Livestock Science ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Short communication

Repeated hormonal treatment and laparoscopic ovum pick-up followed by in *vitro* embryo production in goats raised in the tropics

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ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form
18 March 2014
Accepted 8 April 2014

Keywords:
Goat
Hormonal treatment
Oocyte
Embryo
In vitro

ABSTRACT

The objectives of this study were to evaluate the ovarian response of oocyte donor goats which underwent seven repeated ovulation hormonal treatments and laparoscopic ovum pick-up (Experiment 1), and to compare the *In vitro* production (IVP) of embryos from goats hormonally treated for eighth time to IVP of embryos from females with a single hormonal treatment (Experiment 2). In Experiment 1, 12 goats were subjected to 7 repeated hormonal treatments and laparoscopic ovum pick-up (LOPU) every 2–3 weeks, and the following variables were recorded: number and size of punctured follicles, number of recovered *cumulus*-oocyte complexes (COCs), recovery rate and COCs quality. In Experiment 2, IVP of embryos from goats hormonally treated for eighth time (repeated-treated group, RT) was compared with IVP of embryos from 10 other goats with only one hormonal treatment (single-treated group, ST). In this step, in addition to variables observed in Experiment 1, cleavage, blastocyst rates at Days 7 and 8 of culture, hatched rate and the number of blastomeres per embryo were also evaluated. In Experiment 1, when comparing LOPU sessions, no difference ($P > 0.05$) was verified for number of punctured follicles, number of recovered COCs and recovery rate. However the percentage of large follicles was different ($P < 0.05$) between LOPU 1 (27.7 ± 10.5) and 7 (12.4 ± 12.1), the latter being similar to sessions 2 (13.9 ± 10.9), 3 (13.4 ± 7.9), 4 (13.2 ± 10.7), 5 (22.4 ± 14.7) and 6 (17.7 ± 15.8). The percentage of COCs suitable for IVM was lower ($P < 0.05$) for LOPU 1 (80.6%), 2 (82.4%) and 3 (79.7%) than LOPU 6 (91.3%) and 7 (95.2%). In Experiment 2, total number of follicles aspirated/goat and total number of COCs recovered were similar ($P > 0.05$) for RT (18.0 ± 5.8 and 12.3 ± 3.7, respectively) and ST (19.4 ± 7.8 and 15.8 ± 7.7, respectively) groups. However, the recovery rate was lower ($P < 0.05$) in the RT *vs* the ST group (68.5% and 81.4%). The percentage of small, medium and large follicles was similar ($P > 0.05$), respectively, for RT (28%, 55% and 17%) and ST (20%, 65% and 15%) groups. The percentage of COCs suitable for IVM had no difference ($P > 0.05$) for RT (96.6%) and ST (93.7%) groups. No significant difference was observed between RT and ST groups for: cleavage rate (68.6% *vs* 67.0%), blastocyst rate at Days 7 (27.9% *vs* 31.8%) and 8 (27.9% *vs* 34.1%), hatched rate (33.3% *vs* 53.3%), and blastomeres/embryo (252.9 *vs* 229.8). In conclusion, in goats raised in tropical climate, the IVP of embryos from oocytes obtained by LOPU could be an efficient and suitable method for rapid propagation of genetically

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http://dx.doi.org/10.1016/j.livsci.2014.04.012
1871-1413/

superior animals. Donor goats, even after repeated hormonal treatments followed by LOPU, maintained the ovarian response for oocyte and embryo production, becoming close to reality the term "oocyte permanent donors".

1. Introduction

Goats occupy a very important niche in regions of the world where environmental resources and infrastructure are scarce. In the Northeast Brazil, where 93% of the goat population is concentrated, the flocks still have low productivity rates (Sousa et al., 2011). In order to obtain genetic improvement of these herds, it is important to use reproductive biotechnologies, such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and *In vitro* production of embryos (IVP). The great advantage of IVP is the use of both sex gametes without the inconveniences observed in MOET, as for example: low ovulation rate, premature regression of corpora lutea and low fertilization rate. However, after transfer to synchronized recipients, the survival rate of fresh IVP goat embryos is significantly lower than their *in vivo* counterparts (47% *vs* 71%), probably due to the better oocyte developmental competence obtained during *in vivo* maturation (Cognié et al., 2003).

