding value of non-transgenic mice.

The results of the proliferation of thymus lymphocytes are shown in Figure 64. In basal conditions (Fig. 64A), physical exercise shows neither effect on this function in Ovx-NTg nor in Ovx-3xTg-AD females, whereas, it enhances the same function in both Ovx-NTg and Ovx-3xTg-AD females in response to ConA (p< 0.01) as well as in response to LPS stimulation (p< 0.01 and p< 0.05respectively) (Fig. 64B and C).

With respect to the stimulation index (%) of thymus lymphocyte proliferation in response to ConA as well as in response to LPS (Fig.65A and B), results showed that physical exercise stimulates this function in both Ovx-NTg (p< 0.001 and p< 0.01 respectively) and Ovx-3xTg-AD (p< 0.001 and p< 0.05 respectively) females.

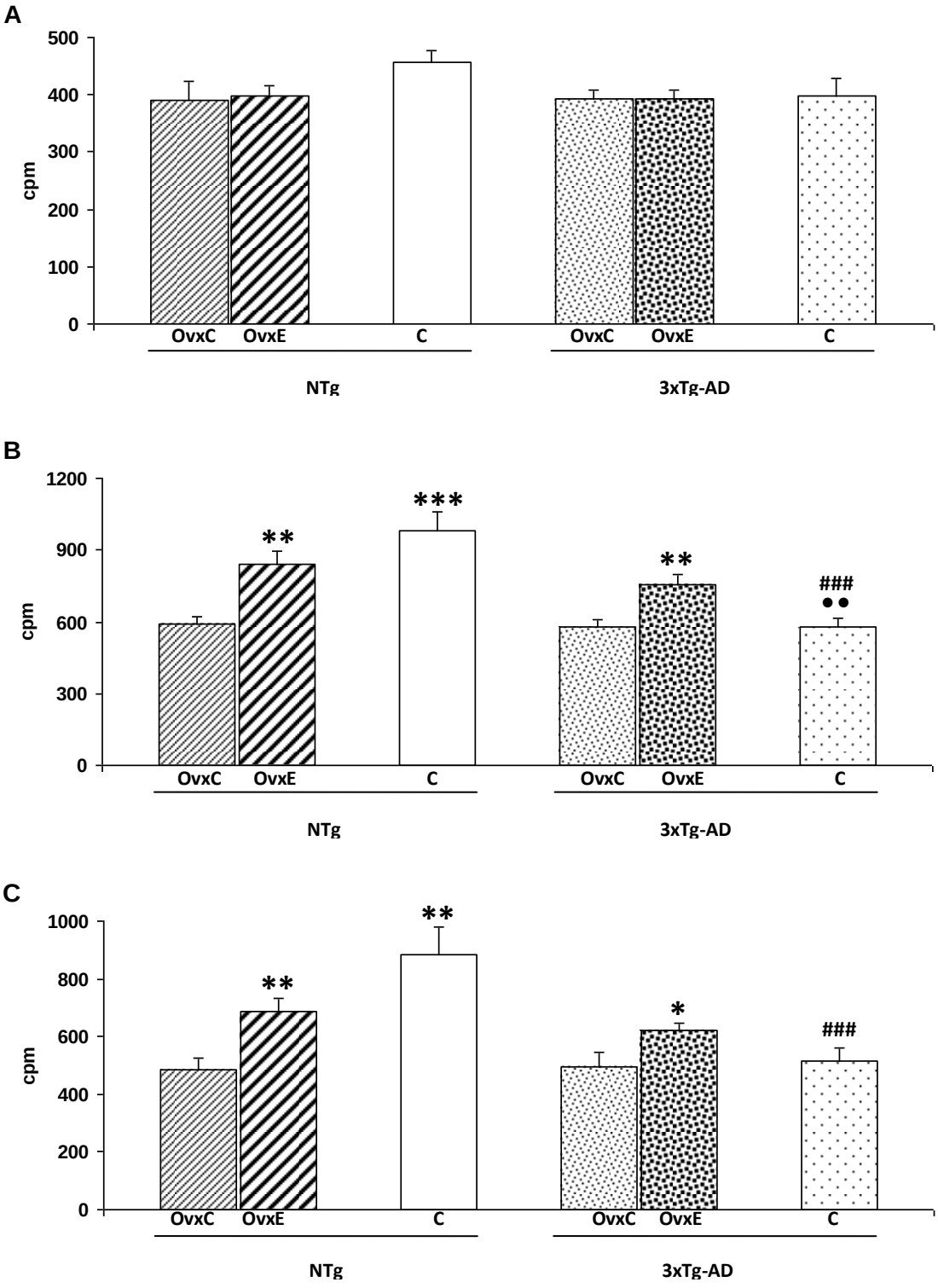


Fig. 64. Effects of physical exercise in proliferation (cpm) of thymus lymphocytes in basal conditions (A) as well as in response to ConA (B) and LPS (C) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value of ovariectomized control mice. •• p< 0.01 with respect to the corresponding value of ovariectomized mice with exercise. ###p< 0.001 with respect to the corresponding value of non-transgenic mice.

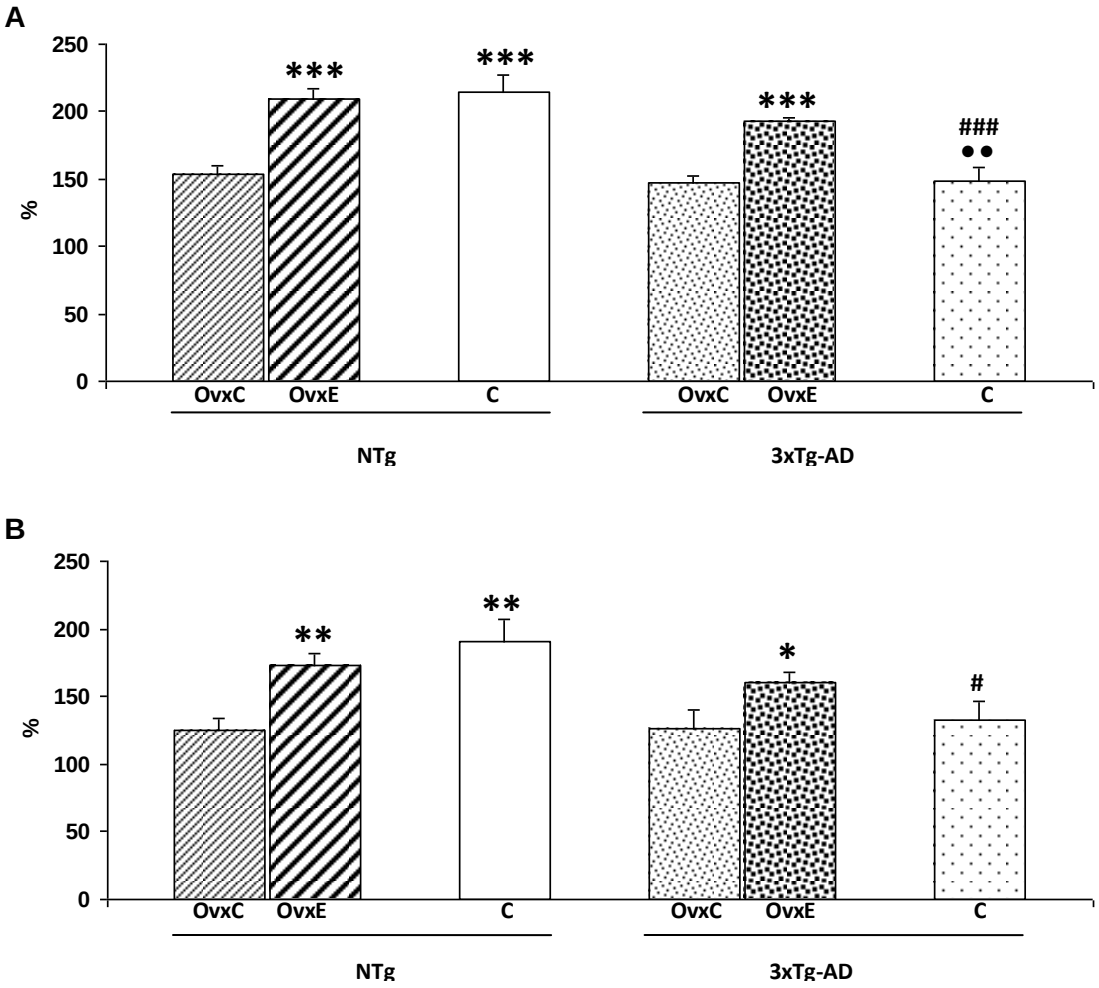


Fig.65. Effects of physical exercise in proliferation stimulation (%) of thymus lymphocytes in response to ConA (A) and LPS (B) from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ with respect to the corresponding value of ovariectomized control mice. #$p< 0.05$; ###$p< 0.001$ with respect to the corresponding value of non-transgenic mice.

Table 21. Effects of exercise on proliferation of spleen and thymus lymphocytes from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice.

