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Ovarian response and embryo gene expression patterns after nonsuperovulatory gonadotropin stimulation in primiparous rabbits does

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ABSTRACT

Ovarian stimulation with equine chorionic gonadotropin (eCG) is largely used in animal reproductive technologies to provide a larger number of oocytes and embryos and to improve the reproductive outcome. However, the consequences of maternal treatment with eCG on embryo gene expression patterns are not widely studied. The aim of this work was to assess the ovarian response (preovulatory follicular population, oocyte maturation, ovulation rate, and serum steroid concentrations), the early embryo survival and gene expression patterns of a panel of quality-genes involved in glucose intake, oxidative stress, apoptosis, proliferation, implantation, and fetal growth in embryos of lactating rabbits treated with eCG. A total of 34 primiparous rabbit does (*Oryctolagus cuniculus*) were randomly distributed at Day 23 postpartum into a treatment group receiving a unique nonsuperovulatory dose (25 IU) of eCG (eCG group; $N = 17$ does); or a control group without eCG treatment previously to artificial insemination (control group; $N = 17$ does). After 48 hours, 8 does of each group were euthanized and their ovarian response was studied. The rest of animals were artificially inseminated and their ovulation was induced with a GnRH analogue. Embryos were recovered 3.5 days later. The oocytes retrieved for *in vitro* maturation showed no differences in metaphase II rate in both experimental groups, although oocyte cytoplasmic maturation, in terms of cortical granule migration rate, was improved in eCG-treated does ($P < 0.05$). The mean number of preovulatory follicles was similar between groups but the ovulation rate was significantly higher in eCG-treated does compared with does not stimulated ($P < 0.05$). No differences were found in serum estradiol and progesterone concentrations of does the day of oocyte and embryo recovery, respectively. However, progesterone:estradiol ratio was slightly increased in eCG group on embryo recovery day ($P = 0.1$). The percentage of embryos recovered at the blastocyst stage was also increased in eCG-treated does ($P < 0.05$), nevertheless, there were no differences in the gene expression patterns of candidate genes *SLC2A4, IGF1R, IGF2R, SHC1-SHC, TP53, PTGS2*, and *PLAC8*; except for the transcripts of *SOD1* mRNA which were downregulated in eCG-derived embryos ($P < 0.05$). In conclusion, the administration of eCG improves ovulation rate, oocyte cytoplasmic maturation, and blastocyst formation in primiparous rabbit does inseminated on Day 25 postpartum. Although it seems not to influence the gene expression patterns studied, a lower antioxidant defense of embryos developed after the maternal eCG treatment is suggested.

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1. Introduction

Ovarian gonadotropin stimulation is routinely administered in animal reproductive technologies for estrus synchronization and multiple ovulation and embryo transfer protocols to encourage follicular growth and generate more developmentally competent oocytes. The action of eCG (equine chorionic gonadotropin) is analogous to that of follicle stimulating hormone (FSH) and luteinizing hormone (LH) enhancing final follicular development. This gonadotropin requires a single administration because it has a relatively long circulating half-life [1]. Therefore, the application of this hormone is largely used in livestock industries as a source of exogenous FSH and LH to increase preovulatory follicular population, female receptivity, and reproductive success, and also in animal laboratory research for supplying oocytes for somatic cell nuclear transfer, animal cloning, and transgenesis [2–4]. In rabbit breeding, eCG is usually applied for improving follicular development and as a method for estrous synchronization in high-yield lactating animals because lactation exerts an inhibitory effect on ovarian function, especially in primiparous does [4,5]. Moreover, gonadotropin stimulation protocols can modify peripheral estradiol (E_2) and progesterone (P_4) concentrations, as well as their ratio (P_4:E_2), and therefore, the environment in which oocytes and embryos are developed [6,7]. Indeed, the eCG administration seems to mediate cytoplasmic oocyte maturation supporting monospermic fertilization and early embryo development [8,9]. However, evidence from other studies indicates that it could reduce embryo development in rabbits [10], and implantation rate, fetal development, and fetal weight in mice [6], and also can provoke ovarian anomalies [11]. Nowadays, the effect of this hormone on embryo quality is still controversial.

