Influence of *in vitro* maturation duration on embryo yield in goats

Phua, A.C.Y., Rahman, M.M., Wan Khadijah, W.E. and Abdullah*, R.B.
Animal Biotechnology-Embryo Laboratory (ABEL), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
*Corresponding author: ramli@um.edu.my

Abstract

Oocytes retrieved from goat ovaries are usually still in immature stage and thus, are not ready for fertlisation. Hence, the oocytes need to be cultured in maturation medium for certain duration before they fertilise. In this experiment, the effect of *in vitro* maturation duration on laparoscopic oocyte pick-up (LOPU)-derived caprine oocytes towards embryo yield (4-cell stage taken as the minimum criterion) was investigated. Separate batches of oocytes were matured for 18 to 20 h, 21 to 23 h, 24 to 26 h and 27 to 29 h, respectively, at 38.5°C under 5% CO_2 in humidified air before they were *in vitro* fertilised and cultured. LOPU-derived oocytes matured for 18 to 20 h improved ($p<0.01$) embryo yield (42.6%). In conclusion, appropriate IVM duration will improve the *in vitro* fertilsation performance in goats.

Keywords: *in vitro* maturation, goat, oocytes, developmental potential

Introduction

The first successful live birth took place from the transfer of *in vitro* matured and fertilised goat oocytes as reported by Crozet *et al*. (1993). Despite improvements made to *in vitro* maturation (IVM) technology over the recent decade, *in vitro* maturation (IVM) is still widely regarded as one of the significantly rate-limiting technologies in that efficiency of blastocyst development remains low (Rho *et al*., 2001; Teotia *et al*., 2001). In IVM technology, many researchers have investigated various factors that influence the ability of oocytes to resume meiosis and reach maturation such as the biochemical regulators of nuclear and cytoplasmic maturation (Park *et al*., 2004), follicle size (Crozet *et al*., 1993), reproductive status of donor goats (Mogas *et al*., 1997), follicle stimulating hormone (FSH)-priming (Mogas *et al*., 1997), cysteamine (Cognie *et al*., 2003) and vitamins (Bormann *et al*., 2003) supplementation, method of culture (Mogas *et al*., 1997), and IVM duration (Rho *et al*., 2001; Cognie *et al*., 2003; Herrick *et al*., 2004). In spite of all attempts made to optimise goat IVM culture conditions, the duration required for goat oocytes to reach Metaphase II still remains an unresolved problem. Knowing the right IVM duration facilitates investigators in timing the preparation of sperm for subsequent insemination. Sperm prepared way in advance before oocyte maturation may result in unsubstantial sperm motility count by the time the oocytes reach maturation, and if prepared a little after that may result in delay due to the amount of time needed for sperm to capacitate and the subsequent hardening of zona pellucida as the oocyte ages (Landim-Alvarenga *et al*., 2002). Various IVM durations for goat *in vitro* embryo production (IVP) ranging from as short as 16 h to as long as 30 h had been documented in the literature with differing results (Rho *et al*. 2001; Cognie *et al*. 2003; Herrick *et al*. 2004). However, most of these studies merely described IVM duration as part of

their IVP practices rather than considering factors affecting the IVM duration such as sources of oocytes from abattoir and LOPU. However, to date, the precise mechanism on how enzymes, factors and chemicals capacitate sperm remains unclear. Thus, this investigation aimed to study the effect of IVM duration on embryo yield in goats. The oocytes matured according to different ranges of duration were *in vitro* fertilised, cultured and then evaluated for embryo yield.

Materials and Methods

Oestrus synchronisation, superovulation and laparoscopic oocyte pick-up (LOPU)

The donor goats used in this study were primarily of mixed Katjang parentage and had already reached sexual maturity. To synchronize oestrous cycle, a controlled internal drug release (CIDR) dispenser was inserted into the vagina and remained for 14 d. At 24 h prior to the removal of CIDR, cloprostenol (Estrumate®, Schering-Plough Animal Health, Australia) was administered intramuscularly at a dose of 125 µg to simulate an oestrogen rush for a rapid and highly visible onset of heat. Upon removal of CIDR, the donor goat was superovulated by administering FSH (70 mg/doe) (Ovagen™, ICPbio, New Zealand) and human chorionic gonadotropin (hCG) (500 IU/doe) (Profasi, Serono Diagnostic, Switzerland). At d 2 after the administration of FSH and hCG, the donor goat was observed for onset of oestrus by a teaser buck. The onset of oestrus marked the urgency to initiate LOPU procedure. The donor goat was sedated and anaesthetised. Anaesthetisation was subsequently maintained while LOPU was in progress. With the grasper holding an ovary, all visible follicles were punctured and the follicular content aspirated via the oocyte retrieval needle.