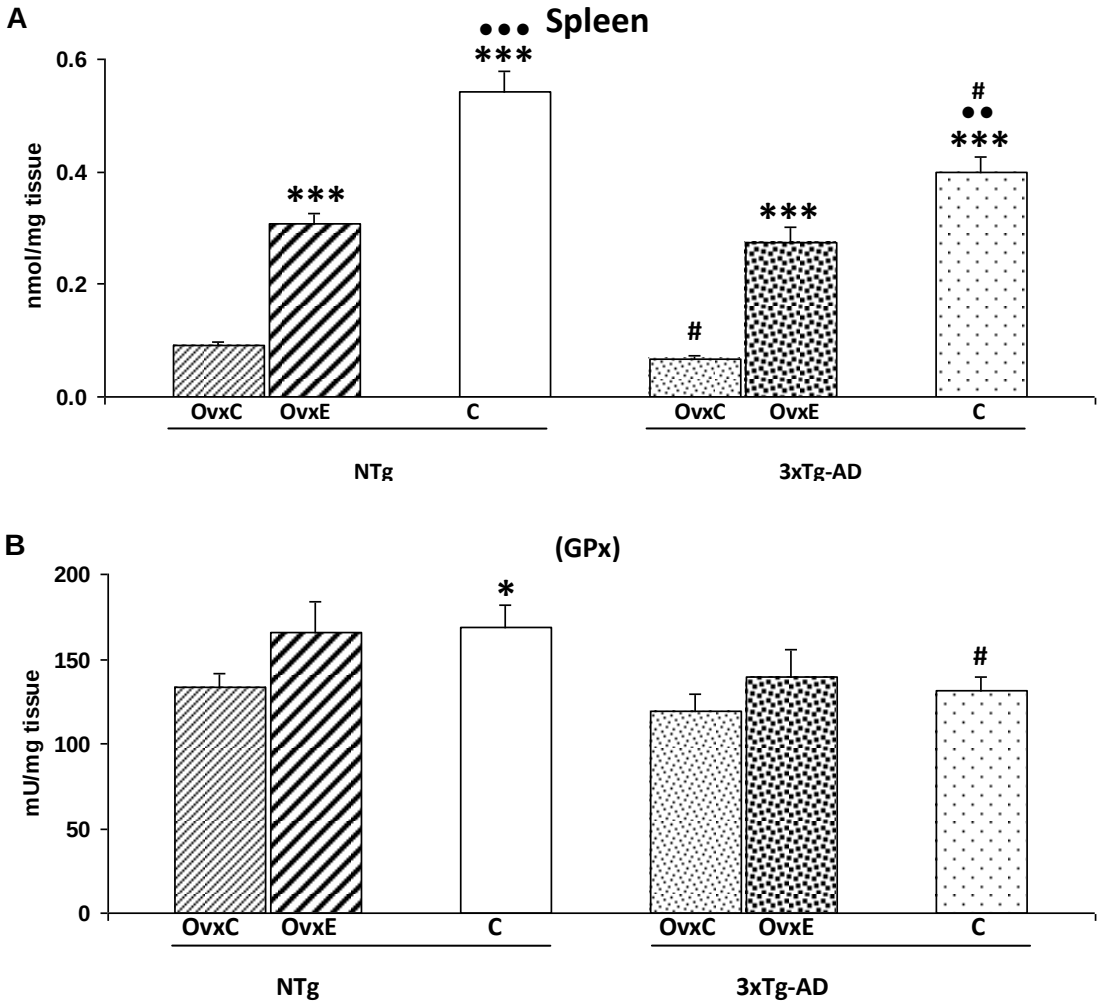
Lymphoproliferation					
Spleen	**(cpm)**			**(%)**	
Mice	**Basal**	**Con-A**	**LPS**	**Con-A**	**LPS**
NTgOvx	435±115	904±107	732±220	218±56	174±57
NTgOvxE	371±37	1122±227*	969±229*	308±37**	261±51**
NTg	521±93$^{••}$	2222±562***$^{•••}$	1331±242***$^{••}$	441±152**$^{•}$	264±71*
TgOvx	395±46	904±333	765±286	226±66	194±65
TgOvxE	391±62	1043±172	935±238	267±12	238±43
Tg	502±69**$^{••}$	1119±232$^{###}$	995±317$^{#}$	225±47$^{##}$	198±56
Thymus	**(cpm)**			**(%)**	
Mice	**Basal**	**Con-A**	**LPS**	**Con-A**	**LPS**
NTgOvx	391±90	589±98	483±124	153±19	125±25
NTgOvxE	398±47	838±155**	689±127**	210±20***	173±26**
NTg	457±55	982±217***	883±277**	215±37***	191±44**
TgOvx	392±44	579±81	496±141	148±16	127±37
TgOvxE	392±45	757±109**	620±70*	193±7***	160±22*
Tg	398±87	576±103$^{••###}$	515±134$^{##}$	148±30$^{••###}$	132±40$^{#}$

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value of ovariectomized control mice. • p< 0.05; •• p< 0.01; ••• p< 0.001 with respect to the corresponding value of ovariectomized mice with exercise. #p< 0.05; ##p< 0.01; ###p< 0.001 with respect to the corresponding value of non-transgenic mice.

Effects of physical exercise on redox state in adult overiectomized 3xTg-AD mice

The results corresponding to the effect of physical exercise on total glutathione (TG) levels and the antioxidant enzyme activities involved in its oxidation-reduction reactions, glutathione peroxidase (GPx) and glutathione reductase (GR) in spleen of adult (9 months) ovariectomized triple transgenic Alzheimer's disease (3xTg-AD) mice are shown in figure 66 and in table 22.

In the spleen (Fig. 66A) the results showed that physical exercise increases TG levels both in Ovx-NTg ($p<0.001$) and in Ovx-3xTg-AD ($p< 0.001$) females, as well as increases GR activity in Ovx-NTg ($p<0.05$) and in Ovx-3xTg-AD ($p< 0.05$) females.



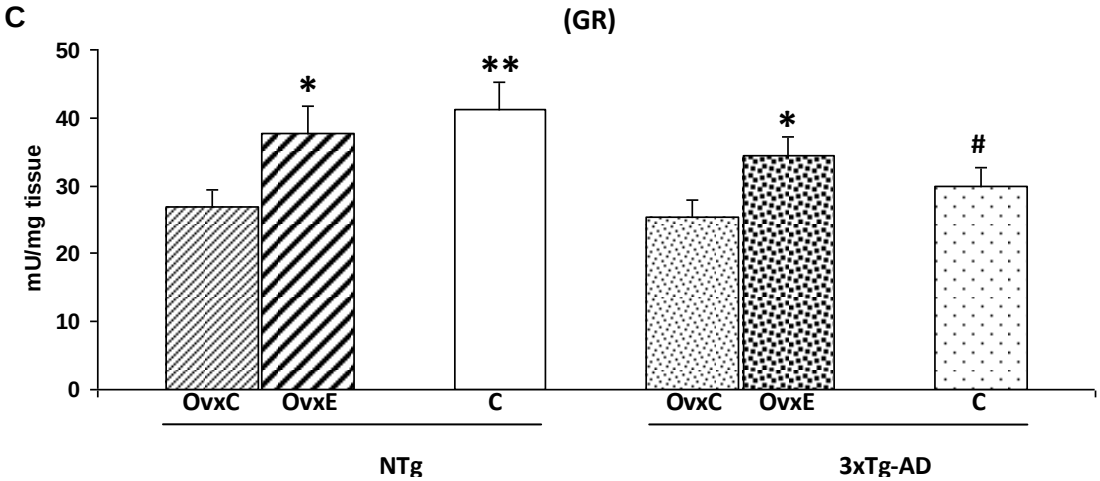


Fig.66. Total glutathione (TG) levels (A), glutathione peroxidase (GPx) (B) and glutathione reductase (GR) (C) activities in spleen from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice. Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value of ovariectomized control mice. •• p< 0.01; ••• p< 0.001 with respect to the corresponding value of ovariectomized mice with exercise. #p< 0.05 with respect to the corresponding value of non-transgenic mice.

Table 22. Effects of physical exercise on the antioxidant defense in spleen from adult (9 months) non-transgenic (NTg) and transgenic Alzheimer Disease (3xTg-AD) ovariectomized female mice.

	Antioxidant defense (Spleen)		
Mice	**TG**	**GPx**	**GR**
NTgOvx	0.09±0.02	133±22	27±7
NTgOvxE	0.31±0.06***	166±52	38±12*
NTg	0.54±0.11***•••	168±37*	41±11**
TgOvx	0.07±0.01#	119±27	25±7
TgOvxE	0.27±0.08***	139±45	35±7*
Tg	0.40±0.08***••#	131±21#	30±8#

Each value is the mean ± standard error of eight values corresponding to eight animals, each value being the mean of duplicate assays. *p < 0.05; **p < 0.01; ***p< 0.001 with respect to the corresponding value of ovariectomized control mice. •• p< 0.01; ••• p< 0.001 with respect to the corresponding value of ovariectomized mice with exercise. #p< 0.05 with respect to the corresponding value of non-transgenic mice.

5. DISCUSSION

5.1. Age-related changes in circadian variations of function and oxidative stress parameters of immune cells

Several studies have described circadian variations of immune parameters, especially in the number of lymphocytes and monocytes (in human blood they show the maximal values during the night and the lowest after waking), as well as in the number of NK cells (which, by contrast, reach their highest level in the afternoon, with a decrease around midnight). Besides, the functions of immune cells have also shown circadian changes. Thus, lymphocyte proliferation, antigen presentation, NK cell cytotoxic activity, gene expression and release of cytokines, among others, show variations along the day (Petrovsky and Harrison, 1998; Buijs et al., 2006; Cutolo et al., 2006; Sephton and Spiegel, 2003). However, the changes in the immune functions at different moments of the day in the same mice have not been studied. This approach is only possible using peritoneal immune cells, which can be obtained without sacrificing the animal. The comparison between these changes in adult and old mice in the same experiment has also been studied for the first time in the present work. Another important fact is that the animals in the present research were studied in the course of their active period of the day (from 8:00 to 20:00h) in which mice were in dark conditions.

The results show, in general, circadian variations in all the functions studied in the case of adult mice, whereas in old animals some functions did not exhibit changes in the course of their active period. Moreover, many functions have shown circadian changes similar to those observed during the ageing process (De la Fuente and Miquel, 2009). In addition, the differences between adult and old in the functions studied were observed only at 8:00 h, but disappeared at 18:00 h.

5.1.1. Age-related changes in circadian variations of function parameters of immune cells

Peritoneal **macrophages** presented an increase of adherence capacity through the day, which is more evident in short time adherence (10 minutes) than in long time adherence (30 minutes). An incubation time of 10 minutes has been shown, in previous studies, to be the best period to observe changes in adherence capacity (De la Fuente et al., 1991). With ageing there is an increase of this function in phagocytic cells from human peripheral blood and from mouse peritoneal suspension. This increase seems to be a consequence of the oxidative state occurring in old subjects. An oxidative stress enhances the expression of adherent molecules. In old animals these changes disappear along the day in the case of the short time adherence studied. Thus, when the adherence in macrophages of adult and old were compared, higher adherence indexes, in old than in adult mice, were observed, especially at 8:00 h. For a short incubation time, the differences between adult and old disappeared.

With respect to the chemotaxis of macrophages, a decrease of this function was observed along the day. This occurs in cells from both adult and old animals, although it was more evident in those of the adults. In agreement with that, it has been observed that human neutrophils showed circadian variation in the chemotaxis capacity with the maximal activity during the day (Muniain et al., 1988). Since this function decreases with ageing in phagocytic cells from humans and mice, macrophages at 18:00h are "older" than in the other hours studied. Thus, when the chemotaxis capacities of macrophages of adult and old are compared, the lower values in old animals are not shown at 18:00 h, the same as in the case of adherence capacity.

