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Influence of heparin or the presence of cumulus cells during fertilization on the *in vitro* production of goat embryos

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ABSTRACT

Considerable research has been focused on *in vitro* production (IVP) of goat embryos to improve its efficiency. In Experiment 1, the effect of the cumulus cells by comparing slaughterhouse-oocytes denuded on purpose (DOP) prior to IVF to intact COC, and the effect of heparin during IVF were assessed. In Experiment 2, oocytes that were already denuded at collection (DOC), DOP and intact COC were studied. Three treatments used oocytes denuded at collection: DOC oocytes were cultured alone for both IVM and IVF; DOC and COC were cultured together for both IVM and IVF or DOC were IVM alone and then mixed with COC for IVF. In other treatments, COC were allocated to four IVF treatments: Intact COC; COC were denuded prior to IVF; COC were denuded and IVF with added cumulus cells; COC were denuded and IVF mixed with intact COC giving two sub-treatments: Denuded oocytes that were IVF with COC; and COC that were IVF with denuded oocytes. After fertilization, all presumptive zygotes were cultured for 8 days. In Experiment 1, the yield of blastocysts as a proportion of total oocytes was greater ($P < 0.05$) for COC that were IVF in the presence of heparin (54%) than without heparin (42%) or oocytes already denuded at collection that were IVF with or without heparin (41%; 38%; respectively). In Experiment 2, the developmental potential of oocytes denuded at collection was reduced (cleavage and blastocyst rates calculated from total oocytes: 34%; 11%, respectively) as compared to COC (77%; 59%, $P < 0.05$). However, when equal numbers of both were mixed at the start of IVM, the rates were not significantly different to COC alone (68%; 45%), but when both were mixed equally only for IVF, the rates were reduced (57%; 40%, $P < 0.05$). Denuded oocytes co-cultured with cumulus cells were not significantly different to intact COC (76%; 55%). The effect of adding COC during IVF to oocytes denuded after IVM was similar to adding cumulus cells to the same type of oocytes. In conclusion, both the use of heparin and the association of oocytes with cumulus cells, either detached or in intimate contact, during IVM and/or IVF significantly improve IVP of goat embryos. Furthermore, some oocytes that are already denuded at collection will develop satisfactorily to blastocysts when matured and fertilized with intact COC.

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1. Introduction

The goat industry has been experiencing a cycle of global growth and is now in need of efficient reproductive biotechnologies in order to sustain worldwide production. In small ruminants, *in vivo* embryo recovery usually requires surgical procedures that impair repeated embryo production from individual donors. *In vitro* embryo production (IVP) has some advantages over *in vivo* recovery such as reliability (Baldassarre and Karatzas, 2004), reproducibility (Stangl et al., 1999), the possibility of collecting oocytes from hormonally stimulated females (Morton et al., 2005), the use of pre-pubertal donors (Baldassarre et al., 2004; Morton, 2008), senile and pregnant females or even in *post-mortem* cases (Baldassarre et al., 2007). Beyond the potential use of IVP in breeding schemes and for patrimonial conservation of genetic diversity, this technique is also required for the establishment of new biotechnologies such as cloning and transgenesis.

Over recent years, considerable research into IVP technology has been undertaken in an attempt to determine which conditions are needed during *in vitro* maturation (IVM), fertilization (IVF) and *in vitro* development (IVD) processes to enhance embryo production. During fertilization, different methods have been described, but specific questions remain to be answered. It has been demonstrated that different supplements such as gonadotropins added to IVM medium may also affect IVF success (Younis et al., 1991), but their benefits alone or in association still need to be clarified. The importance of capacitating agents such as heparin for the success of IVF has been documented in many species. Heparin has been shown to increase fertilization rate in cattle (Parrish et al., 1988) and is thus widely used for sperm capacitation in this species. Similarly, there are some studies demonstrating the role of heparin in goats (Younis et al., 1991; Cox and Alfaro, 2007). However, some reports indicate an adverse effect on fertilization (Malik et al., 1997) or lesser embryo development and quality (Cognié et al., 1995) making its use questionable for goat species.