In vitro maturation (IVM) duration

The effect of IVM duration on LOPU-derived caprine oocytes towards embryo yield (4-cell stage taken as the minimum criterion) was investigated. The LOPU-obtained oocytes were washed 4 times in maturation medium (Ongeri *et al*., 2001) before transferring them to final droplets of maturation medium overlaid with silicone oil. The maturation medium was M199 (Gibco, USA) supplemented with FSH (3 µg/ml), LH (3 µg/ml), oestradiol-17β (1 µg/ml), gentamycin sulphate (25 µg/ml), sodium pyruvate (0.22 mM) and heat-inactivated oestrus goat serum (10% v/v). Separate batches of oocytes were matured for 18 to 20 h, 21 to 23 h, 24 to 26 h and 27 to 29 h, respectively, at 38.5 °C under 5% CO_2 in humidified air before they were *in vitro* fertilised and cultured.

We confined to the normally suggested range of 18-30 h in this study because various IVM durations for goat IVP had been reported as short as 16 h and as long as 30 h; and there had been much debate among researchers as to the duration required for caprine oocytes to reach Metaphase II (Martion *et al*., 1994; Mogas *et al*., 1997; Yadav *et al*., 1997; Rho *et al*., 2001; Bormann *et al*., 2003; Cognie *et al*., 2003; Herrick *et al*., 2004). We revisited the effect of only IVM duration on LOPU-derived caprine oocytes towards embryo yield regardless of other factors. It should be borne in mind that other factors such as follicle size, maturation treatments, sperm preparation and culture systems are all known variables that can affect overall embryo yield (Crozet *et al*., 1995; Keskintepe *et al*., 1996; Pawshe *et al*., 1996; Koeman *et al*., 2003).

Sperm preparation and in vitro fertilisation (IVF)

All sperm preparation was initiated at approximately 1.5 h prior to the end of each of the four batches of IVM duration. The source of semen was from Boer bucks of proven fertility. Three 500-µL straws of frozen sperm suspension were required to obtain sperm microdroplets with sperm density of approximately 1×10^6 to 2×10^6 sperm/mL. All three straws were thawed in a water bath (39 °C, approximately 3 to 4 min) and the thawed semen released into a sterile 15-mL tube. Then, Brackett-Oliphant (BO)-sperm preparation medium was added to the semen to make a total volume of 8 mL. The BO sperm preparation medium consisted of BO medium supplemented with $NaHCO_3$ (37 mM), D-(+)-glucose (13.9 mM), sodium pyruvate (1.25 mM) and gentamycin sulphate (50 µg/mL). The semen was washed twice with the BO-sperm preparation medium via centrifugation (300 × g, 25°C, 5 min). After the supernatant was discarded during the final centrifugation step, BO-IVF medium (330 µL) was gently layered on the surface of the sperm pellet and the tube loosely capped. The BO-IVF medium consisted of BO-sperm preparation medium supplemented with theophylline (5 mM), heparin (25 µg/mL) and heat-inactivated oestrus goat serum (10% v:v). While taking care not to agitate the sperm pellet, the tube was placed at approximately 60° angle to horizontal surface and incubated (38.5 °C, 5% CO_2 in humidified air, 1 h) to allow sperm swim-up and capacitation. After the 1 h sperm swim-up and capacitation, the top layer of the swim-up was used to prepare sperm microdroplets of 80 µL each in a sterile 60-mm culture dish. The dish was then immediately flooded with equilibrated silicone oil. The oocytes (from the IVM culture dish) were washed four times in equilibrated BO-IVF medium to wash off the IVM medium and then transferred to the sperm microdroplets for fertilisation. Each 80 µL sperm suspension droplet contained not more than five oocytes. The dish was incubated (38.5°C, 5% CO_2 in humidified air, 10 h) to allow fertilisation. Whatever that was left from the top layer of the sperm swim-up was used for sperm density assessment via haemacytometer to counter-check that the sperm microdroplets prepared had sperm density of approximately 1×10^6 to 2×10^6 sperm/mL.