The phagocytic capacity has been the most studied function in the context of the circadian variations of the phagocytes. Some studies showed that phagocytosis in blood neutrophils from mice present a rhythmic circadian variation, whose acrophase occurrs in the second half of the dark

period (3:00 h) (Hriscu Hriscu et al., 1998). Moreover, in zebrafish, phagocytosis varied significantly throughout twenty-four hour periods. Phagocytosis of E. coli peaked late in the day (Kaplan et al., 2008). In the ring dove (Streptopelia risoria), the phagocytic index and phagocytic percentage were enhanced during the night, with the maximum at approximately 04:00 hr and the minimum at approximately 18:00 h (Rodriguez et al., 1999). Since the levels of serum melatonin increased during the dark period (from 20:00 to 07:00 hr) with a maximum at 04:00 h, and showed a significant decline during the hours of light with a minimum at 16:00 h in these birds, these circadian changes in the phagocytosis have been shown to be related to those in that hormone (Rodriguez et al., 1999). There are results showing that the phagocytosis capacity of peritoneal macrophages enhances during the dark period in BALB/c mice, with the maximum inert-particle (latex beads) ingestion capacity at 03:30 h (Barriga et al., 2001), this being positively correlated with melatonin serum levels. However, in the present study the phagocytosis of peritoneal macrophages decreased along the day. When cells were in the presence of lymphocytes T and B the decrease started earlier than when only lymphocytes B were present. In agreement with previous results, the latex ingestion capacity of peritoneal macrophages was higher in the presence of only B cells (De la Fuente and Medina, 2005). With ageing there is a decrease of the phagocytosis in cells of humans and mice (citas). In the present study, although the decrease in phagocytosis along the day occurs in cells from both adult and old mice, in the case of adult cells due to their higher decrease, the differences between adult and old were only shown, in general, at 8:00h.

The intracellular levels of superoxide anion increased throughout the day in cells from both adult and old mice. Nevertheless, in general, the values were lower in cells from old mice than in those from adults. Since the levels of intracellular superoxide anion could be indicative of the digestion capacity of phagocytic cells (Victor et al., 2004), the age-related decrease in these levels observed in previous studies (De la Fuente et al., 2004; Arranz

et al., 2010), could show a lower digestion capacity in cells from old animals. In agreement with our results, in human neutrophils incubated by chemotactic peptide, circadian variations along the day have been observed in the superoxide anion levels, with low amounts at 14:00 h (Muniain et al., 1991). In zebrafish, a peak in cellular ROS levels has been observed before dawn (Kaplan et al., 2008). In ring doves, the superoxide anion levels were lower during darkness (minimum at 04:00 hr) and higher during the light period (maximum at 14:00 hr). The increased levels of superoxide anion through the day could be related to melatonin, since both are negatively correlated, and this hormone decreased during the day. Moreover, melatonin shows antioxidant properties (Barriga et al., 2001; De la Fuente and Diaz, 2007), and consequently, its decrease could be related to any increase in oxidant compounds.

Recently, a study on several phagocytic functions of human neutrophils at two different times of day has been carried out, and the results showed that at 15 h several functions of neutrophils such as chemotaxis decrease with respect to their values at 10 h (revised in Mate et al., 2014). One possible reason could be the lower levels of melatonin with the advancing day, but other factors submitted to circadian variations may also be involved (Fernandez et al., 2010).

Peritoneal **lymphocytes** also showed circadian changes in the functions studied.

There was an increase in adherence capacity along the day, similar to that observed in macrophages, and the values in cells from old mice were also higher than those from adults. Nevertheless, unlike phagocytes, the circadian variations of lymphocyte adherence were more relevant in old animals. Moreover, in lymphocyte adherence the differences between adult and old are more marked at 18:00h. Since there are B and T lymphocytes in the peritoneal suspension, and the latter do not adhere to plastic surfaces, the results obtained should be due to the B cells.

The chemotaxis capacity of lymphocytes showed circadian changes similar to those observed in macrophages. Lymphocytes have an age-related decrease of this function, which has also been observed in the present study. Moreover, at 18:00h there were no differences between adult and old mice.

With respect to the proliferation of lymphocytes in response to mitogens, both adult and old mice showed circadian variations in response to ConA, whereas, no changes were observed in response to LPS. Since ConA is a mitogen for T lymphocytes and LPS for B cells, the results showed more relevant circadian changes in the first cells. With ConA, in adult mice, although a decrease in proliferation at 18:00h was observed, a peak of proliferation was observed at 13:00h. In other studies carried out on submaxillary lymph node from rats, a significant diurnal variation in the index of cell proliferation (Neidhart and Larson, 1990) was observed, with a maximal activity during early afternoon (Cardinali et al., 1996). However, the proliferation of murine splenic T cells in response to mitogenic stimuli was maximal at the start of the dark phase in rats (Fernandes et al., 1976).

The proliferation of lymphocytes in response to mitogens decreases with ageing, as has been observed in many studies carried out on human and experimental animals (De la Fuente and Miquel, 2009). In peritoneal lymphocytes this age-related decrease has been also observed (Arranz et al., 2010). In the present study proliferation in cells of old animals was lower than in adult, but the differences between both ages were only observed at 8:00 h with LPS and at 8:00 h and 13:00 h with ConA.

With respect to the **NK activity**, the results of the present study showed that this activity in leucocytes from adult and old mice decreased during the course of the day, with a higher decrease in adult than in old animals. Several studies have shown that the number and activity of NK cells manifest circadian changes in cells from both human and experimental animals. In the peripheral blood of healthy humans, NK activity increases near awakening (Prev, 1988; Levi 1988), with a peak in the morning

(Williams et al., 1976). In rats the activity of NK cells showed a maximum activity in the dark phase (Fernandes et al.,1979; Arjona et al., 2004). Since melatonin release is maximal during the dark phase in men and mice (Goulding and Hall, 1993), the role of melatonin in enhancing NK cell activity could be proposed. In fact, several studies show that melatonin modulates the T helper cell (Th) and NK cell activity (Skwarlo-Sonta et al., 2003).

Although the decrease with ageing in NK activity has been observed in many studies (Solana et al., 2006; De la Fuente et al., 2008; De la Fuente and Miquel, 2009; Arranz et al., 2010), the leucocytes from old animals only showed lower NK activity than those from adults at 8:00 h.

Similar changes to those in mouse peritoneal lymphocytes were observed in a recent study which compared different functions of lymphocytes from peripheral blood of adult and elderly men and women at 10:00h and 15:00h (Mate et al., 2014).

5.1.2. Age-related changes in circadian variations of cytokines released in cultures of peritoneal and blood cells

Cytokines are major mediators of the complex interactions between immune cells, and responsible for the development and resolution of immune responses. The circadian changes in the levels of cytokines have been analyzed in several studies, but in old animals these variations are not known. Moreover, many experiments analyzing cytokine levels have been carried out in plasma or in isolated cells. The data described in the present work are thus of special relevance since this study was performed using unfractionated peritoneal leucocytes, in order to better reproduce the in vivo cytokine response. Moreover, in the same animals the changes in the release of cytokines by total blood cells in response to mitogens have been studied.

IL-2 release levels in cultures of peritoneal leucocytes decreased at 13:00 h and 18:00 h in adult and old mice respectively. Since melatonin modulates IL-2 production (Skwarlo-Sonta et al., 2003), the decrease of Il-2 throughout the day observed in mice, could be due to the decrease of that hormone. Although IL-2 is related to the proliferation of lymphocytes, the circadian variations in both functions are not similar. However, IL-2 changes along the day in a similar way to NK activity.

The decrease of IL-2 with ageing has been observed in many studies in humans and in experimental animals. This decrease coincides with the age-related decline in lymphoproliferative responsiveness to antigens or mitogens and in NK activity (Guayerbas et al. 2002b; Pawelec et al. 2002; De la Fuente and Miquel, 2009; Arranz et al., 2010). In the present work, leucocytes from old animals showed lower IL-2 secretion levels than those from adults, but the differences are statistically significant only at 8:00 h. In our laboratory many experiments demonstrated an impairment in lymphoproliferation, NK activity and IL-2 levels in peritoneal leucocytes from old mice, but all these studies were carried out at 8:00 h (Arranz et al., 2010).

Pro-inflammatory and anti-inflammatory cytokines

In adult mice, **IL-1β** significantly decreased at 13.00 h in response to ConA and then increased with respect to both the values at 8:00 h and at 18:00 h. No circadian variations were observed in IL-1β in response to LPS. Although IL-1β could be related with the proliferation of lymphocytes, especially in the presence of ConA, the circadian changes in both parameters were the opposite. As IL-1β is also a pro-inflammatory cytokine, its increase at 18:00 h seems to show a higher pro-inflammatory state at this time of the day. Ageing is characterized by a chronic low-grade inflammatory status, so-called 'inflamm-aging' (Franceschi and Bonafè 2003; De Martinis et al. 2006), which suggests a loss of homeostasis in cytokine networks that contributes significantly to health loss in old age

(Salvioli et al. 2006). Thus, the results obtained in the present work suggest that in this parameter (IL-1β), adult animals are "older" at the end of their day. However, the values of IL-1β in leucocytes from chronologically old mice are lower than those in adult animals, at least at 8:00h. This decrease in the release of pro-inflammatory cytokine levels in response to a mitogen such as ConA by peritoneal leucocytes from old animals has been observed in previous studies (Arranz et al., 2010).

In old mice there was an increase of IL-1β in response to ConA along the day, but no circadian variations in response to LPS were shown. Thus, differences between adult and old mice were observed only in response to ConA and especially at 8:00h. Leucocytes from old mice showed a lower release of IL-1β than those from adults. Although a reduced LPS-induced IL-1β secretion has been reported in isolated macrophages from aged mice (Chelvarajan et al. 2006), in the present study no differences were observed between old and adult in this cytokine with LPS.

When the release of IL-1β was studied in blood stimulated with LPS, a clear circadian change appeared in adult mice. These animals showed an increase in IL-1β levels at 13:00 h. No changes were observed in old mice throughout the day. However, the levels of this cytokine were lower in old than in adult mice, with significant statistical differences at 13:00h. These differences in the circadian changes of IL-1β observed between the two types of samples studied could be due to both the presence in blood of levels of this cytokine produced in other parts of the organism and by different types of leucocytes and their proportions in peritoneal suspension and in blood.

With respect to the other pro-inflammatory cytokine studied, **TNF-α**, there were no circadian changes in its release by cells from adult mice. Nevertheless, in old animals there were an increase throughout the day in response to ConA and LPS. In response to these mitogens, the release of TNF-α by cells from old animals was smaller than that from adults at 8:00h. This is in agreement with data obtained in mice, which have shown that

macrophages from old animals produce less TNF-α following LPS stimulation than the young (Wang et al., 1995; Renshaw et al. 2002; Boehmer et al. 2005; Chelvarajan et al. 2006).

