In Vitro Embryo Production in Water Buffalo

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Abstract: *In vitro* embryo production (IVEP) is a promising tool with many applications in producing calves from genetically superior animals desired for propagation and in the conservation and revival of endangered species. The techniques of IVEP were adopted from cattle and refined to suit the water buffalo requirements. From the collection of ovaries from a local abattoir and the collection of oocytes by ovum pick up from live animals, gamete storage, collection techniques, handling of ovaries and oocytes to keep the viability and developmental competence, selection of oocytes to the type of culture media and *in vitro* culture condition, and treatment of the sperm cells for *in vitro* fertilization are all-important components of the process that requires careful and precise action to ensure success. Trials on intracytoplasmic injection, the use of sex-sorted sperm cells as a tool for producing sex-predetermined embryos, and the somatic cell nuclear transfer are methods that can be used to produce embryos *in vitro*. This paper provides the important considerations involved in the production of healthy live calves out of *in vitro*-produced water buffalo embryos.

Keywords: *In vitro* embryo production, cryopreservation, vitrification, embryo transfer, somatic cell nuclear transfer, intra-cytoplasmic sperm injection, sexing.

INTRODUCTION

In the Philippines, attempts to produce embryos from follicular oocytes by *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) was successfully demonstrated; resulting in the birth of calves out of embryo transfer (ET) using freshly produced pre-implantation stage crossbred 50:50 river: Swamp embryos [1], and vitrified *in vitro* produced (IVP) river buffalo embryos born both from the river [2] and swamp [3] buffalo recipients, including twins [4]. Embryos produced *in vitro* from *in vitro* matured oocytes and *in vitro* fertilized by density gradient isolated sperm cells [5] also resulted in the birth of healthy live calves. Oocytes retrieved from live animals using ovum pick-up (OPU) and *in vitro* matured, fertilized, and developed into pre-implantation stage embryos [6] also resulted in births of live calves after ET demonstrating the potential of the IVEP as a tool in the production of genetically valued water buffaloes and in overcoming the various reproductive problems that affect the reproduction of this animal species.

In India, live water buffalo calves were also born out of IVP embryos transferred fresh in river buffalo [7], IVP embryos out of hand-made cloning [8], and OPU-derived oocytes [9]. In China, calves were born out of IVP embryos *in vitro* fertilized using sex-sorted sperm cells [10,11], IVP embryos out of somatic cell nuclear transfer (SCNT) [12], and cloned embryos by embryo splitting [13]. In Italy, calves out of IVEP of OPU-derived oocytes were also reported [14-16] with pioneering works by Boni *et al.* [17,18]. Pregnancies of water buffalo out of vitrified buffalo embryos were also reported [19]. In Thailand, the first cloned swamp buffalo produced from adult ear fibroblast cells out of IVEP techniques was also realized [20].

TECHNICAL ASPECT

Complete aseptic conditions are necessary to avoid contamination in the *in vitro* production of water buffalo embryos. Materials and media that come into contact with gametes and embryos should be sterile. Autoclaving at 121ºC for 15 minutes followed by air drying in a low-temperature oven is used for liquid solution and glassware. For plastic materials, ethylene oxide gas sterilization is used. For the preparation of culture media, a laminar flow cabinet is necessary, and prepared media is sterilized by filtration using syringe filters with a 0.2-micron membrane. To minimize stress to the gametes, *in vitro* manipulation procedures are done at appropriate temperatures (35 to 37ºC), pH (7.1 to 7.4), osmolarity (280 to 300 mOsmol), and minimum exposure to UV light. *In vitro* culture is done in a humidified CO_2 incubator at 38-39°C. The sequential

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steps involved in the production of embryos *in vitro* are as follows; 1) collection of oocytes by retrieval from abattoir-derived ovaries or by OPU from live donors, 2) selection of developmentally competent oocytes and IVM, 3) sperm capacitation and IVF, and 4) IVC for embryo development.

Oocytes Collection from Abattoir-Derived Ovary or Ovum Pick Up

To produce water buffalo embryos, oocytes can be retrieved from slaughterhouse-derived ovaries or by OPU from live donors by transvaginal follicular aspiration. Storage temperature, medium, and duration can affect the developmental competence of oocytes [21, 22]. Ovaries stored within 6 hours prior to oocyte collection were found best stored at 25 to 33ºC, with higher temperature detrimental [23]. Longer storage of ovaries is best stored at 15ºC [21]. Ovarian tissue storage at a lower temperature preserves the viability of the ovarian tissue for a longer period. Before oocyte aspiration, washing the ovaries with fresh physiological saline is necessary to wash out any contaminants. Physiological saline: 0.9% sodium chloride in distilled water with 100 µg streptomycin/mL and 100 IU penicillin/mL [2-4] or without antibiotics [22] can be used as ovary storage in a sealed container.

