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Influence of metabolic status on oocyte quality and follicular characteristics at different postpartum periods in primiparous rabbit does

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Abstract

Low reproductive performance of high-yield primiparous animals is closely associated with the metabolic stress caused by a simultaneous gestation and lactation. The aim of this work was (1) to analyze body composition and metabolic environment at three time points along lactation (at parturition time; in the lactation period [Day 11 postpartum]; and in the postweaning period [Day 32 postpartum]) of primiparous rabbit does (Oryctolagus cuniculus) and (2) to investigate the ovarian status at insemination time and the possible link with metabolic environment and with their reproductive performance. To this end, does were either submitted to a semi-intensive reproductive rhythm (Group S, inseminated on Day 11 postpartum) or an extensive rhythm (Group E, inseminated on Day 32 postpartum). Body energy ($P < 0.05$) and protein content ($P < 0.001$) as well as serum leptin ($P < 0.05$) and protein concentrations ($P < 0.001$) increased significantly along the postpartum period. At parturition, body lipid content was significantly lower and serum nonesterified fatty acids concentrations were significantly higher than that on Days 11 postpartum and 32 postpartum. Concerning ovarian status at insemination time, no significant differences were found in mean follicular stages, serum estradiol, progesterone, and prolactin (PRL) concentrations or in prolactin receptor (PRL-R) immunostaining. However, follicles in Group S showed a significantly higher apoptosis index than that of Group E ($P < 0.001$). The nuclear and cytoplasmic oocyte maturation rates of Group S were also significantly lower than that in Group E. In addition, conception rate and prolificacy were improved in Group E ($P < 0.001$ and $P < 0.05$, respectively). In conclusion, in the early postpartum period, metabolic status seems to impact negatively on ovarian follicle and oocyte quality leading to a poor reproductive outcome in primiparous rabbit does.
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Keywords: Follicular atresia; Metabolism; Oocyte maturation; Postpartum; Rabbit

1. Introduction

Rabbits show high sexual receptivity immediately after kindling. Then, it decreases in a nonpredictable trend and another peak appears only after weaning [1]. However, as occurs in cattle [2], insemination of female rabbits during lactation seems particularly negative due to nutritional competition, leading to a

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negative energy balance in these females [3,4]. This phenomenon is well known and particularly significant at the first parity in certain species [5,6]. Despite the rapid increase in doe feed intake during the early postpartum (postpartum) period, the doe is unable to cover its energetic needs for lactation of multiple litters, gestation, and its own growth [7]. Therefore, these animals show a poorer reproductive outcome than that of multiparous animals [2,8,9]. Artificial insemination (AI) around 11 days postpartum is the most common reproductive rhythm in commercial rabbit farms. It adapts well to cycled production, but this approach ignores the reproductive physiology of the females. This rhythm causes, especially in primiparous rabbit does, a pronounced reduction of conception rate mainly due to the low body condition and hormonal antagonism [10,11].

Body reserves status is reflected by changes in some metabolic parameters, such as serum protein, nonesterified fatty acids (NEFAs), and leptin concentrations [12]. They are present in follicles [13–15] and oviduct [16]; therefore, those parameters could affect the competence of the gametes before fertilization, which in turn influences oocyte maturation and presumably embryo survival [17–19]. In addition, during lactation, rabbit does show high prolactin (PRL) concentrations [20]. Prolactin is a hormone produced by the pituitary gland and also by extrapituitary sites such as mammary gland, placenta, and uterus [21]. This hormone is the main responsable of the negative effect of lactation on the reproductive function [22]. The expression of PRL receptor in ovaries in several species [23,24] suggests a direct action of this hormone in follicular development and oocyte quality [25–27].

Most of the negative effects in the postpartum period due to metabolic stress and lactation status, which seem to affect follicle and gamete quality, have been extrapolated from studies in other species [5,28,29]. To our knowledge, no previous studies exist about the possible consequences on rabbit ovaries. In order to explain the observed decrease in reproductive performance, especially in primiparous does, the aim of this work was to study ovarian status at different insemination time points in terms of steroid concentrations and oocyte and follicle features. The evolution of metabolic parameters and body composition during the first postpartum period and the possible relationship of these parameters with the subsequent conception rate and prolificacy to establish the best physiology-based management for rabbit species were evaluated.

2. Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA). All the experimental procedures were approved by the Animal Ethics Committee of the Polytechnic University of Madrid (Spain) in compliance with the Spanish guidelines for the care and use of animals in research [30].

2.1. Animals and experimental design

Along the study, New Zealand × California white rabbit does (Oryctolagus cuniculus) were held on the experimental farm at the Animal Production Department, Polytechnic University of Madrid (Spain). Animals were housed in individual flat-deck cages under a constant photoperiod of 16 h light per day, a temperature of 18 to 22 °C, and a relative humidity of 60% to 75% maintained by a forced ventilation system. They were fed ad libitum a commercial pelleted diet for lactating does (Cunimax; Cargill S.A., Barcelona, Spain).

