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Intrinsic quality of goat oocytes already found denuded at collection for *in vitro* embryo production

Joanna Maria Gonçalves Souza-Fabjan [a,b,*,1], Yann Locatelli [a,c], Nicolas Duffard [a,c], Emilie Corbin [a], Ribrio Ivan Tavares Pereira Batista [b], Vicente José de Figueirêdo Freitas [b], Jean-François Beckers [d], Pascal Mermillod [a]

[a] *INRA, UMR7247, Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, Haras Nationaux, Nouzilly, France*
[b] *Laboratory of Physiology and Control of Reproduction, Veterinary School, State University of Ceará, Fortaleza, CE, Brazil*
[c] *Museum National d'Histoire Naturelle, Réserve de la Haute Touche, Obterre, France*
[d] *Laboratory of Endocrinology, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium*

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ABSTRACT

Although cumulus cells are essential for efficient oocyte maturation, the establishment of protocols that support IVD of embryos obtained from denuded oocytes (DOCs) is important for optimizing the use of reproductive biotechnologies. Thus, this study aimed to establish a protocol for IVD of goat DOC using different strategies of IVM and methods of oocyte activation. Four experiments were performed. Similar developmental competence of slaughterhouse DOC was obtained, regardless of maturation media (complex, semi-defined or simplified). However, the ability to reach the blastocyst stage was affected by the activation method. Denuded oocytes subjected to parthenogenetic activation had greater ($P < 0.05$) development capacity, compared with those undergoing IVF with average cleavage rate of 83% and 75%, blastocyst rate of 49% and 28%, and blastocysts in relation to the cleaved embryos of 59% and 38, respectively. In addition, the quality of embryos evaluated after vitrification/warming was similar between parthenogenetic activation and IVF. Finally, we demonstrated that the coculture of cumulus-oocyte complex (COC) with DOC increased the competence of DOC at a ratio of 1:1 and 1:9 (DOC:COC). We believe that presence of cumulus cells (CCs) is not essential to the meiotic maturation, if at the time of removal of the oocyte from follicular environment, they already acquired competence to development. However, when the oocytes still need to acquire competence, the presence of CC may significantly contribute in their developmental capacity acquisition during IVM. Thus, regardless of the source, these oocytes will require longer time in IVM, contrary to what happens in the absence of CC. In conclusion, although DOC had a lower developmental potential, especially after IVF, they were able to produce blastocysts and the coculture of DOC with COC increased this developmental capacity.

1. Introduction

A high-oocyte quality is the prerequisite for *in vitro* embryo production. On a functional point of view, oocyte quality may coincide with its developmental competence. Sirard et al. [1] described five levels of oocyte competence,

* Corresponding author. Tel.: +55-21-2629-9526; fax: +55-21-2629-2433.
E-mail address: joannavet@gmail.com (J.M.G. Souza-Fabjan).
[1] Present address: Faculty of Veterinary Medicine, School of Health Sciences, Universidade do Grande Rio, Rua Prof. José de Souza Herdy, 1160, CEP 25071 to 202, Duque de Caxias, RJ, Brazil.

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that is, their ability: (1) to resume meiosis; (2) to cleave after fertilization; (3) to develop to the blastocyst stage; (4) to induce a pregnancy and bring it to term; and (5) to develop to term in good health. These abilities may be originated by separate events, and success in the first events does not ensure the success of the subsequent ones.

The interactions between the oocyte and cumulus cells (CCs) that occur uninterruptedly from the formation of the primordial follicle until ovulation play important roles in the acquisition of oocyte competence. The communication maintained between the oocyte and CC by means of an extensive network of gap junctions and other cell communication mechanisms [2], permits the two-way transfer of small molecules such as nutrients microRNA and messengers RNA [3–5]. This is extremely important for obtaining high-oocyte quality, capable of supporting maturation, fertilization, and embryo development. Some evidence shows that factors secreted by CC are very important in promoting oocyte maturation and acquisition of developmental competence. In addition, *in vivo* or *in vitro* induction of the maturation triggered by FSH, leads to the resumption of meiosis in oocytes and also to the expansion of CC [6]. Expanded cumulus matrix is composed of glycosaminoglycans such as hyaluronic acid [7], which are secreted by CC and that may contribute to the microenvironment essential to sperm capacitation and fertilization [8].

It was previously demonstrated in mice [9], rats [10], cattle [11], and pigs [12], that the removal of CC at the beginning of IVM or shortly before IVF is detrimental to fertilization and further embryo development, highlighting the importance of CC for oocyte quality. However, the current follicular aspiration systems lead to loss of CC in approximately 5% of the total oocytes retrieved. It is not known whether these oocytes are denuded randomly by the strength of the aspiration system or because they are originating from follicles already on the way of atresia which weakens the cell connections. A grade classification based on the number of cumulus layers is currently used by many laboratories. Oocytes that are found denuded at collection (grade 3) are considered not suitable and thus routinely discarded. Therefore, if a particular strategy could be applied to the use of denuded oocytes (DOCs) enhancing the overall production of high-quality embryos, it would certainly be a benefit to the industry. Moreover, the denudation of oocytes before IVM, or at least before IVF is necessary for a range of applications. These include germinal vesicle (GV) transfer [13], somatic cell haploidization, cryopreservation of GV stage oocytes [14], and pronuclear transfer [15]. Denudation is also required for the injection of interfering RNA or mRNA into the oocyte to knockout or overexpress specific genes to evaluate their role in IVM or IVF [16]. Despite this need, there are no established protocols for IVM of oocytes denuded at collection (DOC) in goat. Therefore, a method is needed to culture DOC in a system able to positively affect their developmental competence. In addition to this, the mechanisms by which CC improve oocyte maturation are yet poorly understood.

