Reproductive Biotechnology in Buffalo

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Abstract

With the increasing gaps between supply and demand of protein-rich foods (e.g. milk and meat), unabated population growth and unemployment, aggravated my malnutrition and income insufficiency among rural farmers in developing countries particularly in Southeast Asian nations, and the worsening global warming, genetic improvement in livestock and fishery needs to be enhanced and strengthened. The use of reproductive biotechnologies, particularly the embryo *in vitro* production, cryopreservation and transfer technology is a reliable and efficient tool to facilitate genetic improvement eliminating high risk of disease transmission and high cost of live animal and embryo importation. This technology was proven effective in water buffalo.

This technology involves collecting eggs from slaughter house-derived ovaries or by ovum pick-up from genetically superior female, maturing the eggs in incubator, and fertilizing the eggs using semen from genetically superior bull to produce high genetics pre-implantation stage embryos. Embryos are cryopreserved by vitrification in liquid nitrogen, transferred to recipient-native animals that will give birth to purebred, genetically “superior offsprings” that when grown to mature age, will serve as future breeding animals. The “superior offsprings” will also become the source of increased volume of milk and meat necessary for the establishment of dairy and meat enterprises. This will provide employment opportunities and improve the income, health, and well-being of the rural farming families. Genetically superior animals provide the needed protein without raising more animals, thus deal on the issues of livestock’s contribution to climate change and global warming.

In the Philippines, the technology has resulted in the birth of several calves both in using river (Hufana-Duran et al., 2004) and swamp buffalo recipients and in enhancing twinning (Hufana-Duran et al., 2008). The current success rate is 55% retrieval rate by ovum pick-up, 25% blastocyst development for *in vitro* embryo production and 10% calving rate after embryo transfer. If genetically superior females will be used as donors of eggs before breeding them for conception, their extra eggs could be used to produce offsprings using

surrogate mothers, thus, optimizing their reproductive performance and facilitating propagation of genetically superior animals.

This paper presents the use of these reproductive biotechnologies on the production of genetically superior water buffalo in the Philippines.

Keywords: In vitro embryo production, Reproductive biotechnology, Water buffalo, Genetic improvement

1. INTRODUCTION

Artificial insemination through natural or synchronized estrus is the earliest reproductive biotechnique commonly used for the production of genetically superior animal. Using this concept, Cruz *et al*. (1991) attempted to enhance genetic improvement, more particularly for dairy type buffaloes, using superovulation and embryo transfer (ET) and capitalizing on the production potentials of superior animals. However, the response showed a relatively low yield of *in vivo*-derived embryos resulting in a shift of interest to embryo in vitro production (IVP) technology.

Efforts to establish embryo IVP techniques in buffaloes in the Philippines started in 1992. In 1996, an *in vitro*-produced crossbred (male river x female swamp) buffalo embryo was delivered to term by a swamp recipient (Ocampo *et al*., 1996). This calf was a result of *in vitro*-produced embryos freshly transferred to the recipient animal.

Refinements on the in vitro culture systems for water buffalo oocytes and embryos led to improved blastocysts development and resulted to the development of cryopreservation technique for water buffalo embryo by vitrification procedure. This led to the demonstration of international transport of river buffalo embryos from India to the Philippines that resulted to the birth of the first calf born from embryo in vitro produced-vitrified-transferred to both river (Hufana-Duran et al., 2004) and swamp buffalo recipients and in enhancing twinning in water buffaloes (Hufana-Duran et al., 2008).

This paper presents the methodology and the efficiency of the techniques as a promising tool for the production of genetically superior animals for sustainable livestock production.

2. Methodology

Oocyte collection: Oocytes for embryo in vitro production are either collected from ovaries of slaughtered animals or from live animals by ovum pick up (OPU). OPU provides the most reliable source of genetic material to enhance female contribution to genetic progress while abattoir-derived ovaries have very little impact on genetic improvement although these provide a cheap and abundant source of oocytes for propagation of desired breed.

