EFFECT OF SPERM INSEMINATION DURATION ON *IN VITRO* FERTILISATION (IVF) PERFORMANCE IN GOATS

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ABSTRACT The objective of this study was to evaluate the effect of sperm insemination duration during *in vitro* fertilisation (IVF) on the cleavage rate of goat embryos. Goat oocytes were retrieved through the slicing of ovaries obtained from abattoir and laparoscopic oocyte pick-up (LOPU). For *in vitro* maturation (IVM), the collected cumulus oocyte complexes (COCs) were matured and then incubated overnight in CO_2 (5%) incubator at 38.5°C for 24-27 hr (abattoir source) or 18-21 hr (LOPU source). Oocytes were partially denuded and co-incubated with post-thawed sperm with concentration of $1x10^6$ sperm/ml. After 8-14 hr or 18-24 hr of insemination, *in vitro* culture (IVC) of presumptive zygotes were further incubated in CO_2 (5%) incubator at 38.5°C for preimplantation embryonic development. The cleavage of the embryos was observed and recorded daily under inverted microscope. The fertilisation rate with insemination duration of 8-14 hr (39.82%) was higher than that with 18-24 hr (33.59%); however, there was no significant difference ($P>0.05$) between 2 groups of insemination durations. There was significant ($P<0.05$) decrease as the embryo developmental stages increased. Generally, the embryo developmental rates of all stages for insemination durations of 8-14 hr were higher than that of 18-24 hr, which were 49.36 vs. 27.92%; 33.77 vs. 22.29%; 7.32 vs. 2.08% and 0.56 vs. 0.00% for 2-, 4-, 8-cell and morula, respectively. In conclusion, shorter insemination duration may be needed to improve the IVF performance in goats.

ABSTRAK Objektif kajian ini adalah untuk menilai kesan tempoh inseminasi sperma semasa persenyawaan *in vitro* (IVF) ke atas kadar pembelahan embrio kambing. Oosit kambing diperolehi melalui penghirisan ovari yang diperolehi dari rumah sembelihan dan *laparoscopic oocyte pick-up* (LOPU). Bagi pematangan *in vitro* (IVM), kompleks oosit kumulus (COCs) dimatangkan dan kemudian dieram semalaman dalam inkubator CO_2 (5%) pada 38.5ºC selama 24-27 jam (sumber rumah sembelihan) atau 18-21 jam (sumber LOPU). Oosit ditanggal kumulus sebahagiannya dan dieram bersama dengan sperma nyahsejukbeku dengan kepekatan $1x10^6$ sperma/ml. Selepas 8-14 jam atau 18-24 jam diinseminasi, kultur *in vitro* (IVC) zigot andaian dieram seterusnya dalam inkubator CO_2 (5%) pada 38.5ºC bagi perkembangan embrio prapenempelan. Pembelahan embrio diperhati dan direkodkan setiap hari di bawah mikroskop *inverted*. Kadar persenyawaan dengan tempoh inseminasi 8-14 jam (39.82%) adalah lebih tinggi daripada 18-24 jam (33.59%); walau bagaimanapun, tiada perbezaan signifikan ($P>0.05$) ditemui antara 2 kumpulan tempoh inseminasi. Terdapat penurunan yang signifikan semasa setiap peningkatan peringkat perkembangan embrio. Secara amnya, kadar perkembangan embrio bagi semua peringkat perkembangan embrio untuk tempoh inseminasi 8-14 jam adalah lebih tinggi daripada 18-24 jam, di mana 49.36 vs. 27.92%; 33.77 vs. 22.29%; 7.32 vs. 2.08% and 0.56 vs. 0.00% bagi 2-, 4-, 8-sel dan morula, masing-masing. Kesimpulannya, kadar inseminasi yang lebih pendek mungkin diperlukan untuk memajukan prestasi IVF dalam kambing.

(**Keywords**: IVF, goat, insemination duration, embryo)

INTRODUCTION

In vitro embryo production (IVP) technology has been widely used to increase the population of superior genetic merit of livestock animals including goats. To achieve success optimally, sufficient number of oocytes is the prerequisite for various reproductive techniques related to IVP procedures. In order to obtain abundant source of oocytes for large-scale production of embryos by IVP procedure, getting ovaries from abattoir is one of the ways [1]. However, in Malaysia, the number of female goats slaughtered is few and consequently it is quite difficult to carry out proper experiments in goat biotechnologies. Another source of oocytes could be alternatively obtained through laparoscopic oocyte pick-up (LOPU). It has been shown that the ovaries could be hyperstimulated several times and followed by aspiration of oocytes for various embryo techniques [2].