To evaluate metabolic serum parameters, ovarian status, and reproductive performance related to different postpartum periods, a total of 90 primiparous does were used. Litter size of does was equalized to eight kits one day after parturition. Does were randomly allocated in two experimental groups: Group S (n = 45), lactating does inseminated on Day 11 postpartum, following a semi-intensive reproductive rhythm; Group E (n = 45), postweaned lactating does inseminated on Day 32 postpartum, following an extensive reproductive rhythm. In Group S, all animals were estrus-synchronized by transient doe-litter separation before artificial insemination (AI). Biostimulated does were separated from their litters by a metal screen for 24 h before AI, from Day 10 until Day 11 postpartum [9,31]. In Group E, animals were estrus-synchronized by weaning 4 days before AI. In both rhythms, weaning was performed at Day 28 of the lactation period. Artificial inseminations were carried out using a pool of fresh heterospermic semen with more than 20 million spermatozoa in 0.5 mL commercial diluent (Magapor S.L., Zaragoza, Spain). Ovulation was induced by intramuscular injection of 1 μg buserelin (Suprafact, Hoechst Marion Roussel S.A., Madrid, Spain). Conception rate (number of parturitions/number of inseminations × 100) and prolificacy (total born and dead born per doe) were recorded for all animals inseminated.

At parturition time, at Day 11 postpartum and Day 32 postpartum, live body weight (LBW) and estimated body composition were determined in 25 does/group. Metabolic parameters (serum leptin, NEFAs and protein concentrations) were recorded in 15 does/group. Body composition was determined by bioelectrical impedance analysis (BIA) according to multiple regression equations described by Pereda et al. [32] to estimate water, protein, ash, fat, and energy content (%) in relation to live body weight.

To study the ovarian status previous AI, 15 does/group were euthanasized with 30 mg/kg intravenous pentobarbital sodium (Dolethal; Vetoquinol, Alcobendas, Spain) and subjected to laparotomy in order to collect their ovaries. Serum PRL, estradiol (E_2), and progesterone (P_4) concentrations and ovary weight and follicles $\geq$1 mm in the ovarian surface were recorded. One ovary was used for histologic and immunohistochemical assays (follicular population categorization, follicular apoptosis index, and PRL receptor distribution), whereas the other one was used for the oocyte maturation assessment.

2.2. Blood sampling and metabolic and endocrine assay

Blood samples were collected from the margin ear vein into nonheparinized tubes at 0900 to avoid circadian variations. Serum was obtained after centrifugation at 1200 × g for 10 min at 4 °C and stored at–32 °C until analyzed.

2.2.1. Leptin

Serum leptin concentration was determined in duplicate samples using a multispecies leptin RIA kit (LINCO Research, St. Charles, MO, USA), as previously reported in rabbits [33]. Intra-assay and interassay coefficients of variation were 3.1% and 7.3%, respectively. The detection limit after adjusting the standard curve to rabbit values was 0.1 ng/mL HE human equivalent (HE).

2.2.2. Nonesterified fatty acids

Serum NEFA determination was performed in duplicate samples using the acyl-CoA synthetase-acyl-CoA oxidase (ACS-ACOD) method as prepared by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The method is linear over the range from 0.0 to 2.0 mmol/L.

2.2.3. Protein

Serum total protein was determined by the Biuret method, according to Tietz [34]. In alkaline media, cupric ions interact with protein peptide bonds, resulting in the formation of a colored complex. This method is linear up to 13 g/dL.

2.2.4. Estradiol and Progesterone

Serum E_2 and P_4 concentrations were measured in duplicate samples by specific chemiluminiscence methods (CMIA; Abbott Laboratories, Abbott Park, IL, USA). For E_2 detection, purified rabbit anti-estradiol monoclonal antibodies were used. Progesterone detection was achieved by using rabbit anti-progesterone polyclonal antibodies. Intra-assay and interassay coefficients of variation were 6.6% and 7% for E_2 and 5.8% and 6.3% for $P_{4,}$ respectively. The detection limit was 10 pg/mL (E_2) and 0.2 ng/mL (P_4).

2.2.5. Prolactin

Serum PRL concentration was measured in duplicate samples by means of a specific ELISA kit (USCN LIEF, Double Lake, MO, USA). This assay detects both recombinant and natural rabbit PRL. Intra-assay and interassay coefficients of variation were 7.7% and 8.4%, respectively. The minimum detectable dose of rabbit PRL was 0.039 ng/mL.

2.3. Study of follicular population

The number of preovulatory follicles in the ovarian surface $\geq$1 mm in size was first recorded. Then, one ovary per doe was placed into a 4% wt/vol buffered neutral paraformaldehyde solution (pH 7.2 to 7.4). All samples were gradually dehydrated with increasing concentrations of ethyl alcohol (50% to 100% vol/vol). These dehydrated specimens were first embedded in paraffin, prepared by sectioning at 5 μm, and stained with hematoxylin and eosin. In order to study follicle population, histologic sections of each half ovary were examined at the light microscope (Olympus BX40; Olympus, Hamburg, Germany). Rabbit ovarian follicles were categorized into four specific development stages related to the number of layers of granulosa cells according to Arias-Álvarez et al. [35] and Rebollar et al. [36] as primordial, primary, secondary, and antral follicles.

