Tropical Animal Health and Production
https://doi.org/10.1007/s11250-019-01972-5

REGULAR ARTICLES

Influence of eCG and breed on the number of oocytes collected and the production of in vitro embryos of young goats during the reproductive season

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Received: 1 March 2019 / Accepted: 6 June 2019

Abstract

The objective of this study was to determine the effect of breed and equine chorionic gonadotropin (eCG) on ovarian response and in vitro embryo production from young goats. Thirty-one (12 Alpine, 10 Nubian, and 9 Saanen) were randomly assigned into three treatments of eCG (T1, 0 IU; T2, 500 IU; and T3, 1000 IU). Alpine goats showed the highest amount and largest size of follicles ($P = 0.003$). The effect of eCG dose 24 h post application was significant ($P < 0.05$), and was superior in goats undergoing T2. The aspiration rate of cumulus–oocyte complexes (COC) was 34% ($P > 0.05$), except for percentage of denuded oocytes, which obtained the highest number ($P = 0.003$) in the Saanen goats. The same difference was found ($P = 0.02$) in oocytes grade III in T2 and T3, with 42.5 and 37.9% respectively. In vitro embryo production was 80.0% of IVF/cleavage in the Alpine goats ($P = 0.003$). Embryo production was the greatest for T2 (69.2%; $P = 0.004$). T3 goats had higher percentage of morula stage (66.6%; $P = 0.030$). It is concluded that the application of eCG has a significant effect on the ovarian status, and quality and quantity of embryos with a differential response depending on the breed.

Keywords Cumulus–oocyte complexes · eCG · Follicles · Oocytes

Introduction

Assisted reproductive technologies have been suggested as a method to improve the reproductive performance and genetic gain of goat production systems (Amiridis and Cseh 2012). These technologies involve controlling estrus and ovulation, artificial insemination, multiple ovulation and embryo transfer, in vitro embryo production, and cryopreservation of gametes and embryos (Souza-Fabjan et al. 2016, Paramio and Izquierdo 2016). In vitro production of embryos optimizes the generation of embryos and decreases the time of implementation until the birth of the offspring when compared to artificial insemination and traditional embryo transfer (Padilha et al. 2014). This technique has some advantages of reliability (Amiridis and Cseh 2012), reproducibility (Stangl et al. 1999), and the possibility to collect oocytes from hormonally stimulated female (Morton et al. 2005), and juvenile and senile (Baldassarre et al. 2007), infertile, prepubertal, pregnant, or lactating females, and even in some cases postmortem (Paramio and Izquierdo 2016). During in vitro embryo production, vesicle-stage oocytes collected from prepubertal calves and lambs progress through meiosis to metaphase II (MII) at rates similar to oocytes from adult animals (O'Brien et al. 1996). After fertilization, these oocytes are able to direct events in early embryogenesis (O'Brien et al. 1996). These

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Published online: 17 June 2019