There are 3 main steps involved in the method of IVP of embryos, namely maturation of primary oocytes from large antral follicles, fertilisation of matured secondary oocytes with frozen-thawed semen and *in vitro* culture (IVC) of the putative embryos for up to 7 days until the formation of blastocysts. Conventionally, IVP in goats involves the superstimulation procedure in matured female animals either for flushing of embryos and oocytes or LOPU. Various gonadotrophins such as follicle stimulating hormone (FSH), pregnant mare's serum gonadotrophin (PMSG), equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) have been introduced to the hormone regime for superovulation and superstimulation procedures.

A reduction of the period of sperm-oocyte exposure has been suggested to increase the incidence of monospermy [3, 4]. In fact, most of the current IVF systems use a 5-6 hr sperm-oocyte insemination duration [5, 6-9], compared to 12-18 hr insemination duration used in the original porcine IVF systems [10-12]. Such information is not readily available in the literature and this issue is still controversial.

In the present study, we evaluated the efficiency of IVF performance for different insemination durations (co-incubation durations) during IVF procedure in goats.

MATERIALS AND METHODS

Ovary source and animal preparation

The sources of ovaries were from abattoir and LOPU. In order to prepare goats for LOPU, oestrus synchronisation and hyperstimulation of goats is necessary. Oestrous cycle of a donor goat was synchronised using insertion of a Controlled Intravaginal Drug Release device (CIDR, 0.3 g progesterone) into the vagina for 14 days (at 0900 hours on day 0) using a sterile CIDR applicator with water-based lubricant (K-Y Jelly). At 36 hr prior to CIDR removal, a luteolytic treatment of Estrumate® (125 µg cloprostenol) was administered intramuscularly (i.m.; at 0900 hours on day 13) to regress corpus luteum that facilitated initiation of pro-oestrus and eventually resulted in oestrogen surge for the onset of heat (oestrus). PMSG (1500 IU) was administered (i.m.; at 1600 hours on day 14). Upon the removal of CIDR (at 2100 hours on day 14), Ovidrel® (250 IU chorionic gonadotrophin) was administered (i.m.; at 2100 hours on day 14) to stimulate multiple follicular development prior to oocyte-retrieval surgery (at 0800 hours on day 17).

Oocytes recovery

For the abattoir source, the ovary was placed in a Petri dish (90 mm) containing TL-Hepes working solution (5 ml) on a heating stage (38.5ºC). The ovary was held with the help of forceps and each ovary was sliced individually. Checker-board incisions were made along the whole ovarian surface using a quarter section of a stainless steel razor blade held with a sterile haemostat. The COCs in the Petri dish were then examined under a stereomicroscope.

For LOPU source, the donor goat was sedated and anaesthetised and a small incision was made on the disinfected abdominal area and a trocar connected to a CO_2 tank via the CO_2 insufflator was inserted into the incision to insufflate the abdominal cavity with CO_2. Three small incisions (3-5 mm) were made once the peritoneum cavity was expanded. One of the incisions was made near the umbilicus to facilitate insertion of trocar for passing the light probe, one on the right side of lower-ventral abdomen to insert the trocar for passing the grasping forceps and one on the left side of lower-ventral abdomen for passing oocyte retrieval needle.

Using the grasper, the uterine horns were gently manipulated to allow visualisation of each stimulated ovary. Once an ovary was identified, the ovarian ligament was grasped to stabilise the ovary for follicular puncture. The follicular contents were aspirated from all follicles visible on the surface of the ovaries by puncturing follicles with the aspiration needle. The collection tube containing

aspirated fluid (3-5 ml) with COCs was dispensed into a sterile Petri dish (90 mm) for COCs searching under a stereomicroscope.

In vitro maturation (IVM)

A Petri dish (35 mm) with IVM droplets (100 µl) which were overlaid with equilibrated silicone oil was prepared overnight. The dish was kept in CO_2 incubator (5%). Under the stereomicroscope, the COCs were identified and picked up, using a hand-controlled pipette. The COCs from LOPU were then washed in three droplets of flushing medium followed by three droplets of IVM medium, while COCs from ovary slicing were washed with TL-Hepes working solution followed by three droplets of IVM medium. The collected COCs were then matured and incubated overnight in CO_2 (5%) incubator at 38.5°C for 24-27 hr (abattoir source) or 18-21 hr (LOPU source).