Gene expression patterns in early stage embryos rely mostly on the posttranscriptional control of maternal transcripts accumulated during follicular development and oocyte maturation [12]. Consequently, synthetic gonadotropins such as eCG could induce changes in the maternal periconceptional environment that might provoke epigenetic modifications affecting expression of genes that control embryo metabolism and early embryo development. In fact, some important events such as proliferation, differentiation, implantation, or apoptosis in early embryos are mediated by specific expression and quantity of numerous transcripts. Such marks can predict the fertility prognosis, fetal growth, and the health of future offspring [13]. However, the consequences of eCG application on embryo gene expression patterns are not yet elucidated. Candidate genes such as solute carrier family 2 (facilitated glucose transporter) member 4 (*SLC2A4*) and insulin-like growth factor 1 receptor (*IGF1R*) mediate cell proliferation and differentiation from the morula to the blastocyst stage in rabbit [14,15] and mice [16,17]. The *IGF1R* and insulin-like growth factor 2 receptor (*IGF2R*) transcripts are also considered as markers for fetal growth rate [18,19]. On the other hand, an adequate nitric oxide synthase 3 (endothelial cell) (*NOS3*) production by embryos is necessary to provide an adequate blood supply to the site of blastocyst implantation [20]. This together with an upregulation of mRNA transcripts of some genes as prostaglandin-endoperoxide synthase 2 (*PTGS2*) and placenta-specific 8 (*PLAC8*) in blastocysts can be a tool to predict successful pregnancy and delivery in cattle [21]. Moreover, it is known that metabolic and endocrine changes in the periconceptional environment can induce an oxidative stress in embryos and reduce their quality [22]. It is reflected by expression of genes related with antioxidant defense as superoxide dismutase 1 soluble (*SOD1*) and apoptotic pathways associated with oxidative stress as Src homology 2 domain containing transforming protein 1(*SHC1-SHC*) and tumor protein 53 (*TP53*), which upregulation is linked with subsequent increasing of fetal losses and fetal malformations in cattle and mice [23,24].

Taking into account these premises, it should be advisable to use hormone protocols based on the understanding of how they affect the oocyte competence and the yield, quality, and gene expression patterns of early embryos routinely produced in laboratory and farm animals. However, as far as we know, there is scarce literature about the consequences of maternal eCG administration on embryo gene expression patterns [25], and no reports existing in the rabbit specie. In this sense, rabbit is considered as a valuable animal model to study such effects for economic reasons in livestock industries and also for biomedical research, because of the precise timing of ovulation which allows the knowledge of the exact embryonic age and also for the high number and size of embryos available [26].

Thus, the aim of the present work was to determine if a single dose of eCG administered before ovulation can change the relative abundance of a panel of quality genes, involved in important aspects of glucose intake, oxidative stress, apoptosis, mitogenic capacity, implantation, and fetal growth in the rabbit blastocyst. Besides, serum estradiol and progesterone concentrations, preovulatory follicular population, nuclear and cytoplasmic oocyte maturation, ovulation rate, and early embryo development were assessed to increase the understanding of the relationship between maternal periconceptional changes and early embryo responses, and to provide insight to the origin of oocyte and embryo alterations that could be of relevance for the reproductive strategies in animals and humans.

2. Material and methods

Unless otherwise stated, all the chemicals were purchased from Sigma Chemical Company (Spain). Experimental procedures were approved by the Animal Ethics Committee of the Polytechnic University of Madrid (Spain) and were in compliance with the Spanish and European guidelines for care and use of animals in research [27,28].

2.1. Animal housing and experimental design

New Zealand × California white rabbit does *(Oryctolagus cuniculus)* were held on the experimental facilities at the Animal Production Department, Polytechnic University of Madrid (Spain). Animals were housed in individual flat-deck cages under a constant photoperiod of 16 hours of light per day. A temperature of 18 °C to 22 °C and a relative humidity of 60% to 75% were maintained by a forced

ventilation system. Does were fed *ad libitum* commercial diet (Nanta S.A., Madrid, Spain) containing 16.9% crude protein, 15.7% crude fiber, and 2.5% fat; the digestible energy of the diet was 2700 kcal/kg. The animals had free access to water.

To evaluate the ovarian response to the eCG treatment, a total of 34 primiparous lactating rabbit does were used. Day 23 postpartum females were randomly allocated into one of the following two groups: (1) eCG group (N = 17): rabbit does were subjected to a common treatment used in European rabbit facilities based on the administration of a standard dose of eCG (25 IU, im) (Serigan, Lab., Ovejero, Spain) and as previously has been reported by our group [4,29]; (2) control group (N = 17): rabbit does were not treated with eCG.

