Maturation timing and fetal bovine serum concentration for developmental potential of sheep oocytes in vitro

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The success of in vitro embryo production (IVEP) in animals has improved over time, employing a variety of culture media. Here, we assessed the maturation timing and developmental potential of sheep oocytes in vitro at different concentrations of fetal bovine serum (FBS). Cumulus oocyte complexes (COCs) were aspirated from follicles (2-6 mm) of sheep ovaries collected from local slaughter house. COCs were randomly divided into two groups and matured at 38.5°C, 5% CO₂ for 24 h (Group I) and 27 h (Group II). Oocytes cultured for 27 h showed significantly (P<0.05) more maturation than those cultured for 24 h (82 vs. 76%) followed by more cleavage (35 vs. 30%), morula (53 vs. 39%) and blastocyst (17 vs. 11%) percentage. In the second experiment, oocytes were randomly divided into two groups and matured with 10% FBS (Group I) and 20% FBS (Group II) for 27 h supplemented with pyruvate, glutamine, LH, FSH and estradiol. After maturation, oocytes were fertilized by fresh semen for 18 h. Presumptive zygotes in both the groups were again divided into two groups and cultured in 10 and 20% FBS during post fertilization period, respectively. Different FBS concentration in maturation medium did not influence maturation percentage (82 vs. 79%) significantly. Out of culture groups, presumptive zygotes matured in 20% FBS and cultured in 20% FBS during post fertilization period showed significant increase in cleavage percentage (44 vs. 39, 35 and 27%) as compared to other groups but subsequent development to morula (55 vs. 53, 43 and 40%) and blastocyst (20 vs. 17, 16 and 15%) percentage were more in the group matured in 10% FBS and cultured in 20% FBS during post fertilization period.

Keywords: Cumulus oocyte complexes, Embryo, In vitro embryo production (IVEP), IVM

The success rate of in vitro embryo production (IVEP) from slaughter house ovaries has been improved over time to produce large number of embryos, employing a variety of culture media. The IVMFC technique has been standardized in different species and efforts are aimed to improve this technique by reducing the cost of technology and substituting expensive inputs of the in vitro maturation (IVM) process with less expensive and chemically defined inputs. However, the success rate of IVEP is low as compared to in vivo, which on the other hand points to further improvement for its wide use.

IVM is one of the essential steps of IVEP. During IVM, oocytes undergo a series of cytoplasmic changes before resumption of nuclear maturation, leading to variable competence to result in embryo. IVM timing of sheep and goat oocyte varies from 22-32 h. Majority of studies have reported maturation timing of 24 h but some studies have reported maturation timing of 27 and 32 h. It indicates that maturation timing might be playing an important role in both cytoplasmic and nuclear maturation of oocytes for subsequent embryo development. In most of the IVEP studies, IVM medium is supplemented with serum. Serum is known to contain amino acids, hormones, trace elements, growth factors, cytokines, vitamins, heavy metal cations and many other substances needed for oocyte maturation and serves as an osmolyte as well as surfactant. Serum has a biphasic effect, i.e., it inhibits early cleavage divisions but has beneficial effect on development of morulae into blastocyst. Even though a higher number of blastocyst stage is obtained from media supported with serum, these supplementations sometimes cause alterations in the ultrastructure of embryos, impaired compaction, abnormal blastulation, large calf syndrome, aberrant mRNA expression profiles, greater incidences of stillbirths and mortality after birth. Fetal bovine serum (FBS) has been used mostly for maturation of oocytes collected from sheep and goat. However, reports such as the media devoid of FBS showing better maturation effect has made the use of FBS in IVM of sheep and goat oocytes controversial.

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Hence, to get proper maturation (cytoplasmic and nuclear) of oocyte and subsequent developmental stages of embryo, maturation timing, medium supplemented with protein and appropriate concentration of FBS plays an important role. Considering the views given above, there is a need for a systematic study of maturation timing and FBS concentration in the in vitro medium properly standardized for sheep to get better number of viable embryos. Therefore, in the present study, we assessed the maturation timing (24 vs. 27 h) of sheep oocytes in vitro and also observed the developmental potential of oocytes in different concentrations of FBS (10 vs. 20%) in maturation and culture media during post fertilization period.