The appropriate time of cumulus cells removal from oocytes during IVP also remains controversial. In mammals, the effect of cumulus cells on fertilization seems to be species dependent. Partial removal of cumulus cells before IVF decreases sperm penetration in cattle (Zhang et al., 1995), pigs (Suzuki et al., 2000) and the fertilization rate in humans (Tao Tao et al., 2004). Conversely, in different mouse strains cumulus removal did not affect fertilization rates (Vergara et al., 1997). In goats there are reports where oocytes were denuded immediately before IVF (Morton et al., 2005; Katska-Ksiazkiewicz et al., 2007; Rodriguez-Dorta et al., 2007; Freitas and Melo, 2010; Vazquez et al., 2010), just after IVF (Cox and Alfaro, 2007; Shirazi et al., 2010; Romaguera et al., 2011), or even cumulus was partially removed before IVF and oocytes where then completely denuded after IVF (Karami Shabankareh et al., 2011). However, without direct comparison of these procedures, it is difficult to precisely analyze the role and benefits of cumulus cells during fertilization. Furthermore, it is noteworthy to investigate whether any beneficial effect of cumulus cells during fertilization could be mediated by secreted factors, or requires direct contact of these cells with the oocyte (Fatehi et al., 2005).

In general, a great challenge in goat IVP research laboratories throughout the world is the number of goats slaughtered at the same time and consequently the availability of a substantial number of acceptable quality oocytes (Rahman et al., 2007). After retrieval from growing follicles, the oocytes are usually found as cumulus oocyte complexes (COC), within varying numbers of cumulus cells layers. A grade classification (I, II and III) based on the number of cumulus layers and ooplasm morphology has been proposed (Baldassarre et al., 2003). A similar classification system is currently used by many laboratories, and typically only COC with at least one complete layer of cumulus cells are selected for IVM. Oocytes that are found denuded at collection are considered not suitable for IVP and thus routinely discarded. Therefore, if a particular strategy could be applied to the use of denuded oocytes in labor-intensive processes such as ovum pickup from animals of high genetic merit, it would be a benefit to the goat industry.

No previous attempt has been made in goats to analyze the effect of cumulus cell presence, (attached or not to the oocyte) during IVF, nor to evaluate the possibility of using oocytes found denuded at collection for IVP. Therefore, the aims of the present study were to examine the i) effect of including heparin in the fertilization medium ii) influence of cumulus cells both separately or attached to the oocyte during maturation and/or fertilization, and iii) the potential for using oocytes denuded at collection in the *in vitro* production of goat embryos.

2. Material and methods

Except where otherwise indicated, chemicals were purchased from Sigma Chemical Co. (Saint Louis, MO, USA).

2.1. Experiment 1

2.1.1. Experimental design

The objective of this experiment was to determine the effect of the presence or absence of cumulus cells by comparing oocytes denuded on purpose prior to IVF to intact COC, and to determine the effect of the use of heparin in the fertilization medium as well as its possible interactions with the presence of cumulus cells. Consequently, this experiment was a factorial arrangement of two types of oocytes (denuded on purpose × COC) × two IVF media (supplemented with heparin or not) × six replicates.

2.1.2. Aspiration of oocytes

During the breeding season (September and October) ovaries from goats, regardless the stage of estrous cycle, were collected from a local slaughterhouse and transported to the laboratory (Latitude: 46′N) in a thermos box containing saline solution at 30 °C within 3 h after collection. Ovaries were washed in pre-warmed fresh saline (30 °C), and oocytes were aspirated through a 19 gauge short needle from all visible follicles between 2 and 5 mm in diameter into 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) and 40 μg/mL gentamycin.

2.1.3. IVM of oocytes

Cumulus oocyte complexes were isolated under a stereo zoom microscope (Nikon Corporation, Japan) and graded as good, fair or poor. Only good and fair oocytes, *i.e.*, surrounded by at least one complete layer of unexpanded cumulus cells were considered acceptable and used in IVM (Guler et al., 2000). A total of 1447 COC were used. The COC were washed four times and transferred to maturation medium consisting of TCM 199 supplemented with 10 ng/mL epidermal growth factor (EGF) and 100 μM cysteamine in four well petri dishes (Nunc, Roskilde, Denmark) with each well containing 45 to 50 oocytes in 500 μL of maturation medium. COC were incubated for 22 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air (Cognié et al., 2003).