In vitro embryo culture (IVC)

After sperm-oocyte incubation (38.5°C, 5% CO_2 in humidified air) for approximately 10 h, the presumptive zygotes were transferred into a 4-well dish containing equilibrated synthetic oviductal fluid (SOF)-*in vitro* culture medium for washing. The SOF-IVC medium consisted of SOF medium (Keskintepe *et al*., 1998) supplemented with heat-inactivated oestrus goat serum (10% v/v). During the washing step, the presumptive zygotes were pipetted in and out repeatedly to dislodge as much cumulus masses and residue sperm as possible which adhered to the zona pellucida. Finally, the presumptive zygotes were transferred into a 60-mm culture dish containing equilibrated 100 µL droplets of SOF-IVC medium overlaid with silicone oil. Each droplet contained not more than five presumptive zygotes. Under an inverted microscope, each presumptive zygote was observed for presence of two polar bodies. The culture dish containing the presumptive zygotes was then incubated (38.5°C, 5% CO_2 in humidified air) to allow further *in vitro* culture. The daily developmental progress of the presumptive zygotes was monitored. The embryos were transferred to fresh equilibrated 100 µL droplets of SOF-IVC medium overlaid with silicone oil once in every two d.

Statistical analysis

The number of embryos reaching at least the 4-cell stage was recorded and the percentage of embryo yield determined. Statistical differences between proportions of oocytes treated in different IVM duration groups were analyzed using Fisher's exact test in GraphPad Instat Demo (GraphPad Software Inc., USA).

Results and Discussion

Goat oocytes *in vitro* matured for 18 to 20 h improved ($p<0.01$) embryo yield (20/47; 42.6%) (Table 1). This result suggests that most goat oocytes require 18 to 20 h of maturation before they are ready to be fertilised. From 21 h onwards, premature hardening of the zona pellucida may have occurred which makes sperm penetration much more difficult (Landim-Alvarenga *et al*., 2002). The IVM duration result is in agreement with Herrick *et al*. (2004) who reported that the proportion of goat oocytes reached Metaphase II peaks (70 to 80%) at 18 to 20 h of maturation. In addition, Cognie *et al*. (2003) reported that the extrusion of the first polar body at Metaphase II occurred between 16 to 24 h after the start of maturation. In a study on cytoplasmic ultrastructural changes of goat oocytes during meiosis, Rajikin *et al*. (1994) reported that goat oocytes may have attained fertilisable stage after IVM for 20 h in tissue culture medium 199 (TCM199). However, Martino *et al*. (1994) deduced from their result and investigation by others (Song and Iritani, 1987; Le Gal *et al*., 1992) that the time necessary for goat oocytes to reach Metaphase II is about 27 h, independent of the physiological stage of the animal. Rho *et al*. (2001) also confirmed that 27 h of IVM for goat oocytes resulted in significantly higher percentages of nuclear maturation than did 20 or 24 h. On the contrary, Younis *et al*. (1991) reported the best IVM duration to be 24 h. In a study based on chromosomal sequential configuration, Yadav *et al*. (1997) found that the optimal duration of IVM for goat oocytes was 30 h. It is cautionary to consider that the different reports in optimal IVM duration of goat oocytes could probably be due to simultaneous influence of culture media components (Cognie *et al*., 2003), culture conditions (Cognie *et al*., 2003), follicle size (Rho *et al*., 2001) and follicle developmental stage (Yadav *et al*., 1997).

The presented figures for the number of embryos obtained and percentage of embryo yield could have been higher. In the literature, only oocytes with at least one cumulus layer (for example, Ongeri *et al*., 2001), regular round shape and evenly granulated for LOPU and thus scarcity of oocyte samples, naked oocytes (but with regular round shape and evenly granulated cytoplasm as minimum criteria) were also included in this study. Therefore, the percentage of success in embryo yield may be diluted to a certain extent. In conclusion, for *in vitro* production of caprine embryos, LOPU-derived oocytes matured for 18 to 20 h significantly improved embryo yield (42.6%), while abattoir-derived oocytes matured for 22 to 26 hr as reported by Kwong (2012).

Table 1: Cleavage rate of embryos according to *in vitro* maturation (IVM) duration after *in vitro* fertilisation

IVM duration (h)	No. of oocytes	4-cell embryos obtained, % (n)
18 to 20	47	42.6[b] (20)
21 to 23	20	5.0[a] (1)
24 to 26	40	5.0[a] (2)
27 to 29	49	14.3[a] (7)

n: number of embryos; abMeans within a column with different superscripts were significantly different ($p<0.05$).

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