To perform IVP successfully it is necessary to obtain a large number of good quality oocytes that can be achieved by laparoscopic ovum pick-up (LOPU) technique. Thus, donors must be stimulated by gonadotrophins (Avelar et al., 2012). However, to make true the term "oocyte permanent donors", it is essential to have a uniform ovarian response, even after successive hormonal treatments/LOPU. The studies in goats, raised in temperate climate, showed that females have no decrease in oocyte production after several hormonal treatments/LOPU (Pierson et al., 2004). However, it is known that the reproductive activity as well as the ovarian response may be influenced by climatic conditions (Hansen, 2009).

This study aimed to verify, under hot weather conditions, the ovarian response of oocyte donor goats, which underwent successive treatments followed by LOPU, and to compare embryo IVP in goats treated several times to females with a single hormonal treatment.

2. Materials and methods

2.1. Local and climate characterization

The experiment was conducted at the State University of Ceará, located in Fortaleza, Brazil (3°47′38″S, 38°33′29″W). According to Koppen's classification, the climate is Aw, which is characterized by low rainfall and high temperatures. The data of temperature (T) and relative humidity (RH) were daily recorded. The thermal discomfort was assessed by the T and humidity index (THI) and calculated according to the formula defined by Rocha et al. (2008) as: $THI = 0.8 \times T + (RH/100) \times (T - 14.4) + 46.4$. A THI of 72 and below is considered as no heat stress, 73–77 as mild heat stress, 78–89 as moderate and above 90 as severe (Fuquay, 1981).

2.2. Experimental animals

A total of 22 cyclic crossbred Anglo-nubian goats with a mean ($\pm$SD) live weight of 39.2 ± 6.2 kg were used as oocyte donors. Animals were maintained in a semi-intensive system, receiving Tifton (*Cynodon dactylon*) hay *ad libitum* and were subjected to 4 h of daily access to the pasture of this grass. Females were supplemented with concentrate (20% crude protein) and had free access to water and mineralized salt licks.

2.3. Experimental design

In Experiment 1, 12 goats were subjected to seven successive ovarian hormonal treatments followed by LOPU, within two or three weeks-interval. The number and size of punctured follicles, number of recovered *cumulus*–oocyte complexes (COCs), recovery rate and COCs quality were evaluated. In Experiment 2, the same goats of Experiment 1 were subjected to an eighth session three weeks after the seventh session (repeated-treated group, RT) and 10 other goats with a single hormonal treatment (single-treated group, ST) were used. In this step, in addition to variables evaluated in Experiment 1, it was also evaluated: cleavage, blastocyst rate at Days 7 and 8 of culture and hatched rate and the number of blastomeres/embryo.

The IVP of embryos was performed in all 22 goats (RT = 12 and ST = 10) allocated according to the treatment (RT = 4 and ST = 3 or 4 per batch) in three different batches due to logistic (one third of each group at each batch), although one of them was not completed due to problems during oocyte maturation. Therefore, IVF results are presented only for 15 goats in two different batches (RT = 8 and ST = 7).

2.4. Hormonal treatment for ovarian stimulation

Estrous cycles of the 22 experimental goats were synchronized using intravaginal sponges (Progespon, Syntex, Buenos Aires, Argentina) of 60 mg medroxyprogesterone acetate for 10 days (Day 0 = day of sponge insertion). On Day 7, 75-μg D-cloprostenol (Prolise, ARSA S.R.L., Buenos Aires, Argentina) was given and ovarian stimulation started with 180 mg pFSH (Folltropin-V, Vetrepharm, Ontario, Canada) distributed in five injections (40/40, 35/35 and 30 mg), 12 h apart, starting in the morning of prostaglandin application. Thus, the interval from the last FSH administration to LOPU was 24 h. The goats were subjected eight times (RT group) or one time (ST group) to

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hormonal treatment and LOPU procedure every two weeks up to the fourth and every three weeks from the fourth to the last session.