Various techniques were tested for oocyte recovery from the ovaries: follicular aspiration, ovarian slicing, or follicle puncture. Slicing yielded a larger number of retrieved oocytes but is not convenient because of the highly heterogeneous population of oocytes and the high incidence of contamination [23-26]. Follicular aspiration is considered the best method. However, the size of the bore opening of the aspiration needle can damage the surrounding cumulus cells, so an 18-gauge needle with an aspiration medium is appropriate.

The common oocyte holding and washing medium is the pre-warmed modified phosphate-buffered saline (mPBS) with 3mg/mL bovine serum albumin (BSA) [2,3,22]. This washing medium can also be modified with 5% (v/v) heat-inactivated Fetal Calf Serum (FCS) [27]. In some cases, Tissue Culture Medium-199 (TCM199) with 10% FCS, buffered with 25 mM 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 5 mM sodium bicarbonate was also used [28]. Additionally, HEPES-buffered modified Tyrode's medium [29] supplemented with 3 mg/mL BSA, 0.2 mM sodium pyruvate (Sigma) and 50 µg/mL gentamicin sulfate (Sigma) is used [30]. BSA or serum is added in the medium to avoid the oocytes' adherence to the container's surface and to provide energy substrates. Whichever of the above media, the effect on the oocytes is the same, provided the environmental condition is favorable, and the length of operation is short. Time and speed are important in carrying out the *in vitro* manipulation procedures. The shorter the operation in the outside environment, the better the result.

To produce embryos *in vitro* from oocytes of the live donors, OPU is used. While OPU in water buffalo was first reported in Italy [17], it was in 2007 that OPU was practiced in the Philippines [31]. The techniques of OPU in buffaloes are the same in cows [32] as the procedure in water buffalo described earlier [33]. The technique involves ultrasound-guided follicle aspiration to recover oocytes, allowing greater use of genetically valuable females. It is a repeated pick-up of immature oocytes directly from the ovary without any major impact on the health of the female donor. It is applied to cycling donors of all ages, from two-month-old calves to very old cows, except pregnant animals after the third to fourth month of pregnancy [31]. In Thailand, lactating postpartum swamp buffaloes were also used [34].

Various factors can affect the efficiency of OPU. These factors include the physiological condition and estrous cycle of the donor, frequency and season of collection, tools and equipment used, and the skill of the technician. During the follicular phase of the estrous cycle, more oocytes can be collected than during the luteal phase. When OPU is done during the breeding season, better oocyte recovery and a higher developmental competence of the resultant embryos were observed [35-37]. On the frequency of OPU, a once-a-week collection results in the retrieval of a smaller number and lower quality of oocytes and oocytes with expanded cumulus mass and atresia [31]. A twice-a-week oocyte collection was found to be better, allowing maximum recovery of oocytes of suitable quality for embryo production [38] and has no side effects even after twice-a-week collections for over a year [39]. On the other hand, Ferraz *et al*. [40] compared the 7 and 14 days interval of OPU and found that 14 days interval gave a larger ($P < 0.001$) number of ovarian follicles suitable for puncture (15.6 ± 0.7 vs. 12.8 ± 0.4) and an increased ($P = 0.004$) number of cumulus-oocyte complexes (COCs) recovered (10.0 ± 0.5 vs. 8.5 ± 0.3) than in 7 days interval and this was confirmed by Konrad *et al.* [41]. Following a regimen of follicular aspiration sessions every 15 days over a period of five months of work [36], an average of 13.5 ±

5.6 follicles from which 10.2 ± 6.5 structures were recovered and 5.2 ± 3.9 COCs selected continue the IVM process from each animal. However, only 3.1 ± 2.6 COCs/animal/aspiration sessions were considered viable according to the morphological characteristics of the COCs.

Long-term OPU showed that buffalo cows submitted to repeated OPU sampling for a 9-month period demonstrated a decline in follicle recruitment and oocyte collection after the first two months of samplings and after 6-month of samplings a drop in developmental competence was also observed [42]. While different schedules can be used for oocyte recovery, allowing the system to adapt to different practical situations is needed to improve the quality of oocytes. It has been observed that OPU can have its own therapeutic effect on infertile donors, especially those affected by ovarian cysts [31].