2.1.4. Sperm preparation and IVF of oocytes

A batch of semen pooled from two bucks was used throughout experiments. Motile sperm from frozen/thawed semen were separated by centrifugation (15 min 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 and 75 μL were added to each fertilization well, to achieve a final concentration of 1.5×10^6 spermatozoa/mL (Day of *in vitro* fertilization = Day 0).

At the end of IVM, half of the oocytes were placed into 15 mL Falcon tubes containing 2 mL of SOF medium (synthetic oviduct fiuid) supplemented with Hepes (24 μg/mL) and BSA (2 μL/mL), and vortexed for 2 min (medium speed) to remove cumulus oophorus before IVF. These denuded oocytes (denuded on purpose, DOP) were recovered in 35 mm petri plates and then transferred to plates containing washing medium. The remaining COC (cumulus oocyte complex group; COC) were also transferred to similar plates and washed. The washing medium used was SOF medium (pH = 7.3, 280 mOsm), which contained 40 μg/mL gentamycin and 10% of heat-inactivated estrus sheep serum. The COC and the DOP were randomly divided between two fertilization conditions in IVF medium supplemented or not with 5 μg/mL heparin (Calbiochem 375 095). Groups of 45 to 50 oocytes were transferred into four well petri dishes, containing 425 μL of fertilization medium and 75 μL of sperm suspension were added to each well. Sperm and oocytes were co-incubated for 20 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air (Cognié et al., 2003).

2.1.5. IVD of embryos

After fertilization, the COC fertilized with or without heparin were denuded by vortex. The presumptive zygotes from the four groups were washed four times in culture medium (SOF supplemented with 3 mg/mL BSA) to remove spermatozoa and transferred to four well petri dishes containing 25 μL drops of culture medium covered with 700 μL of mineral oil. The presumptive zygotes were incubated for seven days at 38.8 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. After 48 h post-insemination, 10% fetal calf serum (FCS) was added to the culture droplets.

2.2. Experiment 2

2.2.1. Experimental design

The experimental design is shown in Fig. 1. The experiment used oocytes that were already denuded at collection (DOC), oocytes denuded on purpose (DOP) and cumulus-oocyte complexes (COC). Different treatments used the various types of oocytes either separately or in combination only during the IVM (M) or in both the IVM and IVF (F) phases of culture. In one treatment the oocytes were IVF with separate cumulus cells (CC). Three treatments used oocytes denuded at collection: 1. DOC oocytes were cultured alone for both IVM and IVF 2. DOC and COC were cultured together (25 + 25 per well) for both IVM and IVF; 3. DOC were IVM alone and then mixed with COC (25 + 25 per well) for IVF. The other treatments used COC subjected to standard IVM and then allocated to four IVF treatments; 4. Intact COC; 5. COC were denuded prior to IVF; 6. COC were denuded and IVF with added cumulus cells (CC); 7. COC were denuded and IVF mixed with intact COC (25 + 25) giving two sub-treatments: 7a. Denuded oocytes that were IVF with COC; and 7b. COC that were IVF with denuded oocytes. Thus, there were eight IVF treatments each replicated five times with 45 to 50 oocytes per group per replicate (culture well).

2.2.2. Aspiration of oocytes

In the subsequent months, November and December, the same procedure was conducted to recover the oocytes. However, for Experiment 2, all oocytes with a homogenous ooplasm were used, even the ones totally denuded at collection, *i.e.*, quality III (poor) oocytes but with a homogeneous ooplasm. A total of 1697 oocytes were used (1316 COC and 381 already found denuded at collection).