On Day 25 postpartum, the ovaries of nine does from each experimental group were recovered by midventral laparotomy after euthanasia in a hermetically closed CO_2 chamber. Morphologic ovarian features (ovary weight and number of preovulatory follicles) were recorded before using the ovaries for the assessment of oocyte nuclear and cytoplasmatic *in vitro* maturation.

The rest of the does (eight does per group) were weaned and artificially inseminated using a pool of fresh heterospermic semen between 20 and 25 million spermatozoa in 0.5 mL of commercial diluent (Magapor S.L., Zaragoza, Spain). Ovulation was induced by 20 μg gonadorelin im (Inducel GnRH, Lab. Ovejero, León, Spain). Preimplantational embryos were recovered in all animals inseminated 3.5 days later by flushing both reproductive tracts after euthanasia in a hermetically closed CO_2 chamber. To assess the ovulation rate, the number of corpora lutea in the ovaries was counted.

2.2. Blood sampling and serum steroid concentrations

Serum E_2 and P_4 determinations were performed on the day of oocyte and embryo recovery. Blood samples were collected from the marginal ear vein into nonheparinized tubes at 9:00 AM. Serum was obtained after centrifugation at 1200× *g* for 10 minutes at 4 °C and stored at −32 °C until analyzed. Serum E_2 and P_4 concentrations were measured in duplicate samples by solid-phase enzyme immunoassay based on competitive binding method using Rabbit Estradiol and Rabbit Progesterone ELISA Kits, respectively, according to the manufacturer instructions (Endocrine Technologies, CA, USA). The sensitivity of the assays was 5 pg/mL and 0.1 ng/mL for E_2 and P_4, respectively. Intra- and interassay coefficients of variation were 10.5% and 8.5% for E_2, and 8.5% and 7.5% for P_4, respectively. The P_4:E_2 ratio was calculated as P_4 divided by E_2, both in ng/mL, although otherwise the E_2 levels were expressed in pg/mL.

2.3. Oocyte collection and in vitro maturation

The ovaries were placed in PBS at 37 °C and transported to the laboratory. Cumulus oocyte complexes (COC) were obtained by aspiration with a 2 mL syringe and a 25 ga needle from ovarian follicles ≥1 mm in size. A total of 268 COC with compact cumulus cells and homogeneous cytoplasm were washed and placed in 500 μL of maturation medium in four-well dishes (Nunclon Surface, Nunc, Roskilde, Demark) and cultured for 16 hours 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% v:v fetal calf serum, 10 ng/mL epidermal growth factor, and 100 ng/mL insulin growth factor according to previous reports [30,31].

2.4. Assessment of oocyte nuclear and cytoplasmic maturation

After the maturation period, COC were treated for the confocal study as previously reported [32]. 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 paraformaldehyde solution, and stored in PBS at 4 °C. Oocytes were washed with 0.02% v:v Triton X-100 and treated for 40 minutes with 7.5% wt/vol bovine serum albumin (BSA). Then they were incubated for 30 minutes at room temperature with 100 μg/mL *lens culinaris* fluorescein isothiocyanate for cortical granule (CG) staining and for 15 minutes 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 (Leica, TCS SP5, Wetzlar, Germany). Nuclear maturation was measured in terms of metaphase II rate and cytoplasmic maturation in terms of peripheral CG migration. Oocytes that showed CG positioned just beneath the plasma membrane were considered cytoplasmically matured.

2.5. Assessment of ovulation and embryo development

Embryos were recovered by flushing the reproductive tract with warm PBS and 0.1% BSA wt/vol. Morphology of embryos was evaluated immediately after recovery with a stereomicroscope (Nikon, SMZ 800, Tokyo, Japan) and they were then classified on the basis of conventional morphological criteria according to their developmental stage following the guidelines of the International Embryo Transfer Society. Rate of blastocysts recovered and degenerated or retarded embryos (embryos whose stage of development did not correspond to time after ovulation, i.e., zygotes, two- to 16-cell embryos, and early morulae) were referred to the total number of recovered embryos. Blastocysts were immediately snap-frozen in liquid nitrogen and stored at −80 °C for transcript abundance analysis.