Numerous studies have examined the ability of mammalian oocytes and embryos to develop *in vitro* using a wide variety of culture media for IVM, IVF, and IVD [17,18]. The culture systems used for *in vitro* embryo production can be classified according to their formulation in (i) complex media, where serum and/or a somatic cells coculture is used; (ii) semidefined, without somatic cells and in which serum is replaced by purified proteins (albumin, hormones, growth factors,…); (iii) defined in which proteins are replaced by synthetic macromolecules, such as polyvinyl alcohol and polyvinylpyrrolidone [19]. In most of the studies, the basic media were supplemented with hormones and different concentration of serum (10%–20%). In all experiments, maturation media contained one of the following; bovine follicular fluid ([20]), estrous goat serum ([21,22]), estrous sheep serum ([22,23]), estrous cow serum ([24]), or fetal bovine serum ([22]). However, although media supplementation with serum exerts beneficial effects on oocyte maturation, it makes more difficult the interpretation of results, hampers their reproducibility, and introduces a sanitary risk. Thus, this study was designed to establish an *in vitro* protocol for the development of denuded goat oocytes using different strategies during IVM and different methods of oocyte activation.

2. Materials and methods

Except where otherwise indicated, chemicals were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). All the experiments were conducted at the Experimental Unit UEPAO in Nouzilly (France, latitude 47°22′N, longitude 00°41′E). The local ethic committee—Comité d′éthique Center Val de Loire—approved the procedures. Four experiments were performed to examine the possibility of using oocytes already found denuded (grade 3) at collection (DOC) to produce goat embryos *in vitro*.

2.1. Experimental design

2.1.1. Experiment 1

The objective of this experiment was to determine the effect of IVM medium composition on the DOC developmental competence. In total, 411 DOC obtained in four replicates from slaughterhouse ovaries were randomly distributed among three treatments for IVM: maturation medium consisted of TCM 199 supplemented either with (1) 10-ng/mL epidermal growth factor (EGF) and 100-μM cysteamine (EGF medium; simplified); (2) 10% fetal calf serum (FCS), 100-μM cysteamine, and 50-ng/mL ovine follicle stimulating hormone (FCS medium; complex), or (3) 10-ng/mL EGF, 5-UI/mL hCG, 10-UI/mL eCG, 19-ng/mL IGF-1, 2.2-ng/mL FGF, 5-μg/mL Insuline, 5-μg/mL transferrin, 5-ng/mL selenium, 90-μg/mL L-cystein, 0.1-mM β-mercaptoethanol, 75-μg/mL vitamin C, 720-μg/mL glycine, 0.1-mg/mL glutamine, and 110-μg/mL pyruvate, (MIX medium, semi defined). After the IVM, DOC underwent IVF and IVD as described in item 2.2.4, and 2.2.6, respectively. Embryos were examined morphologically and the efficiency of development was evaluated (i) as the percentage of cleaved embryos 2 days pi, and the percentage of blastocysts at 8 days pi, expressed (ii) on the basis of the number of oocytes entering into IVM (iii), or on the basis of the number of cleaved embryos at Day 2. On Day 8, all expanded blastocysts were

transferred into washing plates, washed to remove the mineral oil, fixed, and stained with Hoechst to count their total cell number [25].

2.1.2. Experiment 2

The objective of this experiment was to determine the effects of culture time on the meiotic progression of DOC obtained after laparoscopic ovum pick up (LOPU) or slaughterhouse ovaries submitted to different maturation media (EGF or MIX, both described in experiment 1). Oocytes from both origins were cultured for 18, 22, or 26 hours. At each moment, a third of the oocytes in each well was recovered, washed, denuded of any remaining cumulus cell, fixed, and stained with Hoechst (session 2.3). Each oocyte was ranked as degenerated, GV, intermediate, and MII. Consequently, this experiment was a factorial arrangement of two types of oocytes (LOPU vs. slaughterhouse) × two IVM media (simplified vs. semidefined) × three maturation times (18 vs. 22 vs. 26 hours).

2.1.3. Experiment 3

The objective of this experiment was to determine the intrinsic potential of DOC when submitted to EGF or MIX maturation media and then to either IVF or parthenogenetic activation (PA). A total of 593 DOC were used from slaughterhouse ovaries, in four replicates. After IVF or PA, presumptive zygotes were cultured for 8 days as described below. Embryos were examined morphologically and the efficiency of development was evaluated as in experiment 1. In summary, this experiment was comparing two IVM media (EGF vs. MIX), and two methods of activation (IVF vs. PA). On Day 8, all expanded and hatched blastocysts were evaluated regarding quality through vitrification and warming analysis.

2.1.4. Experiment 4

The experiment used DOC and cumulus-oocyte complexes (COCs). A total of 723 oocytes (290 COC and 433 DOC), obtained in four replicates from slaughterhouse ovaries were IVM, IVF, and IVD. Both COC and DOC were IVM alone, as controls. A mixed group was formed at this time with half COC (n = 25) and half DOC (n = 25). A second mixed group was formed for IVM containing 10% of COC (n = 5) and 90% of DOC (n = 45). After fertilization, the group containing only DOC was transferred to the washing plate and received gentle pipetting to remove spermatozoa. The group containing only COC was vortexed to remove cumulus cells, and transferred to the washing plates. The groups that were mixed during IVM, were separated into DOC which were mixed equally with COC (DOCCOC 1:1), and the opposite (COCDOC 1:1), or in different proportions, DOCCOC 1:9 and COCDOC 1:9 (Fig. 1). The developmental competence of DOC and COC was evaluated by cleavage and blastocyst rate as described in experiment 1.