2.2.3. IVM of oocytes

The COC were washed four times, pooled and then placed, with each well containing 45 to 50 oocytes in 500 μL of maturation medium. The denuded group was submitted to the same process described for COC. A mixed group was formed at this time with half COC ($n = 25$) and half oocytes denuded at collection ($n = 25$). The maturation medium used was EGF + CYST for all conditions.

2.2.4. IVF of oocytes

Semen preparation and the fertilization medium were the same as in Experiment 1, but heparin was added in all treatments (5 μg/mL). Before IVF, all COC were pooled, then allocated at random to treatments and the mechanical manipulation appropriate to the treatment performed prior to IVF. Some of the matured COC were denuded on purpose by vortexing. These denuded oocytes were fertilized alone, or with COC (25/25), or with separate cumulus cells. The COC and oocytes denuded on purpose that were fertilized together were subsequently cultured separately with one culture well containing 25 oocytes denuded on purpose and another with 25 COC. The oocytes that were

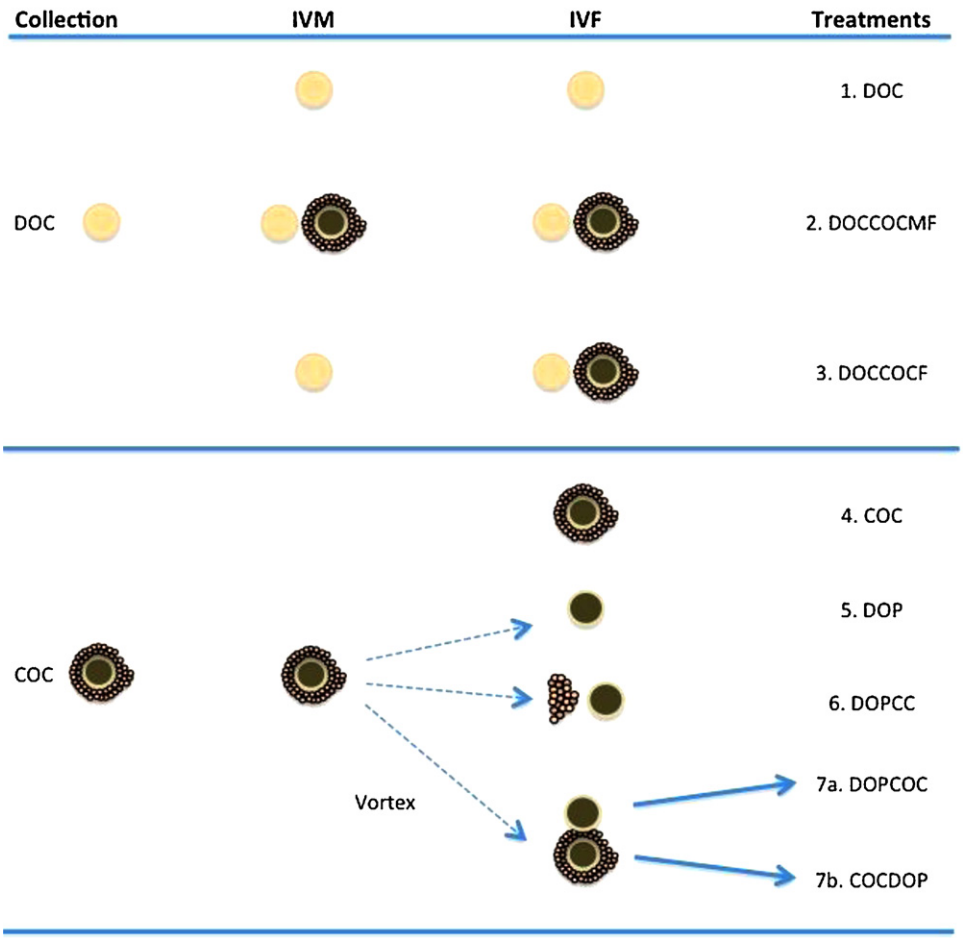

Fig. 1. The effect of different types of oocytes and their culture on the *in vitro* development of goat embryos. Diagram illustrating the treatments used in experiment 2. Oocytes were already denuded when collected (DOC) or cumulus-oocyte complexes (COC) were intact. DOC oocytes were 1. IVM and IVF alone (DOC), 2. IVM and IVF with an equal number (25 + 25) of COC (DOCCOCMF). 3. IVM alone then IVF with an equal number (25 + 25) of COC (DOCCOCF). All COC intact at collection remained intact for IVM and then were: 4. IVF as intact COC (COC), 5. COC were denuded prior to IVF (DOP), 6. COC denuded after IVM and then IVF in the presence of cumulus cells (DOPCC), 7. COC were denuded after IVM and then IVF in the presence of an equal number (25 + 25) of COC giving two sub-treatments 7a. DOP that were IVF in the presence of COC (DOPCOC) and 7b. COC that were IVF in the presence of DOP (COCDOP). COC and DOP oocytes from treatments 7a and 7b were cultured separately following IVF.

already denuded at collection either remained alone for both IVM and IVF or were cultured with COC (25/25) for both IVM and IVF, or only for IVF.

Where cumulus cells were added to oocytes denuded on purpose after IVM (treatment COCCC), the medium remaining after vortexing was centrifuged at 350 × *g* for 10 min and the supernatant removed. The pellet of cumulus cells was suspended in 100 μL of IVF medium and was transferred into the IVF well that contained the oocytes in 325 μL of IVF medium thus making the total volume of medium 425 μL as for the other treatments. Fertilization followed the same protocol as that used in Experiment 1.

2.2.5. IVD of presumable zygotes

