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Effect of Oocyte Maturation Period on Developmental Rate and Sex ratio Distribution of *in vitro* Produced Bovine Embryos

Research Article

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ABSTRACT

It has been suggested that the maturation status of the oocytes at the time of fertilization influences the sex ratio of resulting embryos produced *in vitro*. Therefore, the duration of the oocyte *in vitro* maturation is likely a simple method that leads to *in vitro* production of embryos with the desired sex. For this purpose, the current study was conducted to investigate the effect of *in vitro* maturation culture period of bovine oocytes on the sex ratio of resulting blastocysts. The abattoir ovary-derived oocytes were cultured for various duration of *in vitro* maturation (16 h, 20 h, 24 h, 28 h, and 36 h). After in vitro fertilization and culture of mature oocytes, nested multiplex polymerase chain reaction (PCR) with AMELX and SRY primers was used to determine the sex of blastocysts. The results of this study showed that the cleavage and blastocyst rates were increased by prolongation of *in vitro* maturation (IVM) duration from 16 hours to 24 hours and deceased from 24 hours to 36 hours ($P<0.05$). The sex ratio of male embryos in aged oocytes (36 hours IVM) was significantly higher than in other groups ($P<0.05$). In conclusion, the maturation duration of bovine COCs influences the sex distribution of embryos in the blastocyst stage appearing on day 8, and the male embryos were recovered more than female counterparts by increasing the oocytes culture period.

KEY WORDS bovine, embryo, *in vitro* maturation, oocyte, sex ratio.

INTRODUCTION

Predetermining or skewing the sex ratio of offspring to the favorite sex has always been a desire of livestock owners for their herd. Fertilization of mammalian oocyte with the sexed sperm determines the sex of the embryo and obtaining offspring of predetermined sex. But, the use of sexed sperm for *in vitro* embryo production (IVEP) is expensive and also has low efficiency in IVEP (Steele *et al*. 2020). The sexed semen has lower fertility, lower survival after cryopreservation and leads to a reduction in fertilization, cleavage, blastocyst, and pregnancy rates (Steele *et al*. 2020). Therefore, the use of a simpler method to produce an embryo with desired sex has an immense economic benefits in livestock.

There are various factors including culture conditions (Bredbacka and Bredbacka, 1996; Gutierrez-Adan *et al*. 2001; Peippo *et al*. 2001) duration of sperm-oocyte interaction (Kochhar *et al*. 2003), physiological concentrations of androgens (Macaulay *et al*. 2013), and sperm preparation method (Rheingantz *et al*. 2018; Wolf *et al*. 2018) can all affect the sex ratio of embryos produced *in vitro*. It has been observed that the time of mating or insemination during estrus influence the sex ratio of offspring, and insemi-

nation at the beginning of ovulation produce a larger number of females, and insemination at the end of ovulation resulted, higher male ratio (Wehner *et al*. 1997; Gutiérrez-Adán *et al*. 1999; Rorie, 1999; Martinez *et al*. 2004). Therefore, it has been hypothesized that the oocyte maturation period during IVEP could influence the sex ratio of derived embryos (Dominko and First, 1997). Previously, it has been reported that longer maturation and culture periods were associated with a higher ratio of male blastocysts (Agung *et al*. 2006). However, some studies did not show any differences in the sex ratio of embryos produced through IVEP methods (Dominko and First, 1997; Rizos *et al*. 2008).

There is currently no evidence whether sperm selection is both affected by the ratio of sperm carrying X and Y chromosomes at the time of egg fertilization or affected by the ability of the oocyte to select sperm with a specific genotype. This ability can be obtained by oocyte maturation period during IVEP.

Despite the reduction in developmental rates of resulting embryos from *in vitro* fertilized oocytes earlier or later than the normal time of *in vitro* fertilization (IVF) in cattle (22-24 h after *in vitro* maturation (IVM) initiation), the sex determination of the embryos at the blastocyst stage can be valuable. In other words, the duration of oocyte *in vitro* maturation is likely a simple method that leads to the production of embryos of the desired sex. Therefore, this study was conducted to investigate the effect of *in vitro* maturation culture period of bovine oocytes on the sex ratio of resulting blastocysts.

MATERIALS AND METHODS

In vitro maturation of bovine oocytes

Bovine ovaries were collected from a local slaughterhouse, transported to the laboratory in sterile normal saline solution within 2–3 hours, and prepared for oocyte retrieval. All visible ovarian follicles with a diameter of 2-8 mm were aspirated using a vacuum pump in HEPES-TCM medium supplemented with 10% fetal bovine serum (FBS) and 50 IU/mL heparin. The aspirated cumulus-oocyte complexes (COCs) with evenly granulated cytoplasm and at least three layers of compacted cumulus cells were selected and washed three times. The selected oocytes were randomly divided into experimental groups, according to their IVM duration (16 h, 20 h, 24 h, 28 h, and 36 h) and cultured in maturation medium (bicarbonate#buffered medium 199 supplemented with 0.33 mM sodium pyruvate, 0.05 IU/mL FSH and 10% FBS) at 39 ˚C in 9% CO_2. The trial was replicated five times.

