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Influence of different reproductive rhythms on serum estradiol and testosterone levels, features of follicular population and atresia rate, and oocyte maturation in controlled suckling rabbits

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ABSTRACT

The aim of the current work was to analyze the features of ovarian follicular population and their quality in New Zealand white rabbit does synchronized by 24-h controlled doe-litter separation before artificial insemination (AI) during all their reproductive cycles. Synchronized animals were allocated systematically in two groups. A total of 73 rabbit does (group A) were submitted to a 35-day intensive rhythm (AI on day 4 post-partum [pp] and weaning at 25 days of lactation), and 108 rabbit does (group B) were submitted to a 42-day semi-intensive rhythm (AI on day 11 pp and weaning at 35 days of lactation) during 9 months. At the mid-end of their reproductive life, a total of 26 does (5.4 parturitions), under intensive ($n = 15$) or semi-intensive rhythm ($n = 11$) were either treated in each group with 25 IU eCG 48 h before laparotomy to recover their ovaries ($n = 7$ for group A and $n = 6$ for group B) (according to the Bioethics Committee of the University) or not synchronized with the hormonal treatment ($n = 8$ for group A and $n = 5$ for group B). Blood samples were collected at the moment of ovary recovery; morphometrical parameters, number of total follicles and number of follicles $\geq$1 mm in size in the ovarian surface were recorded. Oocytes from follicles of one ovary were recovered and matured in TCM 199 supplemented with 10 ng/ml

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EGF, 100 ng/ml IGF-I and 10% FCS. The counterpart ovaries were fixed in paraformaldehyde solution for histological studies. Detection of cell apoptosis was determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling (TUNEL) technique. Reproductive performance was affected by the rhythm used, with lower reproductive parameters in the intensive group. The average ovary height and width, the mean number of ≥1 mm follicles and the number of total follicles were similar between groups. Serum concentrations of estradiol (E_2) and testosterone (T) were significantly lower in group A *vs.* B (E_2: 232.4 ± 56.1 *vs.* 399.7 ± 53.0 pg/ml; $P < 0.05$ and T: 1.07 ± 0.10 *vs.* 1.68 ± 0.23 ng/ml; $P < 0.05$). No significant differences were found in follicular population or in the mean follicular apoptosis index between groups. Metaphase II rate was significantly lower in group A *vs.* B (48.5 ± 3.3 *vs.* $67.6 \pm 3.7\%$; $P < 0.01$), as well as the migration rate of cortical granules (12.7 ± 2.7 *vs.* $38.2 \pm 6.6\%$; $P < 0.001$). On the other hand, neither follicular population, nuclear maturation rate nor apoptosis rate were affected by the eCG treatment, but cytoplasmic maturation was higher in animals treated with eCG in group A (29.2% *vs.* 5.5%; $P < 0.01$). In conclusion, rabbit does under transient litter separation during their reproductive life have both their serum estradiol and testosterone concentrations and oocyte quality influenced by the intensive rhythm, leading to a decrease in reproductive parameters. Also, both intensive and semi-intensive rhythms seem to be less receptive to eCG treatment than expected.

1. Introduction

Intensive and semi-intensive reproductive rhythms used in rabbit production systems require the use of lactating rabbit does. However, these females show depressed sexual receptivity and fertility during lactation period (Ubilla and Rebollar, 1995) and require oestrus synchronization methods to increase their lower fertility. Thus, rabbits are usually injected with eCG; this hormonal treatment improves receptivity and increases follicle growth as well as ovulation rate (reviewed by Maertens et al., 1995), but shows reduced efficiency when used repeatedly (Maertens, 1998; Rebollar et al., 2006). In addition, hormone use must be reduced for animal welfare and residues in meat (Castellini, 1996). Then, an alternative method of oestrus synchronization is biostimulation, which can be used in lactating rabbit does. One biostimulation method is lactation control, which consists in separating females from their litters for short periods of time prior to artificial insemination (AI). This transient doe-litter separation improves kindling rates and litter size at birth, with similar results to those obtained when administering hormonal treatments such as eCG before AI in multiparous rabbits, due to a decrease in plasma prolactin concentration and an increase in estradiol levels (Alvariño et al., 1998; Maertens, 1998; Rebollar et al., 2006). Nowadays, little is known about the prolonged effects of continuously controlled lactation in intensive and semi-intensive rhythms on endocrine and reproductive parameters. In that sense, it has been reported that biostimulation induces endocrine changes by stimulating hypothalamus–pituitary–ovary axis activity (Castellini et al., 1998; Ubilla et al., 2000). However, it could affect the hypothalamus–pituitary–adrenal gland axis as a result of the stress factors generated by litter separation, or perhaps induce a condition of behavioural/psychological and/or physiological adaptation that might well reduce fertility (Bonnano et al., 2002).