2.6. RNA extraction, reverse transcription, and quantification of mRNA transcript abundance

Poly (A) RNA was prepared as previously described by Bermejo-Alvarez et al. [33] from groups of 10 to 11 blastocysts of each experimental group (N = 61 and N = 71, in control and eCG groups, respectively) using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions with minor modifications. The reverse transcription (RT) reaction

(Bioline, Ecogen, Madrid, Spain) was carried out using poly (T) primer, random primers, and Moloney murine leukaemia virus reverse transcriptase enzyme in a total volume of 44 μL to produce cDNA. Tubes were heated to 70 °C for 5 minutes to denature the secondary RNA structure and then the RT was completed with the addition of 100 units of Superscript RT enzyme. The samples were subsequently incubated at 42 °C for 60 minutes to allow the reverse transcription of RNA, followed by 10 minutes at 70 °C to denature the RT enzyme. The quantification of all mRNA transcripts was carried out by real-time quantitative RT polymerase chain reaction (PCR). Two repetitions were performed for all genes of interest in each sample. Experiments were conducted to contrast relative levels of each transcript and histone *H2AFZ* that is the housekeeping gene. The PCR was performed by adding a 2 μL of each sample to the PCR mix containing the specific primers to amplify the housekeeping gene, *H2AFZ*, and several transcripts which have been associated with aspects of embryo quality including glucose intake, embryo survival, and fetal growth: *SLC2A4*, *IGF1R*, and *IGF2R*; vascularization for implantation: *NOS3*; pregnancy outcome: *PTGS2* and *PLAC8*; antioxidant defense: *SOD1*; and apoptotic pathways associated with oxidative stress: *SHC1-SHC* and *TP53*. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. Polymerase chain reaction conditions were optimized to achieve efficiencies close to 1 and then the comparative cycle threshold method was used to quantify expression levels as described by Schmittgen and Livak [34]. Quantification was normalized to the endogenous control *H2AFZ*. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

2.7. Statistical analysis

Trial data were analyzed as a completely randomized design using the SPSS program for Windows (SPSS 19.0, SPSS Inc., NY, USA). Means of ovarian weight, number of preovulatory follicles, and corpora lutea, serum estradiol, and progesterone concentrations, P_4:E_2 ratio, and differences in mRNA expression were compared between experimental groups by protected Student *t* test for independent samples. Chi-square test was carried out to compare nuclear maturation and cortical granule migration index of *in vitro* matured oocytes, as well as morphologic categorization of recovered embryos between experimental groups. All results are expressed as the mean ± SEM, and statistical significance was accepted for $P < 0.05$.

3. Results

3.1. Serum estradiol and progesterone concentrations

Serum estradiol (Fig. 1A) and progesterone concentrations (Fig. 1B) were similar between control and eCG groups at oocyte and embryo recovery time, respectively. The P_4:E_2 ratio was similar between eCG and control does the day of oocyte recovery (Fig. 1C). However, P_4:E_2 ratio tended to increase in the eCG does group at embryo recovery time ($P = 0.1$) (Fig. 1C).

3.2. Ovarian weight, follicular preovulatory population, and oocyte maturation

As shown in Table 2, mean ovarian weight in the eCG group was similar to the control group. Significant differences were not found in the mean number of preovulatoy follicles. The percentage of metaphase II oocytes after *in vitro* maturation did not differ between the experimental groups, but percentage of oocytes with correct cortical granule migration was significantly higher in the eCG group compared with the control group ($P < 0.05$).

3.3. Ovulation rate and in vivo embryo categorization

As is depicted in Table 3, ovulation rate, in terms of number of corpora lutea counted on the ovarian surface

Table 1
Oligonucleotic sequences used for amplification of RT-qPCR.

Gene	Primer sequence (5′–3′)	Fragment size, base pairs	GenBank accession number
H2AZ	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
SLC2A4	GGCGGCATGATTTCCTCC GAAGGGCAGCAGGATCAGCT	396	NM_001089313.1
IGF1R	CCCAAGCTCACGGTCATCACTG ATGGGCTTCTCCTCCAAGGTCC	323	EF616472.1
IGF2R	AGCCCTCTGCTGCGTGCTTG TCACGTCGTGCTGCTGGCTG	349	XM_002714965.1
NOS3	CTGCTCCACGTGGCTGAGCC ACATGTGGCCGCGTTCGAGG	390	HM_135204.1
PTGS2	TCCAAGCTGGCCTCACTGATGG AGCATGTGTGTGGCCCGACTTG	169	NM_001082388.1
PLAC8	ACTGGCAGACGGGCGTATGTG AGGGATGCCGTATCGGGTCC	150	XM_002717063.1
SHC1-SHC	GGTTCGGACAAAGGATCACC GTGAGGTCTGGGGAGAAGC	331	NM_001075305
TP53	GTGCTGACCAGGGACACGGC CTGCACCAGGGCAGACCAGC	223	NM_001082404.1
SOD1	GCAGGCAGCAATGGTGTCGC GCCGGCGTTCCCGGTCTTTG	139	HM_001082627.1

Abbreviation: RT-qPCR, reverse transcription quantitative polymerase chain reaction.

