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Goat incubator: can bovine oocytes be matured in the uterine horn of a goat?

Cabra incubadora: os oócitos bovinos podem ser maturados no corno uterino de uma cabra?

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Abstract

We used a goat as a live incubator, along with associated nonsurgical embryo transfer techniques, to perform *ex situ* (*in vivo*) maturation of bovine oocytes. Immature bovine *cumulus*-oocyte complexes (COCs) aspirated from 3-8 mm follicles from slaughterhouse ovaries were randomly split into two groups for *in vitro* (IVM; n = 38) and *ex situ* maturation (ESM; n = 40). IVM was performed for a period of 24 h at 38.5 ºC and with 5% CO_2 in the air of maximum humidity. For ESM, a pre-synchronized nulliparous goat (12 months old) received 40 immature COCs in the uterine horn apiece, via the transcervical route. After 24 h the structures were retrieved through uterine flushing. Analyses of nuclear maturation and lipid quantification were performed on oocytes from both groups. Fluorescent intensity was compared using the Student's t-test. Forty-seven percent of the structures were recovered after uterine flushing (19/40). The nuclear maturation rate was 94.5% (18/19) and 81.6% (31/38) for the ESM and IVM groups, respectively. *In vitro*-matured COCs contained more lipid droplets, expressed as a higher amount ($p < 0.05$) of emitted fluorescent light than *ex situ*-matured COCs (858 ± 73 *vs.* 550 ± 64 arbitrary fluorescence units, respectively). This is the first report to associate nonsurgical embryo transfer techniques and a goat as a live incubator for the maturation of bovine oocytes. We conclude that bovine oocytes can progress meiotically in the uterus horn of a goat and that transcervical transfer of bovine oocytes to a goat's uterus could present an alternative to nuclear maturation.
Key words: COCs. Bovine oocytes. In vitro maturation. Ex-situ maturation. Nonsurgical embryo transfer technique. Goat.

Resumo

Utilizamos uma cabra como incubadora viva, juntamente com técnicas de transferência de embriões não cirúrgicas associadas, para realizar a maturação ex situ (in vivo) de oócitos bovinos. Os complexos cumulus-oócitos bovinos (COCs), aspirados de folículos de 3-8 mm de ovários de abatedouro foram aleatoriamente divididos em dois grupos para maturação in vitro (IVM; n = 38) e ex situ (ESM; n =

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40). A MIV foi realizada por um período de 24 h no meio TCM-199, a 38,5 ºC, e com 5% de CO_2 em umidade máxima. Para o ESM, uma cabra nulípara pré-sincronizada (12 meses de idade) recebeu 40 COCs imaturos no ápice do corno uterino, por via transcervical. Após 24 h as estruturas foram recuperadas através de lavagem uterina. Análises de maturação nuclear e quantificação de lipídios foram realizadas em oócitos de ambos os grupos. A intensidade de fluorescente foi comparada usando o teste t de Student. Quarenta e sete por cento das estruturas foram recuperadas após lavagem uterina (19/40). A taxa de maturação nuclear foi de 94,5% (18/19) e 81,6% (31/38) para os grupos ESM e IVM, respectivamente. Os COCs maturados in vitro continham mais gotículas lipídicas, expressos como uma quantidade maior ($p < 0,05$) da luz fluorescente emitida do que os COCs ex situ (858 ± 73 vs 550 ± 64 unidades de fluorescência arbitrárias, respectivamente). Este é o primeiro relatório a associar técnicas de transferência de embriões não cirúrgicas e uma cabra como incubadora viva, para a maturação de oócitos bovinos. Conclui-se que os oócitos bovinos podem progredir meioticamente no corno uterino de uma cabra, e que a transferência transcervical de oócitos bovinos para o útero de cabra poderia ser uma alternativa à maturação nuclear, visando à redução de lipídios.
Palavras-chave: COCs. Oócitos bovino. Maturação in vitro. Maturação ex-situ. Técnica de transferência de embriões não cirúrgicos. Cabra.

Introduction

Brazil is the largest producer of *in vitro*-generated bovine embryos, producing more than 350,000 embryos per year (VIANA et al., 2017). Despite the continental location of the country, because of the available road-transport and flight connections, *in vitro* embryo production (IVEP) laboratories are located in Centre-South states. Usually, the oocytes are collected from donor cows and sent by road or plane to labs that are often located at great distances from the farm. Embryos are then sent back to be transferred to recipient cows. These logistics increase the total cost of the IVEP process, as well as the risk of reduced pregnancy rates.