2.4. Follicular atresia assay

Strand breaks of DNA occurring during the cell apoptosis process were detected using terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL; In Situ Cell Death Detection Kit, POD, Roche Diagnostics S.L., Applied Science, Barcelona, Spain). Dewaxed, rehydrated sections by standard methods were first carried out. The slides were then pretreated with 20 μg/mL proteinase K working solution for 30 min in a humidified dark chamber at 37 °C. Incubation with the TUNEL reaction mixture took place in a humidified dark chamber at 37 °C for 1 h. After each step of the procedure, sections were rinsed three times in phosphate-buffered saline (PBS). Finally, the slides were covered with Vectashield mounting medium with 4′,6-diamino-2-phenylindole (DAPI) (Vector Laboratories, Ltd., Peterborough, UK). Positive control sections were treated with DNAse I for 10 min at room temperature in a humidified chamber (Roche Diagnostics S.L., Applied Science) before incubation with the TUNEL reaction mixture. For negative controls, samples were just incubated with the label solution of the TUNEL reaction mixture without the enzymatic solution. TUNEL-stained and DAPI-counterstained slides were observed under a fluorescent microscope (F550; Leica, Wetzlar, Germany). Green fluorescence could be visualized only in TUNEL-positive cells. Follicles in medium or advanced stage of atresia were examined according to Kasuya et al. [37], and the percentage of apoptosis expressed is the number of TUNEL-positive follicles divided by the total number of recorded follicles.

2.5. Immunohistochemistry of prolactin receptors

The protocol used was according to that described by García-García et al. [38]. Briefly, after slide deparaffinization and rehydration in graded alcohol, sections were heat-treated in sodium citrate solution (pH 6) to unmask antigen epitopes. Endogenous peroxidase activity was blocked by incubating the slides with 1.5% vol/vol hydrogen peroxide in methanol for 15 min. Nonspecific binding of immunoglobulins was blocked with 3% vol/vol normal equine serum in Tris buffer solution (TBS) at room temperature for 30 min. Primary antibody against prolactin receptor (1:75, Affinity Bioreagents, Golden, CO, USA) was incubated overnight at 4 °C in a humidified chamber. The antibody used in this study specifically recognizes long and short forms of rabbit prolactin receptor (PRL-R). In the negative control sections, incubation with primary antibody was replaced by TBS. After that, samples were incubated for 30 min with biotinylated secondary antibodies (1:400, biotinylated anti-mouse IgG [H + L] made in horse; Vector Laboratories, Ltd.), and subsequently for 30 min with streptavidin–biotin–horseradish peroxidase complex (1:400, Zymed Laboratories Inc., San Francisco, California, USA). After detection with diaminobenzidine, sections were counterstained with hematoxylin, analyzed by the same observer, and photographed under the light microscope (Olympus BX40). To compare the expression of PRL-R in each category between both reproductive rhythms, follicular population was sorted into antral and preantral follicles. Prolactin receptor expression was analyzed using computer-aided image analysis (Metamorph Imaging System 7.5; Universal Image Corp., West Chester, PA, USA). In antral follicles, such program quantifies the follicular area expressing PRL-R. In addition, one threshold for negative staining and two thresholds for positive staining of PRL-R were established for moderate and strong chromatic intensity.

2.6. Oocyte collection and in vitro maturation

The remaining ovaries were placed in PBS at 37 °C and transported to the laboratory. Cumulus-oocyte complexes (COCs) were obtained by aspiration with a 2-mL syringe and a 25-gauge needle from ovarian follicles ≥1 mm in size. Cumulus-oocyte complexes with compact cumulus cells were washed and placed in 500 μL maturation medium in 4-well dishes and cultured for 16 h at 38 °C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium consisted of tissue culture media (TCM-199) with 2 mM L-glutamine, 0.1 mg/mL sodium pyruvate supplemented with 10% vol/vol fetal calf serum (FCS), 10 ng/mL epidermal growth factor (EGF), and 100 ng/mL insulin growth factor (IGF), according to Lorenzo et al. [39].

2.7. Confocal microscopy study

After the maturation period, a total of 301 COCs (Group S, n = 188; Group E, n = 113) were treated for the confocal study. First, cumulus cells were removed in 2 mM hyaluronidase by gentle pipetting. Next, oocytes were treated with 0.5% wt/vol pronase to digest the zona pellucida, fixed in PBS containing 4% wt/vol buffered neutral paraformaldehyde solution (pH 7.2 to 7.4), and stored in PBS. Oocytes were washed with permeabilization solution (0.02% vol/vol Triton X-100) and treated for 40 min with blocking solution (7.5% wt/vol bovine serum albumin). Then they were incubated for 30 min at room temperature with 100 μg/mL lens culinaris fluorescein isothiocyanate (FITC-LCA) for cortical granule (CG) staining and for 15 min at 39 °C with 10 μg/mL propidium iodide for nuclear staining. After that, oocytes were mounted between a coverslip and a glass slide supported by paraffin columns and examined under a confocal laser-scanning microscope (TCS SP5; Leica). Nuclear maturation was measured in terms of metaphase II rate. According to a previous work [40], CG distribution was classified as follows (cf. Fig. 6): (A) peripheral: CGs were adjacent to the plasma membrane, showing they were cytoplasmically matured; (B) cortical: most of the CGs were spread throughout the cortical area, being considered as partially matured; (C) homogeneous: CGs were distributed throughout the cytoplasm, as they did not show cytoplasmic maturation; (D) nonhomogeneous or abnormal: anomalous distribution of CGs compatible with poor quality or degenerated oocytes.

