Prolonging the interval from ovarian hyperstimulation to laparoscopic ovum pick-up improves oocyte yield, quality, and developmental competence in goats

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Abstract

The objective was to evaluate the effect of the interval between ovarian hyperstimulation and laparoscopic ovum pick-up (LOPU) on quality and developmental competence of goat oocytes before and after in vitro maturation (IVM) and intracytoplasmic sperm injection (ICSI). Estrus was synchronized with an intravaginal insert containing 0.3 g progesterone (CIDR) for 10 d, combined with a luteolytic treatment of 125 μg cloprostenol 36 h prior to CIDR removal. Ovaries were hyperstimulated with 70 mg FSH and 500 IU hCG given im 36, 60, or 72 h prior to LOPU (n = 15, 16, and 7 does, respectively). For these groups, oocyte retrieval rates (mean ± S.E.M.) were 24.7 ± 2.9, 54.5 ± 4.7, and 82.8 ± 4.6% (P < 0.001), and the proportions of cumulus-oocyte complexes (COC) with more than five layers of cumulus cells were 29.7 ± 8.3, 37.6 ± 6.9, and 37.3 ± 7.0% (P < 0.001). The proportion of IVM oocytes was highest at 72 h (82.1 ± 2.8%; P < 0.05), with no significant difference between 36 and 60 h (57.3 ± 8.9% and 69.0 ± 8.4%). Cleavage rates of ICSI embryos were 4.2 ± 4.2, 70.9 ± 8.4, and 78.9 ± 8.2% with LOPU 36, 60, and 72 h post FSH/hCG (P < 0.01), with a lower proportion of Grade-A embryos (P < 0.05) following LOPU at 36 h compared to 60 and 72 h (29.7 ± 8.3%, 37.6 ± 6.9%, and 37.3 ± 7.0%). In summary, a prolonged interval from FSH/hCG to LOPU improved oocyte retrieval rate and oocyte quality. Therefore, under the present conditions, LOPU 60 or 72 h after FSH/hCG optimized yields of good-quality oocytes for IVM and embryo production in goats.

Keywords: Oocyte; Embryo; Laparoscopic ovum pick-up; Goat; Developmental competence

1. Introduction

In small ruminants, collection of oocytes after exposure of the ovary by laparotomy has been gradually replaced by laparoscopic puncture of follicles and aspiration of their content. Snyder and Dukelow [1] first described laparoscopic ovum pick-up (LOPU) in sheep; however, the technique was not fully developed until 20 years later in conjunction with development of IVP technologies for livestock production [2–4]. A substantial advantage of LOPU over laparotomy is that the same donor can be used repeatedly, as the procedure is minimally invasive and post-operative recovery is much faster. Moreover, LOPU in combination with IVP of embryos increased the number of offspring produced by
genetically valuable does [3] and enabled production of offspring from does unable to reproduce using AI or multiple ovulation-embryo transfer (MOET), such as prepubertal or aged goats [3,5–8]. Furthermore, LOPU can also overcome limitations frequently associated with MOET, including poor ovulation rate, premature luteal regression, and poor fertilization rates. Despite variations in response to superovulation, LOPU consistently yielded oocytes [2,7,9–14], and may be done repeatedly without ovarian damage or decreased donor fertility [13–15]. Furthermore, with hormonal treatments and LOPU, oocyte retrieval can be maximized (without affecting oocyte quality) as frequently as once weekly [12–14,16,17]. In superovulated goats, oocyte recovery rates ranged from 33 to 80%, with average yields that ranged from 5.6 to 13.4 oocytes per animal [7,9–13,15–17].

The mammalian oocyte acquires the ability to mature and accomplish its developmental competence during oogenesis and folliculogenesis; the proportion of developmentally competent oocytes increases with follicular size [18–22]. Consequently, the timing of ovum pick-up (OPU) affects oocyte developmental competence, as OPU disrupts the oocyte’s in vivo maturation process. In humans [23,24], monkeys [25,26] and pigs [27], OPU was performed 36 h after hCG injection; the majority of the oocytes collected were at metaphase II and were meiotically and developmentally competent. However, goat oocytes collected 36 h after eCG treatment (a common time for collection of oocytes from live donors) were still at the immature stages and required in vitro maturation for 27 h before reaching meiotic competence [10]. Remarkably, there are apparently no published studies regarding the effect of a longer interval (>36 h) between FSH/hCG treatment and LOPU on the quality and developmental competence of goat oocytes. Therefore, the objectives of the present study were to determine the: (1) effect of various fixed intervals between FSH/hCG treatment and LOPU on the quality and developmental competence of goat oocytes; (2) developmental competence of these oocytes after in vitro maturation (IVM) and intracytoplasmic sperm injection (ICSI).