2.2. In vitro embryo production

2.2.1. Aspiration of oocytes

During the nonbreeding season ovaries from goats, regardless the stage of estrous cycle, were collected from a local slaughterhouse and transported to the laboratory within 4 hours after collection in a thermos box containing saline solution (0.9% NaCl) at 30 °C. Ovaries were washed in prewarmed fresh saline (30 °C), and oocytes were aspirated from all visible follicles between 2 and 6 mm in diameter with a 18-gauge short bevel needle connected to a Falcon tube under controlled vacuum (30-mm Hg). The collection tube was previously filled with 3 to 5 mL of HEPES-buffered tissue culture medium 199 (TCM 199) supplemented with 10-IU/mL heparin (Choay, Glaxo Wellcome Production, Notre Dame de Bondeville, France), 4-μg/mL gentamycin, and 1-mg/mL BSA (A 9647).

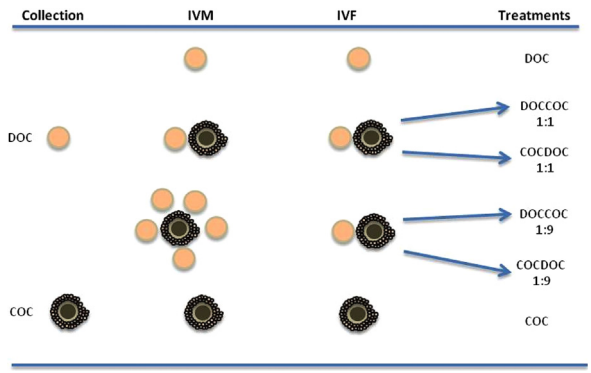

Fig. 1. Effect of different types of oocytes and their culture on the *in vitro* development of goat embryos. Diagram illustrating the treatments used in experiment 4. Oocytes were intact as cumulus-oocyte complexes (COCs) or already denuded at collection (DOC). Cumulus-oocyte complexes and DOC at collection were cultured alone in all steps (COC and DOC), COC were IVM and IVF with an equal number (25 + 25) of DOC and IVD separately (COCDOC 1:1 and DOCCOC 1:1), COC were IVM and IVF within a lower number with DOC (5 COC + 45 DOC) and then IVD separately (COCDOC 1:9 and DOCCOC 1:9).

2.2.2. LOPU in live goats

Laparoscopic ovum pick up sessions were organized on the same day of slaughterhouse-oocytes recovery and conducted in 32 pluriparous Saanen and Alpine goats (alternating 16 goats per week). The animals were housed in free stalls and provided with food and water *ad libitum*. Females received intravaginal sponges impregnated with 45-mg FGA (Chronogest CR, Intervet Schering Plow Animal Health, Angers, France) for 11 days and a prostaglandin intramuscular injection (Cloprostenol, 50 μg; Intervet Schering Plow Animal Health) that was administered 8 days after the FGA sponge insertion. At the same moment, they were stimulated with a total of 16-mg FSH, which was given as twice-daily intramuscular injections in five decreasing dose schedule (4/4/3/3/2 mg). All FSH used in the present study was highly purified porcine FSH, supplied by the Laboratory of Endocrinology, Faculty of Veterinary Medicine, University of Liege, Belgium. Laparoscopic ovum pick up was performed at 24 hours after the last FSH administration and FGA sponges were removed at the end of the procedure.

The goats were deprived of food and water for 24 hours before laparoscopy. Animal suffering was avoided by proceeding under general anesthesia with xylazine (0.5 mg/10 kg of Kensol 2%, Konig, France) and ketamine (25 mg/10 kg of Ketalar Parke- Davis, France), and aspiration procedure was performed as described by

Souza-Fabjan et al. [25]. All ovarian follicles bigger than 2 mm were individually aspirated using an 18-gauge needle (Cook Ireland Ltd., Limerick, Ireland) connected to an aspiration and flushing system. The vacuum pressure was set at 50-mmHg. The collection tube was previously filled with 3 to 5 mL of HEPES-buffered tissue culture medium 199 (TCM 199) supplemented with 10-IU/mL heparin (Choay, Glaxo Wellcome Production, Notre Dame de Bondeville, France), 4-μg/mL gentamycin and 1-mg/mL BSA (A 9647). Once the aspiration was ended, each ovary was gently washed with a heparinized saline solution (25 IU/mL) at 37 °C to prevent possible adhesions. Finally, the trocar orifices were treated with a local antibiotic healing solution. Just after the aspiration, the collection tube was transported to the culture room, where the oocytes were found, selected, and transferred to the washing plate.

2.2.3. IVM of oocytes

Regardless of the source, the oocytes were isolated under a stereo zoom microscope (Nikon Corporation, Japan) and graded as grade 1, 2, or 3. Only denuded (grade 3, DOC) oocytes with homogeneous ooplasm, that is, not presenting one complete layer of unexpanded cumulus cells were used in IVM. The DOCs were washed four times and transferred to 4-well petri dishes (Nunc, Roskilde, Denmark) containing 45 to 50 oocytes in 500 μL of maturation medium. DOCs were incubated for 22 hours at 38.8 °C in a humidified atmosphere of 5% CO_2 in air [25]. *In vitro* maturation experiments varied as specified in the experimental design.