2.8. Statistical analysis

Data were analyzed using the Statistical Analysis Systems (SAS/STAT User's Guide, Release 8.2; SAS Institute, Inc., Cary, NC, USA) and the SPSS program for Windows (SPSS 13.0; SPSS Inc., Chicago, IL, USA). A MIXED procedure was used according to an autoregressive model to analyze repeated measures, including the effect of time (parturition, Day 11 postpartum, and Day 32 postpartum) with respect to LBW, estimated body condition, and serum metabolic parameters. Doe was considered a random effect nested in the treatment. Means were compared using a protected t-test. Means of serum estradiol, progesterone, and PRL concentrations, PRL-R, ovary weight, follicular categorization, and prolificacy (mean total born and dead born per doe) between experimental groups of insemination (11 postpartum vs. 32 postpartum) were compared by protected Student's t-test. Chi-square test was carried out to analyze conception rate (number of parturitions per number of inseminated does), follicular apoptosis index over total number of follicles, and to compare nuclear maturation and CG migration index of in vitro–matured oocytes between experimental groups of insemination. All the results are expressed as the mean ± SEM, and statistical significance was accepted for $P < 0.05$.

3. Results

3.1. Body composition and metabolic status during lactation of primiparous does

3.1.1. Live body weight and body composition

Similar LBW was found among time points measured (3957 ± 53.6 g, 3843.6 ± 34.7 g, and 3769.4 ± 63 g, at parturition, Day 11 postpartum, and Day 32 postpartum, respectively). Estimated body composition is shown in Fig. 1 and energy content in Fig. 2A. Body protein and energy content of does were significantly increased along the postpartum period (body protein: 17.4 ± 0.1%, 17.8 ± 0.1%, and 19.0 ± 0.1%; energy content: 1019.7 ± 16.3 kJ/100 g, 1094.7 ± 10.9 kJ/100 g, and 1139.3 ± 24.3 kJ/100 g; at parturition, Day 11 postpartum, and Day 32 postpartum, respectively). Lipid depots were statistically lower at parturition time compared with that obtained on Day 11 postpartum and Day 32 postpartum (13.3 ± 0.4%, 14.7 ± 0.3%, and 15.6 ± 0.6%, respectively, $P < 0.05$). Body lipid depot tended to be higher in animals undergoing an extensive rhythm than in those submitted to the semi-intensive one ($P = 0.09$). Water content significantly decreased along the postpartum period (63.1 ± 0.4%, 61.0 ± 0.3%, and 59.0 ± 0.6% at parturition, Day 11 postpartum, and Day 32 postpartum, respectively). There were not found significant differences in body ash content (3.1 ± 0.0%, 3.1 ± 0.0%, and 3.1 ± 0.0% at parturition, Day 11 postpartum, and Day 32 postpartum, respectively).

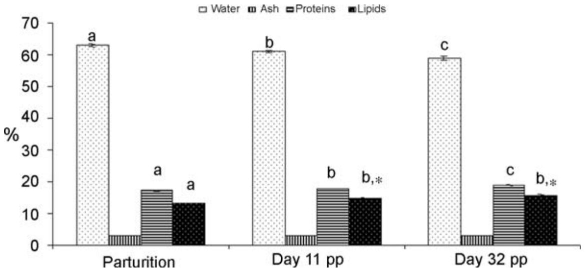

Fig. 1. Estimated body composition measured at different time points along the postpartum (pp) period (at parturition, on Day 11 postpartum [lactation], and on Day 32 postpartum [postweaned]) in primiparous rabbit does. Different letters in the same column indicate differences ($P < 0.05$). *$P = 0.09$.

3.1.2. Metabolic parameters

Comparisons of serum metabolic parameters revealed a significant effect of the postpartum time point (Fig. 2B–D). Serum leptin and protein concentrations increased from parturition to the postweaning period (leptin: 1.6 ± 0.2 ng/mL, 3.0 ± 0.3 ng/mL, and 4.0 ± 0.0 ng/mL, $P < 0.05$; protein: 3.9 ± 0.1 g/dL, 5.2 ± 0.1 g/dL, and 6.0 ± 0.2 g/dL, $P < 0.001$). Serum NEFA concentrations were higher at parturition time ($P < 0.001$) compared with the values obtained on Day 11 postpartum and Day 32 postpartum, which did not show significant differences between them (0.7 ± 0.0 mmol/L, 0.3 ± 0.0 mmol/L, and 0.4 ± 0.1 mmol/L, respectively).