IVEP includes three major steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* development (IVD) of the resulting embryos. Evaluating the effect of different stages of IVEP in the production and quality of blastocysts, Rizos et al. (2002) demonstrated that the intrinsic quality of the oocyte is the main factor affecting blastocyst yields, while the conditions of embryo culture have a crucial role in determining blastocyst quality. During IVM, the oocyte acquires its final developmental competence, impacting further embryo development. Under suboptimal conditions of maturation, changes in oocyte metabolism occur that may decrease success in subsequent steps necessary for the formation of blastocyst. Normally, these metabolic changes lead to higher lipid content that reduces the cryotolerance and cryosurvival of *in vitro*-matured oocytes and *vitro*-produced embryos (PRATES et al., 2014).

An alternative option to reduce the deleterious effects of the *in vitro* environment on oocytes and embryos involves using the reproductive tracts of other species. The oviduct and uterus of rabbits had been formally tested for bovine oocyte fertilization and embryo culture (FUKUI et al., 1983). *In vitro*-fertilized bovine embryos were also cultured *in vivo* in the oviduct of a ewe, which increased their cryosurvival due to a reduction in lipid content (ENRIGHT et al., 2000). However, the reported techniques did not support field applications. In the same way, the intra-follicular transfer of oocytes showed interesting results, but requires specialist skill to transfer oocytes into the follicular antrum (SPRÍCIGO et al., 2016). Thus, our group has proven the feasibility of nonsurgical embryo transfer techniques in small ruminants (FONSECA et al., 2014) and, in this study, we used this new technique to perform the maturation of bovine oocytes in a goat's uterus, whilst aiming to establish an efficient and practical method of *ex situ* maturation for bovine oocytes. This study is the first report to use a goat as a live incubator for the maturation of bovine oocytes.

Materials and Methods

Ethics and animal care

The design for this study was approved by the Animal Care Committee of the Universidade Federal Fluminense (UFF 678/15), and the experiment was conducted in accordance with the principles of the Sociedade Brasileira de Ciência em Animais de Laboratório.

Location and study conditions

The present study was conducted in April 2017, in Coronel Pacheco, Minas Gerais, in southeastern Brazil. The research unit is located at an altitude of 435 m and latitude and longitude of 21º35'S and 43º15'W, respectively. The average annual precipitation and temperature for the area are 1,581 mm^3 and 21 ºC, respectively.

Animal housing and estrus synchronization

A nulliparous ¾ Alpine/Boer goat was used. The animal was housed in a shelter and fed 3 kg of corn silage and a balanced concentrate supplement, according to its nutritional requirements. Water and mineral salt were also offered *ad libitum*. For estrus synchronization, two doses of 37.5 µg d-cloprostenol (Prolise®, Tecnopec LTDA, São Paulo, Brazil) were administered through the laterovulvar route, at 7-day intervals. Cloprostenol administrations were performed between 06:00 a.m. and 07:00 a.m., with estrus starting 48 h after the second cloprostenol dose.

Oocyte recovery, selection, and maturation

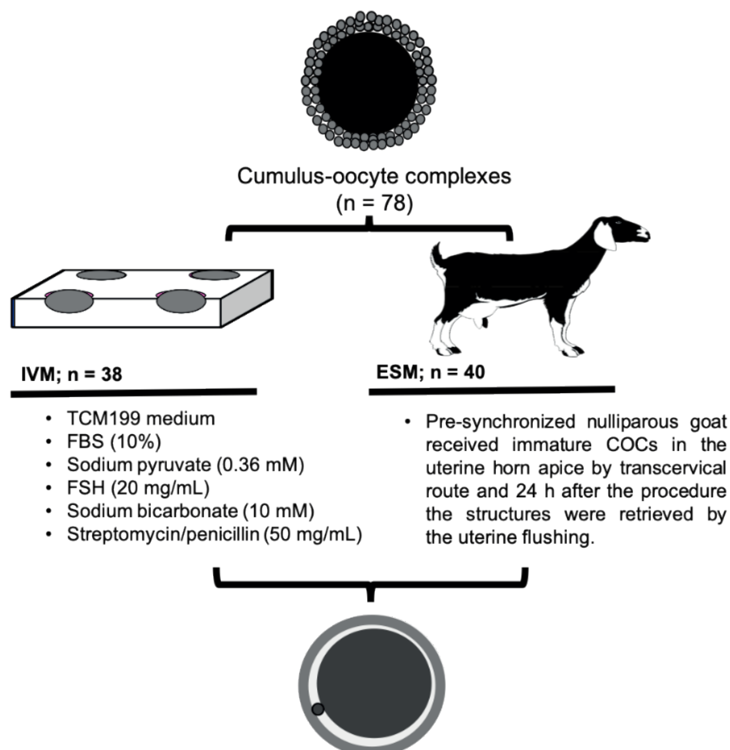
Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory in saline solution (0.9% NaCl with 0.1 g/L streptomycin) at 34-36°C. Follicles with 3-8 mm diameter were aspirated, with COCs with more than three compact layers of *cumulus* cells and oocytes with homogeneous cytoplasm randomly split between two groups for *in vitro* (IVM; n = 38) and *ex situ* (ESM; n = 40) maturation (Figure 1). For IVM, COCs were cultured for 24 h in four well plates containing 400 µL of tissue culture medium (TCM-199; Gibco Life Technologies, Inc., Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS), 20 µg/mL follicle stimulating hormone (FSH; Pluset, Calier, Barcelona, Spain), 0.36 mM sodium pyruvate (Sigma Chemical, St. Louis, MO, USA), and 10 mM sodium bicarbonate and 50 mg/mL streptomycin/penicillin (Sigma Chemical, St. Louis, MO, USA). These were cultured at 38.5 °C in 5% CO_2 and humidified air.

