Cleavage Capability of Water Buffalo Follicular Oocytes Classified by Cumulus Cells and Fertilized In Vitro

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ABSTRACT. Water buffalo (Murrah) oocytes were collected from ovaries obtained from the slaughter house. They were classified according to the character of the cumulus cells under a stereomicroscope, and cultured in 25 mM Hepes buffered Tissue Culture Medium-199 (TCM-199) supplemented with 5% estrous water buffalo serum in an atmosphere containing 5% CO2 in air at 39°C. After 20-24 hr of in vitro maturation, the oocytes were fertilized using capacitated sperm obtained from 4 different bulls. For cleavage the oocytes were cultured at 39°C in TCM-199 supplemented with 1% estrous water buffalo serum and in an atmosphere containing 5% CO2 in air. The good oocytes, with compact and dense cumulus cells cleaved significantly higher (p<0.01, 67.3%, 33/49), than those of fair, partially naked oocytes with thin cumulus layers (27.5%, 25/91) or small remnants of cumulus cells and poor naked oocytes (3/100). A substantial variation in fertilization and developmental rates (16.0% to 43.8%) was observed among 4 different bulls. Late non-surgically into 14 buffalo recipients on day 6 or 7 of their estrous cycle. One recipient was diagnosed to be pregnant by rectal palpation on day 60 and confirmed to be so on day 90 post-estrus.—KEY WORDS: in vitro fertilization, oocyte, water buffalo.

In recent years, viable offsprings have been produced in sheep [3], cattle [4, 8, 9, 11] and pigs [12] by techniques involving the in vitro fertilization (IVF) of ovarian oocytes recovered from the slaughter house and matured in vitro. Methods of IVF and embryo culture in eutherian mammals are now used routinely in livestock production. The water buffalo is one of the most interesting farm animals in getting the emerging technology of in vitro embryo production, especially in tropical and sub-tropical countries. Singh Gurpreet et al. [19] reported that 25 oocytes of 487, obtained from water buffalo, cleaved to the two-cell stage and subsequently one of the two cell embryos developed to eight-cell stage. However, there have been no successful transferable embryos and occurrence of pregnancies following the use of in vitro maturation (IVM) and IVF techniques. The appearance of cumulus cells surrounding the oocytes and that of the ooplasm are correlated, and considered to be the only obvious indicators of an oocyte’s potential to mature in vitro [10]. Shioya et al. [21] showed that normal fertilization rate of oocytes with cumulus cells was significantly higher than that of naked oocytes. However, limited information is available regarding the fertilization and cleavage capability of water buffalo oocytes. The aim of this study is to examine the capability of water buffalo follicular oocytes, classified according to the appearance of cumulus cells, to be fertilized in vitro. To obtain successful pregnancy, 4 different bulls were used when in vitro fertilized oocytes were transferred to the recipients.

MATERIALS AND METHODS

The ovaries of water buffalo were obtained at a New Delhi slaughter house and kept in physiological saline solution supplemented with 100 IU/ml penicillin at 37°C. Time between animal slaughter and recovery of oocytes from the ovaries was 6-7 hr. Oocytes from follicles of 2-4 mm in diameter were aspirated. The follicular contents were then mixed with the same volume of Dulbecco’s Phosphate Buffered Saline and TCM-199 (Earle’s Salt) medium with 25 mM-Hepes buffer supplemented with 3 mg/ml bovine serum albumin. Oocytes were collected in the embryo collector dish [20] and washed several times in the culture medium. The oocytes were classified according to the appearance of cumulus cells under the microscope as follows: Good oocytes had compact and dense cumulus cell layers; Fair oocytes had compact but not dense cumulus cell layers and some were partially naked. Poor oocytes were naked. Degenerated oocytes were naked with degenerated cytoplasm. Oocytes classified as good, fair and poor were cultured in 100 μl drops of TCM-199 (Earle’s Salt) medium which consist of 25 mM-Hepes buffer.
supplemented with 5% heat-treated estrous Murrah buffalo serum. Semen were obtained from 4 different Murrah bulls at the Karnal Dairy Institute in India and diluted to about 10 ml with 5 mM caffeine and 10 \( \mu \)g/ml heparin [16] in a modified defined medium [2].

The extender was removed from the sperm suspension by washing two times (500 × g) for 5 min each. The sperm pellet was resuspended in the same medium as used for washing to give a sperm concentration of 8 × 10^6/ml. The adjusted sperm suspension was diluted to 1:1 with 10 mg/ml crystalized BSA. So, the final sperm suspension for fertilization consisted of caffeine (5 mM/ml) and heparin (10 \( \mu \)g/ml) plus BSA (5 mg/ml) in a volume of 100 \( \mu \)l. Aliquots of sperm were incubated for 1.5–2 hr at 39°C under 5% \( \mathrm{CO}_2 \) in humidified air. Classified oocytes cultured for 20–24 hr were transferred individually into sperm droplets for insemination and incubated for 6 hr. Thereafter the oocytes were transferred to the culture medium for cleavage and development. The medium consisted of 25 mM Hepes buffered TCM-199 supplemented with 5% Murrah buffalo estrous serum. Seventy-two hours after insemination, the culture medium was changed and cumulus cells were removed from the oocytes. These oocytes were cultured with cumulus cells in the same dishes. The culture media was then changed at 48 hr intervals. The oocytes were cultured for 6 days following insemination and were examined for cleavage under a stereomicroscope. Selected embryos were transferred non-surgically to synchronized buffalo recipients on day 6 or 7 (estrus day 0). Recipients were diagnosed for pregnancy on day 60 and 90 by rectal palpation. A statistical evaluation of the differences between treatment groups was made by Chi-square test.