Although the release of this pro-inflammatory cytokine by leucocytes from old mice was higher in resting conditions than that in adult animals, when these cells had to respond to mitogens, the release of TNF-α was lower (Arranz et al., 2010). The increased production of pro-inflammatory cytokines such as TNF-α in resting cells lead to elevations of its circulating levels, which are associated with frailty, morbidity (including dementia and functional disability) and a high mortality risk (Mooradian et al. 1991; De Martinis et al. 2006). However, when an immune stimulus such as LPS is added in vitro, it has been shown that human subjects who at age 85 years produce low TNF-α levels have a more than 2-fold increased overall mortality risk compared to adults with a higher production of TNF-α (van den Biggelaar et al. 2004). As in resting conditions the levels of pro-inflammatory cytokines are increased, this could explain the chronic low grade inflammatory state shown in old animals, but these pro-inflammatory resting leucocytes seem to show a defective inflammatory response upon stimulation, which will ultimately limit the ability to deal of these animals to deal with infection (Arranz et al., 2010).

In the case of TNF-α release by cells in blood stimulated with LPS, adult animals showed a decrease in TNF-α level at 18:00 h with respect to 13:00h, which was no showed in peritoneal cells. This behaviour is not similar to that in humans, since several studies have showed circadian variations in cytokine levels in plasma, with a peak in pro-inflammatory cytokines such as TNF-α during the dark phase of cycle. In fact these cytokines are inductors of sleep. Although the production of macrophage related cytokines (such as TNF-α) seem to increase before sleep, the production of T-cell-related cytokines (such as IL-2) increases during sleep (Perumal et al., 2007).

If there was a relatively similar behavior in circadian changes of this cytokine in adult animals, there were great differences in old mice. Whereas in peritoneal cells appeared an increase along the day, in blood cells a decrease occurred at 13:00 h and 18:00 h with respect to 8:00h. Moreover, comparing the results in old and adult, at 8:00h the lower levels in old animals in the release of TNF-α observed in peritoneal cells disappeared in blood cells, showing even a light tendency to be higher in old mice. As it was previously mentioned, the TNF-α found in blood samples could be also produced by other cells different to immune cells.

The release of the anti-inflammatory cytokine studied, **IL-10**, in adult mice, significantly decreased at 13:00 h, in response to both ConA and LPS. In old animals the levels of IL-10 significantly increased in the course of the day in response to LPS, but no changes were observed with ConA. Comparing both ages, old mice showed lower levels of IL-10 than adults at 8:00h with ConA and LPS. Similar age-related decrease was observed in a previous work also using peritoneal leucocytes (Arranz et al., 2010). In elderly humans, those producing low levels of LPS-induced IL-10, show more mortality risk (van den Biggelaar et al. 2004). Moreover, high levels of IL-10 is a specific marker of longevity in centenarians (Lio et al. 2002), and it has been suggested the key role for raised IL-10 in controlling chronic inflammation and in longevity achievement in mice (Arranz et al., 2010).

When the levels of IL-10 were measured in blood in response to LPS, adult animals demonstrated a decreasing in IL-10 levels in blood in response to LPS at 13:00 h, This is similar to the circadian pattern in IL-10 levels that we obtained in cultures of peritoneal leukocytes. However, old animals showed a decrease in IL-10 levels in plasma at 18:00 h whereas showed an increase at this time in peritoneal cells. Thus, comparing two ages, in blood samples the old mice showed lower IL-10 levels at 8:00 and at 18:00h than adults.

Since in old animals the levels of IL-10 and TNF-α in cultures of peritoneal leukocytes in response to LPS significantly increased in the

course of the day and they are cytokines anti-inflammatory and pro-inflammatory, respectively, seems to have a compensatory relation between both. It is known that TNF-α and related cytokines determine the strength, effectiveness and duration of inflammation, whereas IL-10 and other anti-inflammatory cytokines have the opposite role, limiting and terminating the process (Lio et al. 2003). However, an increased production of anti-inflammatory cytokines or decreased production of pro-inflammatory cytokines have been found to be associated with successful aging and longevity in humans (Lio et al. 2002; 2003; Cederholm et al. 2007). In particular, the polymorphism of the IL-10 promoter associated with high levels of IL-10 is a specific marker of longevity in centenarians (Lio et al. 2002). Moreover, the high basal levels of anti-inflammatory mediators observed in the extreme long-lived mice could have direct beneficial effects for life-span, with high pro-inflammatory cytokines shortening life-span (Arranz et al., 2010). In fact, the key role for raised IL-10, in controlling chronic inflammation and in longevity achievement in mice has been proposed (Arranz et al., 2010).

In the present study, peritoneal leukocytes from adult mice released higher levels of IL-10, IL-1β and TNF-α in response to both ConA and LPS stimulation than those from old animals only at 8:00 h. Previous studies in our laboratory showed an increase of the basal levels of proinflammatory IL-1β, and TNF-α in the old animals, accompanied by a decrease of IL-10. Under LPS and/or ConA stimulated conditions, leukocytes from old mice showed a significantly impaired response with respect to secretion of these cytokines (Arranz et al., 2010). Thus, an inflamm-aging situation is typical in old animals (De la Fuente and Miquel, 2009; Franceschi et al., 1995). This inflammatory profile seems to be mediated by the decrease of anti-inflammatory cytokines such as IL-10, but it was observed at 8:00 h (Arranz et al., 2010). At 18:00 h these differences between adult and old disappear or change. Peritoneal macrophages isolated from rodents generally displayed a decreasing in the production of cytokines IL-1β, TNF-α and reactive oxygen species when stimulated in vitro with mitogen or virus

(Kohut et al., 2004; Alvarez et al., 1996). Some studies showed that stimulated murine alveolar and splenic macrophages produced higher levels of cytokines IL-1β and TNF-α when compared with younger counterparts (Han et al., 1995; Kohut et al., 2004). Immune cells produce proinflammatory compounds to defend the organism against and to destroy pathogens. However, the inflammation has to be controlled by secreating anti-inflammatory compounds to avoid a chronic situation. In the healthy adult there is a balance between inflammatory and anti-inflammatory compounds that is the base of homeostasis**.** With aging the level of pro-inflamatory compounds increase and it is higher than the level of anti-inflammatory compounds, leading to inflammatory stress which is one of the bases of the loss of homeostasis according to the oxi-inflamm-aging theory (De la Fuente and Miquel, 2009).

5.1.3. Age-related changes in circadian variations of oxidative stress parameters of immune cells

Since an oxidative stress is defined as the unbalance between oxidant and antioxidant levels, with higher presence of the first (citas), to analyze circadian changes in the oxidative stress situation of peritoneal leucocytes, the levels of an oxidant such as the extracellular superoxide anion ($O_2^{-}\cdot$), and of antioxidants such as the levels of total glutathione (TG) and the activity of glutathione peroxidase and glutathione reductase were measured.

With respect to the **oxidant** studied, the **extracellular superoxide anion ($O_2^{-}\cdot$)**, this represent the amount of the first free radical produced in the cells that is released. As was mentioned in the introduction section, free radicals and ROS in general, in certain amounts are needed for many physiological processes, which are essential for cell survival, but when the amounts of ROS are very high, they lead to oxidative damage. In the case of superoxide anion, the intracellular levels are used to the digestion of phagocyted material in the phagosomes. However, the extracellular levels can be dangerous since they produce the oxidation of the biomolecules.

The results in the present study showed that peritoneal cells from adult mice released higher levels of this oxidant at 13:00 h under control as well as stimulated conditions, whereas in old mice, in general, no circadian variations were observed in its levels. Old mice showed higher levels of extracellular $O_2^{-}\cdot$ measured in both non-stimulated and stimulated cells at 8h and 13h but not at 18h. This is in agreement with previous results, in which peritoneal cells from old mice release more amount of superoxide anion than those from adults (citas). Moreover, cells from adult mice show as those from old animals, with respect their release of free radicals, at 18:00 h.

To control the excess of free radicals, there are antioxidant defenses. Thus, the balance between ROS and antioxidant levels is the base of a preserved cellular homeostasis typical of the healthy adult**.**

In relation to the **antioxidant defenses**, the circadian variations in the total glutathione **(TG)** and the activity of the enzymes involved in its oxidation reduction cycle; glutathione peroxidase (GPx) and glutathione reductase (GR), in peritoneal leukocyte from adult and old female mice were measured. Cells from adult animals showed a significant decrease in the levels of TG and in the GPx and GR activities in the course of the activity period. These changes are similar to those observed with ageing (De la Fuente and Miquel, 2009; Arranz et al., 2010). Cells from old animals almost maintain the same levels of TG and GPx activity in the course of the day and thus, these parameters did not demonstrate any circadian variations, whereas, GR activity decreased at 18:00 h. Comparing the values between adult and old mice, the peritoneal leucocytes from old mice showed lower levels of total glutathione (TG) and of activity of GPx and GR than those from adults at 8:00 h. The differences decreased in TG levels in the course of the day and disappeared in GPx activity at 18:00 h. Nevertheless, GR activity in old showed lower activity than those from adult mice along the day.

Since with ageing there are more ROS and less antioxidant compounds available, and that oxidative stress is one of the bases of the immunosenescence and the loss of homeostasis according to the oxi-inflamm-aging theory (De la Fuente et al., 2005; De la Fuente and Miquel, 2009), the results obtained on circadian changes in the redox state of peritoneal leucocytes, explain that the immunosenescence of old animals is appreciate at 8:00h, when the oxidative stress situation seems to be more evident. Thus, the involvement of leucocytes in oxi-inflamm-aging (De la Fuente and Miquel, 2009) could increase with ageing and through the day.

It is known that melatonin is a strong antioxidant (Reiter, 1995; Baydas et al., 2001) and stimulates a variety of antioxidant enzymes such as GPx and GR (Pablos et al., 1995). Thus, the decrease of the antioxidants studied through the day and in old mice with respect to adult could be due to the presence of smaller levels of this hormone in these situations. Moreover, GPx activity shows a daily rhythm with a peak after the peak of melatonin, and the pinealectomy, which eliminates the melatonin rhythm, has an inhibitor effect on GPx activity (Giyasettin et al., 2002).

5.1.4. Age-related changes in circadian variations of corticosterone levels

In the context of neuroimmunoendocrine communication, the circadian variations in the neurotransmitter and hormones could be involved in the circadian changes observed in the immune functions through the day (Carrillo-Vico et al., 2007). In this sense, as relevant hormones we have the glucocorticoides. Some studies showed that adrenal glucocorticoids can reset peripheral clocks (Balsalobre et al., 2000), and the adrenal circadian clock regulates the rhythmic release of glucocorticoids into the blood (Oster, et al., 2006). It is known that glucocorticoids inhibit the initial events in the inflammatory response, as well as increases the vascular permeability that occurs following inflammatory insult and decrease leucocyte emigration into inflamed sites (reviewed Perretti and Ahluwalia, 2000). Moreover, they

suppress transcription of many genes encoding pro-inflammatory cytokines and chemokines, cell adhesion molecules and key enzymes involved in the initiation and/or maintenance of the host inflammatory response (Barnes, 1998; Perretti and Ahluwalia 2000 and Smoak and Cidlowski, 2004). Thus, glucocorticoids, which receptors are widely expressed, are anti-inflammatory and immunosuppressive hormones, affecting virtually all immune cells (McEwen et al., 1997; Jin et al., 2009; Agnes and Karen, 2011).

