Full-term Development of *in vitro* Produced-Vitrified Water Buffalo (*Bubalus bubalis* Linn) Embryos

Danilda Hufana-Duran*, Prudencio B. Pedro, Hernando V. Venturina, Peregrino G. Duran and Libertado C. Cruz

Embryo Biotechnology Laboratory, Philippine Carabao Center, Science City of Muñoz, Nueva Ecija 3120 Philippines

Full-term developments of *in vitro* produced (IVP) vitrified water buffalo embryos derived from *in vitro* matured and fertilized oocytes were assessed. Oocytes were matured *in vitro* for 22-24 hours and fertilized by sperm-oocyte co-cultured in a humidified incubator for 6 to 8 hours. Presumptive zygotes were co-cultured with cumulus cell monolayers for development. Resultant pre-implantation stage embryos within 7 days of *in vitro* culture were cryopreserved through vitrification method and post-warming, these were transferred non-surgically to recipients. Out of the 149 IVP vitrified embryos transferred to 79 recipients, a 13.9% (11/79) calving rate was achieved marking a 7.9% (11/149) full-term development of IVP vitrified embryos. Eleven healthy normal calves with average birth weights of 37.0±5.4 kg were born. These results demonstrate the viability of IVP vitrified water buffalo embryos, potential application of the techniques in optimizing the female contribution on genetic progress, and on overall water buffalo genetic improvement.

Key Words: water buffalo, *in vitro* maturation, *in vitro* fertilization, oocytes, spermatozoa, embryo, *in vitro* culture

INTRODUCTION

Assisted reproductive technologies such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET) have been introduced to overcome reproductive inefficiencies and accelerate genetic gain. However, MOET in water buffalo is compromised by the poor ovulatory response of this animal species to hormonal treatment (Karaivanov 1986; Karaivanov et al. 1990; Misra et al. 1990; Cruz et al. 1991). In vitro production of embryos provides an excellent source of embryos for basic research on developmental biology and physiology and for the application of emerging biotechnologies such as nuclear transfer and transgenesis. Our initial report have shown the efficiency of this technique as alternative tool for water buffalo genetic improvement (Hufana-Duran et al. 2004). Furthermore, the application of IVP is an attractive tool for the production of purebred superior genetic animals by interspecific embryo transfer to rescue endangered species or propagate the desired breeds.

*In vitro* production of embryos involves retrieval of oocytes from ovaries of slaughtered animals or live animals through ovum pick up. The oocytes are cultured *in vitro* for maturation in a span of 22-24 h in cattle (Iwata et al. 2004) and water buffalo (Gasparrini 2002), 28-30 h in equine (Dell’aquila et al. 2001), and 16 to 24 hours for sheep and goat (Cognie et al. 2003). In water buffalo, the method was extrapolated from cattle (Totey et al. 1992; 1993a&b; Madan et al. 1994), however, result showed considerable low rate of success compared to cattle (Hasler et al. 1995). Inherent problems of low follicular oocyte population coupled with fragility of buffalo semen

*Corresponding author: danildahd@yahoo.com*
(Madan et al. 1994) and limitations of the in vitro culture systems (Duran et al. 1997) result in low cleavage rate and morula/blastocyst production. However, with the large number of riverine buffaloes slaughtered for meat export in India, in vitro maturation and fertilization of oocytes may prove to be useful for obtaining large number of embryos (Totev et al. 1993b). This possibility is a great opportunity to enhance buffalo genetic improvement in swamp buffalo-dominated countries.

Birth of live calves from in vitro produced buffalo embryos transferred fresh to the recipient animals were reported (Madan et al. 1991; Suzuki et al. 1992). The fresh embryo however, could not survive the outside environment beyond 2 h (Gasparrini 2002). Misra et al. (1990) reported pregnancies and Kasiraj et al. (1993) reported birth of live calves out of in vivo collected buffalo embryos cryopreserved by slow-freezing method. The slow-freezing method, however, is time consuming and requires use of expensive equipment. Vitrification method was found to be a very practical approach for cryopreservation of mice embryos (Kasai et al. 1990) as it allows direct freezing without using any expensive equipment or materials. The efficiency of vitrification system, plus the capacity of in vitro produced buffalo embryos to withstand the stress brought about by the in vitro manipulation and vitrification treatments post alarm on the efficiency of these techniques on buffalo genetic improvement. Assessment of the survivability of in vitro produced buffalo embryos after vitrification and viability to undergo full term development is therefore of great importance, hence, this study.