2. Materials and methods

2.1. Chemicals, reagents and media

All chemicals and media used were from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. For all media, the pH was adjusted to 7.2–7.4 and osmolarity to 280 mOsm/kg.

2.2. Goats

A total of 38 does of mixed local breeds were used. These does were housed on a farm within the university and were fed good-quality Napier grass and pellets, with ad libitum access to water.

2.3. Estrus synchronization and ovarian hyperstimulation

Estrus was synchronized with an intravaginal controlled internal drug release device (CIDR, progesterone, 0.3 g; Pharmacia and Upjohn Ltd., Auckland, New Zealand) for 10 d, with a cloprostenol (Estrumate, 125 µg; Schering-Plough, NSW, Australia) 36 h prior to CIDR removal. At device removal, does were given im treatments of Ovagen (FSH, 70 mg; Immuno Chemicals Products Ltd., Auckland, New Zealand) and Ovidrel (hCG, 500 IU; Serono, Switzerland), and were randomly allocated to undergo LOPU 36, 60, or 72 h later.

2.4. Anesthesia

Feed was removed 24 h prior to LOPU. Anesthesia was induced with 0.22 mg/kg xylazine hydrochloride (Ilium Xylazine-20, Troy Laboratories Pty Ltd., NSW, Australia) given im.

2.5. LOPU

The abdomen and inguinal regions were shaved and scrubbed with Hibiscrub and surgical iodine was applied to the surgical sites. The LOPU procedure was performed with a 4-mm Storz laparoscope (Karl Storz Endoscopes GmbH & Co., Tuttlingen, Germany) attached to a video system. A pneumoperitoneum was created and small incisions (3–5 mm) were made, one on the right and two on the left sides of the lower abdomen to enable trocar insertion. The laparoscope was inserted into the right side of the abdomen (through the trocar sheath). A pediatric grasper and the ovum pick-up needle (FAS Set C2, Gynetics Medical Product, N.V. Hamont-Achel, Belgium) were inserted into the left side of the abdomen. The ovary was held by the grasper and ovarian follicles were individually punctured, flushed, and aspirated by the OPU needle, which was connected to the Cook aspiration and flushing system (V-MAR 5100 and V-Mar 4000, respectively; Cook Australia, Eight Mile Plains, Queensland, Australia). Follicles were classified as small or large (2–3 and >3 mm in diameter, respectively; only large
follies were aspirated. Dulbecco phosphate-buffered saline (DPBS, Dulbecco A, BR0014G, Oxoid Limited, Hampshire, UK) supplemented with gentamycin sulfate (50 μg/mL, G1272, Sigma) and heparin (100 μg/mL, H0777, Sigma) was used as a follicle flushing and oocyte aspiration medium.

2.6. Oocyte retrieval and grading

The cumulus-oocyte complexes (COC) and cumulus-free oocytes (CFO) were collected and rinsed in warm DPBS at 38.5 ºC. The COC were visually assessed and graded according to their cumulus-corona cell investments and morphology of the ooplasm, as described by Rahman et al. [28] (Table 1). Healthy COC or CFO with finely granulated and homogeneous ooplasm (Grades A, B, C, and D) were selected for IVM.

2.7. In vitro maturation

The selected COC and CFO were washed three times in IVM medium, which consisted of M199 with Earle’s salt, sodium bicarbonate, and L-glutamine (Sigma, M-4530) as a base medium, supplemented with ovine LH (10 μg/mL; Sigma L5269), ovine FSH (10 μg/mL; Ovagen), 17β-estradiol (1 μg/mL; Sigma E2257), sodium pyruvate (275 μg/mL; Sigma P3662), gentamycin sulfate (50 μg/mL; Sigma G3632), cysteamine (100 μM; Sigma M9768) and 10% (v/v) heat-inactivated estrus goat serum (EGS). Groups of 10 COCs and CFOs were matured in 100 μL of IVM medium overlaid with light mineral oil (Sigma M8410) for 27 h at 38.5 ºC and 5% CO2 in air.

2.8. Assessment of oocyte maturation

After IVM, COC were treated with 100 μL of hyaluronidase (80 IU/mL) in HEPES-buffered modified synthetic oviductal fluid (mSOF) medium [29]. The COC were visualized under a dissecting microscope and the cumulus-corona cells were carefully stripped off the zona pellucida by sequential denuding through glass pipettes of 250 and 200 μm outer and inner diameters, respectively. Denuded oocytes were assessed (inverted microscope) for maturation. Oocytes with a clear first polar body (PB) were considered as mature and meiotically competent. Mature oocytes with a finely granulated homogenous cytoplasm were selected and cultured in an incubator (5% CO2 and 38.5 ºC) prior to ICSI.

