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The effects of *in vitro* maturation duration on maturation rate and intracytoplasmic sperm injection-derived embryonic development in goats

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ABSTRACT

The aim of the present study was to evaluate the effects of *in vitro* maturation (IVM) duration on maturation rate and intracytoplasmic sperm injection (ICSI)-derived embryonic development in goat embryos. Donor goats were superovulated following oestrus synchronisation prior to laparoscopic oocyte pick-up. The quality of oocytes was scored based on cumulus cell layers, which were graded A, B and C. The oocytes were cultured in IVM medium with two different IVM durations (18-21 h and 22-25 h) for the ICSI procedure. A total of 327 matured oocytes were used, of which 157 and 170 oocytes were used for 18-21 h and 22-25 h IVM duration, respectively. The embryo cleavage rate of Grade A from the 18-21 h IVM group was significantly ($P<0.05$) higher than that of grades B and C. However, in the 22-25 h IVM duration group, the cleavage rates for all grades of oocytes were not significantly ($P>0.05$) different. Regardless of oocyte grades, no significant differences in maturation rates and cleavage rates for all stages of embryonic development between the two groups of IVM durations were observed. The results suggest that both IVM durations have the same potential in ICSI-derived embryonic development.

Key words: Embryonic development, Goats, Intracytoplasmic sperm injection, Maturation rate of oocyte.

INTRODUCTION

The intracytoplasmic sperm injection (ICSI) technique is well known and widely applied as an assisted reproductive technology (ART) to overcome male infertility problems in various animal species (Kimura and Yanagimachi, 1995; Palermo *et al.*, 1992; Wang *et al.*, 2003). There are numerous factors affecting the performance of ICSI, such as quality of sperm, grade of oocyte, culture medium and oocyte activation (Williams, 2002; Zheng *et al.*, 2004; Kato and Nagao, 2009; Nakagawa *et al.*, 2001). Currently, the ICSI performance in goats is low, although information on this area is very limited (Rahman, 2008).

Good-quality oocytes can be obtained from does for ART research studies, using the laparoscopic ovum pick-up (LOPU) procedure. LOPU is a minimally invasive technique and offers faster post-operative recovery than standard laparotomy (Tibary *et al.*, 2005); it is also reported to be an efficient method of providing oocytes (Abdullah *et al.*, 2008; Kong, 2010). Katska-Ksiazkiewicz *et al.* (2007) reported that oocyte quality might influence the success of *in vitro* production of goat embryos. Several researchers obtained a better maturation rate when they cultured oocytes with at least four complete layers (Jimenez-Macedo *et al.*, 2007), two or more complete layers (Rho *et al.*, 2001) or one or two complete layers (Wang *et al.*, 2003) of cumulus cells. The duration of *in vitro* maturation (IVM) may affect fertilisation rates after intracytoplasmic sperm injection (ICSI). For example, oocytes that undergo IVM with a duration time of 22-25 h had a greater survival rate than the longer conventional IVM duration of 27-32 h (Kong, 2010). These findings show that the delayed injection of ICSI may contribute to oocyte ageing, failure of male pronuclear formation and a reduction in the fertilisation rate (Zheng *et al.*, 2004). Maturation duration may be a significant determinant for maximal *in vitro* embryo survival after the ICSI procedure.

At present, the information on this IVM duration in goats is scarce and controversial; it should therefore be clarified and understood before the ICSI procedure in goats can be recommended for routine use in research laboratory projects or application in the goat industry. The objective of this study was to evaluate the effect of two IVM durations on *in vitro* embryonic development in goats.

MATERIALS AND METHODS

Experimental animals and superovulation: The experimental goats were sourced from the Institute of Biological Sciences (ISB) Mini Farm, University of Malaya. Female crossbred goats (Boer × local) were selected as oocyte donors, which underwent oestrus synchronisation and superovulation with an intravaginal controlled internal drug release device (CIDR) for 14 d and were injected with cloprostenol (125 µg Estrumate) 36 h before CIDR removal. Pregnant mare's serum gonadotrophin (PMSG; 1500 IU Folligon) was administered intramuscularly by injection 5 h

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before CIDR removal. The CIDR device was then removed and human chorionic gonadotrophin (hCG; 500 IU Ovidrel) was administered intramuscularly 60 h before performing LOPU. The age of the animals ranged from 12 to 42 months.