observations show that there is a possibility of using juveniles as embryo donors in accelerated breeding programs, with the aim of reducing the generation interval of the damson pathway of selection schemes. The number of ovarian follicles and oocytes from outstanding dams can be increased by hormonal stimulation, extracted and matured in vitro (Paramio and Izquierdo 2016) for further use in breeding programs. The limiting factor in young animals is lower percentage of mature follicles compared to adult females for different species in which in vitro fertilization (IVF) has been carried out (Romaguera et al. 2011; Mendes et al. 2017). Likewise, it is well known that the competence of small follicle oocytes reaching metaphase II after in vitro maturation is lower than that of medium follicle oocytes (Kohata et al. 2013). There are five levels of competition between the oocytes reported, the first being the ability to resume meiosis, to divide after fertilization, to develop in the blastocyst stage, to induce a pregnancy, and to carry it to term and in good health (De Souza-Fabjan et al. 2014; Yang et al. 2016). The gonadotropins which have been used in the treatment of superovulation in goats are porcine or ovine follicle-stimulating hormone (FSH) (Lehloenya 2013), equine chorionic gonadotropin (eCG) (Goel and Agrawal 2005), or a combination between FSH and eCG in regime known as "one-shot" (FSH + eCG) (Baldassarre et al. 2007). The eCG has some advantages such as single administration and low cost. Uribe-Velásquez et al. (2008) reported that the application of eCG after 14 days of intravaginal progesterone applied by a controlled internal drug release in cyclic ewes increased the recruitment of smaller follicles, maximum diameter, and growth rate of large follicles during the first wave of follicular growth. The eCG has been used in almost all programs of synchronization of estrus in goats, with dose level affecting the follicular growth. Studies have shown that the application of eCG can have different effects depending on the dose applied. A dose of 300–400 IU shows that the interval between removal of the device and estrus is affected; intermediate doses of 500–700 IU improves the ovulation rate; doses of 1000–1200 IU cause induction of greater amounts of large follicles (8–10 follicles) and ovulations (6–8 corpora lutea) (Ali 2007). Reporting a higher growth rate of small and large follicles due to the effect of eCG is not the case for medium-sized follicles (Habibizad et al. 2015). The use of eCG in goats could open the possibility of collecting and maturing oocytes from superior does to accelerate genetic progress in this species. As far as the authors are aware, the effect of breed on the differential response to application of eCG in goats has not yet been reported. The objective of the present study was to evaluate the effect of breed and the application of eCG at different doses on the ovarian response and the in vitro embryo production within the breeding season in young goats.

Materials and methods

Location of the experiment

This study was conducted in the Laboratorio de Reproducción Animal de la Facultad de Agronomía de la Universidad Autónoma de Nuevo León, located at 25° 53′ N, 100° 2′ W, 400 masl, and in the Centro de Biotecnología Reproductiva at the Unión Ganadera Regional de Nuevo León, located at 25 ° 36′ N, 99 ° 13′ W, 400 masl.

Experimental animals

This study was approved by the Bioethics and Animal Welfare Committee of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Autónoma de Nuevo León (Act. No. 18), and it was conducted from November through December 2016 (breeding season). Thirty-one goats were used (12 Alpine, 10 Nubian, and 9 Saanen); they had an average body weight of 26.2 ± 0.6 kg, body condition score of 2.9 ± 0.4 (according to a 5-point scale, Mendizabal et al. 2015) with an average age of 10 months. They were fed ad libitum with a feed containing 14% CP and 2.3 MCal of metabolizable energy.

Ovarian stimulation protocol

At the beginning of the experiment, goats were grouped by body weight, body condition score, and breed (4 Alpine, 3 Nubian, and 3 Saanen were assigned by treatment). Prior to the beginning of the application of the eCG, a 5-day estrus synchronization protocol was applied, using vaginal sponges containing 65 mg of medroxyprogesterone acetate (Serigan, Laboratorio Sanfer, México). When the sponge was removed, 7.5 mg of cloprostenol (Prostagenol-D, Laboratorio Prode, Mexico) plus intramuscular application of eCG was applied based on the three treatments that were evaluated: T1: control, 5 ml of normal saline solution (NaCL 0.9%, solution CS, PISA, Hidalgo, Mexico) ($n = 11$); T2: 500 IU ($n = 10$), T3: 1000 IU ($n = 10$) of equine chorionic gonadotropin (eCG) (Novormon® 5000, Virbac®, Guadalajara, Mexico). Prior and 24 h post application of the eCG, it was carried out a transrectal ultrasound (SonoScape, model A5V, China with transducer L761V, 11.0–5. 0 MHz) to determine ovarian status: presence of corpora lutea and follicles.

Oocyte collection by laparotomy

The methodology used for the collection of oocytes was same as that described by Ptak et al. (1999). Goats were deprived of food and water for a period of 24 h, for laparotomy. The goats were sedated with a combination of xylazine hydrochloride 0.6 mg/kg/IM (Procin®, PISA, Hidalgo, México) plus a single