MATERIALS AND METHODS

Oocytes retrieved from ovaries of slaughtered riverine buffaloes and capacitated frozen semen of genetically superior progeny tested bulls in India was used in the production of embryos in vitro. Production of embryos was carried out at the satellite Embryo Biotechnology Laboratory of Philippine Carabao Center in Aurangabad, India. Transfers of embryos were carried out in the Philippines. Detailed procedure of the techniques employed for the production, storage and transfer of the embryos were described in detail in our previous report (Hufana-Duran et al. 2004) with few modifications as follows:

Culture and Holding Medium. Unless specified, culture media used on in vitro embryo production is Medium 199 (Earle’s salts with 25 mM HEPES, GIBCO, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FCS, GIBCO, Grand Island, NY, USA,) and antibiotics (100 IU/mL Penicillin G and 100 µg/mL Streptomycin sulfate). Culture medium was prepared in 100 µL droplets in a Nunc tissue culture dishes (35 x 10 mm, Nunclon 153066, Inter-med., Roskilde, Denmark) covered with mineral oil (Embryo tested, Sigma Chemical Co., Saint Louis, MO) and equilibrated inside a water-jacketed incubator (Forma Scientific 3111 Series) with air temperature of 39°C and 5% CO₂.

Oocyte Collection and In Vitro Maturation. Ovaries were collected from slaughtered riverine buffaloes of various ages and reproductive status then transported to the laboratory in 0.9% sodium chloride solution with 100 units penicillin/mL and 100 µg streptomycin/mL (both from Sigma) at 28° to 33°C. Immature oocytes were collected by aspiration from 2 to 8 mm surface follicles using 18g needle attached to 10 mL sterile disposable plastic syringe. Oocytes with compact cumulus cells and evenly granulated cytoplasm were selected and washed two times in 37°C pre-warmed Phosphate Buffered Saline (PBS) with 3% FBS and two times in pre-equilibrated maturation medium. Oocytes were randomly distributed, 10 in each of 100 µL culture droplets and matured in vitro in a water-jacketed incubator for 22 to 24 h at 39°C and 5% CO₂.

In vitro Fertilization, Embryo Culture and Blastocyst Development. Frozen semen from Murrah buffaloes were thawed at 37°C for 15 sec, dispensed in a sterilized centrifuge tube and layered with 6 mL of freshly prepared 37°C pre-warmed Bracket and Oliphant solution (Brackett & Oliphant 1975) containing 100 µg Na-pyruvate/mL, 10 mM caffeine and 4 units heparin/mL (sperm washing medium). Semen suspensions were washed twice by centrifugation at 800 x g for 8 min discarding the supernatant after each wash. The sperm concentration was determined in a Neubauer chamber and the sperm suspension was diluted 1:1 (v:v) with BO solution containing 10 mg BSA/mL (Fraction V, Wako Pure Chemical Ind., Osaka, Japan) (sperm dilution solution) to form a final sperm concentration of 1 x 10⁶ sperm cells/mL, 5 mM Caffeine, 2 units heparin/mL and 5 mg BSA/mL for IVF medium. One hundred mL IVF droplets were prepared in a culture dish and covered with equilibrated mineral oil. Subsequently, in vitro matured oocytes were partly removed from cumulus cells which were retained and further cultured in maturation medium to develop cumulus cell monolayers for embryo culture. The partly denuded oocytes were washed two times in freshly prepared pre-incubated oocyte washing medium and one time in a dish of IVF medium. Ten oocytes were transferred to each IVF droplet. Sperm-oocyte co-culture for in vitro fertilization was done for a period of 6 to 8 h inside a humidified incubator gassed with 5% CO₂ in air at 39°C.
Approximately at 6 to 8 h of sperm-oocyte co-culture, the oocytes were removed from the fertilization dish, washed four times in pre-incubated culture medium and transferred into the former maturation droplets containing cumulus cells and cultured in vitro for embryo development. On the second day of in vitro embryo culture, cleaved zygotes were separated from the uncleaved ones. Embryo development was monitored daily renewing the culture medium every two days of in vitro culture. Embryos that developed to morula, early blastocyst, blastocyst and expanded blastocyst stages, respectively, were cryopreserved by vitrification method. Embryos were classified and evaluated according to the description of Lindner and Wright (1983).

Cryopreservation by Vitrification Method. The vitrification method described by Kasai et al. (1990) was adopted with few modifications. Vitrification solution used was EFS40 (Ethylene glycol, 40% vol/vol; Ficoll, 18% wt/vol; sucrose, 0.3 M). Details of the protocol are discussed previously (Pedro et al. 1999) and described below.

Subsequently, temperature inside the working room was adjusted to 25°C and all solutions used were equilibrated at the same temperature for at least 3 h. French straws with capacity of 0.25mL were marked at one end with pens of various colors for embryo identification. Embryo identification included rank, age in vitro, developmental stage, date of production, and bull identification. The other end of the straws was cut out with sharp scalpel to form a pointed-shaped open straw to place the embryos.