In the current study no circadian variations were observed in the levels of plasma corticosterone of adult mice. However, the levels of this hormone in control BALB/c mice presented a circadian rhythm with a day-time maximum peak at 16:00 h (Barriga et al., 2001). Some studies showed that serum corticosterone concentrations were significantly lower at 8:00-11:00 h than at 15:00-18:00 h in mice (Kim et al., 2008). In old mice increased levels of this hormone in plasma were observed at 13:00 h and at 18:00 h. In C57BL/6J male mice a distinct age-related circadian pattern was observed. During the light period this was expressed with a relative hypercorticism in animals of 9-months of age and with a relative hypocorticism in those of 16-months of age (Dalm et al., 2005). In aged rats only a brief plasma corticosterone peak was observed at the early scotophase (Cano et al., 2008).

When levels of corticosterone in old and adult were compared, old mice showed higher levels of this hormone in plasma than adults at 13h and at 18h. These results are in agreement with results found in the plasma and adrenal gland corticosterone levels in aged male rats (Cano et al., 2008). Moreover, adrenocorticotropic hormone (ACTH) was also elevated in old male mice as well as in the aged male rats (Dalm et al., 2005; Cano et al., 2008).

Since corticosterone is a glucocorticoid, which acts as anti-inflammatory and immunosuppressive hormone (Agnes and Karen, 2011), its circadian changes are certainly responsible in part for the time-dependent

changes observed in the inflammatory response (Cutolo et al., 2006). In addition, the most relevant hormone involved in the circadian regulation, the melatonin, it is also implicated in the time-dependent inflammatory reaction with effects opposite to those of cortisol. Melatonin is involved in the control of rhythmic adaptations to daily and seasonal cycles, in mammals. Therefore, this hormone acts as a circadian clock, giving a time-related signal to a number of body functions such as the immune response (Srinivasan et al., 2008). In the present work the plasmatic melatonin levels have not been measured, but it is a future aim in our investigation. Moreover, it is known that, in man and mice, the melatonin release is maximal during the dark phase (Goulding and Hall, 1993). Several studies show that, melatonin modulates the T helper cell (Th) and lymphocyte counts (Skwarlo-Sonta et al., 2003). However, other investigations have suggested that melatonin alone does not affect lymphocyte proliferation, but does potentiate the corticosteroid inhibition of lymphocyte proliferation (Rogers et al., 1997). Thus, in human, cortisol and melatonin show an opposite response to the light (Cutolo et al., 2006). Both hormones act trough the decrease of the activation of the nuclear transcription factor (NFκB). Thus, melatonin reduces constitutive NFκB activation in cultured pineal glands (Cecon et al., 2010). In the pineal gland of rats, it has been observed that NFκB increased continuously during the light phase, and a sharp drop occurred when lights were turned off (Cecon et al., 2010). Recently, a diurnal rhythmicity in healthy humans between immune cellular response carried out by Th1 type cells, (which releasing mainly INF-γ, IL-2 and TNF-α) and immune humoral response by Th2 type (which releasing, among others, including IL-10) has been found and related to immunomodulatory actions of cortisol and melatonin. Thus, the IFN-γ/ IL-10 ratio decreased during the early morning and correlated negatively with plasma cortisol and positively with plasma melatonin (Cutolo et al., 2006). Nocturnal sleep favors a shift toward Th1 mediate immune defense, and a circadian peak of the ratio of IFN-γ / IL-10 production in blood samples is found during nocturnal sleep. This peak was completely abolished after the administration of cortisone at 21:00 h in the earlier evening, suggesting that

the suppression of endogenous cortisol release during early sleep plays a mediating role for the Th1 shift (Dimitrov et al., 2004; Kidd, 2003). Thus, cortisol and pro-inflammatory cytokines show a negative relation, since the increase production of inflammatory cytokines induce sleep while anti-inflammatory cytokines prevent sleep induction (Krueger et al., 2003; Krueger et al., 2001). The changes in TNF-α release by blood cells could be related to the levels of corticosterone since, in the present study, old mice showed higher levels of corticosterone in plasma than adults particularly at 13h and at 18h, when there are decreased levels of TNF-α by blood cells. In addition, it was observed that the susceptibility to lethal doses of endotoxin increase dramatically during the resting period of mice (Halberg et al., 1960).

In spite of the relevant effect of corticosterone and melatonin on the circadian changes of the immune functions, other hormones and neurotransmitter can also be involved. This is the case, for example, of the neuropeptide Y (NPY), which shows a modulator role in the immune system (Ferreira et al., 2010) and presents an increase in its levels both at the beginning and at the end of the light portion of the daily photoperiod in rats (Calzá et al., 1990). Upon LPS stimulation, NPY treatment strongly impaired the release of IL-1β (Ferreira et al., 2010). The modulator role of NPY on other cytokines such as TNF-α, as well as the changes of this regulation with ageing has also been shown (De la Fuente et al., 2001).

Other hormone with circadian variations than could affect the immune functions is prolactin (Yu-Lee, 2002). Plasma concentration of prolactin rise following the onset of sleep and its levels fall upon awakening (Frantz, 1978) and lymphocytes express prolactin receptors (Russell and Larson, 1985). Suppression of prolactin secretion in laboratory animals resulted in depressed T cell proliferation, cytokine production (Bernton et al., 1988), and expression of high affinity IL-2 receptors (Mukherjee et al., 1990).

5.2 Redox state in adult, mature and old prematurely ageing mice (PAM) vs non prematurely ageing mice (NPAM)

In the present work we used a model of prematurely ageing mice (PAM) that has been developed in our laboratory. Previous studies showed that PAM, in comparison to the non-prematurely ageing mice (NPAM) suffer a premature immunosenescence. This has been observed in several functions of leucocytes from peritoneum and from immune organs such as spleen. Moreover, PAM showed a brain neurochemistry similar to mice of an older chronological age and display higher levels of anxiety and emotionality with respect to their NPAM counterparts as well as a poor neuromuscular coordination. Moreover, PAM showed shorter life span than NPAM (Viveros et al., 2001; Guayerbas et al., 2002[a]; Guayerbas and De la Fuente, 2003, Viveros et al., 2007).

In addition, as a fact that confirm the theory of oxidation-inflammation of ageing and the possible involvement of the redox state of the immune cells in their function and in the general "oxi-inflamm-aging" of the organism (De la Fuente and Miquel, 2009), peritoneal leucocytes from young and adult PAM showed an oxidative stress situation (Alvarado et al., 2006[a]). Moreover, in spleen and, especially in brain of adult PAM also an oxidative stress with an imbalance between the levels of oxidants and the antioxidant defense, the first being higher than the second, has been observed (Viveros et al., 2007).

Since the redox state in other organs such as liver and heart of adult PAM in comparison to NPAM have not been studied and the age-related changes in this redox state are unknown, in the present study we decided to study the oxidative stress status in organs such as liver, spleen and heart of

PAM as well as in their NPAM counterparts in different ages (adult, mature and old).

In **adult** PAM, the results of the present study showed that the antioxidant defenses such as levels of TG and GSH as well as the GPx and GR activities in liver, as well as TG levels and these enzymatic antioxidants in spleen and heart, showed significantly lower values than those found in their NPAM counterparts. Moreover, the levels of the GSSG (an oxidant compound) and the GSSG/GSH ratio (a marker of oxidative stress situation) (Hwang et al., 1992) in liver of PAM were higher than those in NPAM.

The role of GSH in the organism is involved in two types of reactions during detoxification of reactive oxygen species (ROS). It reacts non-enzymatically with radicals such as the superoxide radical anion, nitric oxide or the hydroxyl radical (Saez et al., 1990; Clancy et al., 1994; Winterbourn and Meto-diewa, 1994; Singh et al., 1996) and GSH also is the electron donor for the reduction of peroxides in the GPx reaction (Chance et al., 1979). The final product of the oxidation of GSH is glutathione disulfide (GSSG).

Previous results in our laboratory showed an age-related decrease in GSH levels in blood leucocyte from women (Arranz et al., 2008) as well as in peritoneal leucocytes from chronological old mice and young prematurely aging mice (De la Fuente et al., 2004; Alvarado et al., 2006[a]; Viveros et al., 2007; Arranz et al., 2010). Moreover, they have also observed lower levels of GSH in brain of PAM with respect to NPAM (Viveros et al., 2007). Other authors have found similar results with respect to the levels of GSH in different tissues. Thus, they observed that GSH level falls in all tissues with age, due to a corresponding fall in glutamate cysteine ligase (GCL), which catalyzes the first and rate-limiting step in GSH biosynthesis to form the dipeptide γ GluCys that is then combined with glycine to generate GSH in a reaction catalyzed by glutathione synthase (GS) (Wang et al., 2003; Liu et al., 2004). Several studies have showed decreased levels of GSH in the brain

as well as in liver and heart from rats with an anxiety-like behavior (Sahin and Gumuslu 2004; Eren et al., 2007; Zafir and Banu, 2009). Decreased levels of both GSH and total antioxidant capacity (T-AOC), a measure of the additive effects of specific antioxidants, such as free sulfhydryl groups, vitamins C and E, uric acid, and bilirubin (Erel, 2004), have also been reported in humans with a state of trait anxiety (Arranz et al., 2007; Eskiocak, 2005). The blood levels of specific antioxidant appear to be decreased in anxiety, suggesting depletion of non-enzymatic antioxidant defences (Hovatta et al., 2010).

Regarding the GPx and GR activities in the organs studied, with lower values in PAM as compared with their NPAM counterparts, the results are in agreement with those found in peritoneal leucocytes of PAM (Alvarado et al., 2006a). In addition, other authors showed that in the brain of mice, the levels of several free-radical scavenging enzymes, including glutathione reductase (GR) superoxide dismutase (SOD) and catalase decrease with ageing (Mo et al., 1995). An age-related decrease of several antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), as well as GPx or GR was also found in liver and heart as well as in the frontal cortex in rats (Djordjevic et al. 2009; Eren et al., 2007). These decreased activities may represent situations where excessive oxidative challenge and high ROS levels have led to consumption of the antioxidant enzymes or depletion of its essential component (Bouayed et al., 2007).