After fertilization, the groups that were already denuded were transferred to the washing plate and received gentle pipetting in order to remove spermatozoa. The other groups were vortexed separately to remove cumulus cells, and transferred to the washing plates.

2.3. Assessment of embryo development and statistical analysis

Embryos were examined morphologically and the efficiency of development was evaluated (i) as the percentage of cleaved embryos 2 days after fertilization, and the percentage of blastocysts on Day 8 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. Data were collected over six (Experiment 1) and five (Experiment 2) replicates. After the use of Kolmogorof-Smirnov test for Gaussian distribution of the data, an arcsine transformation was applied to normalize the data, when necessary. However, means and S.E.M. are presented untransformed. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison posttests were performed. A value of $P < 0.05$ was considered to be statistically significant. Statistical analyses were performed using Prism 5.0a software.

Table 1
Effect of culturing goat oocytes during IVF as intact cumulus-oocyte complexes (COC) or denuding them prior to IVF (DOP), and the absence (–) or presence (+) of heparin in the IVF medium on the *in vitro* cleavage at 2 days post insemination and development at 8 days post insemination of goat embryos (mean ± S.E.M).

Conditions	Heparin	Oocytes (*n*)	Cleavage (%)	Blastocyst production (%) from	
				cleaved oocytes	from total oocytes
COC	–	365	66 ± 4.1[a]	63 ± 4.1[ab]	42 ± 2.9[a]
	+	364	72 ± 2.8[a]	75 ± 3.5[a]	54 ± 2.2[b]
DOP	–	360	64 ± 2.5[a]	59 ± 4.4[b]	38 ± 3.0[a]
	+	358	68 ± 3.5[a]	61 ± 3.7[ab]	41 ± 2.7[a]

Within a column, values with different superscripts differ ($P < 0.05$)

3. Results

3.1. Experiment 1

When the data for oocytes fertilized (IVF) in the presence or absence of heparin were combined, the cleavage rate was similar for COC (69 ± 2.5%) and oocytes denuded on purpose (66 ± 2.2%) but the COC developed a greater proportion of blastocysts than denuded whether this was expressed as a percentage of cleaved oocytes (70 ± 3.0% compared with 60 ± 2.8%; $P < 0.05$) or total oocytes (48 ± 2.3% compared with 39 ± 2.1%; $P < 0.05$). The overall effect of including heparin in the IVF medium compared to no heparin was to increase cleavage rate (70 ± 2.2% compared with 65 ± 2.4%; $P < 0.05$), and the proportions of blastocysts formed from cleaved oocytes (68 ± 2.9% compared with 61 ± 2.9%; $P < 0.05$) or total oocytes (48 ± 2.1% compared with 40 ± 2.1%; $P < 0.01$). The results for the four treatments are presented in Table 1. COC that were IVF in medium supplemented with heparin had the greatest cleavage rate (72 ± 2.8%; NS), and the percentage of blastocysts produced from cleaved oocytes (75 ± 3.5%; NS) and total oocytes (54 ± 2.2%; $P < 0.05$).