Consequently, embryos were washed with PB1 medium (Dulbecco’s phosphate-buffered saline containing 5.56 mM glucose, 0.33 mM pyruvate, 100 units penicillin/mL and 3 mg BSA fatty acid free/mL), exposed to EFS40 and placed on the tip of the pointed-shaped open straw within a period of 5 min and rapidly plunged in liquid nitrogen. Thereafter, straws were kept in liquid nitrogen tank.

Recipient Animals. Recipient riverine buffaloes, in complete confinement system of management, were either treated with Prostaglandin F2 alpha (Prostavet 2 mL im, Virbac Laboratories, France) to induce estrus or they were transferred with embryo after detection of natural estrus. For estrus synchronized recipient animals, functional CL was checked manually by palpation per rectum prior to PGF2 alpha administration. From the 36th to the 72nd hour after the prostaglandin injection, recipient animals were carefully observed every 4 h for estrus symptoms and particularly for the moment of standing heat, using a teaser bull. Signs of estrus considered were either standing-still while mounted, mucus discharge, frequent urination, bellowing, and mounting or a combination of all these manifestations. Symptoms of estrus were confirmed by palpation per rectum of the genitalia. Embryos were transferred to recipient animals on different days after estrus, according to their development (Day 0=onset of observed estrus/onset of IVF). Recipient animals were treated with Lidocaine Hydrochloride (2% im, Ethical Pharmaceutical Co. Pvt. Ltd., India) at varying levels depending on the response while in the chute.

Warming and Transfer of Embryos. Embryo straws were recovered from liquid nitrogen tank and the open pole where embryo(s) were loaded, was directly warmed in 0.5 M sucrose solution in PB1 medium at 25°C for re-expansion within 5 min. Re-expanded embryos were washed in PB1 medium and 1, 2, or 3 embryos were loaded in 0.25 mL French straw for transfer. Same batch of the embryos at various developmental stages were further cultured in vitro after warming to assess the in vitro hatching rate which served as control.

Before the transfer of the embryo(s), the presence of corpus luteum in the ovaries was checked by palpation per rectum. To enhance easy penetration of ET gun into the cervix, a cervix expander (FHK, Japan) was first inserted to the vagina of the recipient animals. After expanding the cervix, the expander was removed and the ET gun was inserted to the entrance of the cervix with an outer sheath. The tip of the outer sheath was then punctured and the gun was inserted into the cervix and pushed gently until it reached the uterine horn ipsilateral to the ovary bearing the CL. The tip of the gun was inserted up to 5 to 10 cm beyond the external bifurcation. Embryo was deposited into the uterine horn.

Pregnancy Diagnosis. Recipient animals were subjected to palpation per rectum for at least 30 d after ET to check the persistency of the corpus luteum present during the transfer of the embryos. Thereafter, confirmation of pregnancy was done by another palpation per rectum at least 45 and 180 d after the transfer. The number of calves born out of the diagnosed pregnant recipient animals was recorded.

Results were analyzed using Chi-square and Fisher’s Exact Test for any significant difference.

RESULTS AND DISCUSSION

A total of 71 IVP vitrified embryos were warmed and cultured in vitro culture for at least 72 h to assess hatching rate. Hatching rate obtained was recorded from 59 (83.10%) embryos. Irrespective of developmental stage, no significant difference (Table 1) was observed on in
vitro survival rate after vitrification and thawing indicating that in vitro produced buffalo embryos are capable of overcoming the stress brought about by the vitrification treatment and maintains development potential after ultra-low temperature storage demonstrating viability for embryo transfer.

Out of the 149 IVP vitrified embryos transferred to 79 recipients, a 13.9% (11/79) calving rate was achieved marking a 7.9% (11/149) full-term development. Eleven healthy and normal calves with average birth weights of 37.0 kg were born confirming our report (Hufana-Duran et al. 2004) demonstrating viability of the embryos, withstanding vitrification stress, and ability to undergo full term development and birth of live calves after embryo transfer.

Madan et al. (1994) obtained 12.5% calving rate after transfer of fresh in vitro produced buffalo embryos to recipient animals. Kasiraj et al. (1993) reported calf birth rate of 23% from in vivo-collected-cryopreserved-thawed water buffalo embryos. In the present study, the embryos are produced in vitro and vitrified for cryopreservation. The quality of the embryos as affected by the stress incurred by in vitro manipulation surely affects the success rate. In vitro produced embryos transferred fresh to the recipient animals were not exposed to cryopreservation stress thus more viable than embryos subjected to cryopreservation. Similarly, embryos produced in vivo were found to have better quality than those produced in vitro (Farin et al. 2001). Improving the in vitro culture system is necessary to improve the embryo quality.