2.9. Sperm preparation

Sperm motility was evaluated under an inverted microscope and motile sperm were selected by swim-up. Briefly, 500 μL of frozen buck semen from the Jermasia breed (a breed developed at the university) of proven quality was thawed and incubated at 38.5 ºC in a humidified atmosphere of 5% CO2 for 60 min in 2 mL of mSOF medium supplemented with 20% (v/v) goat serum (GS). After incubation, the supernatant was removed and centrifuged at 200 × g for 10 min. The sperm pellet was washed twice and re-suspended with an equal volume of mSOF medium containing heparin (50 μg/mL, Sigma, H0777) and incubated for another 15 min in a humidified atmosphere of 5% CO2 at 38.5 ºC.

2.10. ICSI

The ICSI procedure was performed on an Olympus IX71 inverted microscope fitted with Narishige hydraulic micromanipulators (Narishige Scientific Instrument, Tokyo, Japan). The holding pipettes were made from borosilicate glass tubing of 1.0 mm outside diameter (OD) using a Sutter Micropipette Puller (P-97, R.B. Abdullah et al. / Theriogenology 70 (2008) 765–771

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>COC with more than five complete layers of cumulus oophorus and corona radiata (cumulus-corona) cells and finely granulated homogeneous ooplasm</td>
<td>A</td>
</tr>
<tr>
<td>COC with 3–5 complete layers of cumulus-corona cells and finely granulated homogeneous ooplasm</td>
<td>B</td>
</tr>
<tr>
<td>COC with 1–2 complete layers of cumulus-corona cells or COC with 3–5 partially invested cumulus-corona cell layers and finely granulated homogeneous ooplasm</td>
<td>C</td>
</tr>
<tr>
<td>Cumulus-free oocytes (CFO) or oocyte with incomplete investment of cumulus-corona cells (1–2 layers) and finely granulated homogeneous ooplasm</td>
<td>D</td>
</tr>
<tr>
<td>Degenerating oocyte or oocyte with abnormal size, shape and heterogeneous ooplasm or apoptotic oocytes in jelly-like cumulus-corona cells investment</td>
<td>E</td>
</tr>
</tbody>
</table>

Source: Rahman et al. [28].
Sutter Instrument Co., Novato, CA, USA), in combination with a Narishige microforge (Model MF-9) and Narishige microgrinder (Model EG-4, Narishige Scientific Instrument, Tokyo, Japan). The holding pipettes were pulled to an OD of 150 \(\mu\)m and then fire-polished to create an ID of 30–40 \(\mu\)m, with pipette tips bent to an angle of 35°. Injection needles (Humagen, Charlotte-ville, VA, USA) had an ID of 6–7 \(\mu\)m and OD of 8 \(\mu\)m, with a sharp spike and a tip bent at 35°.

An aliquot (1–2 \(\mu\)L) of washed sperm suspension was added to a 5 \(\mu\)L elongated microdroplet of ICSI medium (HEPES-buffered mSOF medium) prepared on the left side of the ICSI dish. A motile sperm was aspirated into the injection needle and brought into a microdroplet containing 10% polyvinylpyrrolidone (PVP), located in the center of the dish. The sperm was immobilized by crushing its tail against the bottom of the dish with the injection needle. The immobilized sperm was then aspirated, tail-first, into the injection pipette. A mature oocyte was orientated with its first PB at the 6 or 12 O’clock position, by the holding pipette in a microdroplet located on the right side of the ICSI dish. The sperm was injected head-first, into the ooplasm of the oocyte (at the 3 O’clock position). During ICSI, ooplasm was aspirated into the injection needle to break the oolemma (and ensure that the needle was in the ooplasm). The sperm was then injected into the ooplasm with a minimal volume (<5 pL) of PVP.

2.11. IVC

Injected oocytes were cultured in vitro in mSOF medium supplemented with 10% heat-inactivated goat serum in 5% \(CO_2\) at 38.5 °C. After 48 h of IVC, presumptive zygotes were cultured in freshly prepared mSOF medium and embryos were cultured for <192 h. Once daily, embryos were assessed and their developmental stage recorded.