The higher levels of GSSG that we have been observed in liver of PAM in the present study agree with the previous results obtained from our group in macrophages of PAM in comparison to those in NPAM (Guayerbas et al., 2002). Findings of other authors in mice showed age dependent increases in GSSG in several tissues including liver, kidney and heart (Rebrin et al., 2003). In addition, since the GSSG/GSH ratio is an indicator of the cellular redox environment state and shows an oxidative stress situation (Schafer and Buettner, 2001), the increase in this ratio observed in liver of PAM confirms the oxidative stress situation of these animals. This higher GSSG/GSH ratio in PAM results of a decrease in the

GSH content and an increase in the GSSG levels, which is characteristic of chronologically older mice (Mo et al., 1995; Rebrin et al., 2003). Another study showed that the GSSG/GSH ratio in human plasma increase with age, this ratio remained constant until 45 years and then decrease linearly thereafter, suggesting that GSH metabolism fails to keep up with oxidizing events beginning in late middle age (Jones et al., 2002).

These results confirm the premature oxidative state of adult PAM and consequently these animals are biological older, at the same chronological age, than NPAM, which we have already observed in the immune cells of those animals (Alvarado et al., 2006; Viveros et al., 2007). Our results are in agreement with those found in other prematurely aging model mice that lacking TR4 nuclear receptor (a key transcriptional factor regulating various biological activities, including reproduction, cerebella development, and metabolism), which exhibited defective oxidative stress defense in embryonic fibroblasts (Lee et al., 2011). In the senescence-accelerated mouse prone 8 (SAMP8), which is a model to probe the effects of aging on biological processes, displays oxidative stress, neural deterioration, altered levels of anti-oxidant enzymes and showed chronic inflammation (Menardo et al., 2012). In addition, PAM are animals with higher levels of anxiety (Viveros et al., 2007) and it is known that subjects with anxiety or depression show premature immunosenescence and oxidative stress situation (Arranz et al., 2007; 2009). Moreover, another model of anxiety in rats that developed by perinatal ethanol exposure, showed an increase in the levels of lipid peroxidation and protein oxidation in the brain, and a reduction in the levels of the endogenous antioxidant glutathione (Brocardo et al., 2012).

With respect to the results obtained in **mature** PAM and NPAM, the results showed that the levels of TG as well as the GPx and GR activities were lower in liver, spleen and heart of PAM compared to their NPAM counterparts. However, the differences were smaller between mature PAM and NPAM compared to those found in adults. Previous results obtained in our laboratory from peritoneal leukocytes of mature PAM showed a

decreasing in the antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities with respect to NPAM. However, the levels of several parameters of oxidation such as levels of extracellular superoxide anion, prostaglandin E2, nitric oxide, GSSG and lipid peroxidation (malondialdehyde, MDA) were higher in PAM than those found in their NPAM counterparts (Viveros et al., 2001, 2007; Alvarado et al., 2006[b]).

The age-related decreases seen in this study in TG and GSH could be due to increased GSH consumption, decreased GSH production or some combination of both. Moreover, the increased consumption of GSH would be consistent with an increase in ROS production with age, since aging is linked to an increase in the production of free radicals (Sastre et al., 2000; McArthur, 1998).

In chronologically **old** PAM, the levels of TG as well as GPx and GR activities in all studied organs were similar to those found in their NPAM counterparts. This could be explained by the age-related decrease in the antioxidant defense affecting NPAM. Thus, in chronologically old animals the oxidative stress situation of NPAM is similar to that in PAM, although these PAM showed this impairment in the antioxidant defense and consequently oxidation state since they are adults. The age-related changes in the antioxidant defenses studied in PAM and in NPAM suggest that NPAM show the typical decrease in these defenses with ageing whereas PAM did not suffer that changes because they show that decrease at adult age. The decrease in TG and GSH observed in the present study could be due, as it was previously mentioned, to an increase in the GSH consumption, a decrease in the GSH production or some combination of both. Since there is an increase in ROS production with age (McArthur, 1998; Sastre et al., 2000; De la Fuente and Miquel, 2009), and PAM produced higher oxidant compounds in adult age, we propose that an increased consumption of GSH would be involved in the premature oxidative stress state and premature aging of PAM. Moreover, since PAM show a shorter life span than NPAM (Vida and De la Fuente, 2013), we

suggest that the premature oxidation in PAM is determinant of the deterioration of the organism, which is shown as a older biological age, that promote a premature death of these animals in comparison to NPAM of the same chronological age. In addition, since the differences in the antioxidant capacity between PAM and NPAM disappear with age, we could suggest that to study the oxidative stress in this model (and possibly in other models) is better in adult animals than in mature and in old animals.

Several studies showed that anxiety is controlled by the nervous system, with gamma-aminobutyric acidergic (GABAergic) and serotoninergic systems playing important roles in its regulation (Weinberger, 2001). In this regard, it has been observed that abnormalities in these neurotransmitter systems in rodents can result in anxiety-like behavior (Gorman et al., 2002; Gingric, 2005). In addition, the alterations in these systems can also alter the function of the hypothalamic-pituitary-adrenal (HPA) axis, which is crucial for the regulation of stress and anxiety-related responses, and also have an impact on emotional response (Mathew et al., 2008). In this context, several studies have shown that oxidative stress-related molecular mechanisms and inflammation may represent unifying factors influencing the aethiology of normal and pathological anxiety (Hovatta et al., 2010). Recent research has shown a close relationship between the anxiety and oxidative stress status in both humans and experimental animals displaying high trait anxiety, as well as in human patients suffering from anxiety disorder (Bouayed and Bohn, 2010, Bouayed, 2011). Thus, the increased oxidative damage in brain, which cause nervous system impairment, contribute to the development of pathological anxiety disorders (Bouayed et al., 2009). Moreover, this oxidative stress situation in subjects with anxiety also can be observed at peripheral levels, especially in the immune system. Thus, there are several studies confirming a positive correlation between the amount of intracellular ROS in immune cells and anxiety-related behavior in mice (Bouayed et al., 2009; Ramalet al., 2008).

5.3. Immune function and antioxidant defenses in male and female triple transgenic mice for Alzheimer´s disease (3xTgAD). Effect of ovariectomy and physical exercise.

We have studied a murine model of triple-transgenic Alzheimer's disease (3xTg-AD) mice harboring *PS1*$_{M146V}$, *APP*$_{Swe}$ and *tau*$_{P301L}$ transgenes. This animal model mimics both amyloid and tau AD neuropathologies in an age-dependent manner in disease-relevant brain regions and reveals the important role of the intraneuronal accumulation of oligomeric Aβ in the etiopathogenesis of the disease (Oddo et al., 2003 [a]). The 3xTg-AD mice present synaptic and cholinergic deficits (Oddo et al., 2003; Oddo et al., 2005), the characteristic reactive gliosis inflammatory profile (Kitazawa et al., 2005) as well as cognition impairment (Billings et al., 2005) and behavioral and psychological symptoms of dementia like behaviors (Giménez-Llort et al., 2007).

Previous studies in our laboratory in old (15 months of age) 3xTg-AD, in which there are Aβ deposits in many cortical regions and tau hyperphosphorilation, showed an impairment of the neuroimmunoendocrine network and increased mortality rate. These animals had altered several functions of spleen and thymus leukocytes, whereas no oxidative stress parameters such as the levels of reduced glutathione and the results were gender specific (Giménez-Llort et al., 2008). Nevertheless, it was not known the immune state and oxidative stress situations at early ages.

Since at 4 month of age there are intracellular Aβ immunoreactivity, and at 9 months of age there are extracellular Aβ deposits in brain of 3xTg-AD mice (Giménez-Llort et al., 2007), in the present work, we proposed to study several peripheral immune functions at those ages. In leucocytes from spleen and thymus from male and female 3xTg-AD and NTg mice, several function, which are markers of biological age and longevity (De La Fuente and Miquel, 2009), were studied. Thus, chemotaxis, natural killer cell

activity, lymphoproliferation both in basal conditions and in response to Con A and LPS mitogens, as well as the levels of IL-2, IL-10, IL-1β and TNF-α in supernatant of cultures of spleen lymphocytes in response to ConA and LPS, were analysed in 3xTg-AD mice versus NTg at 4 (young adult) and 9 (adult) months of age. In addition, the levels of glutathione and the activities of the enzymes involved in its oxidation-reduction reaction GPx and GR in spleen of those young and adult male and female animals were also analysed.

5.3.1. Age-related changes of immune functions and antioxidant defences in male and female 3xTgAD

The regulatory systems, namely the nervous, endocrine, and immune systems, are intimately linked and interdependent, and a neuroimmune-endocrine system allows the preservation of homeostasis and therefore of health (Besedovsky and DelRey, 2007; De la Fuente and Miquel, 2009), as it has been mentioned previously. The communication between these regulatory systems is mediated by cytokines, hormones, and neurotransmitters through the presence of their receptors on the cells of the three systems, and the mediators of the three systems coexist in lymphoid, neural, and endocrine tissues. These facts show the complexity of the regulation not only at general levels, but also at local levels. The ageing process affects all of the physiological systems but especially the regulatory systems and the communication between them.

It is known that ageing results in impairment in the immune response, which is consequence of the imbalance in the oxidant-antioxidant state (De la Fuente and Miquel, 2009). Thus, to study the premature immunosenescence of 3xTg-AD in depth, the changes in function and antioxidant **parameters in thymus and spleen leucocytes from young-adult (4 month of age) and adult (9 month of age)** 3xTg-AD and the corresponding control NTg, **were studied.**

Changes in the neuroendocrine modulation of immune response with ageing, such as the changes in the innervation and irrigation of immune

organs, in the number and affinity of leukocyte receptors for neurotransmitter and hormones, as well as in the intracellular signals and the resulting response of the immune cells to them (Bellinger et al., 2001; De la Fuente et al., 2001; De La Fuente et al., 2004; Puerto et al., 2005), have been observed.

Immune function in adult-young and adult 3xTg-AD mice vs NTg

The results in the present study showed that, at both ages (adult-young and adult), spleen and thymus leucocytes from 3xTg-AD mice exhibited lower chemotaxis, natural killer cell activity, lymphoproliferation in response to Con-A and LPS mitogens than in NTg mice. These results confirm the premature immunosenescence of 3xTg-AD, since previous results showed that those immune parameters decrease with ageing (De la Fuente et al., 2004). This is in agreement with the suggestion of Richartz et al. (2006) that observed a general decline in the immune functions in AD. They found peripheral changes of the immune systems including lymphocyte function and subset distribution, as well as a significant decrease of T and B-lymphocytes. Moreover, in senile dementia Alzheimer type individuals were characterized by decreased T helper cell (Th) activity (Antonaci et al., 1990). In addition, other studies showed a decline in the innate immune cells of AD patients (Fiala et al., 2002). A decreasing in blood NK activity was also observed in AD patients as compared to the control (Higuchi et al., 2010).