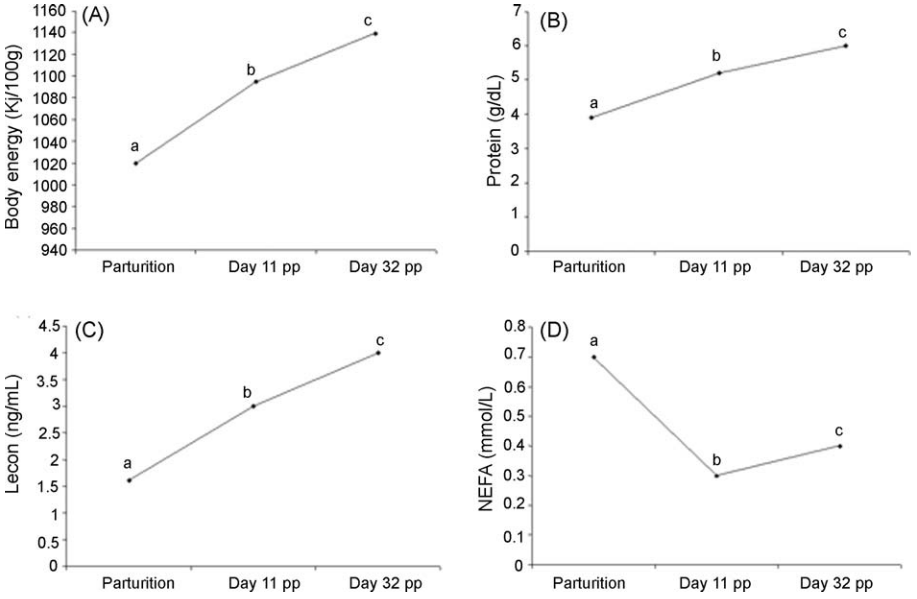

Fig. 2. (A) Body energy content and (B, C, D) serum parameters related to metabolic status measured at different time points along the postpartum (pp) period (at parturition, on Day 11 postpartum [lactation], and on Day 32 postpartum [postweaned]) in primiparous rabbit does. All samples were recovered at 0900. Different letters indicate differences ($P < 0.05$).

3.2. *Ovarian status of primiparous does at artificial insemination time*

3.2.1. *Steroid concentrations*

Serum E_2 concentrations did not show significant differences in both rhythms (16.2 ± 0.8 pg/mL vs. 20.5 ± 5.0 pg/mL, Group S and Group E, respectively). However, P_4 concentrations tended to be higher ($P = 0.07$) in the group inseminated at early postpartum compared with the extensive group (5.5 ± 1.7 ng/mL vs. 2.0 ± 0.6 ng/mL, respectively).

3.2.2. *Features of follicular population and apoptosis index*

Average ovary weight (338.0 ± 11.2 g vs. 361.2 ± 12.8 g) and mean of total preovulatory $\geq$1 mm follicles in the ovarian surface per ovary (10.0 ± 0.9 vs. 10.0 ± 0.7) were similar between groups. A total of 4282 follicles (Group S, n = 2189; Group E, n = 2093) were studied for categorization. No significant differences were found in the mean of primordial, primary, and secondary follicles per ovary between animals from each reproductive rhythm (3.3 ± 0.4 vs. 4.6 ± 1.1; 9.0 ± 1.9 vs. 7.1 ± 1.1, 52.8 ± 7.2 vs. 63.5 ± 13.7, respectively). However, the average number of total antral follicles/ovary were slightly higher in the semi-intensive group compared with that of the extensive one (8.7 ± 1.1 vs. 6.5 ± 1.0, $P = 0.1$). In addition, atresia rate was studied in a total of 952 follicles (Group S, n = 544; Group E, n = 408). Apoptotic cells were localized predominately in antral granulosa layers (Fig. 3A, B), especially near the cumulus oophorus complex (Fig. 3C, D). Mean follicular apoptosis index was significantly higher in the group inseminated in the early postpartum period compared with that of the extensive group (26.8 ± 1.9% vs. 15.4 ± 1.7% respectively).

3.2.3. *Serum PRL concentrations and PRL-R in ovarian follicles*

Serum PRL did not show significant differences either in lactating does (Group S) or in those postweaned (Group E) at AI time (3.8 ± 0.8 ng/mL vs. 7.2 ± 2.6 ng/mL, respectively). Positive immunostaining for PRL-R in oocyte, zona pellucida, cumulus cells, granulosa cells, and theca interna of the follicles at different stages of development was found in doe ovaries of both reproductive rhythms (Fig. 4B, C). Prolactin receptor was not immunolocalized in theca externa cells (Fig. 4B). Control tissue samples showed no positive staining (Fig. 4A). Quantification of PRL-R immunostaining did not show significant differences between Group S and Group E either for moderate (91.5 ± 1.5% vs. 84.9 ± 4.3%, respectively) or for strong chromatic intensity areas (26.6 ± 5.2% vs. 18.7 ± 3.3%, respectively).