The embryo-recipient synchrony is another factor that may have affected the present results. Reports (Xu et al. 1987; Misra and Jashi 1991; Kajihara et al. 1992) have indicated that embryo-recipient synchrony is one major factor that contributes to higher pregnancy rate. Ten of the calves were born from females transferred by natural estrus and only one calf was born from estrus-synchronized recipient. The pre-determined embryo transfer after estrus synchronization may have affected and contributed on the loss of pregnancies among synchronized recipient animals. Estrus observation was conducted by visual observation from the herd. However, the less pronounced signs of estrus in water buffaloes is a limiting factor to accurately determine the perfect timing for embryo transfer. Kasiraj et al. (1993) indicated that buffalo embryos produced in vivo and cryopreserved by slow freezing technique could only tolerate synchrony or asynchrony of -12 h. In cattle, Hasler et al. (1995) reported ±36 h asynchrony did not affect pregnancy rate. In the present study, embryo-recipient asynchrony reached up to -48 h to +24 h based on the occurrence of estrus manifestations among the selected recipients. It was noted that estrus manifestations recurred in about 3 to 4 consecutive days. This makes it difficult to establish the 0 h of the onset of estrus which makes asynchrony considered as one problem that affects the full term development of the IVP vitrified embryos.

Literature in cattle IVP of embryos illustrated many factors that affect the quality and viability of the IVP embryos. Some of these factors are the different protocols employed, medium condition and sources of oocytes, sperm preparation techniques, oocyte maturation and fertilization conditions, embryo environment and others. Further studies is recommended to finely tune the IVP techniques in water buffaloes to further improve the viability, and survivability after embryo transfer.

Table 1. In vitro hatching rate and full term development after non-surgical embryo transfer of in vitro produced vitrified water buffalo embryos

<table>
<thead>
<tr>
<th>Embryo stages</th>
<th>In vitro hatching rate, %</th>
<th>Number of embryos transferred</th>
<th>Full-term development rate, (%)</th>
<th>Average birth weight of resultant calf, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Morula</td>
<td>90.91</td>
<td>18</td>
<td>1 (5.55)</td>
<td>44.0</td>
</tr>
<tr>
<td>b. Early Blastocyst</td>
<td>80.00</td>
<td>24</td>
<td>1 (4.17)</td>
<td>33.0</td>
</tr>
<tr>
<td>c. Mid Blastocyst</td>
<td>75.00</td>
<td>25</td>
<td>1 (4.00)</td>
<td>43.1</td>
</tr>
<tr>
<td>d. Expanded Blastocyst</td>
<td>90.00</td>
<td>34</td>
<td>3 (8.82)</td>
<td>38.8</td>
</tr>
<tr>
<td>e. Combination of b&amp;c</td>
<td>-</td>
<td>16</td>
<td>2 (12.5)</td>
<td>32.65</td>
</tr>
<tr>
<td>f. Combination of c&amp;d</td>
<td>-</td>
<td>16</td>
<td>1 (6.25)</td>
<td>39.0</td>
</tr>
<tr>
<td>g. Combination of b&amp;d</td>
<td>-</td>
<td>16</td>
<td>2 (12.5)</td>
<td>35.7</td>
</tr>
<tr>
<td>TOTAL</td>
<td>83.10</td>
<td>149</td>
<td>11 (7.9)</td>
<td>37.0±5.4</td>
</tr>
</tbody>
</table>

Values in column 2, 4, and 5 are not significantly different (P<0.05)
Average birth weights of calves borne out of in vitro produced-vitrified embryos after embryo transfer ranged from 26-44 kg. Weights of twin births’ is lower (ranging from 26 to 27.4 kg) than single births (ranging from 36 to 44 kg). No large calf syndrome was observed among the calves as birth weights of water buffalo calves born out of artificial insemination ranges from 18kg-47kg (N=136, PCC data).

Embryos that reached morula, early blastocyst, mid blastocyst and expanded blastocyst stages were all resulted to birth of live calf. These results suggest that developmental stage is not a limiting factor on the success of vitrification and embryo transfer of in vitro produced water buffalo embryos as long as the embryos have developed to the advance preimplantation stages.

With the above results, commercial production of water buffalo embryos through in vitro production techniques and cryopreservation through vitrification approach offers a promising tool for buffalo genetic improvement program. The availability of superior genetic materials in India, which are otherwise wasted, could be used to produce buffalo embryos that could be used for embryo transfer in swamp-buffalo dominated countries. Reviews (Wrathall 1995; Stringfellow and Wrathall 1995) and studies (Avery et al. 1993; Galiore 1998) have indicated the safety of international trade and use of embryo transfer. With the sanitary controls imposed on IVF, commercial application of this technique would therefore a great opportunity to enhance buffalo genetic improvement. Improving pregnancy rate through this approach is a challenge for further study.

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