dose of 0.5 ml/SC of atropine sulfate (Tropigenol, Aranda, Querétaro, México) and 20 mg/kg/IM ketamine hydrochloride (ANESKET®, PISA, Hidalgo, México). Exteriorization of the reproductive tract and the ovaries was exposed through a medio-ventral incision of 5 cm for oocyte aspiration through a 20-ml syringe, equipped with an 18-G needle. The oocytes were collected from follicles of 2 to 6 mm in diameter using the BioLife Advantege Embryo Collection Medium (Agtech, KC, USA) added with 2 ml of heparin, which were in water bath at a temperature of 37 °C. Then the content of the syringe was passed into filters Emcon™, where filtering used the media mentioned above and then passed to check boxes (100 × 15 mm) to carry out the identification and classification of the oocytes using a stereoscope microscope Nikon SMZ645 (Nikon Transfromer, Modelo XN, Japón). The cumulus–oocyte complexes (COC) were classified in three categories (grades I, II, and III) according to their homogeneity morphology of the cytoplasm and compaction of the cumulus cells, where it is defined as follows: grade I—oocytes with more than 3 layers of cumulus cells, compact, with homogenous cytoplasm, uniformly granulated; grade II—oocytes with less than 3 layers of cumulus cells with homogenous cytoplasm; and grade III—oocytes with a single layer of cumulus cells, with irregular cytoplasm and dark areas (Padilha et al. 2014). Subsequent to classification, the oocytes were washed 3 times in the maturation media and were placed in tubing Ependorff containing TCM 199 plus 10% fetal bovine serum (*v*/*v*) and 50 μg/ml gentamicin to be sent to the Centro de Biotecnología Reproductiva at the Unión Ganadera Regional de Nuevo León, in a portable incubator of oocytes kept a temperature of 38.5 °C.

In vitro maturation

Arriving at the laboratory, the COC were again classified and washed 3 times and they were passed in groups of more than 2 oocytes to microdrops of 90 μl of IVM medium, which was composed of 4.5 ml tissue culture medium TCM 199 without HEPES, 0.5 ml fetal bovine serum (FBS), 25 mM/mL sodium pyruvate, 25 μg/mL amikacin, 25 μg/mL penicillin, 0.02 UI/m follicle-stimulating hormone (FSH), 5 μg human chorionic gonadotropin (hCG), 1 μg estradiol 17β (E2 17β), 30 μM epidermal growth factor (EGF), and 0.01 mM cysteamine, covered with 3690 μl of mineral oil under 5% CO_2 in air at 38.5 °C with maximum humidity for 24 h (Urdaneta et al. 2004).

In vitro fertilization

Once the oocytes were matured during 24 h, they were prepared to perform IVF. The oocytes were transferred to microdrops of 90 μl of fertilization medium. For each fertilization process, a straw was used, which was thawed at 37 °C for 1 min. A total of 6 straws from only 1 buck were used. The motile sperms were selected by centrifugation in a discontinuous density gradient of Percoll (De Souza-Fabjan et al. 2014), by adjusting the sperm concentration to 4 × 106 cells/mL, adding to the medium a total of 10 μl of semen. The IVF was composed of 4694 μl base medium supplemented with 4 mg/L bovine serum albumin, 0.33 Mm sodium pyruvate, 25 μg/mL amikacin, 25 μg/mL penicillin, 20 μM penicillamine, 10 μM hypotaurine, 2 μM epinephrine, and 5 UI/mL heparin, under mineral oil (De Souza-Fabjan et al. 2014) sperm and oocytes were co-incubated for 16 to 20 h at 38 °C to 39 °C in humidified atmosphere of 5% CO_2 in air.

In vitro embryo culture

At 18 h after fertilization, presumptive zygotes were washed to remove both remnant cumulus cells and attached sperm cell. They were transferred into microdrops of 90 μl of IVC, which was SOF supplemented with BSA fraction V, which was composed of 5 mL base medium supplemented with 2.77 mM myo-inositol, 0.34 mM sodium tris-citrate, and 2 mM glutamine, under mineral oil, incubated at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2. After 24 h, the percentage of cleavage was determined. The total number of oocytes that were put to mature was 87 and of them the presumed zygotes were 42. The morphological evaluation and categorization of embryos were performed, according to the manual of the International Embryo Technology Society, at the 7th day post IVF.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS, version 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The number of follicles before and after ultrasound was analyzed with a linear model that included the effect of breed, dose of eCG, and their interaction. The variables of retrieved oocytes, the quality, and the embryo production were analyzed using the chi-square test (χ^2) to test significant differences between breeds and cGG doses. A significant difference was declared when the P value was < 0.05.