COCs transfer and recovery

Estrus was detected using a teaser buck. At estrus onset (24 h after the second cloprostenol administration), ovaries were checked for dominant/ovulatory follicle identification using transrectal ultrasonography scanning (Mindray Color-M5 VET™, Shenzhen, China), twice daily (two ovulations were detected at 60 h and confirmed at 72 h after the second dose of cloprostenol administration). The uterine horn ipsilateral to the ovulatory follicles was chosen to receive COCs at 84 h after the second cloprostenol dose. For ESM, a 5-mL syringe, with 1 mL of the described medium, and 2 mL of air, was coupled with a Tom Cat catheter. COCs were loaded into the middle column of three, separated by air bubbles in the catheter. The female goat was prepared to receive COCs, as described earlier. COCs were transferred (FONSECA et al., 2014) and recovered (FONSECA et al., 2013) through a transcervical route. Briefly, under sedation and anesthesia, the goat was kept in a standing position and contained within an appropriate box. Cervical immobilization and traction were achieved using Pozzi forceps (FONSECA et al., 2016). A number 8 urethral catheter, with mandrel, was humidified using PBS medium and inserted into the cervix. The cervical rings were gently transposed,

and after the loss of resistance, the urethral catheter was moved laterally to reach the desired uterine horn. The mandrel was removed, the syringe/Tom Cat device was attached to the urethral catheter, the COCs transferred to the uterine horn, and, finally, the urethral catheter removed. Oocyte recovery occurred 24 h after the transfer, and the procedure was identical to that which was previously reported (FONSECA et al., 2013). A total of 180 mL of PBS was used in each uterine horn.

Figure 1. The experimental design of Experiment 1. The effect of the maturation system (*in vitro* maturation - IVM, and *ex situ* maturation - ESM) on nuclear maturation and oocyte lipid accumulation. FBS (Fetal Bovine Serum).



Lipid quantification

For lipid quantification, 15 oocytes from each group were randomly selected. Quantification of lipids was performed using the Nile Red dye technique (LEROY et al., 2005). COCs were completely denuded in a PBS solution with 0.1% hyaluronidase and then fixed in a 500 uL 4% formaldehyde solution at 4 °C, for at least 24 h. These were then transferred to individual Eppendorf tubes (one oocyte/tube) containing 30 μL of a solution of 10 μg/mL Nile Red (Molecular Probes, Inc., Eugene, OR, USA) dissolved in saline solution (0.9% NaCl), with 1 mg/mL polyvinylpyrrolidone. Oocytes were stained overnight, in the dark, and at room temperature. The Nile Red stock solution (1 mg/mL) was prepared in advance with dimethyl sulfoxide and stored at room temperature in the dark. Final concentrations were achieved by diluting the stock with the saline solution. The amount of fluorescent light emitted by the oocyte on the slide was evaluated at 582 ± 6 nm, using an Olympus BX60 light microscope, equipped with an epifluorescence system and a 10 × objective. Images were captured separately using monochrome filters for FITC, with a cooled charge-coupled device camera (Magnafire; Olympus). Images were then imported into Adobe Photoshop (Adobe Systems, Mountain View, CA)

as TIFF files. The area of each oocyte was manually demarcated, and the mean fluorescence calculated using ImageJ software. The results were expressed as arbitrary units of fluorescence.

Nuclear maturation analysis

For maturation analysis, the same oocytes were stained with 10 µg/mL Hoechst 33342, dissolved in physiological saline (0.9% NaCl), with 1 mg/mL polyvinylpyrrolidone. To evaluate the meiosis nuclear stage and the presence or absence of the first polar body, slides were examined using epifluorescent microscopy. Oocytes displaying metaphase II plate were considered mature.