On the other hand, ovulation does not occur spontaneously in the rabbit doe because this is a neuroendocrine reflex triggered by copulation. Thus, the use of AI requires exogenous administration of GnRH analogues that act on the pituitary to release LH. In addition, GnRH has shown to act directly on the ovaries through specific ovarian receptors (Reeves et al., 1980), blocking follicle growth and differentiation (Hsueh et al., 1980), and inducing apoptotic cell death in rat ovaries (Billig et al., 1994). Also, it seems to be involved in ovulation, luteinization and luteolysis in the human ovary (Choi et al.,

2006). In rabbit farms where 35-day intensive rhythms (AI on day 4 post-partum [pp] and weaning at 25 days of lactation) are applied, the use of GnRH analogues is more intense than in 42-day semi-intensive rhythms (AI on day 11 pp and weaning at 35 days of lactation). Both groups of animals are injected repeated doses of GnRH with different frequencies, causing detrimental effects in their pituitary that could affect their ovarian follicular population features, but little is known about it.

On the basis of the above considerations, the aim of this study was to investigate the long-term effects of different reproductive rhythms in rabbits, always synchronized by a transient doe-litter separation, in a non-lactating status, on serum steroid hormones estradiol and testosterone, features of follicular population and atresia rate, as well as oocyte maturation. Also, we have studied the influence of the use of a dose of hormonal treatment (eCG injection) in these animals.

2. Materials and methods

Unless otherwise stated, all the chemicals were purchased from Sigma Chemical Company (Spain).

2.1. Animals and experimental design

A total of 181 rabbit does were held on the experimental farm of the Animal Production Department of the UPM into individual flat-deck cages with a closeable nest box, under a constant photoperiod of 16 h light per day, a temperature of 18–22 °C, and a relative humidity of 60–75% maintained by a forced ventilation system. All animals were fed *ad libitum* with a commercial pelleted diet containing 18% crude protein (CP) and 12.7 g digestible protein (DP) per MJ digestible energy all through the experiment. Nulliparous does were first inseminated at 19 weeks of age and, after their first kindling, does were randomly distributed in two experimental groups during all their reproductive cycles: group A: 73 does inseminated on day 4 pp and weaned on day 25 of lactation; group B: 108 does inseminated on day 11 pp and weaned on day 35 of lactation. Then, animals were separated from their litters by a metal screen for 24 h before AI according to Alvariño et al. (1998), from Day 3 to Day 4 of the lactation period in the intensive group (group A) and from Day 10 until Day 11 in the semi-intensive one (group B). Lactating rabbit does were inseminated in a single dose using a pool of fresh heterospermic semen from bucks selected for growth performance, with more than 20 million spermatozoa in 0.5 ml of a commercial diluent (Magapor, S.L., Spain). To induce ovulation, does were given an intramuscular injection of 1 μg buserelin acetate (Suprefact, Hoechst Marion Roussel, S.A., Spain) at the moment of AI. Twelve days after first AI, does were palpated to check pregnancy. Non-pregnant does were maintained until the following insemination, but replaced if they were infertile after three inseminations. At parturition, number of AI needed to achieve parturition, prolificacy (kits born alive and dead), parturition interval (PI) in days and productivity (number of weaned rabbits per doe and year) were recorded.

A group of 26 non-pregnant, non-lactating and post-weaned rabbits submitted to intensive or semi-intensive rhythm (group A $n = 15$, and group B $n = 11$) during their reproductive life were randomly allocated at the mid-end of their productive cycle (5–7 parturitions) in two groups: one treated with 25 IU of eCG (Ovejero, León, Spain) ($n = 7$ for group A and $n = 6$ for group B), and another one not synchronized with the hormonal treatment ($n = 8$ for group A and $n = 5$ for group B). The hormone was administered 48 h before animals were subjected to laparotomy to obtain their ovaries. All rabbits were anesthetized with 30 mg/kg of i.v. pentobarbital sodium (Dolethal, Vetoquinol, Alcobendas, Spain) before laparotomy. All procedures involving animals were conducted after approval by the animal Bioethics Committee of the Universidad Politécnica (Spain), according to the General Guidelines on Experimentation Animal Care.