Results

Breed effects on the number of follicles extracted per animal were not significant before injection of eCG but were significant after injection ($P = 0.003$), with Alpine showing the highest response (Table 1). After injection with eCG, the number of follicles extracted significantly increased ($P < 0.01$), regardless of dose level (Table 1). It was a differential response between the breeds on the number of follicles extracted

Table 1 Effect of breed and eCG dose on the size of follicles in goats of 10 months after the injection of eCG (means ± SEM)

	No. of goats	No. of follicles	Size of follicles (mm)
Breed			
Alpine	12	117	3.4 ± 0.12[a]
Nubian	10	55	2.5 ± 0.23[b]
Saanen	9	74	2.7 ± 0.18[b]
Treatment (IU eCG)			
T1 = control	11	92	2.64 ± 0.20[b]
T2 = 500	10	98	2.54 ± 0.18[b]
T3 = 1000	10	116	3.36 ± 0.16[a]

[a,b] Means with different superscripts within breed ($P = 0.003$) or treatment ($P < 0.001$) are statistically different

after eCG injection; the Alpine goats showed a higher response than the Nubian and Saanen goats.

In the Alpine goats, a greater growth of follicles was present in comparison to the size of follicles from Nubians and Saanen goats ($P = 0.003$) (Table 1). An effect of the eCG dose ($P < 0.001$) on the size of the follicles was presented, being greater for the goats that received 500 and 1000 IU in comparison to the control (Table 2). The effect of the breed on the quality of the oocytes collected in general was not significant, except for percentage of denuded oocytes (Table 3); the Saanen goats had higher percentage than the Alpine and Nubian goats ($P = 0.003$). The percentage of degenerate oocytes was similar between breeds. The quality of COC collected was not generally affected by the application of eCG with the exception of the percentage of GIII. Compared to the control group, the goats injected with either 500 or 1000 IU had higher percentage of GIII oocytes ($P = 0.020$) (Table 3). Indicator traits of in vitro production of embryos by breed are presented in Table 4. Alpine goats had a better response than Nubian and Saanen goats for the total number of embryos 7 days post IVF/cleavage ($P = 0.003$). Breed differences for the other variables were not statistically different. Injection of eCG significantly increased the total number of embryos 7 days post IVF/cleavage compared with the control group ($P = 0.004$), but the goats injected with 500 IU of eCG had higher response than the goats injected with 1000 IU. Injection of 1000 IU of eCG significantly improved the percentage of embryos that were present at the morula stage ($P = 0.030$) compared to the control goats or goats receiving 500 IU (Table 4).

Discussion

This study is one of the first studies that reports the use of IVF to obtain oocytes from young goats in Mexico. In general, eCG affected the in vitro production of embryos according to the dosage and breed. These results confirm that the administration of eCG improves the activity of the ovary, which is reflected in the recruitment of more small follicles showing an elevated growth rate, a sustained growth of medium and large follicles, and improved development of the dominant and preovulatory follicle, as reported in dairy cattle (De Rensis and López-Gatius 2014).

Breed has been reported to affect ovarian activity and follicular development when ewes are treated for superovulation (Quintero-Elisea et al. 2011). In this study, the Alpine and Nubian goats responded better to the administration of eCG than the Saanen breed on the size of follicles (mm) and a higher percentage of embryos 7 days post IVF. The response to hormonal treatment is multifactorial, where both genetic and hormonal factors are present, such as gonadotrophin type, degree of purity, applied dose, and protocol (Lehloenya 2013). The application of eCG can cause variations in follicular response due to genetic differences between and within breeds (Quintero-Elisea et al. 2011), where the response to eCG is a function of the prolificacy of the breed. Generally, prolific goats have a higher number of follicular waves and respond

Table 2 Effect of breed and eCG dose on size of follicles recorded per animal in young goats during the breeding season (means ± SEM)