In vitro fertilisation (IVF)

Oocytes were partially denuded by sucking in and out from the pipette. The oocytes left with one or two layers of cumulus cells were washed thrice with droplets of IVF medium (100 µl) before being transferred to the insemination culture dish and kept in the CO_2 incubator (5%) to maintain its temperature (38.5ºC) and gas equilibration until required for insemination.

A frozen straw of goat sperm was pre-thawed at room temperature (25ºC, 1 min) followed by thawing in a water bath (37ºC, 3 min). Using a pair of sterile scissors, the straw was cut at both ends and the sperm was slowly released at the bottom of the swim-up tube (15 ml). Without agitating the mixture of sperm and medium, the swim-up tube was carefully held at 60º to the horizontal surface in the CO_2 incubator (5%) and incubated at 38.5ºC in humidified air (45 min) to allow sperm swim-up. The upper layer of the medium containing highly motile sperm (sperm suspension) was aspirated (1 ml) from the swim-up tube and placed in a sterile conical tube (15 ml) and sperm-SOF medium (2 ml) was added to the sperm suspension to make a total volume of 3 ml. With the tube tightly capped, the tube was centrifuged twice for 5 min (200x *g*) and the supernatant was discarded. While conducting the assessment of sperm concentration, the capacitation-treated sperm were incubated in CO_2 incubator (5%). After the sperm concentration has been determined by haemacytometer, sufficient volume of sperm suspension was added to the fertilisation droplets (30 µl) containing 5-10 matured oocytes to yield final concentration $1.0x10^6$ sperm/ml.

In vitro culture (IVC)

The dish containing IVC droplets under silicone oil were equilibrated overnight at 38.5ºC in CO_2 incubator (5%). The culture medium used for *in vitro* embryo culture was KSOM. After 8-14 hr or 18-24 hr sperm insemination duration, all the presumptive zygotes were removed from the insemination droplets and washed, subsequently were transferred to new IVC droplets (100 µl) for culture (38.5ºC, 5% CO_2). Each culture droplet (100 µl) contained 5-10 presumptive zygotes.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) and Duncan's multiple range tests (D-MRT), using the SPSS statistical software package version 17. A probability of $P<0.05$ was considered significant for all statistical tests. Values were presented as mean±SEM.

RESULTS AND DISCUSSION

The fertilisation rate of insemination duration of 8-14 hr (39.82%) was relatively higher than that of 18-24 hr (33.59%) (**Table 1**). The co-incubation of oocytes with sperm for 17 hr was originally established for practical reasons and corresponds to the time for observation of pronuclei. As reported in goat IVF system [13], there was only 10.00% of oocytes fertilised following 1 hr co-incubation with sperm; the cleavage rates were 71.00 vs. 78.00% (3 vs. 17 hr co-incubation) and blastocyst rates were 50.00 vs. 53.00% (3- vs. 17 hr co-incubation), respectively. The cleavage rates in their reports were relatively higher than that of the present study (49.36 vs. 29.32% for insemination durations of 8-14 vs. 18-24 hr, respectively) (**Table 2**). The 6 hr delay in gamete interaction indicated that this duration is probably necessary for sperm to cross the cumulus or that differences in sperm capacitation kinetics exist between IVF systems **(Figure 1(a-d))**.

It was suggested that reducing the oocyte-sperm insemination duration lowers oocyte penetration and fertilisation during bovine IVF [14], but resulted in similar oocyte cleavage rates [15], and either similar [15] or reduced [16] rates of blastocyst formation. Shortening the gamete insemination duration during human IVF increases normal fertilisation [17, 18], embryo development [17, 19 and 20], increased embryo morphology [20-23], implantation and pregnancy rates [21 and 23].

According to Marchal & Matas, penetration of IVM porcine oocytes as well as polyspermic fertilisation occur as early as 2 hr post-insemination. Moreover, it is known that the incidence of acrosome-reacted living sperm, under capacitating conditions, remains constant after 2, 4, and 6 hr of co-incubation with oocytes (Vasquez, 1993). It was suggested that by reducing the sperm-oocyte insemination duration from 12-18 to 8 hr [27] as well as the removal of adherent sperm from the zona pellucida after 6-8 hr of insemination duration [28], decrease the incidence of polyspermy. Grupen *et. al.* ,2000 proposed to decrease the exposure duration of matured oocytes to sperm from 5 hr to 10 min and maintain the oocytes with the zona-bound sperm in a fresh IVF medium drop without sperm for an additional 5 hr incubation; and this modification of the IVF conditions resulted in a higher penetration rate (57.00 vs. 80.00%) and blastocyst development (8.00 vs. 30.00%) as compared to the conventional procedure of gametes co-incubated together for 5 hr. Frozen-thawed sperm cells generate increased amounts of reactive oxygen species (ROS) as by-products of metabolism and cell death [30, 31] which may be related to longer insemination duration. Several studies have shown that ROS plays an important role in mediating sperm-zona interactions as it increases DNA fragmentation [32], modifies the cytoskeleton [33], and produces a loss of fluidity, integrity and competence of the sperm membrane to participate in the membrane events associated with fertilisation [34-36].