2.2.4. Sperm preparation and IVF of oocytes

Two straws of semen from the same ejaculate/buck were used per replicate throughout experiments. Motile sperm from frozen/thawed semen were separated by centrifugation (15 minutes at 700 × g) on 2 mL of Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient (45/90%). Viable sperm were diluted in the appropriate volume of fertilization medium, to achieve a final concentration of 2.0×10^6 sperm/mL. The matured DOCs were washed in fertilization medium. The fertilization medium consisted in synthetic oviduct fluid (SOF) medium (pH = 7.3, 280 mOsm), which contained 10% of heat-inactivated estrus sheep serum, 5-μg/mL heparin (Calbiochem 375 095) and 4-μg/mL gentamycin. Groups of 45 to 50 oocytes were transferred to 4-well petri dishes, containing 450 μL of fertilization medium, and 50 μL of sperm suspension were added to each well. Sperm and oocytes were coincubated for 18 hours at 38.8 °C in a humidified atmosphere of 5% CO_2 in air [26].

2.2.5. Parthenogenetic activation (PA)

After 24 hours of maturation, oocytes were submitted to PA [25]. The oocytes were washed twice in TCM 199 containing 5% FCS and 1-μg/mL gentamycin. Then, oocytes were activated for 5 minutes in the same medium supplemented by 2-mM ionomycin and washed again three times. Finally, they were transferred into the same medium containing 5-mM 6-DMAP during 4 hours to inactivate M-Phase Promoting factor kinase activity. After the activation process, oocytes were washed three times.

2.2.6. IVD of embryos

The presumptive zygotes were washed four times in IVD medium (SOF supplemented with 3-mg/mL BSA) to remove spermatozoa and transferred by groups of 25 into 4-well petri dishes containing 25-μL drops of IVD medium covered with 700 μL of mineral oil. The presumptive zygotes were cultured for 7 days in the same drop at 38.8 °C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. After 48 hours pi, 10% FCS was added to the culture droplets.

2.3. Determination of stage of nuclear maturation

After IVM, oocytes were recovered, washed in TCM 199 medium with 2-mg/mL BSA, transferred within a 10-μL droplet onto a grease-free slide, and dried. They were then fixed in ethanol 100% for at least 3 hours. After drying, oocytes were stained in 10-μg/mL Hoechst 33342 fluorochrome (stains all nuclei) in Vectashield mounting medium (Vector Labs, Burlingame, CA, USA), and overlaid with a cover slip sealed with nail varnish. Slides were stored at 4 °C until they were examined using epifluorescent microscopy to evaluate the meiosis nuclear stage and the presence or absence of the first polar body.

2.4. Vitrification and warming

The ability of the blastocysts to survive cryopreservation was used as a tool to assess their quality [27]. On Day 8 pi, all blastocysts were transferred into washing plates, washed to remove the mineral oil and vitrified as previously described [28] Embryos of the same stage of development were vitrified together in the same straw (young and expanded or hatched blastocysts). Briefly, embryos were kept at room temperature for 5 minutes in PBS supplemented with 20% new-born calf serum (NBCS). Blastocysts were then vitrified in three steps at room temperature as follows: 10% glycerol for 5 minutes, 10% glycerol and 20% ethylene glycol for 5 minutes, and finally 25% glycerol and 25% ethylene glycol for 30 seconds in PBS–NBCS supplemented with 0.4-M sucrose. During the last step, embryos were quickly aspirated into the center part of a 0.25-mL plastic straw (IMV, L'Aigle, France) within 20 to 30 μL of vitrification solution. Embryos were separated by two air bubbles from two surrounding segments of PBS–NBCS containing 0.8-M galactose (about 90 μL each). The straws were sealed with polyvinyl alcohol and immediately plunged into liquid nitrogen.

All straws remained from 7 to 15 days in liquid nitrogen before warming. For warming, straws were held 5 seconds in air followed by 15 seconds in a 22 °C water bath. The straw content was expelled into an empty Petri dish, where embryos were kept for 5 minutes and were stirred gently to facilitate the mixture of the two solutions. Afterward, the embryos were gently placed into another plate containing PBS–NBCS and left for 5 minutes at room temperature. They were subsequently washed in the same medium at 39 °C and placed in culture for 72 hours (SOF medium containing 10% FCS, 500 μL per well in a 4-wells plate, embryos from one straw in each well). Survival rates were evaluated with morphological criteria, on the basis of the integrity of the embryo membranes and the *zona pellucida*

Table 1
Effect of different maturation media on developmental competence of goat embryos produced by IVF exclusively from denuded (grade 3) slaughterhouse oocytes.

Treatment	n	Cleavage (%)	Bl/cleaved (%)	Bl/DOC (%)	Hbl/totBl (%)	Total cells n
EGF[b]	155	52 ± 7.5[a]	36 ± 3.9[a]	19 ± 5.0[a]	55 ± 22.9[a]	184 ± 12.6[a]
FCS[c]	130	51 ± 15.0[a]	41 ± 4.8[a]	21 ± 3.3[a]	52 ± 14.8[a]	190 ± 13.8[a]
MIX[d]	126	60 ± 9.4[a]	39 ± 9.3[a]	23 ± 8.1[a]	55 ± 15.9[a]	179 ± 12.4[a]

Percentages of cleavage, blastocysts (Bl), and hatched embryos in relation to the total blastocysts (Hbl/totBl; mean ± SEM).
n represents the number of IVM oocytes submitted to IVF and IVD.
Within a column, values with letter "a" differ significantly by ANOVA and SNK test ($P < 0.05$).
Abbreviations: DOCs, denuded oocytes; EGF, epidermal growth factor; FCS, fetal calf serum; SEM, standard error of the mean.
[b] EGF: 10-ng/mL epidermal growth factor, and 100-μM cysteamine.
[c] FCS: 10% fetal calf serum, 100-mM cysteamine, and 50 ng/mL ovine follicle stimulating hormone.
[d] MIX: 10-ng/mL EGF, 5-UI/mL hCG, 10 UI/mL eCG, 19-ng/mL IGF-1, 2.2-ng/mL FGF, 5-μg/mL Insuline, 5-μg/mL transferrin, 5-ng/mL selenium, 90-μg/mL L-cystein, 0.1-mM β-mercaptoethanol, 75-μg/mL vitamin C, 720-μg/mL glycine, 0.1-mg/mL glutamine, and 110-μg/mL pyruvate.