	No. of goats	No. of follicles	Size of follicles (mm)		
			Before eCG application	No. of follicles	After eCG application
Breed					
Alpine	12	69	3.47 ± 0.19	108	3.18 ± 0.15[a]
Nubian	10	17	2.05 ± 0.37	38	2.97 ± 0.25[a]
Saanen	9	32	2.52 ± 0.27	42	2.88 ± 0.24[b]
Treatment (IU eCG)					
T1 = control	11	42	2.95 ± 0.27	66	2.64 ± 0.19[b]
T2 = 500	10	32	3.27 ± 0.23	70	3.61 ± 0.19[a]
T3 = 1000	10	46	2.76 ± 0.24	52	2.90 ± 0.21[a]

[a,b] Means with different superscripts within breed or treatment are statistically different ($P < 0.05$)

Table 3 Effect of breed and eCG dose on oocyte quality in young goats during the breeding season

	Breed				Dose of eCG (IU)			
	Alpine	Nubian	Saanen	*P* value	T1 = control	T2 = 500	T3 = 1000	*P* value
No. of goats	12	10	9		11	10	10	
No. of recovered oocytes/no. of follicles (%)	44/155 (28.4)	28/73 (38.4)	26/79 (32.9)	0.921	29/94 (30.9)	40/122 (32.8)	29/91 (31.9)	0.912
Quality of cumulus–oocyte complexes (COC) (%):								
GI	0/44 (0)	0/28 (0)	0/26 (0)	0.651	0/29 (0)	0/40 (0)	0/29 (0)	0.780
GII	3/44 (6.8)	1/28 (3.6)	0/26 (0)	0.752	0/29 (0)	2/40 (5.0)	2/29 (6.9)	0.310
GIII	18/44 (40.9)	11/28 (39.3)	8/26 (30.8)	0.515	9/29 (31.0)[b]	17/40 (42.5)[a]	11/29 (37.9)[a]	0.020
Denuded oocytes (%)	17/44 (38.6)[b]	13/28 (46.4)[b]	16/26 (61.5)[a]	0.003	16/29 (55.2)	16/40 (40.0)	14/29 (48.3)	0.259
Degenerate oocytes (%)	6/44(13.6)	3/28 (10.7)	2/26 (7.7)	0.533	4/29 (13.8)	5/40 (12.5)	2/29 (6.9)	0.299

[a, b] Means within a row with different superscripts are statistically different ($P < 0.05$)

better to exogenous hormonal application (Baril et al. 1989). In this study, goats responded below the average to what has previously been reported in literature as the doses that were used for eCG on the number of follicles were found 24 h post-application of the hormone.

A possible reason for low response could be that the goats used in this study were not in the same phase of the estrus cycle, presenting a random follicular population; this makes the response to the eCG different. It is important that in subsequent studies on the application of eCG in synchronized estrus goats, the follicular wave be homogenized to obtain a uniform follicular growth (Bruno-Galarraga et al. 2015). The increase in follicle size, caused by the administration of 1000 IU of eCG, could be due to the establishment of ideal conditions for follicular growth, such as the presence of FSH and LH receptor sites in the follicle (Baruselli et al. 2004). These results agree with those reported by Salehi et al. (2010), where it was demonstrated that the administration of eCG caused growth and development of the small follicles. In dairy cattle, the eCG treatment resulted in a lower incidence of atresic follicles and recruitment of smaller follicles (≤ 5 mm) with an increase in the growth rate, as well as a constant growth of medium follicles (6–8 mm) and large (≥ 9 mm) (Pacala et al. 2010). As for the quality of the COC, when there was a low number of grade I and II oocytes, an increase in the number of denuded oocytes was observed for the three treatments. This may have been due to inadequate oocyte aspiration, because constant aspiration pressure ranging from 50 to 100 mmHg is required, leading to a loss of cumulus cells in 5% of total oocytes recovered (Rodriguez et al. 2006). It is unknown if these oocytes are randomly stripped by the force of the aspiration system or because they originated from follicles already on the path of atresia that weakens cellular connections (Souza-Fabjan et al. 2016). The greater number of grade III structures (COC) obtained with both eCG treatments may be due to the FSH activity of the eCG, which triggers the proliferation of cumulus cells in the early preantral phase, preventing them from atresia, and induces the synthesis of receptors of LH as well as the expression of steroid hormones (Richards et al. 2002).