Table 1. Fertilisation rate (%, mean±SEM) of goat oocytes for different insemination durations in *in vitro* fertilisation

Insemination duration	No. of matured oocytes	Fertilisation rate (n)
8-14 hr	134	39.82 ± 8.18^{a} (n=68)
18-24 hr	60	33.59 ± 10.97^{a} (n=17)

[a]Mean value within a column with same superscript was not significantly different (P>0.05)

Table 2. Cleavage rate (%, mean±SEM) of goat oocytes for different insemination durations in *in vitro* fertilisation

Insemination duration	Cleavage rate (n)			
	2-cell	4-cell	8-cell	Morula
8-14 hr	$49.36\pm10.41^{a,z}$ (n=46)	$33.77\pm8.76^{a,z}$ (n=28)	$7.32\pm5.06^{a,y}$ (n=10)	$0.56\pm0.56^{a,y}$ (n=2)
18-24 hr	$27.92\pm10.87^{a,z}$ (n=10)	$22.29\pm9.26^{a,yz}$ (n=7)	$2.08\pm2.08^{a,xy}$ (n=1)	$0.00\pm0.00^{a,x}$ (n=0)

[a]Mean value within a column with same superscript was not significantly different (P>0.05)
[yz]Mean values within a row with different superscripts were significantly different (P<0.05)

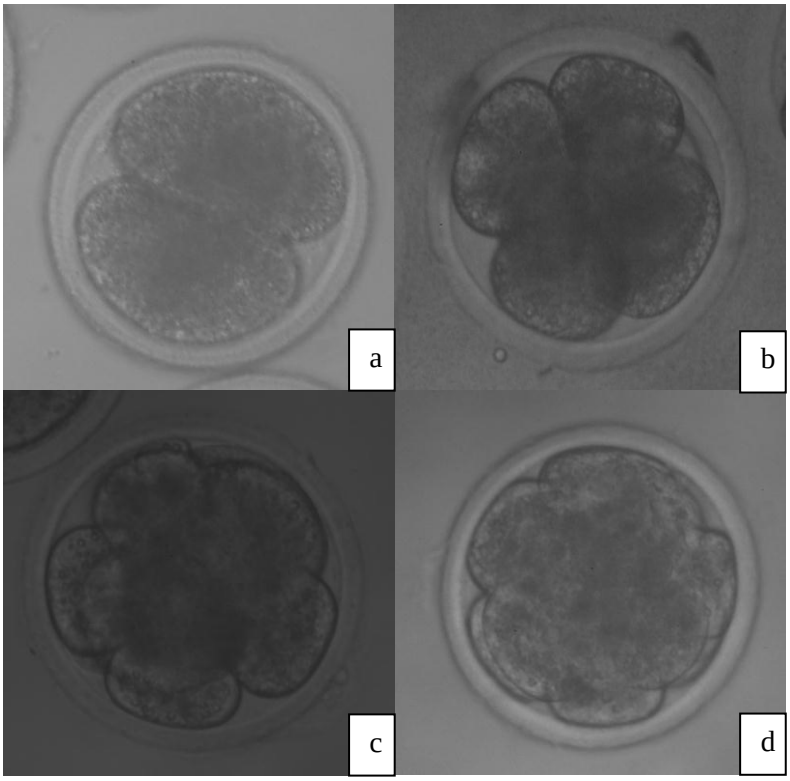

Figure 1(a-d): Embryos at 2- (a), 4- (b), 8-cell (c) and morula (d) obtained from goat IVF.

CONCLUSION

Many factors are involved in the IVF performance in goat including internal and external factors. This research indicates that insemination duration may play important role in the cleavage of goat embryo. It is suggested that shorter insemination durations of 8-14 hr may be better than that of 18-24 hr as consistently shown in our data that the former was higher in the cleavage rates.

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