In vitro embryo production

The matured oocytes were exposed to motile sperm separated by centrifugation of frozen-thawed semen on a discontinuous Percoll density gradient (0.5 mL 40% Percoll over 0.5 mL 90% Percoll) at 700 g for 20 min. Oocytes were cultured in Tyrode's–albumin–lactate–pyruvate (TALP) medium supplemented with 25µg/ml heparin and 6mg/ml BSA and incubated with motile spermatozoa at 1×10^6 spermatozoa/mL at 39 ˚C in 9% CO_2 for 22-24 h. After fertilization, presumptive zygotes were mechanically denuded from their cumulus cells and cultured in synthetic oviductal fluid supplemented with amino acid and BSA (IVC-SOFaaBSA) under mineral oil in 9% CO_2 and 7% O_2, with maximum humidified atmosphere. The oocytes with the pale and disintegrated cytoplasm were recorded as degenerated oocytes and then removed from the culture medium. The culture medium was refreshed on the third day of culture (day 0 defined as the day of fertilization) with IVC-SOFaaBSA containing 5% charcoal-stripped FBS (CSS) and the culture was continued until 8 days post-fertilization. The embryos at blastocyst stage were stored in liquid nitrogen (-196 ˚C) until DNA extraction.

Embryo sex determination

Determination of embryo sex was performed using the invasive method and nested multiplex PCR using *SRY* and AMELX primers. The embryo lysis was performed using NP40/PK method. In this method, a modified form of phenol-chloroform DNA extraction method, a very useful lytic non-ionic detergent (NP40), and proteinase K was used. At first, each blastocyst was placed in 10 µL DNA lysis buffer composed of distilled H_2O (2.5 µL), PCR buffer (2.5 µL) and NP/PK solution (1 µL NP40 10%, 1 µL proteinase K and 48 µl H_2O) (5 µL) and incubated at 50 ˚C for 60 min. Then the extracted DNA was heated at 96 ˚C for 15 min for inactivation of protease K and mixed with the PCR reaction containing 10 mM dNTP, forward and reverse *SRY* and *AMELX* primers, 50 mM $MgCl_2$, Taq polymerase, and H_2O. The sequences of primers are accessible in Table 1.

Table 1 Sequence of used primers for nested multiplex PCR

Gene	Primer sequence (5′−3′)
SRY	F-GTGAAVGAAGACGAAAGGTG
	R-ATAGCTAGTAGTCTCTGTGCCTC
AMELX	F-CCAACACCACCAGCCAAACC
	R-GGTCTTGTCTGTTGCTGGCC

Multiplex-nested single cell polymerase chain reaction was carried out under standard conditions (TECHNE thermocycler, TC-512). The first stage of multiplex PCR (10 cycles) was designed to increase the primers efficiency to binding with template as follows: 30 sec denaturation at 94 ˚C, 1 min annealing at 55 ˚C, and 1 min extension at 72 ˚C, followed by an additional 25 cycles for more specific attachment as follows: 30 sec denaturation at 94 ˚C, annealing at 56.5 ˚C, 45 sec and 30 sec extension at 72 ˚C.

The same PCR conditions above were used to nested PCR step. PCR products were analyzed by electrophoresis on the polyacrylamide gel (Iranian Pyapajuhesh, EPS-600Z) after silver staining for detection of DNA bands in gels.

Statistical analysis

The differences in post fertilization development of embryos between groups were analyzed using One-Way ANOVA (all pairwise multiple comparison procedure; Tukey test). The sex ratio between experimental groups was analyzed using Chi-square. The results were reported as mean ± SEM and differences were considered significant at the level of $P < 0.05$.

RESULTS AND DISCUSSION

The result of the developmental rate of bovine embryos subsequent to different IVM duration is shown in Table 2. The cleavage rate increased significantly from 16h to 24h with increasing the duration of IVM and decreased the cleavage rate significantly when durations of IVM are longer than 24 hours to 36 hours. No significant difference was observed for cleavage rate between groups of 16 *vs*. 36 h or 20 *vs*. 28 h IVM duration periods ($P<0.05$).

A similar trend was observed for blastocyst production as for cleavage rate when different IVM durations were used.

The amount of proportion of female *in vitro* produced embryos decreased with increasing IVM duration of oocytes from 16 to 36 hours and vice versa. However, in this study, despite the difference between the data obtained from the studied groups, there was only a significant difference between 16 h and 36 h groups ($P<0.05$) (Figure 1).

In addition to many factors (Bredbacka and Bredbacka, 1996; Gutierrez-Adan *et al*. 2001; Peippo *et al*. 2001; Kochhar *et al*. 2003; Macaulay *et al*. 2013; Rheingantz *et al*. 2018; Wolf *et al*. 2018), the maturation status of the oocytes at the time of insemination influences the sex ratio of the embryos produced *in vitro*. Nonetheless, the developmental rate of the resulting embryos is also affected.

