Live Births Resulting from Swamp Buffalo Oocytes Matured, Fertilised and Cultured in vitro

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ABSTRACT: In this study, developmental competence of early-stage buffalo embryos resulting from swamp buffalo oocytes matured and fertilised in vitro with murrah buffalo spermatozoa was determined after culture in either: (A) TCM 199 + 10% buffalo calf serum and a cumulus cell monolayer (cumulus cell monolayer) or (B) the amniotic cavity of a developing chick embryo (CEAm). The proportion of early stage embryos developing to morula and blastocyst stages was greater in the cumulus cell monolayer treatment group (35.7%) compared to the CEAm treatment group (10.6%). Non-surgical transfer of these embryos to swamp buffalo recipients (cumulus cell monolayer: n=24 embryos; CEAm: n=6 embryos) resulted in the birth of a male (26 kg) and a female (21 kg) F1 buffalo calves. These results indicated that pre-implantation stage buffalo embryos cultured in cumulus cell monolayer and CEAm are viable to term after transfer to recipient buffalos.

Key Words: Swamp Buffalo, Oocytes, IVM, IVF, Births

INTRODUCTION

Considerable progress has been made in the development of procedures for in vitro maturation/in vitro fertilisation/in vitro culture (IVM/IVF/IVC) of buffalo oocytes, both for murrah and swamp buffalo type buffaloes (Singh et al., 1989; Suzuki et al., 1991; Totey et al., 1992 and Jainudeen et al., 1993). However, in vitro production of pre-blocking stage embryos and subsequent development to the blastocyst stage has been extremely variable. The ability of these embryos to develop to full term is compromised due to low numbers of in vitro produced blastocysts as a proportion of total oocytes collected and a high incidence of embryonic death (Suzuki et al., 1992). One possible constraint has been the absence of a reliable IVC system. For instance, the cumulus cell co-culture system was observed to effectively provide the necessary culture environment for early-stage embryos to develop to the advanced pre-implantation stages only when it is in direct contact with oocytes throughout the maturation period. Similarly, the amniotic fluid of a 72 hr-old chick embryo has been found to support normal mammalian embryogenesis due to the growth factors associated with the developing chick embryos and in the purity of the amniotic fluid itself (Blakewood et al., 1989).

By using these culture systems in the production of pre-implantation buffalo embryos from oocytes that have been matured and fertilised in vitro, it is hoped that a considerable number of embryos can be produced and ultimately develop to full term. In this study, we determined the developmental competence to term of swamp buffalo oocytes matured and fertilised in vitro with murrah buffalo spermatozoa after co-culturing with cumulus cells or in the amniotic cavity of a developing chick embryo.

MATERIALS AND METHODS

Oocyte collection and maturation in vitro

Ovaries were obtained from a local abattoir and transported to the laboratory in a sterile saline solution (0.9% NaCl, w/v) supplemented with antibiotics (0.1 mg/ml penicillin and 500 ug/ml streptomycin) at 35°C within 5 h. Oocytes were aspirated from 3-6 mm follicles using an 18 gauge needle attached to a 10 ml syringe. The selected oocytes with intact cumulus cell investments with evenly granulated cytoplasm, were washed twice and cultured in droplets of TCM 199 + 10% buffalo calf serum supplemented with gonadotropins (FSH, 0.5 ug/ml; LH, 10 ug/ml, Sigma, St. Louis, MO) for 22 h in an incubator (39°C; 5% CO2 in air with high humidity).

Sperm preparation and fertilisation in vitro

Locally processed frozen semen from a murrah buffalo bull was used for in vitro fertilisation. For each trial, two straws containing 0.5 ml semen were thawed in a water bath at 37°C for 15 sec and processed by the swim-up method. Briefly, the 1 ml semen suspension was put in a test tube layered with 2 ml of the fertilisation medium (7 reference) containing caffeine (5 mM) and bovine serum albumin (BSA; 10 mg/ml) and incubated (39°C, 5% CO2 in air) for 1 h. Afterwards, approximately 1.8 ml of the upper portion of the semen suspension was recovered and washed twice in the same fertilisation medium by centrifugation at 1,800 rpm for 10 min. The sperm pellet was re-suspended in the same medium to give an initial sperm concentration of 10 x 106 sperm/ml. Then, a 50 ul aliquot of the sperm suspension was introduced in droplets of 50 ul fertilisation medium.
containing the pre-washed oocytes to co-incubate. The final sperm concentration during fertilisation in 2.5 mM and 5 mg/ml BSA was 5 x 10^6 sperm/ml.

**In vitro culture and embryo transfer**

After 6 hr of sperm-oocyte co-incubation, the oocytes were recovered and cultured in droplets of TCM 199 + BCS and cultured for a total of 30 h. Cleaved embryos at 2- to 4- cell stages were then pooled and cultured in (A) cumulus cell monolayer or for 144 hr or in (B) in the amniotic cavity of a developing chick embryo (CEAm) for 72 h. After 72 hrs, embryos cultured in CEAm were recovered and cultured for an additional 72 hr in droplets of TCM 199 + BCS for continued development. The cumulus cell monolayer was prepared from the oocytes which were denuded free of cumulus cells to form a monolayer. The CEAm culture system was prepared using a 3 day old embryonated chicken egg maintained in a conventional incubator with provisions for adequate temperature (38°C) and humidity (40-70%). At the end of the culture period compacted morula and early blastocyst stage embryos (cumulus cell monolayer: n=24 embryos; CEAm: n=6 embryos) were transferred non-surgically to selected recipients (normally cycling swamp buffalo cows) on days 6 or 7 of their oestrous cycle (three embryos per recipient). Oestrus of the recipients either occurred naturally or was induced artificially by intramuscular injection of Oestrophan (0.25 mg Cloprostenol). Pregnancy diagnosis was performed on days 60 and 90 of gestation by rectal palpation.