(with the exception of hatched blastocysts), and reexpansion of the blastocoel. Furthermore, the percentages of embryos that resumed their development and reached a more advanced developmental stage after culture were recorded (development rate). The embryos were evaluated at 6 hours, 24 hours, 48 hours, and 72 hours [29].

2.5. Statistical analysis

The Lilliefors test was used to verify the data normality. One-way analysis of variance followed by Student Newman Keuls posttest was performed. The variables of maturation kinetics (LOPU vs. slaughterhouse; EGF vs. MIX media, and maturation times: 18 vs. 22 vs. 26 hours) and embryonic survival after vitrification were evaluated using the Fisher test. A value of $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using Prism 5.0a software.

3. Results

3.1. Experiment 1

The maturation media did not affect the developmental competence of DOC, inferred by cleavage, blastocyst, blastocysts in relation to the cleaved, hatched in relation to the blastocyst, and total number of cells per blastocyst data (Table 1). When the data were pooled regardless of the treatment, average cleavage was 54± 10.6%, blastocyst 21± 5.5%, blastocysts in relation to the cleaved 39± 6.0%, hatched in relation to the blastocyst 54± 17.9%, and total number of cells per blastocyst 184 ± 12.9.

3.2. Experiment 2

As expected, the stimulation treatment increased the number of puncturable follicles when compared with nonstimulated ovaries (data not shown). The results of the meiotic progression of DOC obtained from LOPU or slaughterhouse ovaries submitted to different media and times of maturation are shown in Figure 2. A total of 83 and 222 oocytes from the LOPU and slaughterhouse groups, respectively, were matured in either EGF or MIX media. After IVM, no significant difference in degeneration rates was observed between the oocytes from the two sources ($P > 0.05$). However, a higher ($P < 0.05$) percentage of oocytes-resumed meiosis after GV breakdown toward Metaphase II (MII) stage in slaughterhouse group (69%) compared with LOPU-oocytes (54%). In the LOPU group, 33% (27/83) of DOC-resumed meiosis, but did not reach the MII stage, which was significantly higher than 20% (44/222) of slaughterhouse-DOCs.

Both variables (media and maturation time) did not affect the kinetic of the meiotic progression of DOC derived from LOPU. Regardless of medium and maturation time, an overall mean of 87% of total LOPU-DOC resumed meiosis. However, only 54% reached M II stage, while 33% remained in intermediary stages of meiosis. The extension of 8 hours in maturation time (18–26 hours) did not increase the percentage of oocytes in M II stage (56 and 53%), nor degenerated oocytes (16% and 10%). Similar results were also observed in slaughterhouse-DOC, cultured in EGF media. However, slaughterhouse-DOC matured in MIX media had their dynamics of meiotic progression affected depending on maturation time. An increase in the percentage of oocytes in MII over time (18–26 hours) and reduction in the percentage of oocytes in the intermediary stage were observed. However, the extension in maturation time increased the percentage of degenerated oocytes.

3.3. Experiment 3

The maturation medium (EGF vs. MIX) and activation method (IVF vs. PA) did not affect the cleavage rate of DOC (Table 2). However, a significant reduction in the number of putative zygotes that reached the blastocyst stage was observed in the IVF group, compared with the PA group, regardless of maturation medium. Consequently, it was also observed difference in the blastocyst rate in relation to the initial number of oocytes between the two activation methods. Despite this, hatching rate in relation to the blastocyst was similar between the two methods. When the data were pooled regardless of maturation medium, average cleavage was 75% and 83%, blastocyst rate was 28% and 49%, blastocysts in relation to cleaved embryos was 38% and 59%, and hatched blastocysts in relation to total blastocysts was 63% and 64%, for IVF and PA, respectively. The evaluation of survival after vitrification (72 hours) showed that regardless of the medium of IVM and activation methods, the expanded blastocysts obtained from

