PRODUCTION OF RIVER BUFFALO (*BUBALUS BUBALIS)* CALVES BY EMBRYO *IN VITRO* PRODUCTION-VITRIFICATION AND TRANSFER TECHNIQUES IN THE PHILIPPINES

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ABSTRACT

Aimed at producing genetically superior water buffaloes for increased milk and meat purposes, the Philippines through the Philippine Carabao Center endeavoured to develop the advance reproductive biotechnologies. The efficiency of the *in vitro* embryo production in the production of genetically superior animal was assessed. In study 1, the viability of *in vitro* derived vitrified embryos (2n=50) were assessed by non-surgical transfer to river (2n=50) recipients. Study 2 examined the efficiency of swamp buffloes (2n=48) as surrogate mothers of river buffalo embryos, and Study 3 examined the possibility of twinning in water buffaloes by embryo transfer. A Satellite Embryo Biotechnology Laboratory was established in India to secure ovaries from slaughtered river buffaloes and oocytes were collected, matured and fertilized *in vitro* and cultured for embryo development. *In vitro* fertilization was carried out using top three progeny tested bulls from National Dairy Development Board of India. Resultant embryos were cryopreserved by vitrification procedure and transported to the Philippines in liquid nitrogen for embryo transfer. Results in Study 1 demonstrated 16.36% (9/55) pregnancy rate and 10.91% (6/55) calving rate. In Study 2, 12.5% (5/40) pregnancy and 10% (4/40) calving rate were achieved and 23.1% (6/26) calving rate was achieved in study 3 with 3.8% (1/26) twinning rate when embryos are transferred in twos. The results demonstrated efficiency of the technology and its potential application in water buffalo genetic improvement program.

Keywords: *in vitro* embryo production, cryopreservation, vitrification, embryo transfer, buffalo, *Bubalus bubalis*

INTRODUCTION

There are two types of water buffaloes; the river type (2n=50) and the swamp type (2n=48). The river type buffaloes are for milk and meat production while the swamp type is for draft purposes. The Philippines is dominated by swamp-type buffalo called Philippine Carabao. With the advent of farm mechanization and increased inputs in farm operations coupled by environmental calamities that affects agriculture productivity,

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transforming the swamp buffaloes into dairy type animals became an interest to provide the farmers additional source of income from the sales of milk, address malnutrition problems in the rural areas, as well as unemployment problems and food security in the country. Philippines have been an import-dependent when comes to milk and meat products for many years hence production of animals that could produce more milk and meat is given attention.

Artificial insemination (AI) is the earliest reproductive biotechnique used to upgrade the swamp buffaloes. This technique was perceived to be practical keeping in mind the high cost of keeping bulls and high risk of disease transmission associated by natural breeding of live animals. With the Philippine carabao as the breeding base, to produce closed to purebred riverine buffaloes through AI is a long way process requiring about 15 to 20 years of continuous backcrossing. To shorten the process, embryo transfer (ET) which is widely used in cattle and other livestock species (Scherzer *et al.*, 2008) is an effective alternative. To facilitate the water buffalo genetic improvement program, the ET technique was initiated and superovulation and *in vivo* collection of embryos were tried but the poor ovulatory response of buffaloes (Cruz *et al.*, 1991; Misra, 1993) *vis a vis* the cost of hormones limits its practical application. This slowed down its application and instead increased interest on embryo *in vitro* production (IVP). *In vitro* embryo production is a technology that allows production of embryos both from slaughter house derived ovaries or from live animals collection of eggs through ultrasound guided ovum pick up. This technology involves collection of immature eggs from ovarian follicles, maturing the eggs inside a carbon dioxide incubator, *in vitro* fertilization of the matured eggs and further *in vitro* culture for embryo development and vitrification procedure to cryopreserve (Pedro *et al*., 1999). This technology is coupled with the cryopreservation and ET to produce calves of desired genetics. This paper presents the outputs in demonstrating the techniques of embryo *in vitro* production, cryopreservation and transfer in the production of river buffalo calves for milk and meat purposes.