2.5. Laparoscopic ovum pick-up (LOPU)

LOPU started just after sponge removal in the morning of Day 10. Briefly, goats were deprived for 36 h of food and for 24 h of water prior to LOPU. Anesthesia was induced with 2.5% thiopental (Tiopentax, Cristália, São Paulo, Brazil) animal/dose and maintained with 3% isoflurane (Isoforine, Cristália). Follicles of 2–7 mm were punctured using a system comprised by a 5-mm Hopkins laparoscope (Karl Storz, Tuttlingen, Germany) associated to a 22-G needle and a vacuum pump (WTA, Cravinhos, Brazil) regulated to 35 mmHg. The collection medium was HEPES buffered TCM 199 (Nutricell, Campinas, Brazil) supplemented with 20 IU/mL heparin (Hepamax-S, Blausiegel, São Paulo, Brazil) and 40 μg/mL gentamicin sulfate (Sigma-Aldrich, St. Louis, MO, USA). The follicles were classified as small (<3 mm), medium (3–4 mm) or large (>4 mm). After LOPU, each ovary was flushed with 100 mL of heparinized saline solution.

2.6. Assessment of COCs quality and In vitro maturation (IVM)

Except otherwise indicated, chemicals were purchased from Sigma-Aldrich. COCs were graded from GI to GIV as earlier defined (Avelar et al., 2012). In Experiment 2, the selected COCs (GI–GIII) were washed four times and then transferred to four well Petri dishes containing maturation medium consisting of HEPES-buffered TCM 199 supplemented with 10% (v/v) fetal calf serum (FCS; Gibco-Invitrogen, Carlsbad, CA, USA), 10 ng/mL Epidermal Growth Factor (EGF), 100 μM cysteamine, 20 μg/mL FSH/LH (Pluset, Hertape-Calier Barcelona, Spain), 1 μg/mL estradiol-17β, 40 μg/mL gentamicin sulfate and 10% (v/v) estrus goat serum (EGS). In each well, 40–50 oocytes were placed in 500 μL of maturation medium and COCs were incubated for 22–24 h at 38.5 °C in humidified atmosphere of 5% CO_2 in air.

2.7. In vitro fertilization (IVF) and In vitro culture (IVC)

Frozen/thawed semen was used throughout the experiment. Mobile sperm was separated by centrifugation (700*g* for 15 min) on Percoll gradient (45/90%). After, the supernatant was discarded and the pellet was overlaid with 600 μL of HEPES-buffered SOF and 40 μg/mL gentamicin. Sperm was centrifuged at 200*g* for 5 min and supernatant was discarded. The final concentration was adjusted to 2×10^6 spermatozoa/mL in IVF medium, which consisted in SOF medium, supplemented with 5 μg/mL heparin (Calbiochem, Irvine Sci., Santa Ana, CA, USA), 40 μg/mL gentamicin and 10% EGS (IVF medium). Groups of 45–50 oocytes were transferred into four well dishes, containing 450 μL of IVF medium and 50 μL of sperm suspension were added to each well. Sperm and oocytes were co-incubated for 20 h at 38.5 °C in humidified atmosphere of 5% CO_2 in air. After IVF, *cumulus* cells were removed by pipetting several times. The putative zygotes were washed four times in IVC medium (SOF supplemented with 3 mg/mL BSA) and transferred in groups of 25 to four well dishes containing 25 μL drops of IVC medium, added with 10% FCS 48 hpi, covered with mineral oil. The putative zygotes were incubated for 8 days at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 (Souza et al., 2013). Hatching rate was evaluated on Day 8 of culture. In order to determine the number of blastomeres, embryos that reached the blastocyst stage on Day 8 were stained with Hoechst 33342 and observed under inverted fluorescence microscopy (TE2000, Nikon, Kawasaki, Japan).

2.8. Statistical analysis

All data were expressed as the mean ($\pm$ SD) or percentage. It was used one-way ANOVA followed by Tukey's post-test (aspirated follicles and recovered oocytes), unpaired *t* test (blastomeres/embryo) or chi-squared test (recovery and embryo rate). A value of $P<0.05$ was considered to be statistically significant. All analyses were performed using Graph Pad 3.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

The mean values for temperature and humidity were 27.5 ± 2.6 °C and 78.9 ± 8.5%, respectively. Thus, the calculated value for THI was 77.8.

Concerning Experiment 1, the ovarian response is presented in Fig. 1. During the seven sessions of LOPU, a total of 1556 follicles were punctured (18.5 ± 2.7/donor) and 1117 COCs were recovered (13.3 ± 1.8/donor). Thus, the total recovery rate was 71.8%. No difference ($P>0.05$) was found among LOPU sessions for number of punctured follicles, number of recovered COCs and recovery rate. No significant difference was observed in the number of small and medium follicles among the sessions. However, the percentage of large follicles was different ($P<0.05$) between LOPU 1 and 7 (Table 1). Concerning the percentage of COCs suitable for IVM, statistical differences ($P<0.05$) were found between LOPU 1, 2 and 3 *vs* LOPU 6 and 7 (Table 1). At the end of seventh LOPU, macroscopic evaluation of the reproductive tract identified only three donors (25%) with mild adhesions of the omentum to the abdominal wall.