Laparoscopic oocytes pick-up (LOPU) for oocyte retrieval: The LOPU procedure was performed as described by Abdullah *et al.* (2008). Briefly, anaesthesia was induced and the abdomen and inguinal regions were shaved and scrubbed. The LOPU was performed with a Storz laparoscope attached to a video system. A pneumoperitoneum was created and small incisions (3-5 mm) were made, one on the right side and two on the left side 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 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 a Cook aspiration and flushing system. The collected oocytes were rinsed in warm saline solution at 38°C. Subsequently, oocytes were graded (A, B and C) according to the cumulus cell layers and morphology (Fig. 1), as described by Rahman *et al.* (2007), and cultured into IVM medium.

***In vitro* maturation (IVM) of oocytes:** After grading, the oocytes were cultured into the IVM microdroplet for a duration period for IVM of either 18-21 h or 22-25 h. The IVM media consisted of tissue culture medium (TCM)-199 with Earle's salt, L-glutamine and sodium pyruvate, TCM-pyruvate, follicle stimulating hormone (FSH), gentamycin, oestradiol 17β and oestrus goat serum. At the end of the particular IVM duration, the oocytes were removed from the cumulus cells by treating and gently pipetting them, using 0.1% of hyaluronidase in TCM-199 with HEPES. In this study, only the Grades A, B and C oocytes were selected for the ICSI procedure, because of our experience in previous research of low-maturity and low-quality oocytes graded D and E (unpublished data). Next, the matured Grades A, B and C oocytes with the presence of polar body (MII stage) were placed in TCM-199 supplemented with foetal bovine serum (FBS; 30%) inside the incubator (5% CO_2 and 38.5°C) prior to ICSI.

Sperm preparation: The Jermasia goat sperm source was obtained from the frozen thawed sperm. A straw of frozen sperm (0.5 ml) was withdrawn from the liquid nitrogen storage tank and left outside briefly at room temperature (25°C, 1 min), followed by thawing in a water bath (37°C, 1 min). The straw was taken out of the water bath, disinfected with alcohol (70%) and allowed to dry. The equilibrated sperm wash medium (Sp-TALP) was poured (3 ml) into a centrifuge conical tube (15 ml size). Then, the sealed end of the straw was cut and the thawed sperm were inserted into the Sp-TALP (3 ml). The remaining sperm in the straw were released into the Sp-TALP by cutting the other end of the straw, which was plugged with cotton, and gently passing it through the medium. Before mixing the sperm and medium, a drop of thawed sperm was placed on the slide, in order to examine the movement characteristics of the sperm under the inverted microscope. The centrifuge conical tube (mixed sperm and medium) was tightly capped and then the tube was centrifuged (200 × *g*, 15 min). After centrifugation, the supernatant was discarded and the pellets of the sperm were