In carrying out the OPU, antral follicles ≥2.0 mm in diameter are aspirated from each ovary using stainless steel needle with a length of 50 cm [6] to 55 cm [43] long, depending on the availability. The stainless-steel needle has an 18-gauge or 0.1 cm diameter to minimize mechanical damage to the cumulus and granulosa cells surrounding the oocyte. In aspirating the oocytes, the ultrasound echo tip is 5- to 9-MHz [43-45], and the micro-convex transvaginal transducer has a negative pressure of 40 to 110 mmHg depending on the machine [40,42,43]. While a recommended pressure was provided, it is important to check the quality of the aspirated oocytes by setting the negative pressure of the machine to ensure the good quality of the aspirated oocytes. The higher the negative pressure and the longer the collection tubing, the lower quality of oocytes was observed. This was due to the disrupted granulosa and cumulus cells surrounding the oocytes. To minimize this, it is necessary that the needle and tubing are pre-loaded with the collecting medium such as mPBS with 3% BSA, 50 µg/mL gentamycin, and 20 µg/mL heparin or any other oocyte holding medium suitable for aerial condition. In the absence of BSA, 1% Polyvinyl Alcohol (PVA) can be used [6,40].

The combination of superovulation and OPU to recover the oocytes before the onset of estrus was reported that the procedure can be repeated at best every two weeks [14]. Administration of bovine somatotropin [19,40] or gonadotrophins [34], or pharmacologically to synchronize the follicular waves [46] were found to increase the number of medium and large-sized follicles prior to OPU in buffaloes, and these pre-treatments has no effect on embryo production after IVEP.

Hardening of the surface of the ovaries was found as one of the side effects of OPU on the donor animal, which can occur after several months of repeated ovarian puncture. A decline in follicle recruitment and oocyte retrieval was also observed [42]. After OPU, all non-pregnant donors return to estrus within two weeks from the last OPU and can be inseminated successfully or subjected to bull for natural mating.

The potential impact of the OPU and IVEP techniques in the diffusion of ET in the application of water buffalo genetic improvement is obvious [33]. Reports showed 0.43 to 3.3 oocytes/ovary can be retrieved from buffalo ovary [23,25,47,48]. The method of oocyte retrieval, the size of the ovary and the number of follicles and corpus luteum (CL) present, the competence of the person doing the aspiration, the breed and health condition of the donor, and the season of the year when the collection was made affects the number of collected oocytes [35,49,50]. The inherently low follicular reserve in buffaloes also contributed to the number of culturable grade oocytes, making it lower than in cattle [51,52].

Oocyte Screening and *In vitro* Maturation

Oocyte selection is a prerequisite and one important component of IVEP. Oocytes collected from abattoir ovaries or retrieved from OPU are of the heterogeneous population, so the selection of those that are viable is necessary. The selection of oocytes with developmental competence is based mainly on the compaction of the surrounding cumulus and granulosa cells and the granulation or homogeneity of the ooplasm. The presence and quality of cumulus and granulosa cells surrounding the oocytes are essential to deliver and facilitate the transport of signals and nutrients into the oocytes. Depending on the appearance of the surrounding cumulus and granulosa cells, IVM of oocytes is done inside a water-jacketed incubator for 18 to 24 h [23,53]. Interestingly, oocytes surrounded with expanded cumulus cells require a longer period of 20 to 22 h of IVM, while those surrounded by compact cumulus cells (Figure **1**) require a longer period of 24 to 26 h IVM for optimum blastocyst development [54]. The homogeneity of the ooplasm is one important consideration in the selection of buffalo oocytes but does not demonstrate a significant difference in the developmental competence of the oocyte [23].