3.2. Experiment 2

Cleavage and blastocyst development rates are shown in Table 2. Overall, cleavage rate was greater ($P < 0.01$) for the five treatments with intact COC during IVM (75 ± 1.8%) than for the three treatments using oocytes denuded at collection (DOC 54 ± 3.9%). Similarly, these groups of treatments differed ($P < 0.01$) in blastocyst development both as a proportion of cleaved (74 ± 1.8% compared with 57 ± 5.0%) or initial (53 ± 2.0% compared with 33 ± 4.2%) number of oocytes. Oocytes denuded at collection (treatment DOC) had a lesser ($P < 0.05$) cleavage (34 ± 1.5%) and blastocyst production (11 ± 1.9% of total oocytes) rates than all other treatments. The control treatment (intact COC), however, had the greatest cleavage and blastocyst production rates (Table 2).

When oocytes denuded at collection were IVM alone but mixed with equal numbers of intact COC during IVF (treatment DOCCOCF), the overall cleavage rate (57 ± 2.3%) was greater than DOC alone and less than COC alone (77 ± 3.5%; $P < 0.05$) being mid-way between the other two values. The cleavage rate of DOC oocytes mixed with COC during both IVM and IVF (68 ± 1.4%), however, was more similar to that of the control treatment COC (NS). Blastocyst production rate from cleaved oocytes for both these mixed treatments were similar and less (NS) than for COC. The proportion of blastocysts as a percentage of the initial number of oocytes was less for those only IVF with COC than for control (40 ± 1.2% compared with 59 ± 4.4%; $P < 0.05$) but not different than for oocytes both IVM and IVF with COC (45 ± 3.6%).

Oocytes that were intact during IVM but denuded on purpose (DOP) prior to IVF had a cleavage rate of 70 ± 4.3%, and blastocyst production rates of 68 ± 3.0% of cleaved and

Table 2
Cleavage rate and *in vitro* development at 8 days post insemination of goat embryos matured and fertilized in different conditions (Experiment 2).

Treatments[a]	Oocytes (*n*)	Cleavage (%)	Blastocyst production (%) from from	
			cleaved oocytes	from total oocytes
1. DOC	121	34 ± 1.5[c]	32 ± 4.9[b]	11 ± 1.9[c]
2. DOCCOCMF	245	68 ± 1.4[a,b]	67 ± 4.5[a]	45 ± 3.6[a,b]
3. DOCCOCF	275	57 ± 2.3[b]	69 ± 2.7[a]	40 ± 1.2[b]
4. COC	289	77 ± 3.5[a]	77 ± 3.1[a]	59 ± 4.4[a]
5. DOP	230	70 ± 4.3[a,b]	68 ± 3.0[a]	47 ± 3.9[a,b]
6. DOPCC	227	76 ± 4.7[a]	72 ± 3.1[a]	55 ± 4.3[a]
7a. DOPCOC	147	69 ± 2.8[a,b]	76 ± 6.4[a]	52 ± 5.0[a]
7b. COCDOP	163	80 ± 3.0[a]	74 ± 4.0[a]	59 ± 3.5[a]

Within a column, values with different superscripts differ ($P < 0.05$)
[a] Oocytes were already denuded when collected (DOC) or cumulus-oocyte complexes (COC) were intact. DOC oocytes were 1. IVM and IVF alone (DOC), 2. IVM and IVF with an equal number (25 + 25) of COC (DOCCOCMF). 3. IVM alone then IVF with an equal number (25 + 25) of COC (DOCCOCF). All COC intact at collection remained intact for IVM and then were: 4. IVF as intact COC (COC), 5. COC were denuded prior to IVF (DOP), 6. COC denuded after IVM and then IVF in the presence of cumulus cells (DOPCC), 7. COC were denuded after IVM and then IVF in the presence of an equal number (25 + 25) of COC giving two sub-treatments 7a. DOP that were IVF in the presence of COC (DOPCOC) and 7b. COC that were IVF in the presence of DOP (COCDOP). COC and DOP oocytes from treatments 7a and 7b were cultured separately following IVF (mean ± S.E.M.)