In Experiment 2, no significant differences ($P>0.05$) were identified between both groups (RT *vs* ST) for the total number of punctured follicles, total recovered COCs and the percentage of COCs suitable to IVM (Table 2). However, the recovery rate was lower ($P<0.05$) in the RT compared to ST group (68.5% *vs* 81.4%). The percentage of small, medium and large follicles was similar ($P>0.05$), respectively, for RT (28%, 55% and 17%) and ST (20%, 65% and 15%) groups.

The results of IVP in RT *vs* ST group are listed in Table 3. No significant difference ($P>0.05$) was observed between the experimental groups for: cleavage rate, blastocyst rates (at Days 7 and 8), hatched rate as well as number of blastomeres per embryo.

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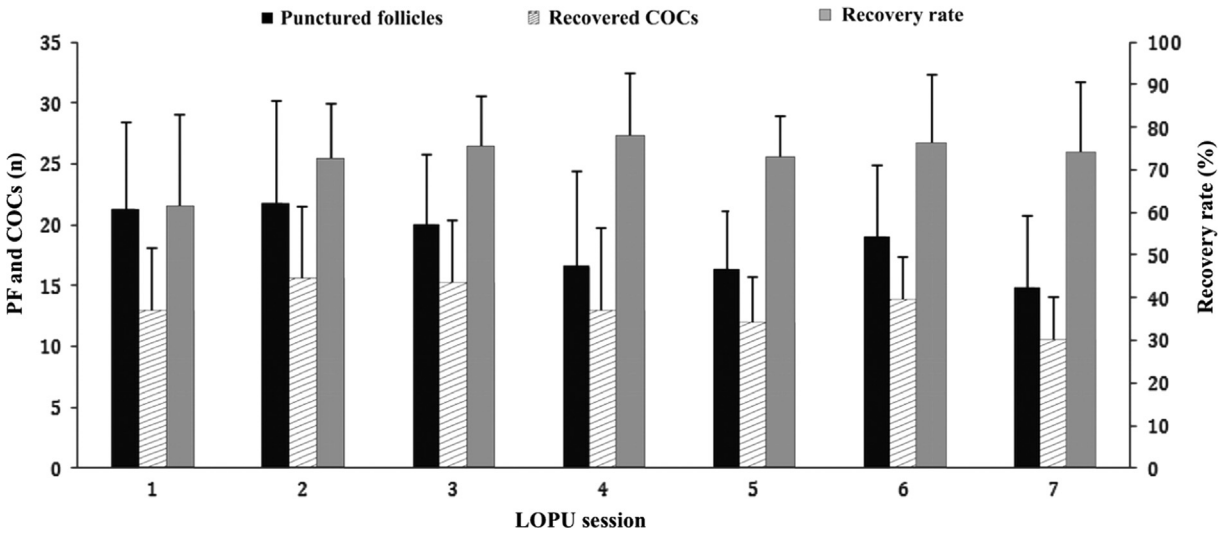

Fig. 1. Number of punctured follicles (PF), recovered *cumulus*–oocyte complexes (COCs) and recovery rate in goats raised in the tropics and subjected to seven sessions of hormonal treatment/laparoscopic ovum pick up (LOPU). No significant difference was found among sessions ($P > 0.05$).

Table 1
Punctured follicles, follicle size, recovered *cumulus*–oocyte complexes (COCs), recovery rate and suitable COCs for IVF from goats hormonally treated for seven times/sessions, raised in the tropics. Mean (± SD) and percentages.