2.12. Embryo transfer

A laparoscopic tubal embryo transfer (TET) technique was used, with a home-made embryo transfer (ET) catheter, a flexible polythene tubing (OD and ID of 1.09 and 0.38 mm, respectively), threaded through a 25-G hypodermic needle. Briefly, a 1-mL insulin syringe (filled with mSOF medium) was connected to the ET catheter and the catheter was flushed until 0.001 mL of medium was left in the syringe. A small column of air (5 mm) was then aspirated into the catheter. Embryos (3–5) embryos were then aspirated from the well of a four-well Nunc dish, resulting in a 10-mm column

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Mean (±S.E.M.) quantity and quality of caprine oocytes retrieved, according to the interval between FSH/hCG treatment and laparoscopic ovum pick-up (LOPU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interval (h)</td>
<td>No. of goats</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>36</td>
<td>15</td>
</tr>
<tr>
<td>60</td>
<td>16</td>
</tr>
<tr>
<td>72</td>
<td>7</td>
</tr>
<tr>
<td>72</td>
<td>7</td>
</tr>
<tr>
<td>96</td>
<td>7</td>
</tr>
<tr>
<td><strong>Within a column, means without a common superscript differed (</strong>(P &lt; 0.001)<strong>); (n = total number of oocytes used.</strong></td>
<td></td>
</tr>
</tbody>
</table>
containing the embryos and the medium, followed by another 5-mm air column. The catheter was threaded through the trocar, gently guided into the oviduct, and the embryos expelled into the oviduct by completely depressing the plunger of the syringe.

2.13. Experimental design

A total of 15, 16, and 7 healthy goats were selected and following ovarian hyperstimulation, were randomly allocated to undergo LOPU at 36, 60, or 72 h after FSH/hCG, respectively. Although 15 goats were intended for LOPU at 72 h post FSH/hCG, only seven healthy animals were selected, due to a problem with health status at the time that the study was conducted. Follicle aspiration, oocyte recovery, maturation and embryo development were recorded. The experiment was replicated at least three times for each treatment.

2.14. Statistical analysis

The effect of interval before LOPU on oocyte quality and developmental competence was determined with one-way ANOVA; significant differences between the means were located with Duncan’s Multiple Range Test. Analyses were carried out with SPSS (Statistical Packages for Social Sciences) for Windows, Version 11.5, SPSS Inc. (2002), Chicago, IL, USA. All data are presented as mean ± S.E.M.

3. Results

There was no significant effect of treatment on the number of follicles aspirated per goat (Table 2). However, the proportion of oocytes retrieved increased (P < 0.001) as the interval from FSH/hCG to LOPU was more prolonged. The fewest oocytes per goat were retrieved at 36 h, and the proportion of Grade-A oocytes was greater (P < 0.001) as the interval from FSH/hCG to LOPU increased (Table 3). Similarly, rates of maturation, cleavage, and morula formation increased significantly as the interval from hormonal treatment to LOPU was prolonged (Table 4). A total of 24 morulae were transferred into the oviducts of five surrogate does (3–5 embryos per doe), but no pregnancy was obtained.

4. Discussion

The data obtained in this study indicated the usefulness of the ovarian hyperstimulation protocol using FSH/hCG and LOPU for OR in goat. In the present study, the number of visible follicles and OR rate seemed better than previous findings in our laboratory [28], especially with 60 and 72 h intervals between FSH + hCG treatment and LOPU. The yields of COC in this study seemed similar to previous reports of 10.4–14.7 follicles/goat and oocyte retrieval rates from 33 to 80% [9–13,15–17]. Using a lower time interval of 24 h between FSH + eCG and LOPU Gibbons et al. [12] retrieved 5.6–8.0 oocytes per doe

Table 3
Mean (±S.E.M.) in vitro maturation of caprine oocytes, according to the interval between FSH/hCG treatment and laparoscopic ovum pick-up

<table>
<thead>
<tr>
<th>Interval (h)</th>
<th>No. of oocytes cultured</th>
<th>No. of oocytes died during IVM</th>
<th>Survival rate (%)</th>
<th>Maturation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>44</td>
<td>0</td>
<td>100.0 ± 0.0 (n = 44)</td>
<td>57.3 ± 8.9ab (n = 24)</td>
</tr>
<tr>
<td>60</td>
<td>89</td>
<td>4</td>
<td>94.8 ± 2.3 (n = 85)</td>
<td>69.0 ± 8.4ab (n = 57)</td>
</tr>
<tr>
<td>72</td>
<td>95</td>
<td>5</td>
<td>88.8 ± 3.2 (n = 90)</td>
<td>82.1 ± 2.8b (n = 76)</td>
</tr>
</tbody>
</table>

a,bWithin a column, means without a common superscript differed (P < 0.001); n = total number of oocytes used.