RESULTS

The total of 536 oocytes were obtained from the 518 ovaries of water buffalo in the 4 groups. Number of oocytes classified as good, fair and poor was 49 (9.5%), 91 (17.0%) and 100 (18.7%), respectively. The cleavage rates of good, fair and poor oocytes were 67.3% (33/49), 27.5% (25/91) and 3% (3/100), respectively. Significant difference (p<0.01) was observed between the good oocytes when compared to the fair and poor ones. Yield of transferable embryos from good, fair and poor oocytes was 18.4% (9/49), 5.5% (5/91) and 0% (0/100), respectively. A substantial variation in fertilization and

<table>
<thead>
<tr>
<th>No. of examination</th>
<th>No. of ovaries</th>
<th>No. of oocytes</th>
<th>No. of cultured oocytes (%)</th>
<th>No of degenerated oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>obtained (%)</td>
<td>Good</td>
</tr>
<tr>
<td>1</td>
<td>67</td>
<td>70(104)</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>115</td>
<td>140(122)</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>160</td>
<td>146(91)</td>
<td>16</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>176</td>
<td>180(102)</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>518</td>
<td>536(104)</td>
<td>49(9.5%)</td>
<td>91(17%)</td>
</tr>
</tbody>
</table>

Table 2. Cleavage of in vitro fertilized buffalo oocytes, and yield of transferable embryos

<table>
<thead>
<tr>
<th>Classification of oocytes from appearance of cumulus cells</th>
<th>Good</th>
<th>Fair</th>
<th>Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of oocytes</td>
<td>49</td>
<td>91</td>
<td>100</td>
</tr>
<tr>
<td>No. of cleaved oocytes (%)</td>
<td>33(67.3)</td>
<td>25(27.5)</td>
<td>3(3.0)</td>
</tr>
<tr>
<td>Yield of transferable embryos (%)</td>
<td>9(18.4)</td>
<td>5(5.5)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

a, b, c: Values with different superscripts are significantly different (p<0.01).
developmental rates (16.0% to 43.8%) was also observed among the 4 different bulls. Late morulae obtained by IVM-IVF were transferred non-surgically to 14 buffalo recipients on day 6 or 7 (estrous day 0) of their estrous cycle. One recipient was diagnosed pregnant by rectal palpation on day 60 and confirmed to be so on day 90 post-estrus.

**DISCUSSION**

Assessment of the appearance of the cumulus oocyte complexes acquired by aspiration of follicles is very important to estimate oocyte quality [21]. The results of this study suggested that it is also very important to classify oocytes according to the cumulus cells for *in vitro* maturation and fertilization of buffalo oocytes. Many factors have been reported to affect maturation and fertilization. The quality, source and preparation of the sperm and oocytes, the culture medium and incubation periods are all important factors affecting *in vitro* fertilization [17].

In this experiment, we dealt with the appearance of the cumulus cells attached to oocytes under the same conditions of Shioya's report [21]. The total number of oocytes, classified as: good, fair and poor oocytes, were quite different from that of bovine oocytes [7]. Only 536 oocytes were obtained from 518 ovaries, thus only a limited number was available for classification. It has been suggested that a few oocytes, that can be obtained from the ovaries could limit superovulation responses in the water buffalo [13]. Most of naked oocytes had germinal vesicles, in which their border with the ooplasm was indistinct. The low cleavage rates of naked oocytes show that naked oocyte may have already degenerated before the start of culture for maturation and thus, the low maturation rate of naked oocytes may not be due to the lack of cumulus cells. A large number of cumulus cells and some direct cell-oocyte contact were necessary for completion of meiosis [18]. Significant difference in cleavage rate between good and fair oocytes indicate that thick, compact cumulus cells are essential for further fertilization of the embryos after *in vitro* fertilization.

The effect of heparin may be enhanced by caffeine [14]. In this study, we used 5 mM caffeine with 10 μg/ml heparin supplemented with BO for capacitation. EL-Menoufy [5] described that the optimum concentration of caffeine for activity of water buffalo spermatozoa was 2–8 mM/ml, and the stimulation of fructolytic activity was achieved when 8–10 mM/ml caffeine was used. However, cleavage rates were very low in this study, although 5 mM caffeine was used for capacitation. These results indicate that the optimum concentration of caffeine and heparin must be determined.

Addition of sperm to maturation medium is absolutely required for cumulus expansion and oocyte maturation, and attainment of normal embryonic development in water buffalo. Addition of hormones has been substituted by estrous cow serum, which has proven to effectively induce maturation, resulting in oocytes with high developmental competence [6]. Although we used the estrous water buffalo serum for IVM, IVF and IVC in this study, our results are far from conclusion, because only a limited number of oocytes were used.

The results of fertilization and embryo development *in vitro* varied, depending on the spermatozoa obtained from 4 different water buffalo bulls. Although such phenomenon is described for bovine bull [22], it is generally unrelated to the bull's *in vitro* fertilizing capability [15]. However, a similar report is not available for the water buffalo bull. Therefore, it is necessary for determining the optimum heparin concentration and/or number of
sperm for IVF to test water buffalo bull individually.

Although we did not use the co-culture system with oviduct cells [18] or granulosa cells [1] for in vitro maturation, fertilization and culture, at least 14 transferable embryos were produced by the simple co-culture system [8]. However the cleavage rate of in vitro matured and fertilized oocytes was very low (14.3% to 48.7%). It is, therefore, suggested that further research should be conducted to obtain the optimum conditions for IVM, IVF and IVC of water buffalo oocytes.

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