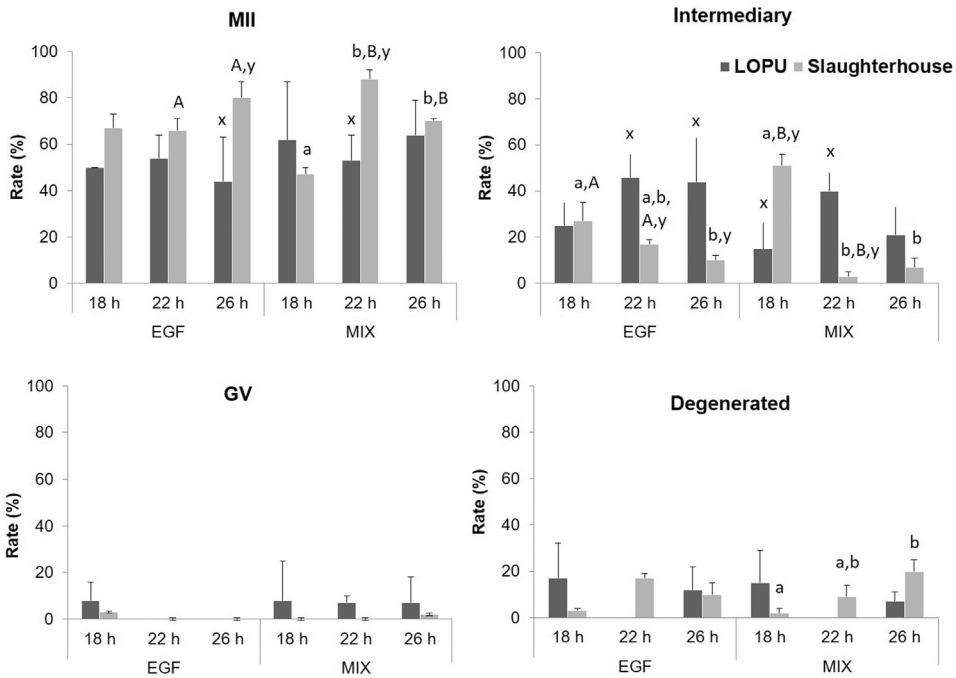

Fig. 2. Effect of maturation time (18, 22, and 26 hours) on the meiotic progression of low quality (grade 3) goat oocytes obtained after laparoscopic ovum pick up (LOPU) or from slaughterhouse ovaries, and submitted to different maturation media (EGF: 10-ng/mL epidermal growth factor and 100-μM cysteamine; or MIX: 10-ng/mL EGF, 5-UI/mL hCG, 10-UI/mL eCG, 19-ng/mL IGF-1, 2.2-ng/mL FGF, 5-μg/mL Insuline, 5-μg/mL transferrin, 5-ng/mL selenium, 90-μg/mL L-cystein, 0.1-mM β-mercapto, 75-μg/mL vitamin C, 720-μg/mL glycine, 0.1-mg/mL glutamine, and 110-μg/mL pyruvate); a,b differ between the maturation time (18, 22, and 26 hours) at the same maturation medium and oocyte origin; A,B differ between the maturation systems (EGF and MIX) at the same maturation time and oocyte origin; x,y differ between the oocyte origin (LOPU and slaughterhouse) at the same blastocyst stage and method of embryo production (mean ± SEM).

different groups have similar cryosurvival and thus quality (Table 3). Similar results were observed when the survival of hatched blastocysts from different IVM medium was compared. However, the activation method of DOC from EGF maturation medium, significantly affected the survival of hatched blastocysts after vitrification (57 ± 21.5 vs. 82 ± 10.2, respectively, for IVF and PA groups).

3.4. Experiment 4

Cleavage and development to the blastocyst stage rates are shown in Table 4. The coculture of COC with DOC in the ratio of 1:9 reduced significantly the cleavage rate of the COCDOC 1:9 group, in comparison with other groups. However, the ability of cleaved embryos to develop to the blastocyst stage was not affected, it was similar for the COC and COCDOC 1:1 groups, and higher than DOC, DOCCOC 1:1, and DOCCOC 1:9 groups, whereas the group COCDOC 1:1 was similar to DOCCOC 1:1 and DOCCOC 1:9. Despite the beneficial effect of coculture with COC on the development of DOC, there was no difference in any of the evaluated parameters between the three DOC groups (DOC, DOCCOC 1:1, and DOCCOC 1:9). Higher development of oocytes to the blastocyst stage was observed in COC and COCDOC 1:1 groups. However, the latter group did not differ from COCDOC 1:9, DOCCOC 1:1, and DOCCOC 1:9.

Table 2
Effect of maturation medium and activation method (IVF or submitted to parthenogenetic activation, PA) in denuded (grade 3) slaughterhouse goat oocytes on embryo developmental competence.

IVM system	Method	n	Cleavage (%)	Bl/cleaved (%)	Bl/DOC (%)	Hbl/totBl (%)
EGF[c]	IVF	150	77 ± 5.0[a]	38 ± 2.6[a]	29 ± 3.4[a]	68 ± 8.3[a]
	PA	138	77 ± 6.7[a]	61 ± 3.1[b]	47 ± 4.4[b]	66 ± 6.7[a]
MIX[d]	IVF	151	72 ± 5.3[a]	38 ± 6.8[a]	27 ± 5.7[a]	59 ± 2.5[a]
	PA	154	88 ± 4.0[a]	57 ± 3.0[b]	51 ± 3.5[b]	54 ± 3.0[a]

Percentages of cleavage, blastocysts (Bl), and hatched embryos in relation to the total blastocysts (Hbl/totBl; mean ± SEM).
n represents the number of IVM oocytes submitted to IVF/PA and IVD.
Within a column, values with different superscripts differ significantly by ANOVA, and SNK test ($P < 0.05$).
Abbreviations: DOC, denuded oocytes; EGF, epidermal growth factor; SEM, standard error of the mean.
[c] EGF: 10-ng/mL epidermal growth factor, and 100-μM cysteamine.
[d] MIX: 10-ng/mL EGF, 5-UI/mL hCG, 10-UI/mL eCG, 19-ng/mL IGF-1, 2.2-ng/mL FGF, 5-μg/mL Insuline, 5-μg/mL transferrin, 5-ng/mL selenium, 90-μg/mL L-Cystein, 0.1-mM β-mercaptoethanol, 75-μg/mL vitamin C, 720-μg/mL glycine, 0.1-mg/mL glutamine, and 110-μg/mL pyruvate.

Table 3
Reexpansion and hatching rates of vitrified and warmed blastocysts derived from denuded (grade 3) slaughterhouse goat oocytes submitted to different maturation media and fertilized (IVF) or submitted to parthenogenetic activation (PA; mean ± SEM).