MATERIALS AND METHODS

Experimental Design. Frozen embryos (2n=50) were produced at the Satellite Embryo Biotechnology Laboratory in India by complete *in vitro* maturation, fertilization and culture for embryo development. They were transported to the Philippines in liquid nitrogen for ET. To assess the viability and normalcy of the resultant calves, Study 1 was conducted at the National Gene Pool of the Philippine Carabao Center where river buffaloes were used as recipient animals. To examine the potential use of swamp buffaloes as surrogate mothers and assess the full-term development of 2n=50 embryos in 2n=48 recipient womb, Study 2 was carried out. Study 3 was designed to evaluate the possibility of twinning using *in vitro*-produced vitrified embryos.

Embryo in vitro Production, Cryopreservation and Transfer. Oocytes were collected from the ovaries of genetically superior females identified by visual assessment using body and udder sizes and conformation. Animals with high milk potentials were characterized to have deep, wide udder with balanced proportions and teats that are equal in size. The cleft and attachment points or ligaments are large and strong that shows healthy udder that can produce as much milk as possible. For animals with high meat production

potential, selection was based on these phenotypic characters; animal with a deep, barrel-shaped body, broad chest, straight legs, hooves that grow at the proper angle and a well-proportioned appearance were considered. For bull semen use for the *in vitro* fertilization process, frozen semen from the Top 3 progeny tested bulls of National Dairy Development Board of India were purchased and used.

The detailed procedure on *in vitro* embryo prodution, cryopreservation and ET were presented and discussed (Hufana-Duran *et al*., 2004; 2007; 2008). Briefly, ovaries were collected, stored in a sealed thermos with physiological saline and brought to the laboratory. Ovaries were washed with physiological saline thoroughly to remove blood and contaminants then cumulus-oocyte complexes (COCs) were aspirated using 18 gauge needle attached with 10 ml sterile plastic syringe. Aspirates were pooled in sterile 50 ml centrifuge tubes. COCs surrounded by >3 layers of compact cumulus cells and with granulated cytoplasm were selected and *in vitro* matured for 22 to 24 h in TCM 199 (Earle's salts with 25 mM HEPES, Gibco-BRL, Life Technologies Inc., Grand Island, NY, USA) containing 10% FBS (Gibco) and antibiotics (100 unit penicillin/ml and 100 µg streptomycin/ml) covered with mineral oil (Embryo tested, Sigma). Culture medium was prepared in 100 µl droplets in Nunc tissue culture dishes (35 x 10 mm, Nunclon 153066, Inter-med., Roskilde, Denmark) covered with mineral oil and equilibrated in an incubator (Forma Scientific 3111 Series) with 5% CO_2 in air at 39°C. *In vitro* maturation, fertilization, and culture of the oocytes for embryo development was carried out in the same incubator.

For *in vitro* fertilization (IVF), IVF medium is made with Brackett and Oliphant solution (BO solution, Brackett and Oliphant, 1975) containing 10 mg/ml Bovine serum albumin (A6003, Sigma), 10 mM caffeine (C4144, Sigma) and 4 units/ml heparin (H3393, Sigma) prepared in 25 µl droplets in 35 x 10 mm sterile culture dish, covered with mineral oil and equilibrated at least 1 h inside an incubator prior to use. At 21 h after initiation of oocytes in vitro maturation, IVF was initiated with frozen semen from Murrah buffaloes were thawed at 37ºC for 15 seconds, dispensed in a sterilized centrifuge tube and layered with 6 ml 37ºC pre-warmed BO solution (sperm washing solution) containing 1.25 mm Na-pyruvate, 13.9 mm glucose, and 50 µg/ml gentamycin solution. Semen suspensions were washed two times by centrifugation at 800 x g for 8 minutes 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 to form a final sperm concentration of $1x10^6$ sperm cells/ml, 5 mm caffeine, 2 units heparin/ml and 5 mg BSA/ml for IVF medium. Subsequently, *in vitro* matured oocytes were partly removed from cumulus cells which were retained in the culture droplets to develop cumulus cell monolayers for embryo co-culture system. Oocytes were washed twice in freshly prepared pre-incubated oocyte washing medium and once in a dish of IVF medium. Ten oocytes were transferred to each IVF droplet. Sperm-oocyte co-culture for IVF was done for a period of 6 to 8 h inside an incubator with 5% CO_2 in air at 39ºC. Then 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 embryos were separated from the uncleaved ones and the culture medium renewed. In Study 3, renewal of *in vitro* culture medium was

carried following the method of sequential media system (Hufana-Duran *et al*., 2008b). Embryos that developed to morula, early blastocyst, blastocyst and expanded blastocyst stages, respectively, were cryopreserved by vitrification method as described by Kasai *et al.* (1990); Pedro *et al*. (1999).