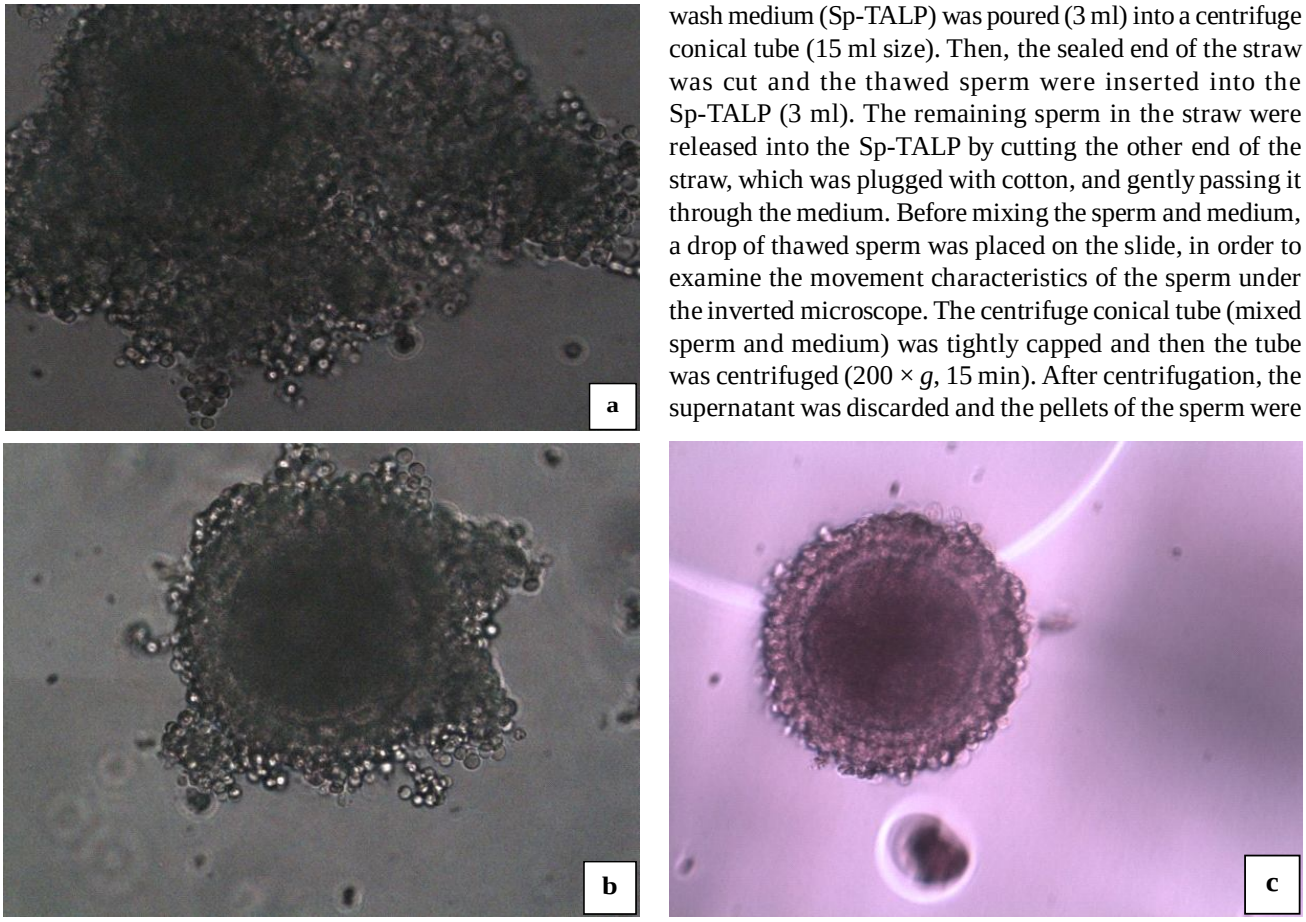

FIG 1. Morphology of different grades of goat oocytes: (a) Grade A, (b) Grade B, and (c) Grade C.

loosened by gently pipetting with a micropipette, before placing them (100-200 µl) at the bottom of the centrifuge conical tube with fresh capacitation medium (2 ml). The sperm were allowed to capacitate inside the sp-TALP medium containing 50 µg/ml heparin for 60 min. At the end of the sperm capacitation process, three quarters by volume of the supernatant was discarded. The remaining supernatant containing pellets of sperm was gently loosened and withdrawn into the ICSI dish for the ICSI procedure. The remaining sperm pellets were kept inside the CO_2 (5%) incubator.

Intracytoplasmic sperm injection (ICSI): The ICSI procedure was carried out as described by Abdullah *et al.* (2008), except that different media were used. In this study, the medium used for sperm was sp-TALP, and for oocytes TCM-199 HEPES + FBS (30%) was used.

Oocyte activation and *in vitro* culture (IVC): The post-ICSI oocytes were kept for 30 min in the TCM-199 + FBS (30%) inside the incubator (5% CO_2 and 38.5°C) before being activated for 5 min with Ca^{2+} ionophore (5 µm) and for 4 h with 6-dimethylaminopurine (6-DMAP). After activation, post-ICSI oocytes were rinsed with IVC medium (KSOM) three times before the real culture was placed in the incubated KSOM medium inside the incubator (5% CO_2 and 38.5°C). The embryos were evaluated at day 2, 5, 7 and 9 for embryonic development.