2.2. Collection of blood samples and hormone analysis

Blood samples were collected from the margin ear vein into non-heparinized tubes, kept at room temperature for 1 h and then at 4 °C overnight. Serum was obtained after centrifugation at $1200 \times g$ for 10 min and stored at −20 °C until analyzed.

Serum estradiol 17-β (E_2) and testosterone (T) levels were measured in duplicate samples by specific chemiluminiscence methods (CMIA, Abbott Laboratories, Abbott Park, IL, USA), using purified rabbit

and rat monoclonal antibodies, respectively. Intra- and inter-assay coefficients of variation were 2.3 and 2.4 for E_2, and 1.9 and 3.7 for T. The detection limit for both hormones was 10 pg/ml. Serum E_2:T ratio was calculated as E_2 divided by testosterone, both in ng/ml, although otherwise the E_2 level was expressed in pg/ml.

2.3. Study of the follicular population and atresia

Ovaries were recovered, using one of them for histological studies and the other one for maturation assessment. Length and diameter of ovaries were first recorded. Then, ovaries in two halves were placed into a 4%-buffered neutral paraformaldehyde solution (pH 7.2–7.4). All samples were gradually dehydrated with increasing concentrations of ethyl alcohol (50–100%). These dehydrated specimens were first embedded in paraffin, prepared by sectioning at 6 μm, and stained with hematoxylin and eosin. In order to study follicle population, a pair of hystological sections of each half ovary were examined at light microscope (Olympus BX40, Hamburg, Germany). Rabbit ovarian follicles were categorized into four specific development stages related to the number of layers of granulosa cells, according to Rebollar et al. (2008).

In addition, paraffinned sections of remaining half ovaries were used for detection of cell apoptosis using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling (TUNEL; In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH) method. Section dewaxing and rehydration by standard methods were first carried out. The incubation with the TUNEL reaction mixture was performed in a humidified dark chamber at 37 °C for 1 h. The first developing step was the addition of 50 μl Converter Peroxidase (In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH) for 30 min in a humidified dark chamber at 37 °C. The final developing step was incubating preparations with 3,3′-diaminobezidine tetrahydrochloride (DAB) at room temperature, for approximately 3 min in the dark. After each step of the procedure, sections were rinsed three times in PBS. Counterstaining with hematoxylin was carried out. To preserve the samples until their visualization, they were dehydrated in ethanol, washed in xylene and finally mounted with Depex®. To achieve positive controls, slides were treated with DNAseI (DNAse I, Roche Applied Science, Germany) after the permeabilisation treatment. For negative controls, samples were just incubated with the label solution of the TUNEL reaction mixture without the enzymatic solution. Visualization of the labeled samples was performed under an optic microscope (20×). Positive apoptotic cells were considered those presenting a dark brown pigmentation, and negative those with a blue coloration. Follicles in medium or advanced stages of atresia were examined according to Kasuya (1995), and the percentage expressed is the number of TUNEL-positive follicles, divided by the total number of recorded follicles per ovary.

2.4. In vitro maturation

The remaining ovaries were placed in PBS at approximately 37 °C for transport to the laboratory. Cumulus–oocyte complexes (COC) were recovered by aspiration under a stereoscopic microscope and ≥1 mm follicles were recorded for each ovary. Following several washes, COC of each female were transferred into 4-well plates (Nunc™, Roskilde Life Technologies, Denmark) containing 500 μl of maturation medium, consisting in TCM 199 + 10 ng/ml epidermal growth factor (EGF) + 100 ng/ml insulin growth factor (IGF) + 10% foetal calf serum (FCS) and 100 IU penicillin–streptomycin (Lorenzo et al., 1997). Plates were then incubated for 20 h in 5% CO_2 in humidified air at 38 °C. After that, matured oocytes were denuded by a brief incubation in 2 mM hyaluronidase and subsequently washed in PBS medium. Zona pellucida was digested with 0.5% protease and treated with 4% paraformaldehyde, 0.02% Triton X-100 and 7.5% BSA. Oocytes were incubated with 100 μg/ml fluorescein isothiocyanate of *lens culinaris* (FITC-LCA) for cortical granules (CG) staining and with 10 μg/ml propidium iodide for nuclear staining, and observed under a confocal laser-scanning microscope (Leica, TCS SP5). According to a previous work (Arias-Álvarez et al., 2007), CG distribution was categorized as follows: (1) homogeneous: CG were distributed throughout the cytoplasm, as they did not show cytoplasmic maturation; (2) cortical: most of the CG were distributed at the cortical area; (3) peripheral: CG were distributed adjacent to the plasma membrane forming a monolayer, as they were cytoplasmic matured.