OPU from live donors is an extremely versatile technique because it can be applied to donors of all ages from two-months-old calves to very old cows with exception of pregnant animals after the third or fourth month of pregnancy and animals with severe ovarian hyoplasia or in the immediate post partum before ovarian

activity is restored (Galli *et al*., 2001). OPU allows the repeated pick-up of immature ova directly from the ovary without any major impact on the donor female. Use of genetically valuable females at a very early age may substantially increase genetic progress.

To collect oocytes by OPU, a 51cm long 1 cm diameter stainless steel needle is aligned to a vaginal probe to aspirate the oocytes from the follicles. The needle is attached to 50 ml collecting tubes by silicon tubing anchored to vacuum pump (e.g. Fujihira Co. Ltd., Japan) set to produce a negative pressure of 60 mmHg during aspiration. Prior to aspiration, the collecting needle and tubing is pre-loaded with modified Dulbecco's Phosphate Buffered Saline (mPBS) with 3% Bovine Serum Albumin (BSA) or 1% Polyvinyl Alcohol (PVA), 50 ug/mL gentamycin and 20 ug/mL heparin.

The follicular aspirates is maintained at 38ºC in a warming block and transported to the laboratory in same condition. Cumulus-oocyte complexes (COCs) are retrieved by an EnCom filter (Fujihira, Japan) discarding the aspiration medium and washing the follicular aspirates with a pre-warmed mPBS with 3% BSA or 1% PVA without heparin.

In ovary collection from slaughter house, physiological saline (0.9% sodium chloride with 100 µg streptomycin/mL and 100 IU penicillin/mL) at 28 to 33ºC is best for storage and handling of water buffalo ovaries if collection of oocytes is done within six hours after collection (Hufana-Duran, 2008). For longer period, storage in 15ºC preserves the viability of the oocytes (Atabay et al., 2010). To collect the oocytes, an 18 gauge needle connected to a sterile 10 mL plastic syringe is use to aspirate the oocytes from 2 to 8 mm ovarian follicles.

Oocyte Selection and In Vitro Maturation: In selecting oocytes for *in vitro* maturation (IVM), oocytes are selected by compaction of cumulus-corona investment and homogeneity of the ooplasm. Studies showed that oocytes with a compact cumulus require a longer (24 to 26 h) period of IVM and those with loose cumulus require a shorter (20 to 22 h) period of IVM for optimum blastocyst development. Oocytes with a diameter of <100 µm lacked developmental competence evident by the failure to develop to MII after IVM while oocytes with a diameter $\geq$100 µm developed to MII and cleaved after IVF. Optimum cleavage (96.8%) and blastocyst development (27.0%) is observed in oocytes with $\geq$120 µm. The size of the donor follicle is linearly correlated with oocyte developmental competence with follicles $\geq$ 6 mm containing highly developmentally competent oocytes. Brilliant Cresyl Blue (BCB) test showed a significantly higher BCB negative in oocytes with compact cumulus cells (39.1%) compared to the oocytes with loose cumuli (8.6%) indicating that some oocytes with very compact cumulus cells are still at the growing phase of development while most of the oocytes with loose cumulus are at the development phase.

The medium used to wash and culture oocytes for in vitro is Medium 199 (Earle's salts with 25 mM HEPES, Gibco-BRL, Life Technologies, Inc., Grand Island, NY, USA) containing 10% fetal bovine serum

(FBS; Gibco) and antibiotics (100 units penicillin/ml and 100 mg streptomycin/ml, both from Sigma Chemical Co., St. Louis, MO alternatively, Gentamycin sulfate at concentration of 50 µg/ml could be used). Culture medium is prepared in 100 µl droplets in Nunc tissue culture dishes (35mm x 10 mm, Nunclon 153066, Inter-med., Roskilde, Denmark) covered with mineral oil (Embryo tested, Sigma) and equilibrated in a humidified incubator (Forma Scientific 3111 Series) gassed with 5% CO_2 in air at 39ºC. Addition of pyruvate, 10 ng/ml EGF, 0.02 units FSH/ml, and 1 µl estradiol/ml in oocyte in vitro maturation medium improves maturation and blastocysts development rate.