Session	Punctured follicles mean (*n*)	Follicle size (%)			Recovered COCs mean (*n*)	Recovery rate	Suitable COCs rate (%)
		Small	Medium	Large			
1	21.3 ± 7.1 (255)	25.0 ± 11.0	47.3 ± 10.0	27.7 ± 10.5[a]	12.9 ± 5.1 (155)	60.8 ± 21.6	80.6 ± 5.5[a]
2	21.8 ± 8.3 (261)	32.8 ± 13.5	53.3 ± 11.7	13.9 ± 10.9[ab]	15.6 ± 5.9 (187)	71.6 ± 12.7	82.4 ± 3.4[a]
3	20.0 ± 5.7 (240)	32.0 ± 11.4	54.6 ± 10.5	13.4 ± 7.9[ab]	15.2 ± 5.1 (182)	75.8 ± 11.7	79.7 ± 4.4[a]
4	16.5 ± 7.9 (198)	38.4 ± 16.9	48.4 ± 17.2	13.2 ± 10.7[ab]	12.9 ± 6.8 (156)	78.8 ± 14.4	87.2 ± 4.3[ab]
5	16.3 ± 4.8 (195)	33.3 ± 15.4	44.3 ± 18.5	22.4 ± 14.7[ab]	11.9 ± 3.8 (143)	73.3 ± 9.6	85.3 ± 5.0[ab]
6	19.0 ± 5.9 (228)	22.0 ± 9.6	60.3 ± 14.8	17.7 ± 15.8[ab]	13.9 ± 3.4 (167)	73.2 ± 16.0	95.2 ± 2.3[b]
7	14.9 ± 5.8 (179)	34.7 ± 21.8	52.9 ± 23.5	12.4 ± 12.1[b]	10.6 ± 3.4 (127)	70.9 ± 16.4	91.3 ± 3.7[b]

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

Table 2
Punctured follicles, recovered *cumulus*–oocyte complexes (COCs), recovery rate and suitable COCs for IVF from goats hormonally treated for eighth time (repeated-treated group, RT) compared with goats with only one hormonal treatment (single-treated group, ST), raised in the tropics. Mean (± SD) and percentages.

Group	*n*	Punctured follicles mean (*n*)	Recovered COCs mean (*n*)	Recovery rate (%)	Suitable COCs rate[1] (*n*)
RT	12	18.0 ± 5.8[a] (216)	12.3 ± 3.7[a] (148)	68.5[a]	96.6[a] (143)
ST	10	19.4 ± 7.8[a] (194)	15.8 ± 7.7[a] (158)	81.4[b]	93.7[a] (148)

[a,b]Means within a row with different superscripts are different ($P < 0.05$).
[1] COCs that were subjected to IVM (grades I–III).

Table 3
In vitro embryo production from goats hormonally treated for eighth time (repeated-treated group, RT) compared with goats with only one hormonal treatment (single-treated group, ST), raised in the tropics.

Group	Oocytes (*n*)	Cleavage rate (*n*)	Blastocyst rate at Day 7 (*n*)	Blastocyst rate at Day 8 (*n*)	Hatched rate[a]	Number of blastomeres per embryo
RT	86	68.6 (59)	27.9 (24)	27.9 (24)	33.3	252.9 ± 39.6
ST	88	67.0 (59)	31.8 (28)	34.1 (30)	53.3	229.8 ± 123.6

No significant difference was found between groups ($P > 0.05$).
[a] (Hatched blastocysts at Day 8/total blastocysts at Day 8) × 100.

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4. Discussion

In the present study, the THI confirmed that experimental animals were under mild to moderate heat stress. It is well known that under heat stress, an integrated cascade of physiological responses is set in motion, which helps ruminants to maintain homeostasis and physiological equilibrium (Farooq et al., 2010). However, the physiological mechanism used by animals in this experiment would have been sufficient to reduce, or even eliminate, the effects of heat stress on the ovarian response to hormonal treatments; since the response was similar to that observed in animals raised in temperate and cold regions (Pierson et al., 2004).

Regarding Experiment 1, the number of punctured follicles, recovered COCs and recovery rate were similar for LOPU sessions 1–7. These values corroborated those reported in goats in temperate (Baldassarre et al., 2002) and tropical climate (Avelar et al., 2012). COCs recovery rate presented low variability and 71.8% observed was close to the results of other authors working with goats (Baldassarre et al., 2002; Pierson et al., 2004; Avelar et al., 2012). It is reasonable to assume that repeated LOPU up to seven times does not adversely affect female response, and thus the goats were able to maintain the number of follicles and recovered COCs throughout the seven sessions. Interestingly, the interval between ovarian stimulation and oocyte recovery can be reduced to periods as short as 4 days, without affecting follicular development and oocyte quality (Gibbons et al., 2007). Moreover, intervals from 5 to 14 weeks promoted no differences on the number of follicles aspirated and oocytes recovered (Pierson et al., 2004).