Table 1
Effects of management on rabbit reproductive performance and productivity. No. of AI: number of AI needed to achieve the corresponding parturition. PI: parturition interval. [1]Number of weaned rabbits/doe and year. Means in rows with different letters differ ($P < 0.05$). Values are means ± S.E.M.

	Group A (intensive rhythm)	Group B (semi-intensive rhythm)
Parturitions (*n*)	182	220
No. of AI	1.70 ± 0.04^{a}	1.39 ± 0.04^{b}
Prolificacy		
Dead born	1.20 ± 0.18	1.05 ± 0.16
Born alive	8.23 ± 0.27	8.90 ± 0.24
PI (days)	56.0 ± 1.21^{a}	50.1 ± 1.10^{b}
Productivity[1]	51.5 ± 2.66^{a}	63.3 ± 2.42^{b}

2.5. Statistical analysis

A GLM procedure was used to study the effect of reproductive management (intensive or semi-intensive), with respect to reproductive parameters (Statistical Analysis System 8.2; 2001). Means were compared using a protected *t*-test. The rest of parameters were analyzed using SPSS 13.0 (SPSS, Inc, Chicago, IL.). Then, follicular population and morphometrical parameters were analyzed by one-way ANOVA. A Chi-square test was carried out to analyze nuclear and cytoplasmic maturation. Apoptotic follicular index and serum hormonal levels were statistically studied by *t*-test. Statistical significance was considered when $P < 0.05$.

3. Results

3.1. Reproductive performance

Reproductive parameters of the does were affected by the rhythm used (Table 1). The number of inseminations required by animal to become pregnant was higher in group A (1.70 ± 0.03 *vs.* 1.39 ± 0.03 AI; $P < 0.0001$). Prolificacy (born alive and dead) was not significantly influenced by the rhythm, obtaining a mean of 8.6 ± 0.8 born-alive and 1.1 ± 0.1 dead-born kits. In addition, parturition interval in group A was longer compared with the semi-intensive group (56.0 ± 1.4 *vs.* 50.9 ± 1.38 days, respectively; $P < 0.05$.) Productivity estimated by number of kits weaned per doe and year was higher in rabbits inseminated on day 11 pp ($P < 0.05$), with a mean of 12 weaned kits more than does inseminated on day 4 pp.

3.2. Hormone assay

Mean serum concentration of E_2 and T in group A was lower than in group B ($P < 0.05$), as well as the ratio E_2:T, but statistical differences in this parameter were not found (Fig. 1a). Hormonal eCG treatment did not influence E_2 and T blood levels (Fig. 2a).

3.3. Morphometrical analysis and recovered oocytes for IVM

The average height and width of ovaries were similar between groups (Table 2). Also, no significant differences were found in the mean number of ≥1 mm follicles in the ovarian surface and in the number of total follicles in the ovarian surface. On the other hand, the mean number of recovered oocytes was higher for group B than A (9.6 ± 0.8 *vs.* 8.7 ± 0.8; NS), but the number of selected oocytes for IVM was similar between groups (8.1 ± 0.8 *vs.* 8.0 ± 0.7; NS), given that the number of discarded oocytes tended to be higher for group B (2.4 ± 0.8 *vs.* 0.7 ± 0.2, $P = 0.08$).

Non-eCG-treated animals in group A presented a tendency to have a higher number of recovered oocytes per ovary ($P = 0.07$) and oocytes selected for maturation ($P = 0.06$) than eCG-injected ones (Table 2). Haemorragic follicle number was higher in treated animals, but this finding did not reach statistical significance.