**STATISTICAL ANALYSIS**

The data were analysed by Chi-Square test, with a probability level of P<0.05 being considered significant.

**Results**

Both of IVC systems used were able to support three to four successive cleavages of the initially cultured 2-4 cell stage buffalo embryos. However, the proportion of embryos reaching the compacted morula and blastocyst stages was greater for the cumulus cell monolayer compared to the CEAm system (35.7% and 10.6%, respectively). Of the 66 embryos derived from CEAm system, 60 were recovered from the amniotic cavity and after another 72 hrs of culturing in droplets of TCM 199 + 10% BCS, 7 of these embryos developed to the compacted morula and blastocyst stages.

Following embryo transfer, two pregnancies were detected on day 60 and later confirmed on day 90 by rectal palpation. This resulted in a male calf (335 days gestation) derived from the cumulus cell monolayer group and a female calf (321 days gestation) derived from the CEAm group.

**DISCUSSION**

Data presented in this study have shown that swamp buffalo oocytes matured and fertilised in vitro with murrah buffalo spermatozoa could develop to pre-implantation stage when co-cultured with cumulus cell monolayer or in CEAm and result to live birth after transfer. The usefulness of these coculture systems have been demonstrated in other mammalian species (Goto et al., 1988; Blakewood et al., 1989,1990; Ocampo et al., 1993 Madan et al., 1994b) The ability of cumulus cell monolayer to support embryo development is reflective of the secretory products synthesised during active cell mitotic division (Wiemer et al., 1991) whereas, the purity of the amniotic fluid, the growth factor(s) associated with the growing chick embryo and the ability of a living chick embryo to actively regulate its own environment makes it a good alternative physiological system for mammalian embryo development (Blakewood et al., 1989; Ocampo et al., 1994). The apparent ability of the chick embryo to continue its growth in a warm, moist atmosphere that does not require careful monitoring of CO₂ concentration in air makes it a viable option in situations where a CO₂ incubator is not available.

In conclusion, early stage buffalo embryos derived from fertilisation of swamp buffalo oocytes by murrah buffalo spermatozoa can acquire developmental competence when cocultured with cumulus cell monolayer or in CEAm. The identification of factor(s) responsible for the support of buffalo embryogenesis would be beneficial for the future development of a simple defined embryo culture for the specific investigation of factor(s) needed for a better understanding of the physiological requirements of buffalo oocytes/embryos. Moreover, the 20% pregnancy rate obtained in this study could be reflective of the effects of the environment on the viability of transferred embryos during transport from the laboratory to local villages where embryo transfer was undertaken. Although maximum care during handling and transport of the embryos was exercised, there is a need to develop an efficient freezing protocol of in vitro derived buffalo embryos to improve pregnancy rates following embryo transfer of buffalo embryos in village setting.
Table 1. Results of culturing cleaved buffalo embryos.

<table>
<thead>
<tr>
<th>Embryo culture System</th>
<th>No. of 2-4 cell stage embryos cultured / (recovered from amniotic cavity)</th>
<th>Stage of development attained after culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: cumulus cell monolayer</td>
<td>70</td>
<td>45 (64.25)b</td>
</tr>
<tr>
<td>B: CEAm fluid</td>
<td>66 (60)*</td>
<td>53 (88.3)a</td>
</tr>
</tbody>
</table>

*a,b* Values with different superscripts differ at P<0.005

* stage of embryos obtained after recovery from the amniotic cavity (72 hrs culture)

** stage of embryos obtained after another 72 hrs of culture in TCM 199 + BCS

Table 2. Results of transferring buffalo embryos.

<table>
<thead>
<tr>
<th>Origin of embryos</th>
<th>Recipient caracow no.</th>
<th>Estrous cycle (day)*</th>
<th>No. of embryos transferred</th>
<th>Stage of embryos**</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>cumulus cell monolayer</td>
<td>1</td>
<td>7***</td>
<td>3</td>
<td>3 EB</td>
<td>- returned to oestrus</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>2 CM</td>
<td>- male calf was born (26 kg)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6</td>
<td>3</td>
<td>1 EB, 2 ExpB</td>
<td>- returned to oestrus day 20</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>3 CM</td>
<td>- returned to oestrus day 22 of cycle</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>1 EB, 2 ExpB</td>
<td>- cyst palpated on horn bifurcation</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>3 CM</td>
<td>- returned to oestrus on day 21 of cycle</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>6</td>
<td>3</td>
<td>2 CM, 1 ExpB</td>
<td>- returned to oestrus on day 20 of cycle</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6</td>
<td>3</td>
<td>2 EB, 1 ExpB</td>
<td>- returned to oestrus on day 21 of cycle</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>6***</td>
<td>3</td>
<td>2 CM, 1 EB</td>
<td>- female calf was born (21 kg)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>1 CM, 2 EB</td>
<td>- returned to oestrus on day 20 of cycle</td>
</tr>
</tbody>
</table>

* day 0 of oestrus cycle  ** CM – compacted morula; EB early blastocyst; ExpB expanded blastocyst

*** natural oestrus
REFERENCES


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