A decrease was observed in the percentage of large follicles when comparing the first to the seventh LOPU. However, the recovery rate was not affected as previously demonstrated in goats by Gibbons et al. (2007), providing a similar recovered COCs mean within all seven sessions. As expected, the percentage of small and medium follicles was not different throughout the seven sessions, since the goats received the same hormonal treatment before LOPU and overall presented no significant differences in the variables evaluated. The percentage of COCs suitable for IVM was lower in sessions 1, 2 and 3 in comparison with sessions 6 and 7. In fact, we have no plausible explanation for this occurrence. Perhaps this was a consequence of an overall improvement in the aspiration system.

Although occasional adhesions of the omentum to the abdominal wall were observed after LOPU, no moderate or severe lesions that could adversely affect further LOPU were verified. The number of punctured follicles, recovered COCs, follicle size and COCs suitable for IVM did not differ between RT and ST groups and all these parameters were similar to reports in the literature, demonstrating that RT group was able to preserve their response to several hormonal treatments. However, a lower recovery rate was obtained in the RT group (68.5%) in comparison with the ST group (81.4%). Thus, punctures may have potential detrimental effects on the ovaries, as a result of multiple repeated penetrations by the collecting needle.

The competence to development was similar between embryos produced from RT and ST groups. The developmental potential of embryos in terms of blastocyst yields depends on its intrinsic quality but also on the conditions to which they are subjected (Rizos et al., 2002). The cleavage rate was similar between RT and ST groups, reaching a mean rate of ~68%, similar to earlier reports in goats (Souza-Fabjan et al., 2014). Recent results have demonstrated that a good blastocyst rate can be achieved from oocytes derived from abattoir (38–54%; Souza et al., 2013) or in live animals recovered by LOPU (34%; Leoni et al., 2009), similar to 28–34% obtained in the present study. Although not significantly different, hatched rate of ST was 20% higher than RT, suggesting a possible effect on embryo developmental kinetics.

Embryo quality was evaluated on the basis of the number of cells in blastocysts at Day 8, which were similar for the RT (253 cells) and ST (230 cells) groups. This methodology has been widely used as a reliable indicator of embryo quality in farm animals (Farin et al., 1995). The average obtained was similar to 109–270 (Baril et al., 1996) and slightly higher than 187 cells, all reports in goats (Souza-Fabjan et al., 2014). These data indicate that it is possible to produce high quality goat embryos with similar results from RT or ST females.

Repeated oocyte recovery followed by IVM, IVF and IVC has the potential for producing more offspring from genetically valuable goats than traditional MOET procedures (Baldassarre et al., 2002). Thanks to its less invasive nature, the procedure can be repeated more times than laparotomy, normally used for embryo recovery in goats. In addition, it is an interesting approach to overcome problems, such as variability of the superovulatory response, the poor fertilization associated with high ovulatory responses, and early regression of corpora lutea, verified in more than 30% of donors subjected to MOET (Cognié et al., 2003). However, the costs and inefficiencies of the IVP system might restrict its use to special situations.

Indeed, literature comparing MOET and IVP embryos in the same study on goats is scarce. A decade ago it was demonstrated that after transfer to synchronized recipients, the survival rate of fresh IVP goat embryos was significantly lower than their *in vivo* counterparts (47% *vs* 71%), probably due to the better oocyte developmental competence obtained during *in vivo* maturation (Cognié et al., 2003).

In conclusion, in goats raised in tropical climate, embryo IVP from oocytes obtained by LOPU could be an efficient and suitable method for rapid propagation of genetically superior animals. It is noteworthy that goats maintained the quanti–qualitative ovarian response to repeated hormonal treatments followed by LOPU, even under heat stress, becoming close to reality the term “permanent oocyte donors”.

Conflict of interest

There is no conflict of interest.

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Acknowledgments

The authors are grateful to CNPq (Grant No. 304621) (Brasília, Brazil) and FUNCAP (Grant No. 2161900/11) (Fortaleza, Brazil) for the financial support.

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Please cite this article as: Sanchez, D.J.D., et al., Repeated hormonal treatment and laparoscopic ovum pick-up followed by in *vitro* embryo production in goats raised in the tropics. Livestock Science (2014), http://dx.doi.org/10.1016/j.livsci.2014.04.012