ICSI-derived embryos using Hoechst staining: In order to investigate the embryonic development stage, the cleaved embryos were stained with Hoechst (Fig. 2). The number of nuclei in the blastomeres was determined using an epifluorescence microscope. Generally, a few droplets of PBS(-) (100 µl) and fixative solution (100 µl) were mixed and overlaid with mineral oil on the petri dish. The cleaved embryos were washed in PBS(-) and then in fixative solution on the heated stage of the stereomicroscope. Then, the embryos were placed in the last droplet of fixative solution for 5 min at 25°C. Four small drops of Vaseline wax were placed on the centre of the glass slide. After 5 min, the embryos were transferred to the slide and mounted with the coverslip. The excess fixative solution was slowly aspirated out from the embryos before they were mounted with the coverslip. The coverslip was sealed with adhesive (cutex), and the side of slide was labelled and refrigerated at 4°C before being examined under an epifluorescence microscope.

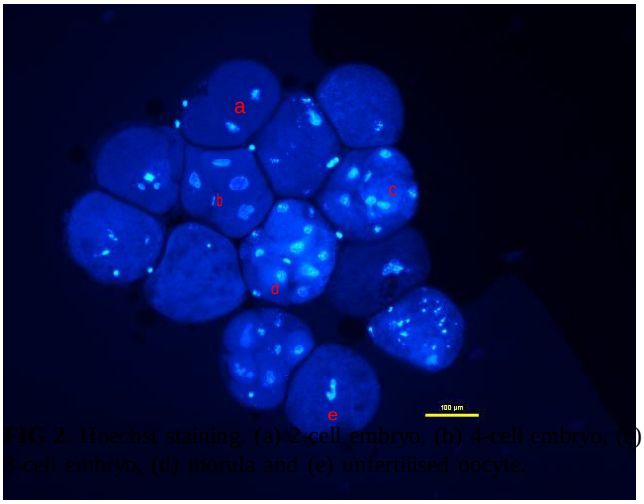

FIG 2. Hoechst staining. (a) 2-cell embryo, (b) 4-cell embryo, (c) 8-cell embryo, (d) morula and (e) unfertilised oocyte.

FIG 2. Hoechst staining. (a) 2-cell embryo, (b) 4-cell embryo, (c) 8-cell embryo, (d) morula and (e) unfertilised oocyte

Counting the cell number and viability of the mature oocytes: Oocytes were removed from cumulus cells using hyaluronidase (0.1%) to help in visual examination of matured oocytes. Only the matured oocytes (MII meiotic stage) were selected for the ICSI procedure. Parameters such as number of retrieved oocytes, maturation rate and grades of oocytes were determined.

Statistical analysis: The data obtained were presented as mean ± standard error of the mean (SEM). The effect of IVM duration on quality of oocytes and embryonic development was determined by one-way ANOVA. The significant difference of means was 5% and was compared with Duncan's Multiple Range Test. The analysis was carried out with SPSS (version 17.0, IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Only oocytes from Grades A, B and C were selected for subsequent ICSI experiments. Two different IVM durations (18-21 h and 22-25 h) were assigned for the ICSI procedure. The cleavage rates for different grades of oocytes

TABLE 1. Cleavage rate (%, mean ± SEM) of ICSI derived embryos at different IVM duration for different grades of oocytes

IVM duration	Grade of oocyte	*No. of injected oocytes	Cleavage rate (%)			
			2-cell	4-cell	8-cell	Morula
18-21 h	A	62	59.20±7.23^{b} (n=42)	47.41±7.15^{b} (n=33)	33.05±7.18^{b} (n=23)	11.93±5.22^{b} (n=6)
	B	32	31.38±7.92^{a} (n=20)	20.46±6.17^{a} (n=13)	10.92±5.12^{a} (n=6)	-
	C	63	35.95±7.34^{a} (n=34)	24.45±5.89^{a} (n=22)	9.28±3.21^{a} (n=12)	1.01±0.71^{a} (n=2)
22-25 h	A	54	57.52±8.02 (n=43)	43.71±8.00 (n=37)	31.24±7.18 (n=26)	3.98±1.78 (n=6)
	B	49	41.11±6.99 (n=29)	31.39±6.79 (n=24)	26.94±6.25 (n=20)	1.94±1.37 (n=2)
	C	67	46.41±7.03 (n=44)	37.06±6.44 (n=37)	13.71±3.26 (n=22)	4.32±2.05 (n=7)

*No. of injected oocytes was based on MII stage oocyte used for ICSI, in respective grade; n, number of observations; ^{ab}Mean values within a column with different superscripts were significantly different ($P<0.05$).

with respective IVM duration are presented in Table 1. A total of 327 matured oocytes were used, of which 157 and 170 oocytes were used for 18-21 h and 22-25 h IVM duration, respectively. At the 18-21 h IVM duration, Grade A oocytes showed significantly (P<0.05) the highest cleavage rates for all stages of development, followed by Grades B and C. At the 22-25 h IVM duration, cleavage rates for all grades of oocytes did not differ significantly (P>0.05) among the grades.