IVM system	Method	Blastocyst development stage (n)	Postwarming blastocyst survival, %				Hatched blastocysts[c] %
			6 h	24 h	48 h	72 h	
EGF[d]	IVF	Expanded (11)	$36 \pm 10.5^{a,x,A}$	$36 \pm 10.5^{a,x,A}$	$36 \pm 10.5^{a,x,A}$	$45 \pm 14.2^{a,x,A}$	$27 \pm 12.5^{a,x}$
		Hatched (30)	$37 \pm 12.7^{a,x,A}$	$40 \pm 13.5^{a,x,A}$	$57 \pm 19.8^{a,x,A}$	$57 \pm 21.5^{a,x,A}$	—
	PA	Expanded (19)	$42 \pm 10.4^{a,x,A}$	$63 \pm 17.2^{a,x,A}$	$63 \pm 17.2^{a,x,A}$	$58 \pm 14.6^{a,x,A}$	$58 \pm 14.6^{a,x}$
		Hatched (28)	$36 \pm 2.2^{a,x,A}$	$68 \pm 19.8^{b,x,A}$	$82 \pm 10.2^{b,x,A}$	$82 \pm 10.2^{b,x,A}$	—
MIX***	IVF	Expanded (12)	$17 \pm 9.6^{a,x,A}$	$25 \pm 10.5^{a,x,A}$	$33 \pm 21.0^{a,x,A}$	$42 \pm 21.5^{a,x,A}$	$33 \pm 14.2^{a,x}$
		Hatched (26)	$35 \pm 6.6^{a,x,A}$	$46 \pm 10.7^{a,x,A}$	$58 \pm 5.0^{a,x,A}$	$69 \pm 9.4^{a,x,A}$	—
	PA	Expanded (23)	$48 \pm 1.1^{a,x,A}$	$39 \pm 11.4^{a,x,A}$	$61 \pm 20.0^{a,x,A}$	$52 \pm 11.1^{a,x,A}$	$48 \pm 14.4^{a,x}$
		Hatched (40)	$25 \pm 4.9^{a,x,A}$	$58 \pm 21.5^{a,x,A}$	$65 \pm 21.6^{a,x,A}$	$63 \pm 18.5^{a,x,A}$	—

() number of blastocysts evaluated.
Within a column, values with different superscripts differ significantly by Fisher test ($P < 0.05$).
a,b differ between the method of embryo production (IVF vs. PA) at the same blastocyst stage and maturation medium.
x,y differ between the maturation medium (EGF vs. MIX) at the same blastocyst stage and method of embryo production.
A,B differ between the blastocyst stage (expanded vs. hatched) at the same maturation medium and method of embryo production.
Abbreviation: SEM, standard error of the mean.
[c] The hatching rate was calculated based on the number of vitrified blastocysts, just in the groups that were expanded before vitrification.
[d] EGF: 10-ng/mL epidermal growth factor, and 100-μM cysteamine;***MIX: 10-ng/mL EGF, 5-UI/mL hCG, 10-UI/mL eCG, 19-ng/mL IGF-1, 2.2-ng/mL FGF, 5-μg/mL Insuline, 5-μg/mL transferrin, 5-ng/mL selenium, 90-μg/mL L-cystein, 0.1-mM β-mercaptoethanol, 75-μg/mL vitamin C, 720-μg/mL glycine, 0.1-mg/mL glutamine, and 110-μg/mL pyruvate.

4. Discussion

The interaction of CC with the oocyte provides the local production of glycosaminoglycans, steroid hormones, nutrients, and other factors to support the oocyte maturation [30,31]. However, depending on the biotechnological purpose, CC removal before IVM can be required. In addition, the systems used for follicular aspiration are leading to a loss of cumulus cells in approximately 5% of the total oocytes recovered. Therefore, when obtaining DOC, it is not possible to be sure whether these oocytes are denuded randomly by the strength of the aspiration system or if they are originating from follicles already on the way of atresia. Moreover, it is known FSH does not select follicles and thus acts in both healthy and in those undergoing regression by atresia. Hence, establishment of protocols that support the preimplantation development *in vitro* of DOCs would be useful for optimizing the efficiency of reproductive biotechnologies, and enable greater use of genetic material of high-economic value females. Our results show that DOC are able to develop *in vitro* to the blastocyst stage regardless of maturation medium and coculture with COC during IVM, but it is influenced by the activation method. In a previous study of our group using COC grade I and II, we also observed that the developmental competence is not affected by the maturation medium [25]. These results suggest that despite the addition of molecules such as EGF, hCG, eCG, IGF-1, FGF, Insuline, and vitamin C, which have been shown individually to exert a beneficial effect on embryonic development [32–35], their association during IVM does not act in synergy to increase the blastocyst rate of DOC and COC. From our own results, it can be concluded that supplementation of the TCM199 medium with EGF and cysteamine is sufficient to support the IVM, IVF, and IVD to the blastocyst stage of goat DOC. Corroborating this finding, some studies have also reported good results of embryonic development when goat COC were matured in TCM199 medium supplemented only with EGF and cysteamine [26,36,37].

Denuded oocytes derived from slaughterhouse and LOPU show differences in IVM kinetic, regardless of IVM media. Eighteen hours are sufficient for IVM of DOC coming from LOPU, regardless of maturation medium, whereas slaughterhouse DOC required 22 hours. Considering that the hormonal treatment used in this study for obtaining the DOC from LOPU-induced follicular growth synchronization, it would be expected that the DOC derived from LOPU might present more synchronized maturation, in comparison with the DOC derived from slaughterhouse. However, this was not observed when grade I and II COC were used [26]. This difference can be attributed to the competence of oocytes at the time of removal from the follicular environment. We believe that presence of CC is not

Table 4
Cleavage rate and *in vitro* development at 8 days after insemination of goat embryos derived from cumulus enclosed (COC) or denuded (DOC) oocytes matured and fertilized in different conditions (mean ± SEM).