In this study, the cleavage and blastocyst rates were increased by prolonged IVM duration from 16 to 24 h or decreased from 24 to 36 h ($P<0.05$). This result was consistent with the results of Agung *et al*. (2006) study that reported the highest and lowest cleavage rate at 22 and 38h, respectively (Agung *et al*. 2006). The results of the current study showed that the sex ratio of male embryos increased when oocytes were exposed longer than 36 h IVM period.

Table 2 Effect of duration of *in vitro* maturation (IVM) on post-fertilization development of bovine embryos

Duration of IVM (h)	Oocyte No.	Cleaved embryos No. (%±SEM)	Blastocyst No. (%±SEM)
16	280	148 (53.1±1.1)^{a}	47 (16.5±1)^{ad}
20	227	149 (65.4±1)^{b}	53 (23.8±2.4)^{ac}
24	402	308 (76.4±1.1)^{c}	146 (38.2±2.4)^{b}
28	320	217 (68.3±0.3)^{b}	83 (28.4±2.5)^{c}
36	257	142 (55.7±1.3)^{a}	35 (13.7±0.5)^{d}

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

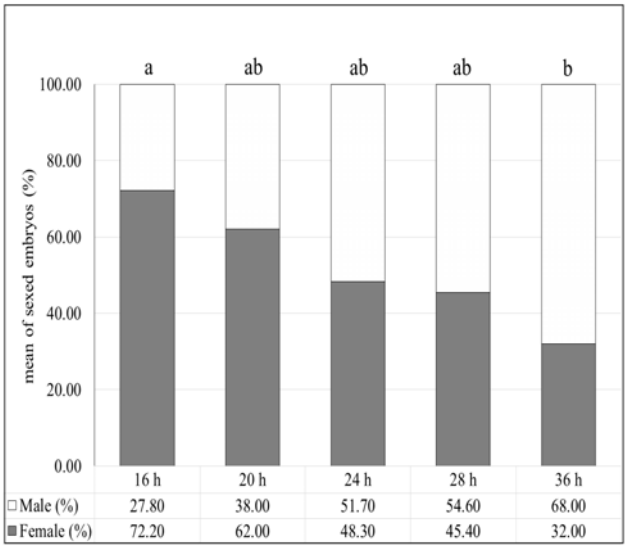

Figure 1 Effect of duration of IVM on the sex ratio of bovine embryos
The means within the same column with at least one common letter, do not have significant difference (P>0.05)

It was in agreement with the results of Dominko and First (1997) study who stated that delaying in oocyte fertilization up to the 8 h after the first polar body extrusion enhanced the sex ratio of male embryos. Other studies also documented that delaying insemination increased the proportion of male embryos (Dominko and First, 1997; Gutiérrez-Adán *et al*. 1999; Agung *et al*. 2006).

It was determined herein that there is no difference in the fertility potential of sperm according to its sex chromosome bearing, so the probability of fertilization is the same with each of the sperm bearing the X or Y chromosome (Rahman and Pang, 2020). To explore the question: Can the oocyte maturation conditions affect the penetration of spermatozoa based on its sex? The current and earlier reports describe that the capacity of the oocyte for preferentially selecting sperm of one sex over another is dependent on their maturational status, and prolonged IVM allows M II-arrested oocytes to penetrate Y-chromosome bearing sperm more effectively than X-chromosome bearing sperm (Gutierrez-Adan *et al*. 2001; Kochhar *et al*. 2003; Agung *et al*. 2006).

A possible reason for the tendency of sex ratio of embryos to male with the increasing IVM duration is the molecular structural difference of male and female embryos because the aged oocytes provide better survival conditions for male embryos (Avery *et al*. 1989; Marquant-leGuienne *et al*. 1992; Tarín *et al*. 2002; Miao *et al*. 2009). In other words, non-structurally developed oocytes-derived female embryos can survive up to the blastocyst stage and different tolerances of male and female embryos have been established in many studies (Dallemagne *et al*. 2018). The determination of embryos sexing at the blastocyst stage herein, describes that the difference in sex ratio under different IVM oocyte duration could influence. A study conducted in 2008 by Rizos to investigate the effect of the duration of oocyte maturation on the sex ratio of blastocysts showed that maturity of the oocyte at the time of the interaction and timing of gamete interaction does not affect the sex ratio of the embryos produced *in vitro*, but it can affect the kinetics of the early cleavage divisions (Rizos *et al*. 2008). Contrasting results of currents study to Rizos *et al*. (2008) study might be the time of sex determination of embryos in 2-cell or blastocyst stages. In fact, what matters is the sex of the embryos in the blastocyst stage, so the ratio of embryos can be changed by increasing or decreasing the duration of *in vitro* maturation of the oocytes, despite a decrease in the percentage of embryos produced.

CONCLUSION

In conclusion, our results indicated that the maturation duration of bovine COCs has an effect on the sex distribution of embryos in blastocyst stage appearing on day 8, and higher ratio of male embryos recovered than females when oocytes maturation period extended up to for 36 h.

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