Regardless of oocyte grades, no significant (P>0.05) differences were observed in maturation rates and cleavage rates for all stages embryonic development, whether at 18-21 or 22-25 h IVM duration (Table 2). In addition, it was found that the first polar body formations for 18-21 h IVM duration were neither as clear nor as fully visible as protrusions as those of 22-25 h IVM duration (Fig. 3).

Kong (2010) reported that IVM duration at 22-25 h gave a better maturation rate than 26-29 h (71.6% versus 38.7%, respectively). Another report showed the maturation rate at 16-24 h was 21.0-72.0% after initiation of the maturation process (Cognie *et al.*, 2003). In the present experiment, non-significance (P>0.05) of maturation rates was observed in both IVM durations, the 18-21 h IVM duration showing numerically higher maturation rates than the 22-25 h IVM duration (97.34±7.86% vs. 89.01±2.41%, respectively). This indicated that the IVM duration at 18-21 h has same ability to mature oocytes as the 22-25 h duration. Moreover, in the present study it was found that the maturation rates improved on those of the previous study by Kong (2010) at 22-25 h, the maturation rates being 89.0% and 71.6%, respectively. In the IVM duration at 18-21 h, Grade A oocytes showed significantly (P<0.05) the highest cleavage rates for all stages of development, followed by Grades B and C. This shows that the oocyte quality in conjunction with cumulus cells can promote the necessary maturation for embryonic development. Previous studies obtained a better maturation rate when a higher quality of oocytes was cultured with at least four complete layers (Jimenez-Macedo *et al.*, 2007), with two or more complete layers (Rho *et al.*, 2001) or with one to two complete layers (Wang *et al.*, 2003) of cumulus cells.

At the 22-25 h IVM duration, the cleavage rates for all grades of oocytes were not significantly different (P>0.05). The cleavage rates at the two-cell stage for Grades A and B were generally found to be lower (57.5% and 41.1%) than previous data (64.5% and 64.5%), respectively, as reported by Kong (2010). However, for Grade C, the cleavage rate at two-cell stage was slightly higher (46.4%) in the present study than in previous data (44.6%) reported by Kong (2010). These differences may be due to the number

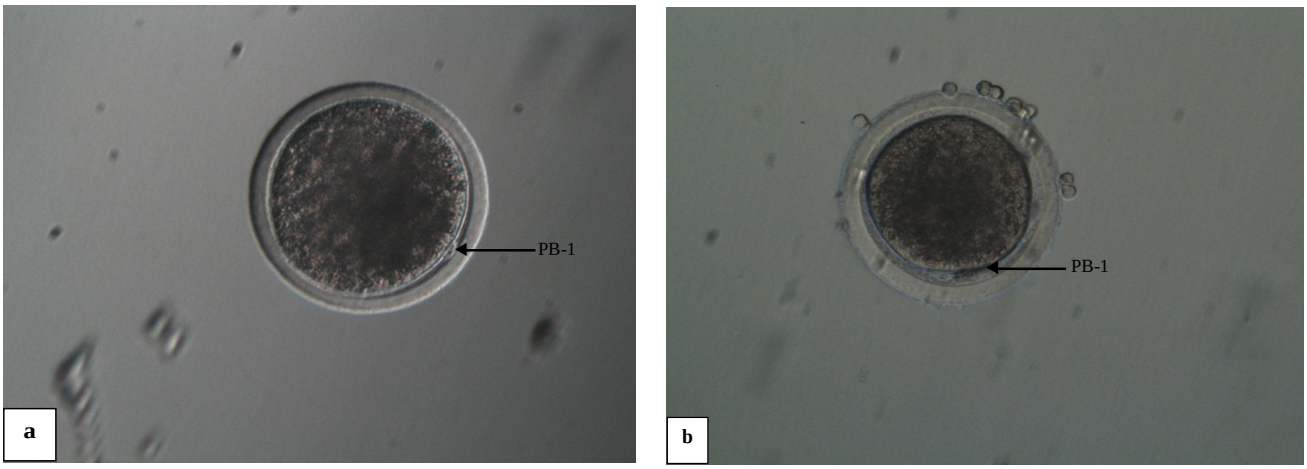

FIG 3. Morphology of matured oocyte with presence of first polar body: (a) at IVM duration 18-21 h, and (b) at IVM duration 22-25 h.