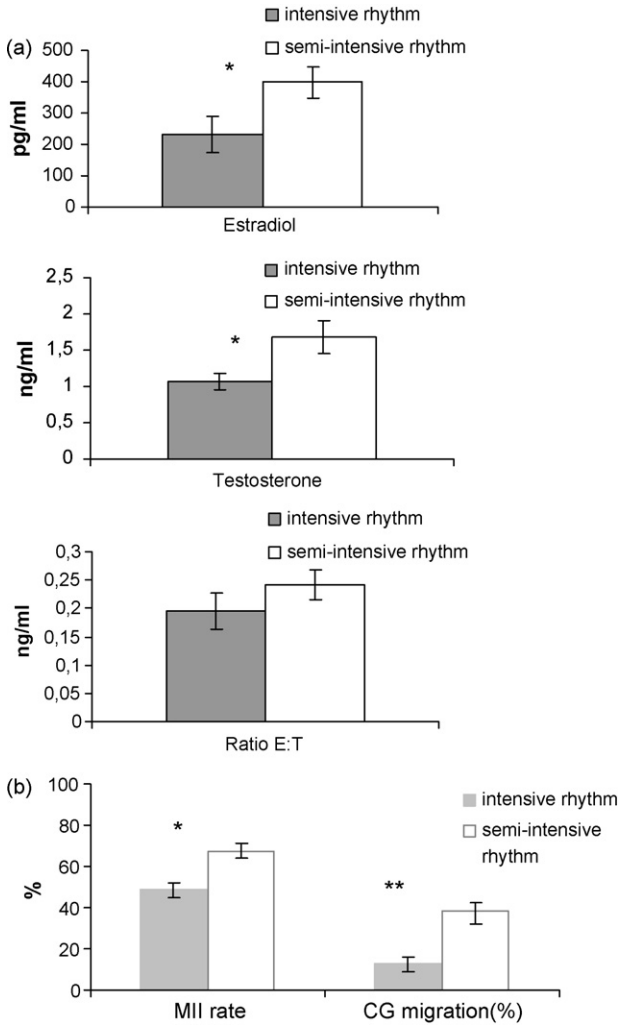

Fig. 1. (a) Serum hormonal concentrations in intensive (group A) and semi-intensive rhythm (group B). The values are means ± S.E.M. (*$P < 0.05$); (b) nuclear (MII rate) and citoplasmyc maturation (cortical granules migration, CG) of oocytes from rabbit does under intensive (group A) and semi-intensive (group B) rhythms. The values are means ± S.E.M. (*$P < 0.01$; **$P < 0.001$).

3.4. Follicular population

As depicted in Table 2, no significant differences were found in the mean number of primordial, primary and secondary follicles between groups. However, the average number of total antral follicles tended to decrease in group A *vs.* group B (3.5 ± 0.4 *vs.* 5.7 ± 1.2; $P = 0.1$). In addition, follicular population was not affected by the hormonal treatment.

3.5. Apoptosis rate

The study of the atresia rate was carried out in a total of 414 follicles ($n = 214$, group A; $n = 200$, group B, Fig. 3a and b). Mean apoptosis index of follicles was higher for group A compared to group

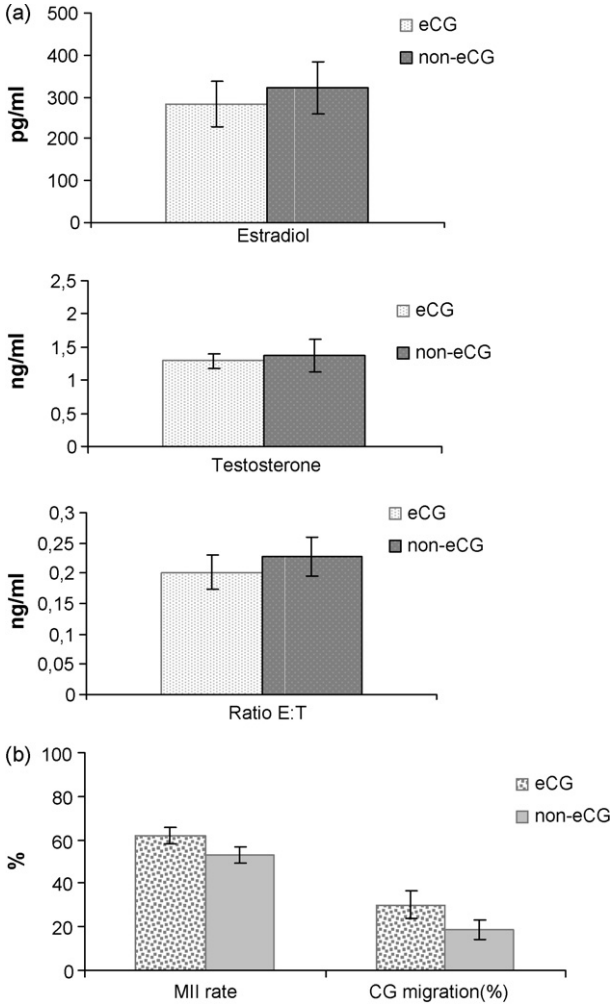

Fig. 2. (a) Serum hormonal concentrations in treated and untreated animals with eCG. The values are means ± S.E.M. (b) Nuclear (MII rate) and citoplasmyc maturation (CG migration) of oocytes from rabbit does in treated and untreated animals with eCG. The values are means ± S.E.M.