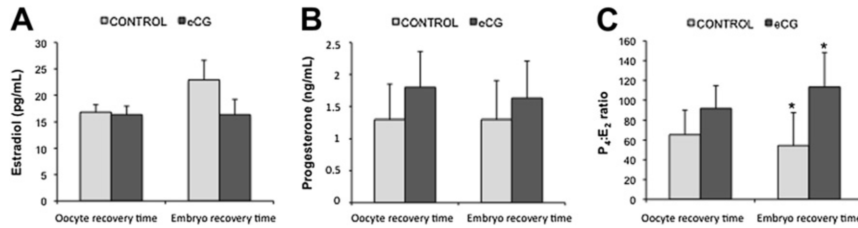

Fig. 1. Serum estradiol (A) and progesterone (B) concentrations and progesterone:estradiol (P_4:E_2) ratio (C) from rabbits treated with eCG (eCG group) or nontreated (control group) at oocyte recovery time (48 hours after eCG administration; N = 9 does per group) and at embryo recovery time (3.5 days after artificial insemination; N = 8 does per group). Error bars represent SEM. * P = 0.1.

was higher in the eCG group compared with the control group ($P < 0.05$). A total of 147 embryos were recovered (N = 72 and N = 75 in control and eCG groups, respectively). The average number of embryos recovered per doe was similar between groups (8.0 ± 2.6 vs. 8.7 ± 3.0, in control and eCG groups, respectively). The percentage of blastocysts was higher in the eCG group than that observed for the control group ($P < 0.05$). The percentage of degenerated or retarded embryos was lower in eCG-treated does compared with nontreated does ($P < 0.05$).

3.4. Transcript abundance of quality-related genes in blastocysts

The relative abundance of the transcripts studied is shown in Figure 2. Transcripts of *SOD1* mRNA were downregulated in the eCG group compared with the control group ($P < 0.05$). There were not differences in the gene expression patterns of *SLC2A4, IGF1R, IGF2R, NOS3, PTGS2, PLAC8, SHC1-SHC*, and *TP53* in blastocysts of both experimental groups.

4. Discussion

This study provided evidence of the ovarian response and gene expression patterns of rabbit blastocysts when an eCG treatment was used before induction of ovulation and AI as a reproductive strategy of ovarian stimulation. The results demonstrated that first administration of eCG improved ovulation rate, oocyte cytoplasmic maturation, and the blastocyst yield in primiparous does. It seemed not to have an influence on gene expression patterns in blastocysts compared with untreated females; but a lower antioxidant defense of early embryos developed after eCG administration was suggested.

In agreement with previous results, the current work demonstrated that rabbit does treated with a stimulatory dose of eCG did not modify the number of preovulatory follicles although their ovulation rate was significantly improved compared with nontreated animals [9,35]. In this sense, Rebollar et al. [4] showed similar serum FSH and LH concentrations in eCG treated and nontreated lactating rabbits before AI. Also similar LH peak was observed in such study. However, in current work the differences in ovulation rate and oocyte competence might have been because of the eCG-enhanced responsiveness of granulose cells to LH by an increase of mRNAs encoding LH receptor and epidermal growth factor-like factors, as occur in pig [36] and mice [37,38]. Nuclear maturation rate was similar in oocytes regardless of the treatment, which indicates that eCG might not be significantly determined by the capacity of the oocyte to achieve metaphase II stage before ovulation. However, our results show that the developmental competence acquired at the end of follicular development could be enhanced in oocytes of eCG-treated does compared with those not treated, because a significant improvement in cytoplasmic maturation of oocytes were found in such group, as it was previously described in the cat [8]. Moreover, it is known that the developmental competence of oocytes is certainly determined by the rate of development to the blastocyst stage [39]. In this line, the higher ovulation rate in eCG-treated rabbits was also accompanied with a significantly higher rate of blastocysts recovered in such animals in agreement with previous reports [9]. According with these findings, Rebollar et al. [5] showed that prolificacy was improved in primiparous does treated first time with eCG compared with control does. However, high degrees of variability in gonadotropin responses associated with the oocyte competence of oocytes, embryos, and the reproductive outcomes are

Table 2
Ovarian features and oocyte maturation in control and eCG-treated rabbits does 48 hours after eCG administration.