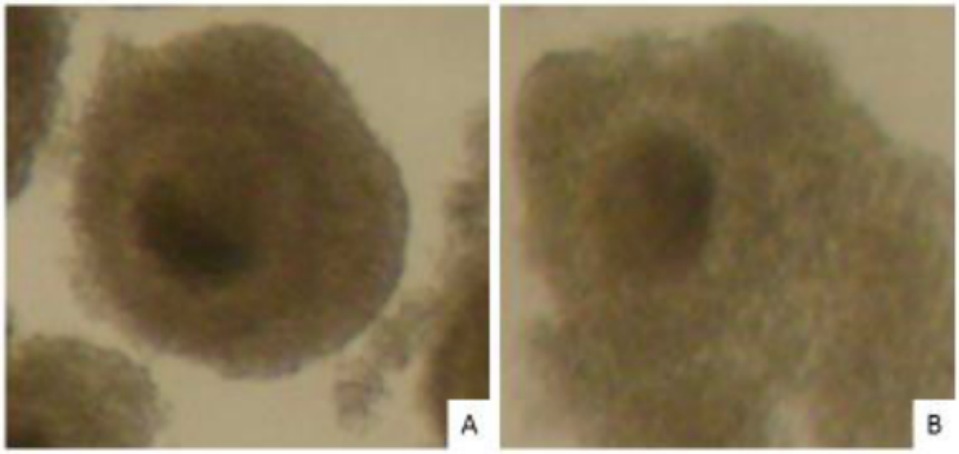

Figure 1: Water buffalo oocytes are surrounded by **A**) compact and **B**) loosened cumulus cells [33].

Studies addressing various factors to improve the IVM rate of water buffalo oocytes were carried out; external parametric indicators to selected developmentally competent oocytes [54,55], type of culture media [27, 56], supplements in the culture medium [57-60], and type of serum [61,62]. The quality of oocytes is important in ensuring the production of viable embryos *in vitro*.

The developmental competence of the oocyte dictates its ability to develop to preimplantation stages after IVF. A decrease in developmental competence is due to insufficient cytoplasmic maturity brought about by the limitations in the IVC environment.

The donor follicle's size and the oocyte's diameter are positive indicators of developmental competence in oocytes [23]. The size of the donor follicle is linearly correlated with oocyte developmental competence, with follicles ≥ 6 mm containing highly developmentally competent oocytes. Oocytes from less than 2 mm follicles are likely at the growing stage and lack developmental competence. These oocytes have a diameter <100 µm that, when matured *in vitro*, fails to develop to MII. The oocytes with a diameter ≥100 µm developed to MII and cleaved after IVF, but those with a diameter ≥120 µm have the highest developmental competence, evidenced by higher blastocyst development [54].

Brilliant Cresyl Blue (BCB) is used to identify oocytes that are at the growing stage [23], a stage when the oocyte is accumulating the developmental competence to undergo meiosis. BCB studies [23,63] showed that oocytes with highly compact cumulus cells are still at the growing phase of development, thus, requiring a longer period of oocyte maturation [23,54].

The IVC environment and condition are critical factors in producing water buffalo embryos *in vitro*. It is inevitable to provide the needed nutrient support that signal and enhances the mechanisms to acquire the developmental competence of the oocyte. Culture media, its components, pH, and osmolality play an important role. There are three types of culture media used in buffalo IVEP; the simple, the complex, and the chemically-defined media. The simple media are bicarbonate-buffered with physiological saline containing pyruvate, lactate, and glucose that differ in energy sources and ion concentration [64]. The complex media is added with vitamins, amino acids, and purines and has the basic components of the simple media. The chemically defined media are entirely free of animal-derived components, including serum, have known chemical components, and represent the most consistent and purest cell culture environment that is suitable for the *in vitro* cell culture [15]. TCM 199, mSOF, RPMI-1640, Ham's F-10, MEM, CR1aa, and CR2aa are used as basic media and are supplemented with either follicular fluid [62,66], hormones [61,66], serum [64], growth factors [59,60,62], antioxidants [58,67], and to control bacterial contamination was provided with appropriate level of antibiotics [23]. Chemically defined media formulations were used with promising results [68]. For maximum oocyte IVM, supplementation of TCM 199 with serum, Follicle Stimulating Hormone, and cysteamine is optimum in producing maturation and subsequent embryonic development after *in vitro* fertilization [58,69]. TCM 199 medium supplemented with one of the following sera: superovulated buffalo serum, buffalo estrus serum, steer serum, or FBS at 5 to 10% concentration resulted in the maturation of oocytes, but cleavage and blastocyst rates are higher ($P<0.05$) in Superovulated Buffalo Serum [61]. Using the best

culture system as described above has resulted in the birth of live calves [2,10-12, 70,71].

The environment inside the incubator is important in ensuring embryo production. A humidified environment at 38.5°C and 5% CO_2 with a relative humidity of 95% is optimum for efficient IVC using TCM 199-based medium [23], but the provision of 5% O2 is needed in modified Synthetic Oviductal Fluid (mSOF)-based culture [60]. A temperature of 36.5°C could significantly decrease the maturation rate of oocytes [72]. The increase of oxidative stress was a major factor affecting the *in vitro* embryo development of water buffalo oocytes and embryos [57], which occurs when the culture condition is not favorable. The significant lipid content and abundance of cytoplasmic granules found in ultrastructural studies in water buffalo oocytes [23,73] probably renders the buffalo oocytes and embryos more sensitive to oxidative damage.