B (75.9 ± 2.9 *vs.* 67.0 ± 6.0), but no significant differences were found between them (Table 2). Also, apoptosis rate was unaffected by the eCG treatment (72.1 ± 18.0 *vs.* 70.5 ± 12.0).

3.6. Nuclear and cytoplasmic maturation

A total of 211 COC ($n = 122$, group A, and $n = 89$, group B) were examined to study nuclear and cytoplasmic maturation (Fig. 4a and b). As depicted in Fig. 1b, Metaphase II rate was significantly lower in group A *vs.* B ($P < 0.01$), as well as cytoplasmic maturation measured in terms of peripheral cortical granule migration ($P < 0.001$). Nuclear maturation rate was unaffected by the eCG treatment (Fig. 2b); however, oocytes of animals treated in group A showed higher rate of CG migration than non-eCG-injected ones (28.0 ± 9.1 *vs.* 5.5 ± 3.0%; $P < 0.01$). See Table 2.

Table 2
Morphometric ovarian parameters, folicular population, apoptosis rate and oocyte maturation of rabbit does under different productive rhythms (intensive and semi-intensive) treated or untreated with eCG. Means in rows with different letters differ ($P < 0.05$). Values are means ± S.E.M.

	Group A (intensive rhythm)		Group B (semi-intensive rhythm)	
	Untreated with eCG	Treated with eCG	Untreated with eCG	Treated with eCG
Ovary height (mm)	17.1 ± 0.6	16.9 ± 1.4	15.8 ± 3.5	17.8 ± 2.4
Ovary width (mm)	6.6 ± 0.4	7.7 ± 0.7	6.8 ± 0.6	8.4 ± 0.5
Mean of ≥1 mm follicles	9.0 ± 1.0	7.3 ± 1.6	7.6 ± 0.7	8.6 ± 0.9
Mean of total follicles	10.6 ± 1.9	11.4 ± 2.2	10.8 ± 1.8	13.4 ± 1.5
Mean of haemorrhagic follicles	1.63 ± 1.0	4.1 ± 2.5	3.2 ± 1.7	4.8 ± 2.3
Mean of recovered oocytes	10.5 ± 1.0	6.7 ± 0.9	9.6 ± 0.8	9.4 ± 1.6
Mean of IVM selected oocytes	9.4 ± 1.1	6.7 ± 0.9	8.6 ± 1.0	7.4 ± 1.0
MII rate (%)	43.3	56.8	67.5	67.7
CG migration (%)	5.5[a]	29.2[b]	44.4	32.1
Mean of primordial follicles	75.7 ± 17.3	79.0 ± 30.7	97.7 ± 43.3	87.3 ± 22.0
Mean of primary follicles	3.5 ± 0.9	4.5 ± 1.2	6.7 ± 2.5	5.17 ± 1.6
Mean of secondary follicles	6.0 ± 0.8	10.8 ± 2.1	12.5 ± 4.8	10.8 ± 2.7
Mean of antral follicles	3.6 ± 0.6	3.3 ± 0.8	6.8 ± 2.0	4.6 ± 3.3
Apoptotic follicles (%)	76.3 ± 4.8	75.3 ± 2.2	65.0 ± 16.1	68.0 ± 5.8