Vitrification of embryos was carried out in a room at 25°C. French straws (0.25 ml) with embryo identification and one end cut out with sharp scalpel to form a pointed-shaped open straw was used to load the embryos. 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 (ethylene glycol, 40%, v/v; ficoll, 18%, w/v; sucrose, 0.3 M) and placed on the tip of the pointed-shaped open straw (Hufana-Duran *et al*., 2004) within a period of 30 seconds and rapidly plunged in liquid nitrogen. For embryo transfer, recipient animals were either treated with prostaglandin F2 (PGF2) alpha (Prostavet 2 ml i.m., Virbac Laboratories, France) to induce estrus or they were transferred with embryo after detection of natural estrus. Recipient animals were treated with lidocaine hydrochloride (2% i.m., Ethical Pharmaceutical Co. Pvt. Ltd., India). For embryo warming, the pointed tip of straw where embryo(s) were loaded was directly warmed in 0.5 M sucrose solution at 25ºC for re-expansion within 5 minutes. Reexpanded embryos were washed in PB1 medium and loaded in 0.25 ml French straw for transfer.

During transfer, the presence of corpus luteum was checked by palpation per rectum. A cervix expander (FHK, Japan) was first inserted to the vagina of the recipient animals for easy penetration of ET gun into the cervix. After the expander was removed, the ET gun was inserted to the entrance of the cervix with an outer sheath. The gun was inserted into the cervix when the tip of the outer sheath was punctured. Then it was 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 and calving rate. Recipient animals were checked of the persistency of the corpus luteum present during the transfer of the embryos. Confirmation of pregnancy was done by another palpation per rectum at least 45 and 180 days 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

Assessment on the viability and full-term development of *in vitro* derived vitrified embryos (Study 1) showed that from a total of 55 recipient animals, 9 (16.36%) were diagnosed pregnant and 6 (10.91%) gave birth to live, healthy and normal calves after an average of 312.8±2.99 days of gestation (Table 1).

In Study 2 (Table 2), a total of 40 swamp buffaloes (2n=48) served as recipient animals of river (2n=50) *in vitro* produced and vitrified embryos. Pregnancy rate was 12.5% (5/40). Four healthy calves were delivered normally, presenting a 5.0% (4/80) full-term development rate and 10% (4/40) calving rate. Of the calves delivered to term, three (75%) were delivered alive and healthy, while one (25%) was a stillbirth caused by dystocia.

In Study 3 (Table 3), embryos transferred to recipient animals have resulted in 3.8% (1/26)

Table 1. Live birth of *in vitro*-derived-vitrified-warmed buffalo embryos after non-surgical embryo transfer.

No. of recipient animals	No. became pregnant, n (%)	No. of recorded abortions, n (%)	No. of live births, n (%)
55	9 (16.36)	3 (5.45)	6 (10.9)

Table 2. Pregnancy and calving rate after non-surgical embryo transfer of 2n=50 vitrified *in vitro* derived riverine embryos to 2n=48 swamp buffalo recipients.

Number of recipients	Pregnancy rate, %	Calving rate, %		
		Normal	Stillbirth	Total
40	5 (12.5)	3 (75.0)	1 (25.0)	4 (10.0)

twinning rate and 19.2% (5/26) single births. The overall calving rate was 23.1% (6/26), which was higher than the study 1 and previous report (10.9%, Hufana-Duran *et al.*, 2004; 13.9%, Hufana-Duran *et al*., 2005a). Improvement on the success rate was accounted to the improvement of the culture system (use of sequential media system, Hufana-Duran *et al*., 2008b) and sperm treatment (Hufana-Duran *et al*., 2005b), the use of recipients undergoing spontaneous estrus, and the transfer of embryos at different embryonic stages that suits the synchrony of the *in vivo* environment.