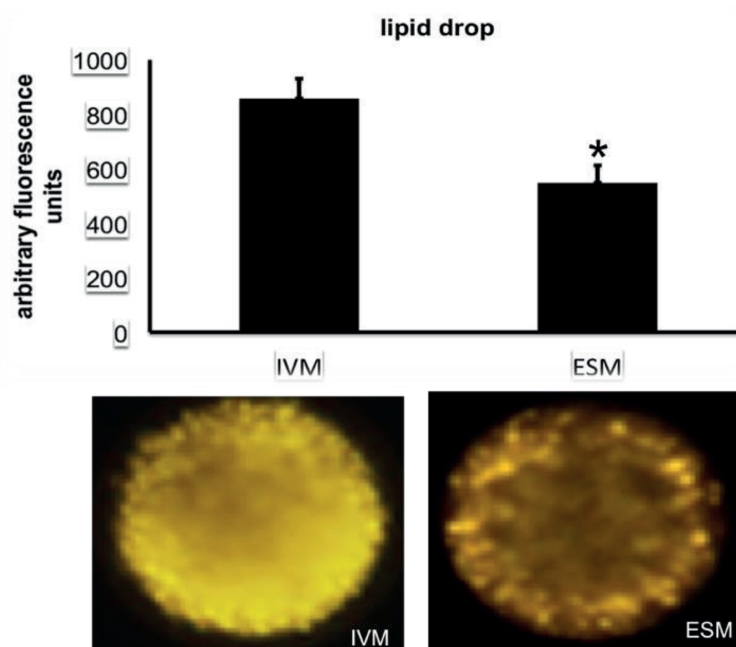
Statistics

Lipid quantity was inferred by the measurement of fluorescent intensity of individual oocytes using ImageJ program, with this intensity being compared using Student's t-test. Values are shown as means ± standard error means (S.E.M.), with a significance level of $P < 0.05$.

Results

Forty-seven percent of the structures were recovered after uterine flushing (19/40). All of those were in the same horn as they were originally transferred, while there were no oocytes in the contralateral horn. The cumulus cells expansion was similar in both groups. The nuclear maturation rate was 94.5% (18/19) and 81.6% (31/38) for ESM and IVM groups, respectively. Figure 2 shows the fluorescent intensity of IVM and ESM oocytes after Nile red staining. *In vitro*-matured oocytes contained more lipid droplets - expressed as a higher amount ($P < 0.05$) of emitted fluorescent light (858 ± 73 arbitrary fluorescence units) - than *ex situ*-matured oocytes (550 ± 64 arbitrary fluorescence units).

Figure 2. The effect of the maturation system on oocyte lipid accumulation. '*' represents a significant difference between the two groups ($P < 0.05$). Fluorescent intensity was compared using the Student's t-test. Provides a representative image of *in vitro*-maturation (IVM; n = 15) and *ex situ*-maturation (ESM; n = 15) oocytes, after Nile red staining.



Discussion

This is the first report to associate nonsurgical embryo transfer techniques with a goat as a live incubator for the maturation of bovine oocytes. Despite the average oocyte recovery rate and the implementation costs for the technique, the results of this strategy are promising. Although widely used, especially in Brazil, the suboptimal *in vitro* culture environment restricts the efficiency of IVEP. During IVP, more than 90% of the oocytes collected from follicles larger than 3 mm in cattle can complete nuclear maturation. However, few of them are competent for cytoplasmic maturation. Consequently, only 30 to 40% of the oocytes reach the blastocyst stage after IVF and IVD (SIRARD et al., 2006). Additionally, when compared to embryos produced *in vivo,* those that reached the blastocyst stage are of inferior quality in terms of cryoresistance, ultrastructure, and lipid content (FAIR et al., 2001).

The results of the present study demonstrate that the use of goats as incubators might offer a valid alternative for intracellular lipid reduction during oocyte maturation. Consequently, depending on the *in the vitro* culture system, this strategy offers an alternative for lipid reduction in embryos and cryotolerance modulation. High neutral lipid content, especially for triglycerides stored in the lipid droplets in the cells, has been suggested as a major cause for the low cryotolerance of *in vitro*-produced embryos (POLGE et al., 1974). Excessive endogenous neutral lipid accumulation affects the dehydration-rehydration equilibrium during embryo freezing and thawing (VAJTA et al., 1999), triggering ice crystal formation and exacerbating the deleterious effects of cryopreservation. It is important to highlight that we believe there are important advantages in the use of goat as a model, such as the low cost of maintaining a goat and its easy of handling compared to other productive species (e.g., cow). Moreover, the success of oocyte/embryo transfer and recovery in the goat species is well recognized (FONSECA et al., 2013, 2014, 2016).

We conclude that bovine oocytes might progress meiotically in a goat's uterus, resulting in relative low lipid content, and without compromising nuclear maturation. The transcervical transfer of bovine oocytes to a goat's uterus for *ex situ*-maturation could provide an alternative approach to increasing the viability of embryo produced *in vitro*. However, further studies are required to improve oocyte recovery rates and to fully evaluate fertilization and embryo development.

Conflict of Interest Statement

There are no conflicts of interest regarding authorship or publication of this article.

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