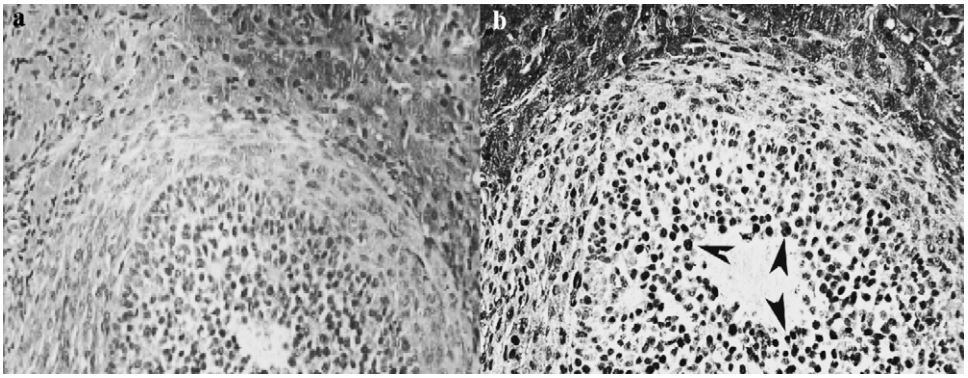

Fig. 3. Assessment of apoptosis. Analysis of DNA strand breaks by TUNEL in ovarian rabbit follicles. (a) Follicle negative for TUNEL reaction; (b) antral follicle with granulosa cells positive for TUNEL staining.

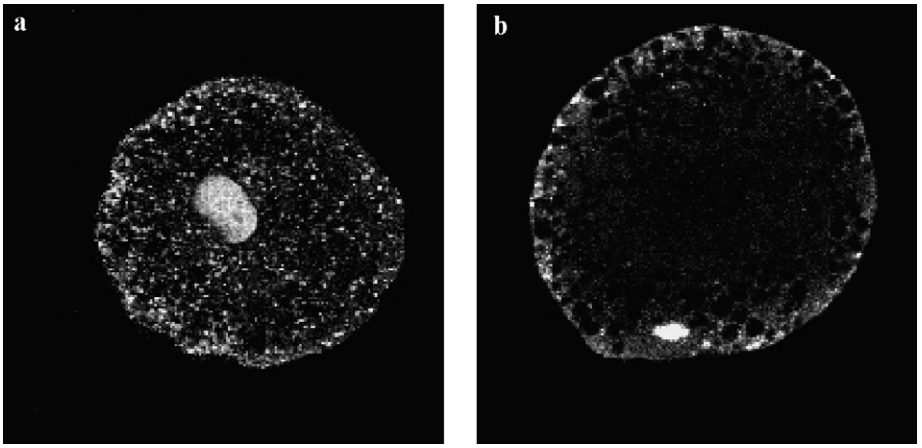

Fig. 4. Nuclear and citoplasmyc maturation (CG migration) of oocytes from rabbit does. (a) Oocyte showing germinal vesicle stage (VG) and homogenous CG distribution; (b) oocyte showing metaphase II stage and cortex migration of the CG.

4. Discussion

Probably, there is not an optimum reproductive rhythm for rabbit farms (Castellini, 1996). In these animals, sexual receptivity is aphasic, being very high immediately after kindling and weaning (Theau-Clément et al., 1990). From the animal physiology point of view, the use of AI after weaning compared with insemination at 11 days pp could be more adapted to doe physiology (Castellini et al., 2003). Our results confirm that reproductive performance seems to be affected in continuous intensive rhythms compared with semi-intensive ones, as reported by other authors (Cervera et al., 1993; Rebollar et al., 2006). In the present work, does of group A required more inseminations per gestation than B-group rabbits to become pregnant, so the number of pregnant females in the first group was lower than in the second one. Together with this finding, we observed that parturition intervals was longer than theoretical or expected in both reproductive managements, also showing differences between them; intervals were around 21 days higher than expected in the intensive group and 8 days higher than expected in the semi-intensive group. Hence, the results obtained lead to a better-estimated productivity (number of kits weaned per year and doe) in the rabbit does of the semi-intensive rhythm group.

Follicular population in rabbits is characterized by not a very large total number of primordial and small developing follicles due to the relative thinness of the ovarian cortex. On the other hand, they possess a higher number of mature follicles (Kranzfelder et al., 1984, reviewed by Arias-Álvarez et al., 2007). In our experiment, slightly lower population of antral follicles was found in the group under intensive management that could be responsible of the relative lower serum E_2 levels of the intensive group compared with the semi-intensive one. Measurements of E_2 levels have been commonly used to assess follicular growth and its steroidogenic capacity (Wallach and Noriega, 1970). In this way, the decrease in hormonal serum levels may indicate lower follicle steroidogenic activity. This reveals poorer ovarian activity in those animals, which is also reflected in poor oocyte quality and, ultimately, in reduced reproductive parameters. However, E_2 levels reported in our work were higher than those showed by younger animals of our farm (data not shown), and higher than those reported by other authors (De Turckheim et al., 1983; Brecchia et al., 2006; Rebollar et al., 2006). Probably, estradiol values are elevated as a result of the non-lactating and post-weaning status of animals of this experiment. Most of the studies carried out in rabbit does occurred during the lactation period, when high plasma concentrations of prolactin are shown to diminish plasma E_2 concentrations. Nevertheless, previous reports have demonstrated transient changes in plasma concentrations of E_2 after doe-litter separation, and in the pituitary LH response to exogenous GnRH administered at the time of AI after biostimulation (Ubilla et al., 2000). Thus, further studies are required to elucidate if a long exposure of rabbit does to biostimulation methods in intensive or semi-intensive rhythms during all their reproductive life, with repeated doses of GnRH stimulation, may cause marked and persistent changes in ovarian function that could be responsible for elevated estradiol levels as reported in the present work.