47 ± 3.9% of total oocytes. All these values were less (NS) than for the control treatment COC but greater ($P < 0.05$) than for oocytes denuded at collection (DOC). In the treatment where cumulus cells (CC) were added during IVF to oocytes denuded after IVM, the cleavage (76 ± 4.7%) and overall blastocyst production (55 ± 4.3%) rates were greater than for oocytes denuded at collection (DOC; NS) and slightly less than those of the control treatment COC (NS). The effect of adding COC during IVF to oocytes denuded after IVM was similar to adding CC to the same type of oocytes (Table 2). In both cases, the blastocyst production rate was greater than for oocytes denuded during IVF but the differences were not significant. COC that were IVF in the presence of denuded oocytes had similar cleavage and blastocyst production rates to the control treatment (COC).

4. Discussion

The use of heparin as a capacitating agent can help embryo development. The hypothesis for the present experiments was that the presence of cumulus cells during fertilization could improve fertilization rate and help the oocyte during early post fertilization events. For the first time it is reported in goats that using oocytes with cumulus cells during IVF improved developmental potential, as compared to oocytes denuded before IVM.

Greater numbers of blastocysts were obtained from COC fertilized in the presence of heparin than those for COC fertilized without heparin, suggesting that the addition of heparin to the fertilization medium improves sperm capacitation of frozen-thawed goat sperm. These results support those of previous studies demonstrating the role of heparin in goat IVP (Younis et al., 1991; Cox and Alfaro, 2007). Conversely, an adverse effect on fertilization (Malik et al., 1997) or a lesser viability of embryos after transfer (Cognié et al., 1995) was also reported when using this capacitating agent. Poulin et al. (1996) attributed the limited success of IVM/IVF technique in producing live goat offspring to date to the use of heparin for buck sperm. It is noteworthy to describe that in the current study, no positive effect for heparin was observed when oocytes were denuded (DOP) before IVF and that was the procedure used earlier by our group for previous studies (Poulin et al., 1996). Results of the present study showed that the effects of the presence of cumulus cells and the inclusion of heparin in the IVF medium were additive and resulted in improved development of oocytes to blastocysts. The fertilization rate was rapidly improved by adding heparin to the IVF medium at values that varied between 2.5 and 10 μg/mL, depending on the male (Cox et al., 1995). In the present study, a similar concentration was used (5 μg/mL) and the mean cleavage rate was 75% in the COC + heparin groups in both experiments, 21% greater than previously described in goats (Katska-Ksiazkiewicz et al., 2004). However, a much greater concentration of heparin (50 μg/mL) was used, that may have negatively influenced the results in this previous study.

Information available on the role of cumulus oophorus during fertilization and the effect of these cells on subsequent embryo development in goat is currently limited. The appropriate time of cumulus cell removal from oocytes during IVP also remains controversial. We have previously reported that the cumulus oophorus was routinely removed before IVF (Cognié et al., 2004; Rodriguez-Dorta et al., 2007). However as demonstrated in the present study, the presence of cumulus cells and heparin during fertilization allowed better oocyte development than when oocytes were denuded before IVF, even though the cleavage rate was not affected. In a previous study, employing a similar methodology in oocytes of cattle, removal of cumulus cells from COC before IVF decreased the cleavage rate compared with intact COC. As in the present study, on the basis of the total number of oocytes at the onset of culture, the percentage of blastocysts that were formed at Day 9 was less when denuded oocytes were fertilized compared with oocytes fertilized within COC (Fatehi et al., 2005). It is possible that the presence of cumulus cells during fertilization could help the oocyte during early post fertilization events, such as sperm nucleus remodeling, and, in turn, affect developmental competence. Cumulus cells maintain the oocyte under meiotic arrest preventing oocyte ageing (Eppig, 1989), participate in the induction of meiosis by transducing the LH signal to the oocyte (Mattioli and Barboni, 2000), are important for efficient cytoplasmatic maturation of the oocyte (Mori et al., 2000) and protect oocytes against oxidative stress during oocyte maturation (Tatemoto et al., 2000). Cumulus cells also participate to the maintenance of greater concentrations of reduced glutathione in oocyte. This glutathione is necessary for correct processing of sperm chromatin configuration changes after IVF (de Matos et al., 2002). All these positive effects of cumulus cells are probably responsible for the greater blastocyst rates achieved by the oocytes denuded on purpose in comparison to the oocytes already denuded at collection, because the latter underwent IVM without cumulus cells support.