TABLE 2. Cleavage rate (%, mean ± SEM) of ICSI derived embryos at different IVM duration regardless of grade of oocytes

IVM duration	*No. of injected oocytes	Maturation rate¶	Cleavage rate (%)			
			2-cell	4-cell	8-cell	Morula
18-21 h	157	97.34±7.86 (n=157/169)	42.17±4.48 (n=96)	30.78±3.88 (n=68)	17.75±3.30 (n=41)	4.31±1.83 (n=8)
22-25 h	170	89.01±2.41 (n=170/195)	48.34±4.26 (n=116)	37.39±4.09 (n=98)	23.96±3.89 (n=68)	3.41±1.01 (n=15)

*No. of injected oocytes was based on oocytes used for ICSI; n, number of observation; ¶no. of matured oocytes/no. of oocytes recovered.

of samples used. In previous studies by Rahman (2008), who used FSH + hCG treated donor goats, the cleavage rates of Grades A, B and C were higher (84.0%, 66.7% and 57.8%, respectively) than in the present study. The difference between these experiments may be due to the types of hormone used, FSH being more effective than PMSG (Armstrong *et al.*, 1983; Nuti *et al.*, 1987). In addition, PMSG was found to disrupt normal fertilisation (Moor *et al.*, 1985) and also to reduce the fertilisation, embryonic development and pre-implantation stages of development (Evans and Armstrong, 1983).

Regardless of the oocyte grades, no significant ($P>0.05$) differences in maturation rates and cleavage rates were found for all stages of embryonic development between 18-21 h and 22-25 h IVM duration (Table 2). This indicated that the IVM duration at 18-21 h had the same fertilisation and embryonic developmental ability as the 22-25 h IVM duration. There was no significant difference ($P>0.05$) in development between IVM durations. It is well known that oocyte maturation is involved in nuclear and cytoplasmic maturity in order for ICSI to succeed (Kahraman *et al.*, 2000). During the experiments, the first polar body formation morphology for 18-21 h IVM duration was neither as clear nor as fully visible as a protrusion as those of 22-25 h IVM duration. This is probably because the mature oocytes that underwent 18-21 h of IVM duration were 'young' or early matured. However, this suggestion requires further study. In addition, the extrusion of the first polar body occurred before the oocyte was able to be fertilised, namely before the cytoplasm was completely maturated, in which case the resumption of meiosis was likely to fail or be incomplete (Kubiak, 1989). It has been suggested that the formation of the first polar body may reflect an asynchrony between nuclear and cytoplasmic maturation (Eichenlaub-Ritter *et al.*, 1995), which would affect the ability of cells to support pronuclear formation after ICSI. The maturation of nuclei is easier and faster than of cytoplasm (Krisher, 2004). The degree of cytoplasm maturation will determine the developmental competence of IVM oocytes to undergo further embryonic development (Combelles *et al.*, 2002; Inoue *et al.*, 2008). If the oocytes are exposed to IVM culture for an inadequate duration, their development can be impaired. The kinetics of immature and IVM oocytes were demonstrated during meiotic maturation and have a close relationship with spindle assembly. In humans, the spindle configuration anomalies are associated with reproduction failure (Szczygiel and Kurpisz, 2001). However, studies of the spindle assembly and maturation duration have yet to be performed.

However, if the oocytes were quickly matured *in vitro*, the first polar body would be extruded early and would complete the nuclear and cytoplasm maturation during IVM; the possibility of developing to blastocyst stage would also be clearer (Dominko and First, 1997). The prolongation of metaphase II arrest before ICSI leads to a reduction in successful fertilisation and embryo development (First *et al.*, 1988).

It can be inferred that the IVM duration for goat oocytes at 18-21 h has same capacity for embryonic development as 22-25 h. Grade A oocytes give higher cleavage rates at 18-21 h than similar specimens at Grades B and C. However, Grades B and C also could be used for an *in vitro* production programme (IVM and ICSI), in order to obtain good embryonic development.

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