	Control (N = 9)	eCG (N = 9)
Ovary weight (mg)	317.0 ± 23.3	346.0 ± 26.1
Mean preovulatory follicles per ovary (N)	6.9 ± 0.3	7.8 ± 0.5
Oocytes with metaphase II (%)	70.4	59.2
Oocytes with CG migrated (%)	25.8^a	42.9^b

Data are mean ± SEM. Within rows, values with different superscript letters differ significantly ($P < 0.05$).
Abbreviation: CG, cortical granule.

Table 3
Ovulation rate and embryo yield in control and eCG-treated rabbits does 3.5 days after ovulation induction and artificial insemination.

	Control (N = 8)	eCG (N = 8)
Ovulation rate per doe, N	14.2 ± 0.6^a	19.3 ± 1.8^b
Embryos recovered, N	72	75
Blastocyst, N (%)	61 (84.7)^a	73 (97.3)^b
Retarded or degenerated embryos, N (%)	11 (15.3)^a	2 (2.7)^b

Data are mean ± SEM. Within rows, values followed by different superscript letters differ significantly ($P < 0.05$).

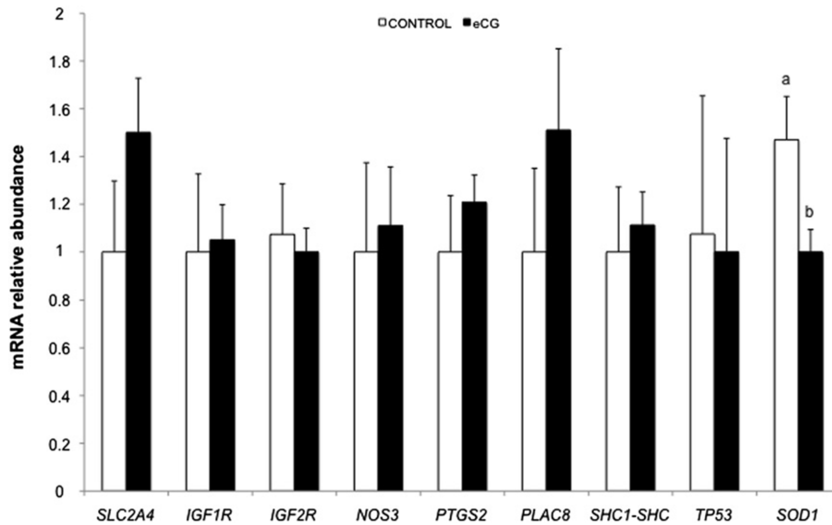

Fig. 2. Relative poly (A) mRNA abundance of the genes studied in blastocysts recovered from rabbits does treated with eCG (eCG group; N = 8) or nontreated (control group; N = 8) 48 hours before ovulation induction and AI. Embryos were recovered 3.5 days after AI. Bars with different letters differ significantly ($P < 0.05$). Error bars represent SEM.

reported in the literature. It seems to depend on the physiological status of the animals, age, breeding, and hormonal preparation. In this way, other authors have reported that hormonal synchronization with eCG is associated with a decrease in oocyte competence in ewes [40] and rabbits [10,29] and with oocyte structural abnormalities in heifers [41,42]. These observations can be explained by the prolonged double activity of FSH and LH of the eCG preparation, which can induce premature luteinization of follicles in cattle [43] causing ovulation of aged oocytes [44] and adversely affects the endometrial receptivity for implantation in rabbit [45]. In this sense, the serum E_2 and P_4 concentrations obtained in the present work were similar to previously reported by our group [46] and no signs of early luteinization was found in the eCG-treated does because on the day of oocyte recovery the P_4:E_2 ratio was similar between experimental groups. Nevertheless, a slight increase in the P_4:E_2 ratio in the eCG-treated does was found at embryo recovery time, which matches with the findings of Rebollar et al. [5]. High concentrations of circulating progesterone in the early luteal phase have also been associated with an advancement of conceptus elongation and higher pregnancy rates in cattle [47].