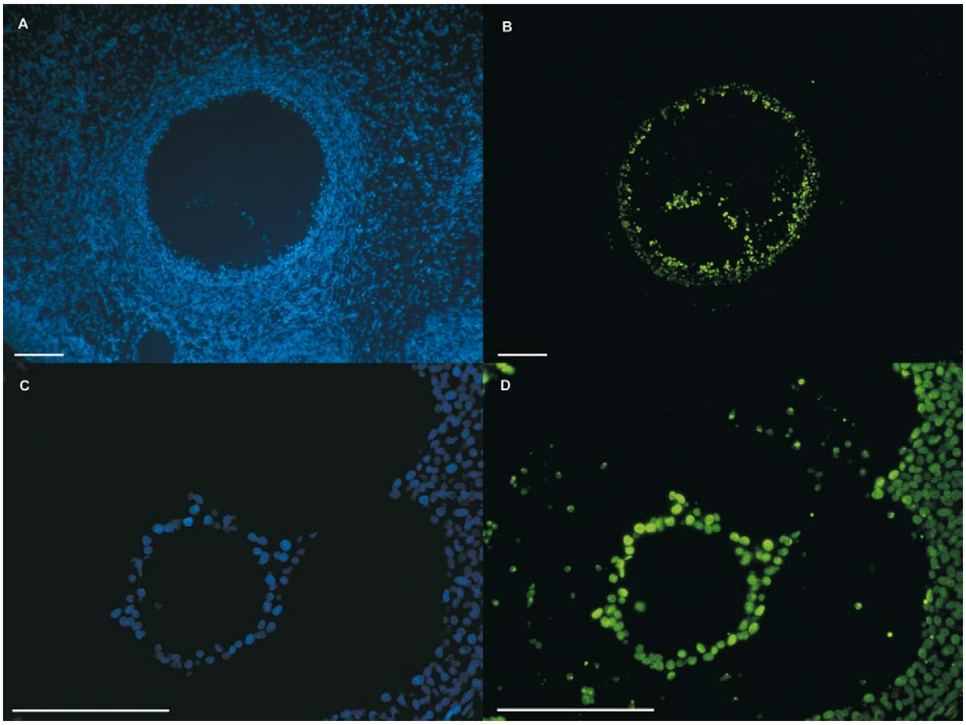

Fig. 3. Follicular apoptosis at insemination time measured by TUNEL in ovarian sections of primiparous rabbit does under semi-intensive (Group S) or extensive (Group E) rhythms. (A, C) Control sections showing ovarian cells marked with 4′,6-diamino-2-phenylindole (DAPI). (B) Follicular apoptotic cells labeled. (D) Detail of apoptotic cumulus cell. Scale bar = 200 μm.

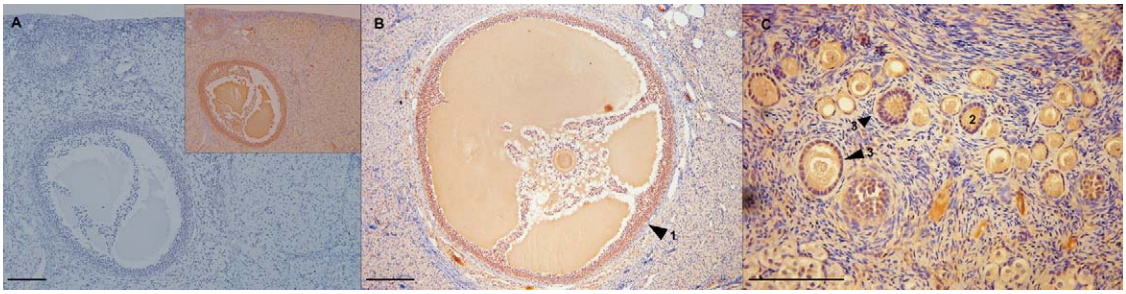

Fig. 4. Detection of PRL-R (arrowheads) by inmunohistochemistry in ovarian sections of primiparous rabbit does under semi-intensive and extensive rhythms. (A) Negative control; (B) antral follicle (1) with strong staining of PRL-R in granulosa cells; (C) PRL-R in oocytes (2) and granulosa cells (3) in preantral follicular population. Scale bar = 200 μm.

