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Short Communication

In vitro maturation of sheep oocytes in different concentrations of mare serum

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The aim of the study was to determine the optimum concentration of the mare serum (MS) for sheep *in vitro* oocyte maturation. Sheep ovaries were collected from a local abattoir and transported within 1 h to the laboratory in a warm saline solution (30 – 35°C), supplemented with 100 IU penicillin G and 100 µg streptomycin sulfate/ml. Cumulus-oocyte complexes (COC's) were obtained by slicing of follicles, washed in TCM-199 modification with $NaHCO_3$ and supplemented with 50 µg/ml gentamycin, and 0.25 mM sodium pyruvate without any serum supplementation. The COC's were randomly divided into four groups. Group 1 (n = 105) COC's were fresh control and cultured in TCM-199 medium without serum supplementation. Group 2 (n = 108) COC's were washed five times and cultured in TCM-199 medium supplemented with 10% MS. Group 3 (n = 112) COC's were washed five times and cultured in TCM-199 medium supplemented with 15% MS. Group 4 (n = 114) COC's were washed five times and cultured in TCM-199 medium supplemented with 20% MS. After 38 - 42 h of IVM, oocytes were denuded with the aid of 0.1% hyaluronidase and passing them through a fine pipette, fixed for 24 – 48 h in a mixture of acetic acid and alcohol (1:3) at room temperature, stained for 10 min with 1% (w/v) orcein in 45% acetic acid and examined for the evidence of different stages of maturation. Significantly higher ($p < 0.05$) maturation rates of oocytes (69 – 72%) were observed in all concentrations of mare serum compared to those without serum supplementation. However, no significant difference was observed between the 10, 15 and 20% serum supplemented group.

Key words: Sheep; Mare serum; Follicle slicing; Oocyte maturation; Ovary.

INTRODUCTION

In vitro maturation (IVM) is one of the essential steps in the *in vitro* fertilization (IVF) process. Several workers have studied different aspects of IVM in mammalian oocytes (Pawshe et al., 1996; Ghasemzade- Nava and Tajik 2000; Wani et al., 2000; Roa et al., 2002; Kharche et al., 2005). In most of the studies, the basic medium is supplemented with hormones and different concentrations of serum. The maturation mediums with the selection of protein supplements and hormones for IVM play an important role in subsequent IVF and *in vitro* development (Pawshe et al., 1996). Maturation of the oocytes include two aspects: nuclear and cytoplasm maturation (Sun and Nagai, 2003). *In vitro* maturation oocyte provide an excellent opportunity for cheap and abundant embryo for carrying out basic research and for the application of emerging biotechnologies like cloning and transgenic (Nadi et al., 2002). In 1980s and early 1990s, the heat treatment of bovine serum (Fetal Bovine Serum) was routinely practiced in IVM medium (Gordon 1997). It was found essential to supplement the holding and culture media with 1 - 5% serum to prevent the zona pellucida hardening (Downs et al., 1986). Serum added to the oocyte culture medium provides a source of albumin that balances the osmolarity and acts as a free radical scavenger (Thompson, 2000).

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Sheep oocytes have also been studied for different aspects of maturation (Wani, 2002; Roa et al., 2002). In all the experiments maturation media were supplemented with fetal bovine serum (FBS) (Wani et al., 2000; Ghasemzade- Nava and Tajik, 2000), estrous sheep serum (ESS) (Ghasemzade- Nava and Tajik, 2000) and Human serum (Thomson et al., 1992) as protein supplement. Hormone supplementation varied in all experiments.

To our knowledge, MS has not been used in maturation medium for sheep oocytes. The purpose of this study was to determine the efficiency of different mare serum concentrations on *in vitro* maturation of sheep oocytes. The present experiment was planned during the natural breeding season for Iranian sheep (July–November) to determine the optimum concentration of MS to be used for maturation of the oocytes.

MATERIALS AND METHODS

Two mares (3 and 4 years old) were observed for the occurrence of diestrus at the veterinary hospital of Tehran. Mare serum was collected by filtering serum through Millipore filters (with a pore size of 0.22 µm). The Mare serum was heat inactivated at 56°C for 30 min in a water bath, dispensed into 1 ml aliquots and stored at –20°C until used (Rao et al., 2002; Kharche et al., 2005, 2006). Sheep ovaries were collected from a local abattoir and transported within 2 h to the laboratory in a warm saline solution (30 – 35°C), supplemented with 100 IU penicillin G and 100 µg streptomycin sulfate/ml. All visible antral follicles with a 2 – 6 mm diameter were slicing (Kharche et al., 2006). The COC's were washed 10 times in TCM-199 (Cat. No31100, Gibco) modification solution with $NaHCo_3$ and supplemented with 50 µg/ml gentamycin (Cat. No. G 1264, Sigma), and 0.25 mM sodium pyruvate (Cat. No. P4562, Sigma) without any serum supplementation. The COC's were randomly divided into four groups. Group 1 (n = 105) COC's were taken fresh control and cultured in TCM-199 medium without serum supplementation. Group 2 (n = 105) COC's were washed five times and cultured in TCM-199 medium supplemented with 10% MS. Group 3 (n = 110) COC's were washed five times and cultured in TCM-199 medium supplemented with 15% MS. Group 4 (n = 112) COC's were washed five times and cultured in TCM-199 medium supplemented with 20% MS. Ten to 15 cumulus intact sheep oocytes were transferred into a 50 µl drop of each group of the culture media (control medium or serum supplemented media) under warm mineral oil (Cat. No. M 8410, Sigma) in a tissue culture dish (35 ×10 mm), equilibrated for 2 h in a CO_2 incubator before the oocytes were added. These oocytes were cultured for 38 - 42 h at 37°C under humidified atmosphere of 5% CO_2 in air. Oocytes were denuded after 40 h following culture by treating with TCM-199 containing 0.1% hyaluronidase (Cat. No. H 4272, Sigma) and passing them through a fine pipette. Oocytes were fixed for 24 – 48 h in a mixture of acetic acid and alcohol (1:3) at room temperature, stained for 10 min with 1% (w/v) orcein in 45% acetic acid and examined for evidence of different stages of maturation under a phase contrast microscope. The different stages of maturation examined based on chromosomal configuration were assigned to germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase-I (M-I) and metaphase-II (M-II) categories. A chromosome configuration was designated as GV, when having a single large nucleus with uniformly distributed filamentous chromatin subsequently condensing to form a ring of condensed chromatin around the compact nucleus. In the GVBD category, the nucleolus and nuclear membrane disappeared and chromosomes appeared as condensed and coiled up filaments. The metaphase- I stage was recognized by the appearance of paired chromosomes, while in the metaphase-II emission of first polar body, resulting in the formation of haploid set of chromosomes in the oocytes was observed.