With respect to the cytokines network, the results we obtained in leucocytes of 3xTg-AD mice in response to ConA and LPS showed a decrease in the levels of IL-2 and IL-10, an increase in those of IL-1β and TNF-α. These results are in agreement with those observed with ageing (Arranz et al., 2010). Previous results in our laboratory from old (15 months of age) 3xTg-AD mice demonstrated an increase in the release of TNF-α by spleen leukocytes (Giménez-Llort et al., 2008). Some studies showed that the stimulation by recombinant amyloid-beta peptide induces the production of the pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, and TNF-β (Pellicano

et al., 2010). A significant decrease in IL-10 production was found in AD patients after stimulation of peripheral blood mononuclear cells with β-amyloid compared with age-matched healthy controls (Speciale et al., 2007). Results on human showed a significant increase in interferon gamma (IFN-γ) secretion by mononuclear cells (Huberman et al., 1994) and in IL-1β in AD patients as compared to the control (Higuchi et al., 2010).

Since some studies reported an involvement of systemic immunity in AD patients in the progression of the illness (Pellicano et al., 2010), the results obtained in 3xTg-AD mice, which showed the premature impairment of the peripheral immune cell functions, could corroborate that involvement of the immune system in the progression of the disease.

Antioxidant defense in adult-young and adult 3xTg-AD mice vs NTg

It is known that the oxidative injury is central in the pathogenesis of AD, even before the appearance of amyloid deposits (Selkoe, 2001; Resende et al., 2008). It is found that Aβ produces oxidative injury and pro-oxidants increase Aβ production. Thus, in AD brains the levels of expression and activities of several antioxidant enzymes are altered with an increase in the appearance of markers of oxidative stress (Butterfield et al., 2007; Choi et al., 2005; Chauhan and Chauhan, 2006). Moreover, multiple studies also showed an increase in the levels of lipid peroxidation and protein, DNA, and RNA oxidation in the brain of patients of AD (Lovell and Markesbery, 2007). In addition, it is found that the microglial cells produce neurotoxins in response to Aβ exposure in AD (Streit, 2002), which activate the inflammatory cells in AD brain, results in the secretion of cytokines, such as IL-1 and TNF-α, and chemokines as well as the release of reactive oxygen species and nitrogenous compounds, all of which exacerbate the pathology (Akiyama et al., 2000; Grammas, 2002).

With respect to the oxidative stress parameters analyzed, the results obtained in the present study showed both young adult and adult transgenic mice, compared with the corresponding NTg animals, show a decrease in

spleen antioxidant defenses, such as total glutathione (TG) levels and activity of GPx and GR antioxidant enzymes. These changes in antioxidants compounds are characteristics of prematurely and chronologically aged subjects (De la Fuente and. Miquel, 2009; De la Fuente et al. 2005; De la Fuente et al. 2011).These results agree with those obtained in the mouse model of streptozotocin-induced experimental dementia of Alzheimer's type, which displayed a decrease in the levels of GSH and its dependent enzymes, GPx and GR, activities as compared to the control (Javed et al., 2011). Thus, there is premature immunosenescence in the 3×Tg-AD mice, which is underpinned by an oxidative stress state. These facts confirm the premature ageing of transgenic mice and explain their early mortality. However, in old (15 months of age) 3xTg-AD mice similar levels of GSH were obtained in the spleen and thymus leukocytes to those found in NTg mice (Giménez-Llort et al., 2008). These results confirm the premature oxidative stress state in 3xTg-AD mice, which is detected at peripheral levels, and in adult mice.

If we compare the immune and antioxidant parameters between mice at 4 and 9 months, the results showed, in general, that 3xTg-AD adult (9 months of age) mice demonstrated impairment in functional activity and antioxidant defense more than those found in young-adult mice. This impairment is also observed in NTg with exception in chemotaxis index of female spleen leukocytes, levels of IL-1β and TNF-α in cultures of spleen leukocytes, which agree with the results obtained in rats (De La Fuente et al., 2004).

Gender related changes in immune function and antioxidant state in 3xTg-AD and NTg mice

There are gender differences in the changes of the neuroimmunoendocrine network with ageing, it is documented that females live longer than males in a great range of animal species, including humans (Rosenblitt et al., 2001).

The results obtained in the present study showed that male mice, particularly 3xTg-AD, demonstrated impairment in functional activity and antioxidant defense more than those found in females. These findings are agree with those found in previous studies in our laboratory that showed significant changes in the immune functions of old 3xTg-AD male mice. Thus, a higher impairment of natural killer cell activity as well as a smaller release of IL-2 by spleen and thymus leucocytes in male 3xTg-AD mice than in females was observed (Giménez-Llort et al., 2008).

Several studies reported that the immune system in females works more efficiently and effectively than this in males (Aspinall, 2000). In vitro stimulation of lymphocytes with phytohemagglutinin, a mitogen used to elicit cytokine production from immune competent cells, found that females produce more Th2 (IL-4, IL-10) cytokines, responsible for secretion of antibodies (Whitacre et al., 1999), than males (Giron-Gonzalea et al., 2000), as well as show stronger antibody (Weinstein et al., 1984) and cell-mediated immune responses (De la Fuente et al., 2004). Furthermore, females have a higher percentage of T lymphocytes within the total lymphocyte pool (Bouman et al., 2004), and have more active circulating polymorphonuclear leucocytes and macrophages (Spitzer et al., 1996[a]; Spitzer et al., 1996[b]). Scotland et al. (2011) showed that the numbers of leucocytes occupying the naive peritoneal and pleural cavities is higher in female than in male mice and rats, comprising more T- and B-lymphocytes as well as macrophages. Female resident macrophages exhibit greater Toll-like receptor expression, as well as enhanced phagocytosis and NADPH oxidase-mediated bacterial killing (Scotland et al., 2011).

Oestrogens have been suggested to be responsible for this higher immunocompetence of females (De la Fuente et al., 2004; Keller et al., 2001), since immune cells such as T cells, macrophages, and monocytes possess oestrogen receptors (Bird et al., 2008). Our results imply that adult females, of reproductive age, have a more active immune system and antioxidant defences than age matched males.

5.3.2. Immune function and antioxidant defense in ovariectomized (Ovx) NTg and 3Tg AD female mice, and their comparison with males

It is known that menopause results from reduced secretion of the ovarian hormones oestrogen and progesterone, which takes place as the finite store of ovarian follicles is depleted. This is a normal event for women or it can also be induced by surgery, chemotherapy, or radiation (Nelson; 2008). Ovariectomy in rodents constitutes a good model for mimicking human oestrogen loss and thus the menopausal situation. This ovariectomy in mice and rats showed impairment in the sensorimotor abilities, exploratory capacities, premature immunosenescence as investigated in organs, such as spleen and axillary nodes, as well as in peritoneal leucocytes (De La Fuente, 2004; Baeza et al., 2010; Fernández-Tresguerres, 2004; Baeza et al., 2011).

Since ovariectomy seems to cause a deterioration of homeostasis, and its effect in NTg and 3xTg-AD mice has not been studied, in the present work the function and antioxidant parameters above mentioned were analysed in ovariectomized animals. Thus, the effect of ovariectomy on chemotaxis, natural killer cell activity, lymphoproliferation (both in basal conditions and in response to Con A and LPS mitogens) were studied in leukocytes from spleen and thymus as well as the levels of IL-2, IL-10, IL-1β and TNF-α, in cultures of spleen lymphocytes in response to ConA and LPS. In addition, the levels of glutathione and the activities of the enzymes involved in its oxidation-reduction reaction GPx and GR in spleen of adult 3xTg-AD ovariectomized mice were analysed. All these parameters were studied in female adult *(9 month of age) NTg and 3xTg-AD ovariectomized mice and in the corresponding sham groups as well as in male NTg and 3xTg-AD mice.*

The results in the present study showed that NTg ovariectomized mice exhibited impaired functional activity and antioxidant defense compared to those found in their non-ovariectomized animals. These results are agreed

with those obtained in our laboratory from rodents and humans (De la Fuente, 2004; Fernández-Tresguerres, 2004; Arranz et al., 2008; Baeza et al., 2010; Baeza et al., 2011). Peritoneal macrophages and lymphocytes from old ovariectomized females showed that the chemotaxis index, NK activity and proliferation in response to Con A and LPS mitogens were significantly impaired with respect to the corresponding sham animals. In addition the ovariectomized mice exhibited lower levels of IL-2 and IL-10 in supernatants of cultured cells stimulated with the mitogen ConA with respect to sham females (Baeza et al., 2011). Similar results have also been obtained in axillary nodes and spleen from rats (De La Fuente et al., 2004) and mice (Baeza et al., 2010). Since many of these parameters are markers of biological age and longevity (Alonso and De la Fuente, 2008), *ovariectomy produces a premature ageing, which has been proposed previously* ***(Baeza et al., 2010).***

It is known that oestrogens are responsible for the higher immunocompetence of females, since oestrogen-deprivation might decrease several immune functions (Keller et al. 2001; De la Fuente et al., 2004). Moreover, oestrogens appear to play a central role in the immune response and immune-mediated diseases (Cutolo et al., 1995). Recent studies have shown the presence of oestrogen receptors on the cells involved in the immune response, namely thymocytes, T cells, macrophages, monocytes and endothelial cells (Cutolo et al., 1995; Bird et al., 2008). In addition, hormone replacement therapy by oestrogens and natural phyto-oestrogens in ovariectomized rats, which showed the impairment in leucocyte functions (chemotaxis, lymphoproliferative response to the mitogen, the release of IL-2 and the NK cell activity) improved their immune response (Baeza et al., 2009).

The results obtained in the present study showed that adult ovariectomized mice exhibited impairment in antioxidant defense (levels of total glutathione and GPx and GR activities) compared to those found in their non-ovariectomized animals. Previous results showed that ovariectomy caused no alteration in GSH levels, but it increased those of GSSG, with the

subsequent rise in the GSSG/GSH ratio, and did not significantly alter the catalase and GPx activities (Baeza et al., 2011). The antioxidant deficit has been related to impaired immune responses, leading to frequent and severe infections (Knight, 2000). Glutathione (GSH) is the principal intracellular non-protein thiol and plays a major role in preservation of the intracellular redox state. It acts as a nonenzymatic reducing agent, preventing oxidative stress in most cells, tissues, and organs (Dröge, 2002). It was shown that the administration of N-acetylcysteine, thiolic compound acting as GSH precursor, in postmenopausal women, improved the immune function and oxidative stress parameter parameters as compared with the control group (Arranz et al., 2008).