3.2.4. Nuclear and cytoplasmic oocyte maturation

As shown in Fig. 5, in line with the follicular parameters measured, oocyte quality was also affected by the reproductive rhythm. Primiparous does of the extensive group showed a significant increase in the rate of oocytes reaching metaphase II (MII) compared with that of does inseminated in the early postpartum period (84.3 ± 3.4% vs. 48.6 ± 3.6% respectively). The percentage of oocytes presenting peripheral CG migration was also statistically higher in the extensive group than in the semi-intensive one (73.5 ± 4.1% vs. 21.3 ± 2.9 respectively, $P < 0.001$) (Fig. 6A). Animals inseminated at the early postpartum period (Group S) showed slightly more oocytes with incomplete CG migration (20.2 ± 2.9% vs. 12.4 ± 3.1%, $P = 0.08$; Fig. 6B) and significantly more oocytes with homogeneous CG distribution after in vitro maturation (27.7 ± 3.2% vs. 13.3 ± 3.2%; Fig. 6C); such oocytes were not considered as cytoplasmic matured. Besides, in Group S, an increase of abnormal, nonhomogeneous CG distribution was found compared with that of oocytes from does inseminated in the postweaning

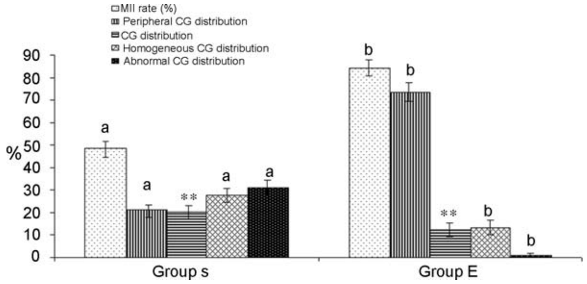

Fig. 5. In vitro oocyte maturation in primiparous does inseminated under semi-intensive or extensive rhythm. Nuclear and cytoplasmic maturation was measured in terms of metaphase II rate (MII) and cortical granules migration. Different letters in the same column indicate differences ($P < 0.05$). $^{**}P = 0.08$.

Table 1
Reproductive parameters in primiparous rabbit does inseminated in the early postpartum (semi-intensive rhythm) or in the postweaning (extensive rhythm) period.

	Reproductive rhythm	
	Semi-intensive (Group S)	Extensive (Group E)
Conception rate (%)	45.1 ± 6.4^{a}	85.2 ± 8.8^{b}
Prolificacy (n)		
Total born per doe	9.3 ± 0.6^{c}	11.4 ± 0.6^{d}
Dead born per doe	2.0 ± 0.6^{c}	0.2 ± 0.6^{d}

Values are means ± SEM. Means in rows with different letters differ: $^{a,b}P < 0.001$; $^{c,d}P < 0.05$.

period (30.9 ± 3.3% vs. 0.9 ± 0.8%, $P < 0.001$; Fig. 6D).

3.2.5. Reproductive performance

The average conception rate and prolificacy of primiparous does was strongly affected by the reproductive rhythm used (Table 1). Animals inseminated in the early postpartum period (Day 11) showed about 40% lower conception rate compared with that of does in the extensive group (inseminated at Day 32 postpartum) ($P < 0.001$). Also, extensive group does had more kits live born (around four kits) than did females of the semi-intensive group.

4. Discussion

There is no doubt that complex interactions between energy requirements, lactation, and ovarian function can modulate fertility and prolificacy of the females during the early postpartum period, but these ovarian mechanisms have not been described properly in the rabbit. When physiologic conditions are optimal in rabbit does (nonlactating, sexually receptive, medium fat females), conception rate can reach values over 90% [41]. However, in contrast with the thought of high reproductive capacity of rabbits, this study shows that conception rate of primiparous does inseminated in the early postpartum period is very low (45%), in agreement with previous reports [7,42–45]. Additionally, lower prolificacy values were obtained in the same group, in contrast with other authors, who found no difference in this parameter [41,44]. In other species, such as dairy cattle, body condition score is also affected by stage of lactation and parity order, which reflects in blood hormone and metabolite fluctuations [5]. In this sense, our study confirmed that a better body composition in terms of higher body protein, energy, and lipid content is associated with changes in metabolic signals, such as an increase in serum protein and leptin concentrations. This improvement may be associated with an increase in the reproductive outcome, according to other authors [7,11,12,45]. All of these findings strongly suggest that AI after weaning is the best management, at least for primiparous does, not affecting the global productivity of the females, as reported previously [11,43].

Prolactin effects are related to the presence of its receptor. In this study, PRL-R appears in different ovarian localizations, including granulosa cells and oocytes of antral follicles, according to previous results [23,38]. This finding evidences that PRL acts directly on rabbit follicles modulating follicular health and development [21]. However, we found no differences in PRL-R immunostaining and serum PRL concentration

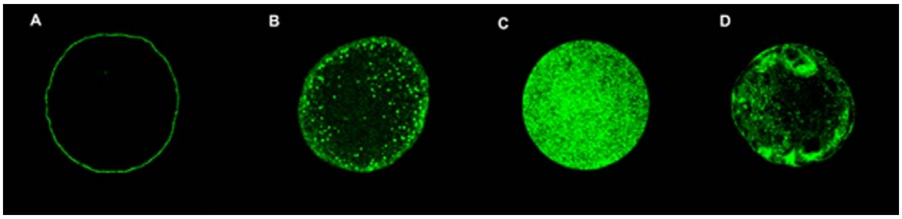

Fig. 6. Cytoplasmic oocyte maturation related to cortical granule (CG) migration: (A) Peripheral CG distribution; (B) cortical CG distribution; (C) homogeneous CG distribution; (D) nonhomogeneous or abnormal CG distribution. Oocyte diameter is around 80 μm.