Table 4 Effect of breed and eCG on the percentage of embryos produced in vitro in young goats during the breeding season

	Breed				Dose of eCG (IU)			
	Alpine	Nubian	Saanen	*P* value	T1 = control	T2 = 500	T3 = 1000	*P* value
No. of goats	12	10	9		11	10	10	
Oocytes IVM/total oocytes recovered (%)	38/44 (86.4)	25/29 (86.2)	24/26 (92.3)	0.514	25/29 (86.2)	35/40 (87.5)	27/29 (93.1)	0.985
Cleavage/oocytes MIV (%)	15/38 (39.5)	11/25 (44.0)	16/24 (66.7)	0.204	17/25 (68.0)	13/35 (37.1)	12/27 (44.4)	0.906
Total production 7 days post IVF/cleavage (%)	12/15 (80.0)[a]	6/11 (54.5)[b]	1/16 (6.3)[c]	0.003	4/17 (23.5)[c]	9/13 (69.2)[a]	6/12 (50.0)[b]	0.004
Morule/total production (%)	4/12 (33.3)	3/6 (50.0)	0/1 (0)	0.499	1/4 (25.0)[b]	2/9 (22.2)[b]	4/6 (66.6)[a]	0.030
Initial blastocyst/total production (%)	4/12 (33.3)	2/6 (33.3)	1/1 (100.0)	0.620	3/4 (75.0)	3/9 (33.3	1/6 (16.7)	0.721
Blastocyst/total production (%)	4/12 (33.3)	1/6 (16.7)	0/1 (0)	0.421	0/4 (0)	4/9 (44.4)	1/6 (16.7)	0.204

[a, b, c] Means within a row with different superscripts are statistically different ($P < 0.05$)

From a functional point of view, the quality of the oocyte can coincide with its degree of development, since the communication that exists between the oocyte and the cells of the conglomerate is made through an extensive network of unions and other mechanisms of cellular communication which small bi-directional molecules such as micro RNA and messenger RNA transfer (Tanghe et al. 2002; Sasseville et al. 2009). This is an important factor to consider when obtaining high quality of COC capable of withstanding maturation, fertilization, and embryonic development (Lihua et al. 2010; De Souza-Fabjan et al. 2016). The interaction between the oocyte and its surrounding cumulus cells is critical for the maintenance of oocyte developmental competence (Tanghe et al. 2002; Avelar et al. 2012). It is mentioned that the presence of cumulus cells during in vitro maturation is essential to improve the rate of blastocysts (Anguita et al. 2009). This work may have affected the quantity and quality of embryonic structures obtained. Regarding the effect of eCG on embryo production, with a higher percentage of embryos 7 days post IVF in the treatment of 500 IU, it can also be suggested that the gonadotropins act in various stages of development, including the final maturation of the oocyte and follicular steroidogenesis (De Rensis and Gatius 2014). The result of the previous statement has a profound effect on the oocyte, the early embryo, and their respective microenvironments (Paramio and Izquierdo 2016). Therefore, ovarian overstimulation in goats can be used to increase the number of oocytes available to produce embryos in vitro, as they achieve adequate cellular maturation both nuclear and cytoplasmic (Malhi et al. 2008). It is concluded that (i) the quantity and size of follicles in the ovary were affected by the application of eCG and (ii) the total production 7 days post IVF was higher for goats that received 500 IU of eCG with a differential response in the goat breeds considered in this study.

Acknowledgments The authors thank CONACYT for the support of scholarship granted to the first author within the program of Masters in Animal Science Agronomy-Veterinary Graduate of the UANL, also for the support program for scientific and technological research, PAICYT-UANL-CT249-15, and for the financing of the project, and to Dr. Juan Francisco Villarreal Arredondo, for his infinite support to carry out this project. The authors are therefore very grateful to Prof. Dr. Nicolas Lopez-Villalobos for his substantial input in providing an English text version of the present manuscript.

Compliance with ethical standards

This study was approved by the Bioethics and Animal Welfare Committee of the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Autónoma de Nuevo León (Act. No. 18).

Conflict of interest The authors declare that they have no conflict of interest.

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