In the present study we have also investigated the effect of ovariectomy on the immune function and antioxidant parameters of ovariectomized triple transgenic Alzheimer's disease (Ovx-3xTg-AD) mice. The result showed there are no significant differences in the functional and antioxidant parameters, with exception in the levels of TG, between the Ovx-3xTg-AD mice and their none-ovariectomized counterparts. This could be explained by the impairment that control female 3xTg-AD mice already have in the immune function and oxidative stress parameters (Javed et al., 2011; Maté et al., 2014). It is difficult to decrease more a state very immune-depressed, as it is improve very much a very healthy immune function. A similar fact was observed in this study with PAM, since old PAM showed immune function and oxidative stress parameters similar to those found in old NPAM. Both Ovx-3xTg-AD and none-ovariectomized 3xTg-AD mice showed similar levels in the parameters studied to those found in males. This is in agreement with previous results obtained in our laboratory that showed the chemotaxis index significantly impaired in ovariectomized animals, resulting in values similar to those found in males (Baeza et al., 2011).

5.3.3. Effects of physical exercise on immune functions and antioxidant defenses in adult male and female NTg and 3xTg-AD mice and in ovariectomyzed mice

Effect of physical exercise in adult male and female NTg and 3xTgAD mice

The 3xTg-AD mice we studied showed impairment in function and oxidative stress parameters with respect to NTg, which confirmed the results found in old (15 months of age) 3xTg-AD mice (Giménez-Llort et al., 2008). Different strategies have been performed to improve this impairment in 3xTg-AD mice such as environmental enrichment (Arranz et al. 2011). Several strategies of life style have been proposed to improve the immune response in subjects with chronological and premature immunosenescence as diets supplemented with appropriate amount of antioxidants (revised in De la Fuente et al., 2011) have been used in elderly men and women (De la Fuente et al., 2007; Arranz et al., 2008), PAM (Alvarado et al., 2006) or double transgenic mice for AD (Marin et al., 2013).

Among other strategies, regular physical exercise, which has a number of beneficial physiological effects, including the modulation and stimulation of the immune functions (Astrand, 1997; Sugiura et al., 2000; Hoffman and Pedersen, 1994; Roy and Pang, 1994), has been proposed to improve immune response in subject with immunosenescence (De la Fuente et al., 2005; De la Fuente et al., 2011).

In the present work the effects of moderate physical exercise (voluntary running wheel) on several functions of lymphocytes, which decrease with ageing, such as proliferation in response to the mitogens ConA and LPS, the antitumoral NK activity and migration capacity to the focus of infection (chemotaxis) in thymus and spleen as well as the antioxidant defense (TG levels and GPx and GR activities) in spleen from female and male NTg and 3xTg-AD mice were studied.

Effects of physical exercise on immune functions and antioxidant defences in adult NTg mice

In NTg adult mice, the results of the present study showed in general that moderate exercise enhanced the leucocytes function parameters in spleen and thymus and improved the antioxidant defense in spleen. These results agree with many previously observed in mice and in humans. Although an excess of physical exercise can deteriorate the immune response, a moderate and training exercise, in general improve immune functions, especially those of innate immunity. Thus, swimming exercise for 90 min/day over a period of 20 days enhances phagocytic functions (chemotaxis capacity, phagocytosis capacity, and superoxide anion production) of peritoneal macrophages compared with those of sedentary control mice (Ferrandez and De la Fuente, 1999). Moreover, the treadmill running exercise and voluntary running exercise for 8-12 weeks enhance phagocytic and elimination activities in peritoneal macrophages as well as lymphocyte proliferation in response to concanavalin A (Sugiura et al., 2001). Several other studies showed that moderate exercise was associated with increased proliferative response to PHA in adult mice and improved ConA-induced IL-2 production in old rats (Kohut et al., 2004.). Besides the enhancement of immune cell functions, it is found that moderate exercise causes an increase of number of cells such as, total leukocyte count, granulocyte, monocyte, lymphocyte and natural killer cell count, total T cell count, and helper:suppressor cell ratio. Moreover, cell proliferation in response to mitogens, serum immunoglobulin levels, and in vitro immunoglobulin production were increased by that exercise (Roy and Pang, 1994). In addition, several kind of intense exercise such as the forced running (on a flat floor for 60 min a day, during 5 days per week for 12 weeks) in young mice (4 weeks) resulted in an increase in the spleen and thymus weight, an increase in phagocytic index of peritoneal macrophages to latex beads and an increase in the proliferation of spleen cells induced by ConA (Sugiura et al., 1993). Some studies in neutrophil from adult human males showed that chronic moderate exercise diminished the neutrophil

apoptosis and maintained the redox balance, by increasing neutrophil GSH which in tern prevented the elevation in the production levels of ROS (Syu et al., 2011). In general, moderate exercise has anti-inflammatory effects, which reduce the risk of diseases **(Gleeson et al., 2011)**. In addition, it was reported that the NK cell activity is increased when measured immediately after or during both moderate and intense exercise (bicycle exercise) of a few minutes (Pedersen and Goetz, 2000).

Effects of physical exercise on immune functions and antioxidant defenses in adult 3xTg-AD mice

In 3xTg-AD adult mice, the results showed that the moderate exercise used enhanced the leucocyte function parameters in spleen and thymus and improved the antioxidant defense in spleen. García-Mesa et al. (2011), have found in 3xTg-AD young (4 months of age) mice, that after one month of getting free access to a running wheel, several benefits of this aerobic physical exercise on synapse, redox homeostasis, and general brain functions. Exercise treatment improved cognitive function and behavior as well as decreased psychological symptoms of dementia. Oxidative stress was decreased and antioxidant defences were induced (García-Mesa et al., 2011). In addition, the results obtained in the present work are agreed with those observed in 3xTg-AD old mice (15 months of age) after they had exposed to enriched environment that began in the adulthood (6 months of age) and lasted for 5.5 months. With enriched environment animals carried out more voluntary physical exercise than the corresponding control without that enrichment. Those animals showed improvement in lymphocyte functional activities such as chemotaxis and natural killer cytotoxicity (Arranz et al., 2011).

The effect of moderate physical exercise was more significant in males than in females and in 3xTg-AD than in NTg mice. This could indicate that moderate physical exercise improves the immune functions and redox state in the individuals suffering a decline in those functions and

redox state as mentioned previously, such as in males as compared with females and in 3xTg-AD as compared with NTg mice. In general this fact occurs with all the strategies of life style studied (De la Fuente et al., 2011).

Effects of physical exercise on immune functions and antioxidant defenses in adult Ovariectomized 3xTg-AD mice

In a previous study carried out by the research group of De la Fuente (2004) it became clear that the lack of sex steroids caused by ovariectomy seriously impaired key immune functions (De la Fuente et al., 2004) and consequently accelerated immunosenescence. Regarding sex hormones, it has been established that physiological levels of oestrogens stimulate the immune response, probably due to the presence of their receptors in certain immune cells including T cells, monocytes and macrophages (Bird et al., 2008). In the present work, we have found that the impairment of the immune functions as chemotaxis capacity, antitumoral NK activity and proliferation of lymphocytes in response to mitogens, in adult ovariectomized NTg as well as 3xTg-AD mice can be improved by moderate physical exercise. Our results are in agreement with several studies that showed the moderate training exercise leads to clear benefits of the immune system with improvement of its functions, such as T cell function, antibody production, macrophage responses, cytokine modulation and ratio naïve/memory cells (Giraldo et al., 2009; Karanfilov et al., 1999). The effects of physical exercise in the immune system are carried out in the context of neuroendocrineimmune communication, and concretely in the stress situation that occurs with physical exercise. Thus, exercise influences the immune response through changes in the sympathetic nervous system (SNS) and/or the hypothalamic-hypophysial-adrenocortical axis (HHA) (Ortega, 1994; Ortega, 2003). It is well known that physically active people are at a lower risk of illness, and that habitual exercise is an effective mean of preventing or delaying chronic diseases, exercise is recommended for the treatment and prevention of a large number of diseases(Pedersen and Saltin,

2006). Moreover physical activity has been proposed as another lifestyle factor to modulate immune response improving health and quality of life in old subjects (De la Fuente et al., 2005;Polidori et al., 2006).

Moreover, the result present results showed that moderate exercise improved some antioxidant defense in spleen such as, the levels of total glutathione (TG) and the antioxidant enzyme activity glutathione reductase, that involved in its reduction reaction (GR). A review by the group of Northoff et al. (1994) is clearly supported that although regular training or habitual physical exercise seems to decrease the capacity of phagocytes for releasing oxidants and leads to an adaptation of antioxidant mechanisms.

Some studies suggested that physical exercise could decrease the increased levels of ROS of phagocytes from old subjects by increasing antioxidant defenses (De la Fuente et al., 2005;De la Fuente et al., 1995).

6. CONCLUSIONS

The following conclusion can be obtained from the three objectives proposed

1. Functions and antioxidant defences of peritoneal leucocytes from adult mice show circadian changes, which are similar to those that occur with ageing. These circadian variations are not shown in old mice. The values of many parameters in adult mice at 18:00h are similar to those in old animals. The circadian changes in pro-inflammatory and anti-inflammatory cytokines in adult and old mice, are different when they are released by peritoneal or blood leucocytes. In adult animals there are no circadian variation in the levels of plasma corticosterone, whereas in old mice, which show higher levels than adult animals, an increase appears in this hormone along the day.

2. The organs (heart, spleen and liver) from adult prematurely ageing mice (PAM) show decreased antioxidant defences (total glutathione levels, glutathione peroxidase and reductase activities) in comparison with the corresponding NPAM. The oxidative stress state (GSSG levels and GSSG/GSH ratio) is higher in the liver of PAM than in that of NPAM. Antioxidant defences and oxidative stress decrease and increase, respectively with ageing in PAM and NPAM. These changes are more pronounced in NPAM and for this reason the differences between both groups decrease with ageing.

3. In 3xTg-AD (Tg) mice with respect to NTg animals the functions of thymus and spleen leucocytes are impaired, as well as the spleen antioxidant defences being decreased. This occurs in both males and females and at 4 and 9 months of age. Ovariectomised NTg mice show an impairment of leucocyte functions and antioxidant defences, with values similar to those in sham 3Tg animals. However, ovariectomy does not affect the parameters studied in 3Tg mice. Voluntary physical exercise improves functions and antioxidant defences in male and female NTg mice as well as in ovariectomised NTg and Tg animals.

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