Materials and Methods

In vitro oocyte maturation

Sheep (breed Bannur) ovaries were collected from local slaughter house in normal saline solution (NSS) fortified with antibiotics and carried to the laboratory at 35-37°C within 3 h of collection. Ovaries were washed 5-6 times in NSS and rinsed in 70% ethyl alcohol for 2-3 min to eliminate surface organisms. Oocytes were aspirated from follicles (2-6 mm) with the help of 20G needle attached to 5 mL syringe containing oocyte collection media (OCM) (TCM-199 + BSA (3 mg/mL) + 5% FBS + heparin (10 μg/mL). Aspirated oocytes (excellent and good quality) were selected for IVM. Cumulus oocyte complexes (COCs) (15-20 numbers) were matured in 35 mm petridish and incubated in CO₂ incubator at 38.5°C, 5% CO₂ and 95% RH in 100 μL of maturation media (TCM-199 + 10% FBS + BSA (3 mg/mL)+ pyruvate (4 mM) + glutamine (0.68 mM) + gentamycin (50 μg/mL)+ FSH (5 μg/mL) + LH (5 μg/mL) + estradiol (1 μg/mL)) under paraffin oil for 24 h (Group I) and 27 h (Group II). The maturation rate was assessed based on the degree of cumulus expansion and extrusion of the first polar body by aceto-orecin staining method. From the result of first experiment, in the second experiment, oocytes were randomly divided into two groups and matured in maturation media with 10% FBS (Group I) and 20% FBS (Group II) for 27 h.

In vitro fertilization (IVF)

IVF was performed by collecting fresh semen from the ram with the help of electro ejaculator. semen after collection was washed twice with washing medium {Fert- TALP+heparin (10 μg/mL)+pyruvate (1 mM)} by centrifuging at 2000 rpm for 5 min. After wash supernatant was removed and pellet was reconstituted in fertilization medium [Fert- TALP+fatty acid free BSA (4 mg/mL) + heparin (10 μg/mL)+pyruvate (1 mM)+ BME (100X) (1%)+ MEM (50X) (1%)] and final sperm concentration was adjusted to 2-3×10⁶ sperms/mL which was assessed through Neubauer’s chamber. The sperm suspension after process was kept in 5% CO₂, 38.5°C and 95% RH till matured oocytes were washed 4-5 times in fertilization medium. Finally in vitro matured oocytes (15-20 numbers) were inseminated with 100 μL of processed spermatozoa and fertilization was carried out by co-incubation of sperm and oocytes for 18 h in fertilization medium in the same temperature and gaseous condition described for maturation.

In vitro culture (IVC)

Following 18 h co-incubation, presumptive zygotes from both maturation groups of second experiment were divided into two groups each and cultured in 10 and 20% FBS during post fertilization period at the same temperature and gaseous condition described for maturation and fertilization in 100 μL of culture media [TCM-199+10% FBS/20% FB+BSA (3 mg/mL) +pyruvate (4 mM)+glutamine (0.68 mM) +gentamycin (50 μg/mL)+BME (100X) (1%)+MEM (50X) (1%)] to get embryos of different developmental stages from 2-cells to blastocysts stage. Presumptive zygotes of first experiment were cultured in similar way discussed above with 10% FBS during post fertilization period. Cleavage rates were recorded on day 2 (48 hpi) of culture and stages of embryonic development were evaluated every 24 h. Blastocyst development was recorded on day 7 (day 0=day of IVF). Finally, there were four groups under observation. Group I (matured in 10% FBS and cultured in 10% FBS); Group II (matured in 10% FBS and cultured in 20% FBS); Group III (matured in 20% FBS and cultured in 10% FBS); and Group IV (matured in 20% FBS and cultured in 20% FBS). In this study, cleavage percentage was calculated from the number of oocytes fertilized whereas morula and blastocysts percentages were calculated from the number of embryos cleaved.

Statistical analysis

The results of the study were analysed by one way ANOVA, wherever necessary to reveal the significant differences among the groups.
Results
The result (Table 1) showed that the maturation percentage was significantly ($P < 0.05$) higher in oocytes matured for 27 h (82%) than 24 h (76%) in terms of cumulus expansion and extrusion of first polar body (Fig. 1a and b). Maturation of oocytes for 27 h also showed increase in cleavage percentage than 24 h (35 vs 30%) but it was not significant. However, subsequent development to morula (53 vs 39%) and blastocyst (17 vs 11%) stages was significantly more in 27 h than 24 h (Table 1). In the second experiment, oocytes were matured in two concentrations of FBS (10 and 20%) as well as cultured again in the same two concentration of FBS (10 and 20%) during post fertilization, thus there were four groups under observation. Result of this study (Table 2) reflects that out of four culture groups, oocytes matured in 20% FBS and presumptive zygotes cultured in 20% FBS (Group IV) showed significant increase in cleavage percentage (44 vs 39, 35 and 27%) as compared to other three groups but subsequent development to morula (55 vs 53, 43 and 40%) and blastocyst (20 vs 17, 16 and 15%) stages were more in the group matured in 10% FBS and cultured in 20% FBS (Group II) (Fig. 1c).