Sperm Capacitation and In Vitro Fertilization: To enhance sperm-capacitation and enable the spermatozoa to penetrate the oocyte, sperm motility enhancer such as heparin and caffeine (Nandi *et al*., 1998; Hufana-Duran, 2008) are used.

In vitro fertilization is carried out by sperm-oocyte co-culture in BO solution (Brackett and Oliphant, 1975) supplemented with 10 mM caffeine, 4 units heparin/mL and 5 mg BSA/ml prepared in 35 mm x 10 mm tissue culture dishes as 50 µL droplets covered with mineral oil. Prior to sperm-oocyte co-culture, the fertilization droplets are equilibrated inside a water jacketed incubator at 39ºC and 5% CO_2.

Frozen semen from genetically superior buffalo bull are thawed at 37ºC for 15 sec, dispensed in a sterile centrifuge tube and layered with 6 mL of freshly prepared 37ºC pre-warmed BO solution containing 10 mg BSA Fraction V (Wako Pure Chemical Industries, Osaka, Japan or Sigma A6003)/mL (BO medium, sperm washing medium). Semen suspensions are washed two times with BO medium by centrifugation at 800 x *g* for 8 minutes discarding the supernatant after each wash.

The sperm concentration is determined in a sperm counting chamber (Neubauer chamber) and the sperm suspension is adjusted to $2x10^6$ cells/mL by adding BO medium (sperm dilution solution). Then the IVF droplets are diluted 1:1 v/v with sperm dilution solution to form an IVF medium with final sperm concentration of $1x10^6$ sperm cells/mL, 5 mM caffeine, 2 units heparin/mL and 5 mg BSA/mL. Subsequently, *in vitro* matured oocytes are partly removed from cumulus cells. Detached cumulus cells are retained on the droplets to develop cumulus cell monolayer for embryo co-culture.

The partly denuded oocytes are washed two times in freshly prepared pre-incubated oocyte washing medium and one time in a dish of IVF medium. Ten oocytes are transferred to each IVF droplet. Sperm-oocyte co-culture for IVF is done for a period of 6 to 8 h inside a humidified incubator gassed with 5% CO_2 in air at 39ºC.

In Vitro Culture for Embryo Development: The methods of IVC of embryos by co-culture with cumulus cells described in bovine by Hamano and Kuwayama (1993) and adopted in water buffalo by Hufana-Duran *et al.,* (2004) or the sequential media containing pyruvate and lactate and different concentration of serum and

presence of glucose as described by Hufana-Duran (1996) and Tsuzuki *et al*., (1998) on the IVC of bovine embryos are used.

To prepare the in vitro culture with cumulus cell co-culture, *in vitro* matured oocytes are slightly denuded from their cumulus cells on IVC droplets and the detached cumulus cells are allowed to settle at the bottom of the dish. The IVC medium is removed and the cumulus cells are washed twice with the IVC medium by pipetting. In sequential media system, after 6-8 hrs of sperm-oocyte co-culture, oocytes are washed to remove excess sperm cells and incubated from 0 to 72 hr in 1% PL medium (TCM 199 with 1% FBS, 0.4 mM sodium pyruvate (P) and 5 mM sodium lactate (L)). At 72 hr of IVC, the culture medium was replaced with 15% PL medium (TCM 199 containing 15% FBS and same concentration of sodium lactate and sodium pyruvate) and incubated for until the 8^{th} day of in vitro culture.

Approximately at 6–8-h of sperm–oocyte co-culture, the oocytes are removed from the fertilization dish, washed four times in pre-incubated culture medium and transferred into the IVC droplets containing cumulus cells and cultured in vitro for embryo development. On the second day of in vitro embryo culture, cleaved embryos are separated from the uncleaved ones. Embryo development is monitored daily and the culture medium renewed every 2 days. Embryos that developed to morula, early blastocyst, blastocyst and expanded blastocyst stages, respectively, can be cryopreserved by vitrification method or transferred to ready recipient animals.