Table 4
Embryo development (mean ± S.E.M.) of in vitro matured caprine oocytes microinjected with frozen-thawed sperm, according to the interval between FSH/hCG treatment and laparoscopic ovum pick-up

<table>
<thead>
<tr>
<th>Interval (h)</th>
<th>No. injectedA</th>
<th>Survival rate (%)</th>
<th>Fertilization rate (%)</th>
<th>Cleavage rate (%)</th>
<th>Morula (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>19</td>
<td>81.3 ± 12.0 (n = 15)</td>
<td>85.4 ± 8.6 (n = 11)</td>
<td>4.2 ± 4.2a (n = 1)</td>
<td>0a (n = 0)</td>
</tr>
<tr>
<td>60</td>
<td>50</td>
<td>75.8 ± 5.8 (n = 37)</td>
<td>80.3 ± 6.6 (n = 29)</td>
<td>70.9 ± 8.4b (n = 21)</td>
<td>9.5 ± 4.6b (n = 3)</td>
</tr>
<tr>
<td>72</td>
<td>74</td>
<td>84.6 ± 5.7 (n = 62)</td>
<td>74.7 ± 12.6 (n = 48)</td>
<td>78.9 ± 8.2b (n = 45)</td>
<td>32.1 ± 15.0b (n = 21)</td>
</tr>
</tbody>
</table>

AWithin a column, means without a common superscript differed (P < 0.01 for cleaving embryos and P < 0.05 for morula). n = total number of oocytes or embryos.

A SHAM test controls were performed with 5, 7 and 2 IVM oocytes from 36, 60, and 72 h post FSH/hCG groups. None of these oocytes were activated by the microinjection with ICSI medium only. Few IVM oocytes were used for SHAM test because of small number of IVM oocytes available for ICSI.
and 5.5–8.8 oocytes per ewe. Therefore, increasing the interval between FSH/hCG and LOPU significantly improved both the mean number of oocytes retrieved per goat and rates of retrieval.

The capacity of the oocyte to acquire developmental competence occurs during the final phases of follicular development [20]. Oocyte developmental competence is the capacity of a mature oocyte to support fertilization, preimplantation embryo development and implantation [30]. This developmental ability is also associated with cumulus expansion, which increases with follicle size and decreases with increasing granulosa cell atresia [19,20,22,31–35]. Visual assessment of morphological features remains the most important criterion for selection of COCs before maturation to select developmentally competent goat oocytes during oocyte retrieval. Therefore, COC were graded according to the cumulus-corona cell investments and appearance of the cytoplasm. Oocyte quality improved when LOPU was performed at 60 h versus 36 h after FSH/hCG treatment. However, delaying LOPU to 72 h post-FSH/hCG treatment did not improve the mean percentage of good quality oocytes relative to LOPU at 60 h; perhaps a more prolonged interval to LOPU increases oocyte atresia.

In this study, it was noteworthy that increasing the time interval between FSH/hCG treatment and LOPU had a positive effect on IVM of oocytes, as evidenced by higher maturation rates at 60 and 72 h compared with 36 h. Although higher number of oocytes were retrieved and cultured at 72 than 60 h group, there was no significant difference in maturation rates between 60 and 72 h groups. That there was less maturation in the group retrieved 36 h post-FSH/hCG group was probably because oocytes had not acquired full meiotic competence and cytoplasmic maturation. The present goat oocyte maturation rate was higher than our previous study [28]. The present maturation rates obtained from 60 and 72 h FSH/hCG and LOPU groups were nearly similar with that of Gall et al. [36] and Samaké et al. [37] who obtained 96 and 100% maturation rates from hormonally treated and estrus synchronized slaughtered does, but not from LOPU. In this study, delaying LOPU enhanced not only IVM of oocytes, but also improved their ability to develop into embryos. Most oocytes developed to the 8 to 16-cell stage, but few formed a morula or blastocyst. A few embryos were obtained from oocytes retrieved 36 h after FSH/hCG, but the yield of morulae was significantly less than those from 60 or 72 h post FSH/hCG. Additional efforts to improve IVM and IVC conditions for production of goat embryos is on-going in our laboratory, including IVM of oocytes by co-culture with goat oviduct epithelial cells or granulosa cells, and production of embryos from dysmorphic goat oocytes through ICSI.

In summary, a prolonged interval from FSH/hCG to LOPU improved oocyte retrieval rate and oocyte quality in goats. Under the present conditions, LOPU at 60 or 72 h after FSH/hCG optimized yields of good quality oocytes for IVM and embryo production.

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