or in serum E_2 concentration and antral follicular population at AI time between both reproductive rhythms. This could be a consequence of the estrus synchronization method of lactating does used in this study. Transient doe-litter separation allows a reduction of PRL concentrations at day of AI in the group inseminated in the early postpartum period; as a result, serum E_2 concentrations and follicular development improve as in the extensive group [9,46]. Measurements of serum E_2 levels have been used to assess antral follicular growth and their steroidogenic capacity [47]. However, the serum estradiol concentrations of lactating rabbit does are widely variable [48]; not reflecting the follicular status at a specific time point related to the apoptosis index [49], as is here also reported. Slightly higher concentrations of P_4 found in the semi-intensive group could also be adversely acting on oocyte maturation, gamete transport, and fertilization [50,51]. However, further specific studies are warranted in order to deepen our knowledge regarding steroid action on the ovary in the early postpartum period in rabbits. Besides, other factors could also be locally acting on the follicular enviroment, altering ovarian status and reproductive performance.

Some metabolic markers change according to body composition and energetic balance in animals, which also seem to affect reproductive function [12]. Non-esterified fatty acid concentrations generally point to mobilization of body lipids and are higher when negative energy balance is more pronounced [5,15]. Also, they have a proapoptotic effect on granulosa cells in vitro [52]. Body fat, energy, and protein requirements become particularly accentuated in the late pregnancy period [3] because of the development of the gravid uterus [6] and the strong fetal protein turnover [53]. That situation could give rise to higher serum NEFA concentrations at parturition time compared with subsequent postpartum time points determined in this study. In contrast with the results obtained in other species [15,54], but in agreement with previous result in rabbits [55], neither lactating does on Day 11 postpartum nor postweaned does showed differences in NEFA concentrations. Therefore, in the rhythms studied in this work, NEFAs do not appear to be the main factors affecting ovarian function in rabbits. However, serum protein and leptin concentrations increased through the postpartum period, reaching the highest values in the extensive group. Knowing that both groups were fed ad libitum with the same diet, lower concentrations of these metabolic parameters in the early postpartum period were probably related to doe lactation status and lower body condition. In this sense, leptin is a hormone for which blood concentrations could reflect the amount of stored body fat and energy [56]; it influences rabbit reproductive function by modulating ovarian physiology through its receptor [6,33]. Leptin receptors have been detected in granulosa cells of follicles, oocytes, and oviducts [13,57–60]. In this sense, previous studies show that recombinant leptin in rats reduces incidence of follicular apoptosis [61]. In contrast, leptin deficiency in Ob/Ob mice is associated with impaired folliculogenesis, which results in increased follicular atresia [62]. In the current study, follicular apoptosis rate was higher in lactating primiparous does inseminated at the early postpartum period than in those under extensive rhythm. Thus, an increase in serum leptin concentrations on postweaning period may be one of the endocrine factors involved in the reduced incidence of follicular apoptosis in such group. In the current work, apoptotic cells were localized predominately in the antral granulosa layers, mostly near the cumulus oophorus complex, in agreement with previous reports [49,63,64]. As a consequence, the developmental potential of oocytes may be affected [65].

Oocyte maturation implies both nuclear and cytoplasmic maturation including CG migration [66,67]. The physiologic status of the animals is determining for oocyte developmental competence [68], as it is acquired during folliculogenesis, before the onset of meiosis [69]. In contrast with this premise, in the bovine model [70], it was recently reported that there was no effect of day postpartum on oocyte quality or in fertility [71]. However, other reports clearly show that conditions related to early lactation negatively affect oocyte quality and endocrine parameters of dairy cattle, effects that are especially pronounced after the first labor [5,15,72,73]. In the current study, oocytes matured in vitro from the group inseminated in the early postpartum period showed a decrease in both nuclear and cytoplasmic maturation rates compared with that of the oocytes of the extensive group. Additionally, in line with the follicular apoptosis results obtained, the percentage of oocytes with abnormal distribution of CG (considered as degenerated) was also higher in does inseminated in the early postpartum period, matching the results of Dominguez [72] in cattle. Therefore, the higher follicular apoptosis index associated with the lower oocyte quality obtained in such group may be an indicator of the impact of metabolic stress on the acquisition of oocyte developmental competence [74] and subsequent fertility [2].

Insemination after weaning improves (1) estimated body composition and energy content; (2) serum leptin and protein concentrations; (3) health of follicular populations in the ovary, showing lower apoptosis rate; (4) oocyte quality, by enhancing oocyte maturation; and (5) as a consequence, conception rate and prolificacy are improved. Therefore, in this article we are reporting for the first time in rabbits some physiologic basis to support that insemination after weaning is the best management for primiparous does because it seems to fit better in the metabolic status and reproductive physiology of such animals. This should be considered when choosing strategies with a view to improving reproductive performance and economic benefits in rabbit farms. Finally, this study shows that rabbits can be a valuable model to study the effect of maternal metabolism on fertility.

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