Follicular atresia is characterized by a rapid loss of mainly granulosa cells via apoptosis (Palumbo and Yeh, 1994; Tilly, 1996). Follicles undergo apoptosis based on their cytological features occurring in parallel with the positive TUNEL reaction (Kasuya, 1995; Kim et al., 1998). This is why in our work TUNEL assessment was accompanied by hematoxylin and eosin histological study. In agreement with other studies (cow: Yang and Rajamahendran, 2000; rabbit: Hutt et al., 2006), we have observed apoptotic cells commonly located adjacent to and within the follicular antrum. Apoptosis of theca cells was associated with follicles exhibiting a large number of TUNEL-positive granulosa cells; it was never detected in the oocytes of antral follicles. These results of TUNEL-positive granulosa cells were frequently visualized within the antral follicles in rabbit (Hutt et al., 2006) and mouse ovaries (Kim et al., 1999). However, apoptotic granulosa cells were detected in an appreciable number of follicles considered to be healthy by morphometric criteria according to Jolly et al. (1997) in ewes. In that way, it has been reported that the proportion of atretic antral follicles is fairly constant during fertile life and varies among species; in rabbits, 60% of the antral follicles are atretic (Kranzfelder et al., 1984). Probably we cannot find any difference in apoptosis index between groups because these animals continuously produce mature follicles that become atretic if ovulation is not stimulated by coitus (Kranzfelder et al., 1984; Boiti, 2004).

Regarding to the *in vitro* oocyte maturation, current results in metaphase II and CG migration rates are according to other studies reported in rabbits (nuclear: Schulz et al., 1985; cytoplasmic: Arias-Álvarez et al., 2008). In mammalian oocytes, CG migration has been used as a criterion for the assessment of cytoplasmic maturation. CG positioned just beneath the plasma membrane render the oocyte ready for fertilization by triggering the cortical reaction (Wang et al., 1997). Our study has shown a significant decrease in both nuclear and cytoplasmic maturation rates in oocytes of the intensive group animals compared to the semi-intensive ones; this may possibly be explained by the slight differences in follicular population features and in hormonal blood levels, as follicular influence on oocyte quality and subsequent potential is evident (Sirard, 2001).

Finally, it has been reported that eCG treatment usually increases ovulation rate by promoting growth of large follicles and by reducing the number of small follicles, increasing their atresia rate (reviewed by Driancourt, 2001). Also, the number of haemorrhagic follicles increases with excessive stimulation of the ovaries (Boiti et al., 1995). In our experiment, the means of total and haemorrhagic follicles were higher for eCG-treated groups, but no statistical differences were found in both means with respect to the other group. Moreover, hormonal blood levels and apoptotic follicular rate were not affected by the eCG treatment. All these data suggested no responsiveness to the hormonal treatment in these animals. However, in the intensive rhythm group, animals treated with eCG showed higher rate of oocyte CG migration and higher mean of secondary follicles than non-eCG-treated ones. Conversely, they shown slightly low E_2 levels and E_2:T ratio in serum than the rest of animals. Thus, other paracrine/autocrine factors could be locally acting to reduce steroidogenesis. We can hypothesize that in the current work, the high E_2 levels in serum in both groups may reveal irregular maintenance of a functional hypothalamic–pituitary axis unable to respond appropriately to the eCG treatment.

In conclusion, rabbit females synchronized by transient doe-litter separation during their reproductive life under an intensive rhythm (IA at Day 4 pp) have their endocrine levels of estradiol and testosterone affected, as well as their oocyte quality, which is reflected in their poor reproductive performance. Also, both groups seem to be less receptive to eCG treatment than expected; maybe the functioning of their HPO system under these intensive rhythms is not reset to its normal level after continous biostimulation cycles and/or GnRH stimulation. Therefore, additional studies should be undertaken to elucidate this hypothesis.

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