After it was established in the present study, the importance of cumulus cells, it was ascertained whether the possible beneficial effect of cumulus cells during fertilization could result from factors secreted by the cells into the media or if the maintenance of gap junction communication between cumulus cells and oocytes was important for optimal fertilization. The understanding of the nature and diversity of compounds that transfer between the cumulus cells and the oocyte *via* gap junctions is limited (Gilchrist and Thompson, 2007). It is reasonable to assume that a positive effect occurs when denuded oocytes undergo fertilization with cumulus cells added in the same IVF well, because the group of oocytes denuded on purpose cultured with cumulus cells has similar cleavage and blastocyst rates to intact COC when compared to oocytes denuded on purpose. These findings were contrary to those previously reported in cattle, when denuded oocytes fertilized together with cumulus cells exhibited a greater cleavage rate, but the blastocyst rate was not different from that of denuded oocytes fertilized in control medium without cumulus cells support (Fatehi et al., 2005).

In general, a great challenge in research aimed at improving IVP in goats is the lack of adequate numbers of ovaries to obtain good quality oocytes (Rahman et al., 2007). Oocytes that are found denuded at collection are usually considered not suitable for IVM and thus

discarded. In cases of recovering oocytes from live and valuable does by ovum pick up, a labor-intensive process, denuded oocytes that are often discarded could represent a loss of valuable genetic material. Therefore, an experiment was designed to evaluate the possibility of using these oocytes to produce some additional blastocysts from a given female. Blastocyst rate from the COC group reached 59%, whereas only 11% of oocytes found denuded at collection reached the blastocyst stage. Hence, "mathematically" it can be projected that if 50% of each type of oocytes are used an average blastocyst rate of 35% could be obtained if the two groups were mixed equally. However, 45% of oocytes reached the blastocyst stage when COC and oocytes denuded at the collection underwent IVM and IVF together and 40% when COC and denuded oocytes at the collection were matured separately and mixed equally in IVF. Furthermore, when oocytes were matured separately a lesser development rate compared to intact COC was occurred. This indicates that co-culturing denuded oocytes with COC for maturation purposes could be an important tool to improve the quality of matured oocytes and the ability to develop to blastocyst after IVF. However, further research is still necessary to affirm that is possible to rescue the developmental competence of denuded oocytes when using similar methodologies and which steps are determinant and what are the mechanisms involved. A preliminary success was reported for producing goat embryos with ICSI using oocytes of heterogeneous and lesser quality (Rahman et al., 2007). During retrieval of oocytes for IVM, it may be reasonable to propose a new category for grading oocytes because the denuded oocytes with a homogeneous ooplasm may be mixed to COC from IVM, to produce some additional blastocysts.

5. Conclusions

It is concluded that i) the inclusion of heparin in the fertilization medium improves the *in vitro* production of goat embryos ii) the association of oocytes with cumulus cells, either separately or in intimate contact, during maturation and/or fertilization improves *in vitro* production of goat embryos iii) some oocytes that are already denuded at collection will develop satisfactorily to blastocysts when matured and fertilized in the presence of intact cumulus-oocyte complexes. The present study has demonstrated a substantive advance in goat IVF and high rates of blastocyst were achieved depending on the treatment.

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