Discussion
The present study was undertaken first to examine the effect of duration (24 h vs. 27 h) on maturation of oocytes. In this study, 24 h maturation showed higher maturation percentage than some previous studies which could be due to factors like breed differences, selection of COCs for maturation or higher number of quality oocytes recovered by aspiration method or more appropriate culture condition. Generally, most of the immature oocytes collected from mammalian ovaries fail to develop up to the preimplantation stage following in vitro maturation, fertilization and culture (IVMFC). This failure has been attributed to factors like quality of oocyte and the culture conditions. Oocytes undergo a series of cytoplasmic changes before resumption of nuclear maturation, that result in embryo after fertilization. Hence, 27 h maturation timing might be suitable for these cytoplasmic and nuclear changes than 24 h maturation. Therefore, the present study showed significantly higher maturation in 27 h than 24 h. Similar finding has already been reported in goat previously. In this study, cleavage percentage was not influenced by maturation timing. However, subsequent development to morula and blastocyst were significantly more in 27 h than 24 h.

![Fig. 1](image)

**Figure 1**—(a) Immature oocytes; (b) In vitro matured oocytes; and (c) Developmental stages of embryo.

<table>
<thead>
<tr>
<th>Maturation timing</th>
<th>Maturation (%)</th>
<th>Oocytes cultured</th>
<th>Cleavage (%)</th>
<th>4-8 cells (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>76.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>260</td>
<td>79 (30.38)</td>
<td>57 (72.15)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31 (39.24)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9 (11.39)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>27 h</td>
<td>82.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>416</td>
<td>144 (34.61)</td>
<td>115 (79.86)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76 (52.78)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25 (17.36)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripts in the same column differ significantly at $P < 0.05$

<table>
<thead>
<tr>
<th>Maturation medium (FBS)</th>
<th>Maturation (%)</th>
<th>Culture medium (FBS)</th>
<th>Oocytes cultured</th>
<th>Cleavage (%)</th>
<th>4-8 cells (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>82.21</td>
<td>10%</td>
<td>416</td>
<td>144 (34.61)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>115 (79.86)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76 (52.78)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 (17.36)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20%</td>
<td>79.74</td>
<td>10%</td>
<td>394</td>
<td>106 (26.90)&lt;sup&gt;h&lt;/sup&gt;</td>
<td>75 (70.75)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46 (43.39)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16 (15.09)&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripts in the same column differ significantly at $P < 0.05$
Previous researchers have reported that duration of exposure to maturation medium has little effect on the cleavage rate but markedly influences the rate of development to the blastocyst stage\textsuperscript{15}. Different media used for \textit{in vitro} maturation support maturation significantly at different rates\textsuperscript{4}.

In the second experiment, from the four groups under observation it was observed that though oocytes matured in 20\% FBS and presumptive zygotes cultured in 20\% FBS during post fertilization period (Group IV) showed significant increase in cleavage percentage as compared to other three groups, the subsequent development to morula and blastocyst percentage were more in the group matured in 10\% FBS and cultured in 20\% FBS (Group II). There may be several possible reasons to decrease subsequent developmental stage of morula and blastocyst in group IV other than groups though this group had shown significant increase in cleavage percentage. One of the most appropriate reason might be that increased accumulation of lipid, mainly polyunsaturated fatty acids, in embryos because of more concentration of FBS in maturation and culture condition, predispose the embryo to oxidative injuries\textsuperscript{16}. Moreover, serum supplementation has also been shown to inhibit cell division and induce mitochondria-mediated apoptosis\textsuperscript{17}.

The present study has shown that 27 h maturation timing and 10\% FBS in maturation medium has favourable effect on \textit{in vitro} maturation and embryo production in sheep and subsequently, 20\% FBS in culture medium during post fertilization period improved the percentage of morula and blastocysts.

\textbf{Conflict of interest}

None of the authors have any conflict of interest to declare.

\textbf{References}