Although the number of blastocysts obtained in eCG-treated does was higher than in the control does, the transcripts of candidate genes related to some aspects of metabolism and development were similar in both experimental groups. The *SLC2A4* acts as a survival factor being necessary for blastocyst formation in rabbit because embryos have access to maternal glucose via *SLC2A4* [14,15,48]. Therefore, cell proliferation and differentiation mainly from the morula to blastocyst stage in rabbit are mediated by specific expression of glucose transporters as *SLC2A4*. The *IGF1R* is generally considered to mediate most biological effects of *IGF1* and *IGF2* [49], which also acts as a mitogenic factor on rabbit [14,15] and mice embryos [17]. Consequently, similar expression of both transcripts could indicate a similar capacity of proliferation and differentiation of blastocysts developed in both experimental groups.

To predict the chance of those blastocysts for implantation, successful pregnancy, and delivery, *NOS3, PTGS2*, and *PLAC8* transcripts were assessed. Endothelial *NOS3* is required to provide an adequate blood supply to the site of blastocyst implantation [20]. Prostaglandins are also involved in the modulation of the endometrial implantation site [50], as a result of an intense proliferation of trophoblastic cells [51,52]. Moreover, an invasion specific gene (*PLAC8*), is upregulated in blastocyst resulting in calf delivery, suggesting a potential role of this gene in placenta development and fetus maternal interphase [21]. In this way, similar mRNA levels between our experimental groups might indicate no significant effect of eCG on the expression of transcripts involved in such events in preimplantational rabbit embryos. In accordance with present results, Edwards et al. [25] did not observed any differences on embryo quality or embryo gene expression after eCG treatment in mice. In line with these findings, *IGF2R* gene expression was also similar between groups in the current work. It is a maternal imprinted gene that regulates the growth of developing embryo *in utero* modulating *IGF2* availability [53]. A previous report also showed that the number of survival embryos at midpregnancy did not vary with the eCG treatment in rabbit [9]. In contrast, higher number of degenerated embryos and decreased pregnancy rates, fetal weight, and fetal maturity were found in rabbits and mice when stimulation with FSH or eCG was used [35,54]. Some authors have postulated that it could be because of an acceleration of endometrial maturation as a consequence of a premature luteinization more than an effect on embryo quality [45,55]. However, as it is mentioned before, in the present work no sign of early luteinization was observed.

Nevertheless, in our study, the gene expression of *SOD1* was increased in the control group with regard to the eCG

group. It is involved in antioxidant defense and serves to protect against superoxide anion toxicity [56]. Our finding might reflect a higher capacity of the control blastocysts to maintain the balance between oxygen radicals and antioxidants compared with blastocysts of eCG-treated does [57]. In fact, the development of all embryos from *SOD1*-deficient oocytes was arrested at the two-cell stage [58]. In mice embryos, the repeated treatment with gonadotropins induced oxidative stress [59]. In rabbits, it has been reported increased fetal mortality [9] and reduced fertility rate of rabbit does when more than four eCG administrations are applied [5,54,60,61]. However, the *SHC1-SHC* and *TP53* expression was not upregulated in embryos of any experimental group in the current work. Then, we can assume that the first administration of eCG in rabbit does did not significantly stimulate such apoptotic pathways associated with oxidative stress in their blastocysts [23,24], but taking into account the literature and the differences in gene expression of *SOD1* in the present work we could not discard other oxidative stress-induced apoptotic pathways at blastocyst stage [62] in primiparous does. Such oxidative events might be implicated in the reduced prolificacy and fertility rates observed in females when eCG administration is used repeated and consecutive times. This is the first work that investigated this relationship but further studies are warranted in order to deepen our knowledge regarding gene expression patterns and oxidative stress when hormonal treatments are used in the reproductive protocols applied in animals and humans.

5. Conclusion

This research allowed us to obtain for the first time a molecular sketch about how rabbit embryos respond to the maternal administration of eCG for ovarian stimulation. The current work showed that the first application of eCG increased ovulation rate, improved oocyte cytoplasmic maturation, and preimplantation embryo development. It seemed not to modify studied embryo-gene expression patterns related to glucose intake, proliferation, and differentiation, apoptotic-induced pathways, implantation, and fetal growth. However, lower antioxidant defense of blastocysts produced after the eCG treatment was suggested, but further studies are needed to shed light on the mechanisms influencing the successful reproductive outcome when this kind of treatment is applied in animal reproductive technologies.

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