Treatments[d]	n	Cleavage (%)	Blastocyst production (% pi) from	
			Cleaved oocytes	Total oocytes
COC	175	67 ± 4.5^{a}	71 ± 4.7^{a}	45 ± 2.0^{a}
COCDOC 1:1	95	65 ± 5.5^{a}	$61 \pm 1.8^{a,b}$	$39 \pm 4.7^{a,b}$
COCDOC 1:9	20	36 ± 4.0^{b}	71 ± 14.4^{a}	$25 \pm 5.0^{b,c}$
DOC	170	68 ± 1.2^{a}	28 ± 2.7^{c}	19 ± 2.2^{c}
DOCCOC 1:1	96	64 ± 3.7^{a}	$42 \pm 5.8^{b,c}$	$26 \pm 4.0^{b,c}$
DOCCOC 1:9	167	68 ± 2.2^{a}	$39 \pm 2.9^{b,c}$	$26 \pm 2.2^{b,c}$

n represents the number of oocytes.
Within a column, values with different superscripts differ significantly ($P < 0.05$) by ANOVA followed by SNK. pi: at Day 8 after insemination.
Abbreviation: SEM, standard error of the mean.
[d] Oocytes were intact as cumulus-oocyte complexes (COC) or already denuded at collection (DOC). COC and DOC at collection were cultured alone in all steps (COC and DOC), COC were IVM and IVF with an equal number (25 + 25) of DOC and IVD separately (COCDOC 1:1 and DOCCOC 1:1), COC were IVM and IVF within a lower number with DOC (5 COC + 45 DOC) and then IVD separately (COCDOC 1:9 and DOCCOC 1:9).

essential to the meiotic maturation, if at the time of removal of the oocyte from follicular environment, they already acquired competence to development. However, when the oocytes still need to acquire competence, the presence of CC may significantly contribute in their developmental capacity acquisition during IVM. Thus, regardless of the source, these oocytes will require longer time in IVM, contrary to what happens in the absence of CC.

Surprisingly, the cleavage rates obtained in experiment 3 for DOC submitted to IVF were approximately 20% more than in experiment 1. In fact, there is no plausible explanation for such difference. Perhaps, it is more challenging to acquire repeatability using DOC than intact COC. The development rate of DOC artificially activated (PA) was higher than for those submitted to IVF, regardless of IVM medium. Han et al. [38] reported that incomplete cytoplasmic maturation of oocytes causes a decrease in male pronucleus formation, monospermic fertilization, and embryonic development. In addition, the CC can promote oocyte fertilizability by preventing changes in the oocyte, such as precocious *zona pellucida* hardening, which are unfavorable for normal fertilization. Spontaneous hardening of the mouse *zona pellucida* occurs during oocyte maturation either *in vivo* [39] or *in vitro* [40] and is caused by precocious exocytosis of cortical granules [41]. *In vitro* zona hardening occurs when DOCs are matured under serum free conditions [42]. Downs et al. [43] demonstrated that in mice both serum deprivation and removal of the CC during oocyte IVM result in an alteration of the *zona pellucida* which is manifested as an increased resistance to proteolytic digestion and sperm penetration. In pigs, mechanical CC removal before IVM also resulted in premature cortical reaction [44]. Thus, this difference in the competence development of DOC artificially activated in comparison to IVF can be attributed to problems in the interaction between the spermatozoa and oocyte, male pronucleus formation and monospermic fertilization. Despite this difference, the inference of the quality of the analysis of blastocyst survival rate after cryopreservation shows that embryos derived from IVF and PA are similar.

During maturation, the COC may secrete soluble regulatory factor(s) that in turn may be favorable to DOC. These factors include progesterone in bovine COC [45], the EGF-like ligands amphiregulin, epiregulin, and betacellulin in primate COC [46], and vascular endothelial growth factor [47]. Cumulus cells also secreted factors induce the nuclear and cytoplasmic maturation of DOC when cocultured with CC. The beneficial role of different somatic cells during coculture has been investigated for decades. Although the mechanisms are not well elucidated, it is suggested somatic cells are involved in the depletion of potentially harmful substances and they may positively modify medium components, among others [27]. Our data, together with those of previous reports, report that the intact COC exert beneficial effect on DOC during IVM, resulting in higher development of DOC to the blastocyst stage, regardless of the proportion of DOC/COC used in IVM. However, they do not significantly increase the blastocyst production rate compared with the control group (DOC groups). In addition, a deleterious effect on the development competence of COC was observed in the proportion of 1:9 (COC: DOC). Previously, Luciano et al. [48] reported that the presence of intact COC during IVM is able to maintain the glutathione (GSH) concentration in DOC significantly higher than in DOC matured alone and similar to the GSH content of DOC matured in presence of cysteamine. Several studies have shown that an increase of GSH exerts a beneficial effect on the blastocyst production due to participation in sperm decondensation and male pronucleus formation, during fertilization [49–51]. Thus, we hypothesize that a lower proportion of COC mixed to DOC (1:9) during IVM possibly reduces the GSH intracellular concentration in COC, which can then reduce their competence for fertilization and development. This may explain the reduced rate of cleavage observed in the COC-DOC 1:9 group.

4.1. Conclusions

In conclusion, under our experimental conditions, similar maturation, fertilization, and embryo development rates were obtained when using complex, semidefined, or simplified maturation media in slaughterhouse DOC. Laparoscopic ovum pick up and slaughterhouse-derived DOC may have different IVM kinetics, with the latter requiring more time, depending on the IVM medium. Parthenogenetically activated DOC present higher development capacity compared with DOC submitted to IVF. However, the quality of embryos obtained in the two methods was similar, as demonstrated by survival rates after vitrification and thawing. Coculture of DOC with COC during maturation plays beneficial effect on the development rate of DOC. Finally, although they are less competent for development than COC, DOC (grade 3 oocytes) can be used to produce some additional embryos from genetically valuable females collected by LOPU or after culling.

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Competing interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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