Cryopreservation of embryos by vitrification: The vitrification method described by Kasai (1996) with few modifications to suit the water buffalo embryos was performed (Pedro et al., 2004). Vitrification solution used was EFS40 (ethylene glycol, 40%, v/v; ficoll, 18%, w/v; sucrose, 0.3 M). Subsequently, temperature inside the working room is adjusted to 25ºC and all solutions used are equilibrated at the same temperature for at least 3 h. French straws with capacity of 0.25 ml are marked at one end with pens of various colors for embryo identification. Embryo identification includes rank, age in vitro, developmental stage, date of production, and bull identification. The other end of the straws is cut out with sharp scalpel to form a pointed-shaped open straw (Fig 1.) to place the embryos. To vitrify the embryos, embryos are 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 are kept in liquid nitrogen tank until a recipient is ready for embryo transfer.

Only Rank A embryos are considered for transfer. Embryo straws are recovered from liquid nitrogen tank and the open pole where embryo(s) are loaded, is directly warmed in 0.5 M sucrose solution in PB1 medium at 25 8C for re-expansion within 5 min. Re-expanded embryos are washed in PB1 medium and either 1, 2 or 3 embryos are loaded in 0.25 ml French straw for transfer.

3. Results and discussion

Embryo transfer: To prepare a recipient animal, recipient buffaloes are either treated with prostaglandin F2 (PGF2) alpha (Prostavet 2 ml i.m., Virbac Laboratories, France) to induce estrus or they are transferred with embryo after detection of natural estrus. For estrus synchronized recipient animals, functional CL is checked manually by palpation per rectum prior to PGF2 alpha administration. From the 36th to the 72nd hour after the prostaglandin injection, recipient animals are carefully observed every 4 h for estrus symptoms and particularly for the moment of standing heat, using a teaser bull. Signs of estrus considered could either standing-still while mounted, mucus discharge, frequent urination, bellowing and mounting or a combination of all these manifestations. Symptoms of estrus are confirmed by palpation per rectum of the genitalia. Embryos are transferred to recipient animals on different days after estrus, according to their development (Day 0 is onset of observed estrus/onset of IVF). To restrain and avoid animal movement, recipient animals are treated with lidocaine hydrochloride (2% i.m., Ethical Pharmaceutical Co. Pvt. Ltd., India) at varying levels depending on the response while in the chute.

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

Pregnancy diagnosis and calving: Recipient animals are subjected to palpation per rectum for at least 30 days after ET to check the persistency of the corpus luteum present during the transfer of the embryos. Thereafter, confirmation of pregnancy is done by another palpation per rectum at least 45 and 180 days after the transfer.

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Fig. 1 . A) French straw with cotton plug. B) a French straw after removal of the cotton plug and trimming the other end to make an open pole where embryo is loaded (C) for vitrification.

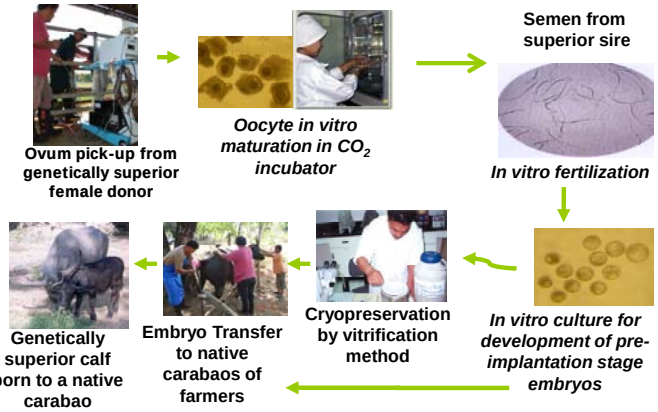

Fig. 2. Schematic illustration of the production of genetically superior animals by embryo in vitro production, vitrification, and transfer techniques

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