The results of the study also showed that recipient animals in spontaneous or natural estrus have higher pregnancy (29% vs. 8.5%) and calving rate (22.6% vs. 5.1%) than those recipients with synchronized estrus (Table 4) with overall calving rate of 14.0%. The low success rate is attributed by the difficulty to determine the perfect time of ET brought about by the lack of overt signs of estrus in water buffalo species that resulted in asynchrony between embryo and the recipient animal.

Overall, the results demonstrated a promising tool to facilitate genetic improvement in water buffaloes. With futher refinement, success rate could be improved. The herein results confirmed earlier reports showing that compared to cattle, the *in vitro* embryo production in water buffaloes has a lower rate of success (Totey *et al*., 1992; 1993a, b; Neglia *et al*., 2003). However, by applying several modifications such as the separation and use of motile sperm for IVF (Hufana-Duran *et*

Table 3. Twin calf production out of non-surgical embryo transfer of vitrified water buffalo embryos produc *in vitro.*

Parameters	Total (%)
Number of embryos transferred	58
Number of recipients	26
Calving rate [% per recipients]	
Singlet	5 (19.2)
Twins	1 (3.8)

Table 4. Pregnancy and calving rate after embryo transfer of *in vitro* produced vitrified water buffalo embryo (2n=50) to river (2n=50) and swamp (2n=48) recipients.

Recipient	Nature of estrus	Pregnancy rate, % (n/n)	Calving rate (%)
River	Spontaneous	28.1 (16/57)[a]	21.1 (12/57)[a]
	Synchronize	8.3 (2/24)[b]	4.17 (1/24)[b]
Swamp	Spontaneous	40.0 (2/5)[c]	40.0 (2/5)[c]
	Synchronize	8.6 (3/35)[b]	5.7 (2/35)[b]
Sub-total	Spontaneous	29.0 (18/62)[a]	22.6 (14/62)[a]
	Synchronized	8.5 (5/59)[b]	5.1 (3/59)[b]
Total		19.0 (23/121)	14.0 (17/121)

Figures in the same column and row with different superscripts are different (P<0.05).

al., 2005b), selection of good quality oocytes for IVM and improvement of culture medium suitable for buffalo oocytes and embryos (Hufana-Duran, 2008), success rates were enhance to a similar level to that achieved in cattle.

In the cryopreservation of embryos, the vitrification technique was considered practical considering the efficiencies achieved (Hufana-Duran *et al.*, 2004) and the absence of any sophisticated equipment. The most important consideration for a successful vitrification was the type and concentration of the vitrification medium, exposure time, and the material where embryos are loaded. In this procedure, an open-poled 0.25 ml French straw was used in loading embryos to allow direct contact of the liquid nitrogen and ultra-rapid vitrification.

Non-surgical transfer of embryo to recipients undergoing natural or spontaneous estrus were done both in river and swamp buffaloes. However, success rate in terms of pregnancy and calving rates were higher from animals that underwent natural than those that underwent synchronized estrus (Hufana-Duran *et al.*, 2004; Hufana-Duran, 2008). Embryo-recipient asynchrony was suspected to be a major problem as shown by the delayed ovulation among animals manifesting estrus symptoms after estrus synchronization. Ovulation synchronization needs consideration and emphasis in order to improve the success rate of ET.

With the above developments and results, the PCC is exploring more applications and a wider scope of reproductive biotechnologies. At the same time, ovum-pick up activities was initiated to use high genetic live females as source of oocytes in order to optimize the female contribution on genetic progress. The use of sexed semen for IVF and AI is another concern that is being work out and considered for application.

Indeed, the use of reproductive biotechnology has contributed significant impact to Philippines buffalo development program. Strengthening research linkages and collaborations with foreign partners are undertaken to further improve and facilitate the development and application of reproductive biotechnologies for genetic improvement.

CONCLUSION

The embryo *in vitro* production, vitrification and transfer techniques is a potential tool to produce live healthy calves and allows international transport of genetically superior germplasm eliminating the high risk of disease transmission and high cost of live animal importation for genetic improvement programs. This technology offers a high potential in facilitating livestock genetic improvement, rescuing endured genetically superior females, and optimizing the reproduction performance. Further studies to improve efficiencies could lead to profitable livestock production activities.

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