Statistical analysis

Data was collected over 10 replicates. The percentages of M-II stage oocytes were calculated in different groups. The collected data were analyzed by Chi square Test. The $P < 0.05$ level was considered significant. (Sigma Stat, Jandel Scientific, SanRafael, CA, USA Sigma).

RESULTS AND DISCUSSION

The results have been presented in Table 1. The maturation rate of the Sheep oocytes in media without protein supplemented was 50% as shown by the control group. However, the protein supplemented in the form of 10% MS significantly ($p < 0.05$) increased the maturation rate to 69%. The maturation rate further increased with 15 and 20% MS supplementation to 70 and 72%, respectively. A significant difference ($p < 0.05$) in the *in vitro* maturation rate was recorded between the serum supplemented and non supplemented medium. The maturation rate between 10, 15 and 20% MS supplementation did not differ significantly (Table 1).

The IVM rates of 69 - 72% in different level of serum were lower compared to published reports (Roa et al., 2002; Ghasemzadeh-Nava and Tajik, 2000; Wani, 2000, 2002). Experiments on *in vitro* maturation of caprain oocytes using TCM-199 supplemented with EGS, revealed a maturation rate of 50% (Pawshe et al., 1996) and 57.6% (Mogas et al., 1995). Mogas et al. (1997) reported the addition of FSH, LH and estradiol-17 µl as well as EGS in TCM-199 medium to increase the maturation rate to 72.4 and 64.1% in adult and pre pubertal goats, respectively. Similarly Pawshe et al. (1996) also reported a maturation rate of 62.6% following the supplementation of hormones and EGS. In the present study a 69 – 72% maturation rate with mare serum was recorded in sheep oocytes with hormone supplementation. Tajik and Shams Esfandabadi (2003) reported an even higher maturation rate of oocytes in culture medium containing EGS without hormones. Our results are in agreement with the finding of Ghasemzadeh-Nava and Tajik (2000) who compared the effect of FBS and ESS and concluded that ESS could support the *in vitro* maturation of ovine oocytes slightly better. The values were 70, 68 and 61% maturation rates for 10, 15 and 20% FBS, respectively, compared to 82, 81 and 72% maturation rates for 10, 15 and 20% ESS, respectively. Some reports indicate (Kharche et al., 2006) that the addition serum enhances maturation, and development of *in vitro*-matured oocytes, our results do no support this results. And maturation of follicular oocytes is normally arrested at the prophase-I of the first meiotic division and the oocyte remain in the dormant stage, called a dictate nucleus. At this stage, nuclear ma-

Table 1. Effect of different concentrations of mare serum on the *in vitro* maturation rate of sheep oocytes.

Groups	MS (%)	No. of oocyte	GV (%)	GVBD (%)	M-I (%)	M-II (%)
1	0	105	16	10	18	53 (50)a
2	10	108	14	6	14	74 (69)b
3	15	112	11	9	13	79 (70)b
4	20	114	6	9	17	82 (72)b

Values in columns with different superscripts (a, b, c, d) differ significantly ($p < 0.05$).
GV, germinal vesicle; GVBD, germinal vesicle breakdown M-I, metaphase-I; M-II, metaphase-II.

terial is enveloped and the resulting structure is called a germinal vesicle. The oocytes remain at this stage until the onset of puberty. Under the influence of gonado-tropins and particularly in response to the LH surge, oocytes resume meiosis just before ovulation. These results in the disappearance of the nuclear membrane and germinal vesicle breakdown, followed by chromo-some condensation with the occurrence of the M-I stage. Subsequently, upon extrusion of the first polar body, the oocytes reach the M-II stage and remain at this stage until penetration by the spermatozoa. Oocytes remained in the GV stage from the onset to 6 – 8 h of culture. The GVBD occurred between 7 and 9 h and the metaphase-I became established within 12 – 18 h. Finally most oocytes reach the metaphase-II stage after 27 h (at 38.5^{o}C) Resumption of meiosis and subsequent sequen-tial configuration are species specific. The configuration of meiotic chromosomes at the time of co-culture of oocytes with spermatozoa for *in vitro* fertilization has a direct influence on the final success of fertilization. The M-II stage which is also known as the second phase of meiotic arrest, is consider as having have completed nuclear maturation of the oocytes required for successful fertilization of oocytes. Obviously, nuclear maturation of oocytes along with cytoplasm maturation is important at the completion of meiotic division for success of fertilization. Therefore, the *in vitro* maturation process is supposed to be completed when the highest percentage of M-II oocytesis observed.

To our knowledge, this report is the first paper about used MS in maturation medium for sheep oocytes. The results indicated that no significant differences are between different concentrations of serum.

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