In water buffalo, attempts to culture pre-antral follicles were initiated [74,75] to address the limited number of oocytes and several potential applications for transgenesis. Microdissection was used to isolate the preantral follicles from abattoir-derived ovaries, and a 1% trypsin digestion of ovarian cortical slices was implemented [76]. Small and medium-sized follicles were cultured [74] and later studies used medium and large-sized (150-500 µ) follicles [76-79]. The growth of preantral follicles cultured in groups was better than those cultured individually. Supplementation of growth factors and hormones in the culture medium enhanced growth [74,80]. The co-culture or addition of cumulus cells or oviductal mesenchymal cells supported the optimum development and survivability [76,77,81].

Preantral follicles cultured with antral follicles support higher survival, growth rate, and antrum formation. Nitric oxide at lower concentrations positively affected the development of buffalo preantral follicles [82,83]. Preantral follicle culture from 5 to 9-month buffalo fetuses showed follicle growth, and although the follicular architecture was preserved in nearly 17% of the follicles, antrum formation failed [75]. Difficulty in isolation of preantral follicles, fewer numbers, poor growth in culture, and poor subsequent development of retrieved oocytes are some of the problems encountered in preantral follicle culture [75,81].

In Vitro Fertilization

In vitro fertilization or IVF in water, buffalo can be done by two methods; 1) Sperm-oocyte co-culture for IVF [2] and 2) intracytoplasmic sperm injection (ICSI) [84]. In sperm-oocyte co-culture for IVF, sperm cells must undergo capacitation to gain the ability to penetrate and fertilize an egg. This capacitation event involves unmasking the receptors on the sperm itself, so it becomes capable of reacting to the genital tract of the female [85]. During capacitation, the glycoprotein coat of the sperm cells is removed to uncover the receptors needed to recognize the chemicals in the female reproductive tract that, in turn, initiate hyperactivation as well as morphological changes involved in acrosome reaction [86]. In ICSI, a micromanipulation system is used to inject the sperm head of a single spermatozoon into the cytoplasm of a mature oocyte. This technique bypassed the acrosome but required activation to effect fertilization. The ICSI technique is best used when using cryopreserved sperm cells with low-post thaw motilities and sex-sorted sperm cells. Using ICSI, improvement in the pronuclear formation and cleavage rate was observed [84], with a blastocyst development rate of 17.0 to 29.0% [85].

During IVF, the medium employed must be capable of providing the secondary oocyte and the capacitated sperm with the conditions that permit sperm penetration. The use of 2X detoxified uterine fluid in Riggers Whitten Whittingham medium at pH 7.4, and 37ºC incubation temperature induces sperm capacitation [88]. Different media were described that could enhance *in vitro* sperm capacitation like Fert-TALP [27,89], but Brackett and Oliphant medium [89] was found better [91,92]. The addition of sperm motility enhancers such as theophylline [23] or 2.5 mM caffeine and 10 µg/mL heparin [2,7,47,93,94] or a mixture of phenylephrine, hypotaurine, and epinephrine [59] were found efficient in enhancing IVF in water buffalo.

The presence of dead sperm cells in the IVF environment is detrimental and can decrease the success rate of IVF. Using frozen-thawed semen for IVF is best when separation of the dead sperm cells from the live population is employed. To separate the live sperm cells, swim-up procedures can be used [89], but ion-exchange filtration was found better [95]. Additionally, the use of discontinuous density gradients of percoll [59] or silica particles [5] can also be used using the centrifugation technique.

Sperm concentration is an important factor in the success of IVF. Polyspermy was observed when sperm concentration was high, while low sperm concentration could result in a low fertilization rate. Optimum sperm concentration for IVF in buffaloes is carried out at 1 to

$2x10^6$ sperm cells/mL [33]. Usually, sperm concentration is determined and adjusted to 2 to $4x10^6$ sperm cells/mL sperm dilution solution. Then the IVF droplets are diluted 1:1 v/v with sperm dilution solution to form an IVF medium with a final sperm concentration of 1 to $2x10^6$ sperm cells/mL, 5 mM caffeine, 2 unit heparin/mL, and 5 mg BSA/mL. In carrying out the IVF, cumulus cells surrounding the *in vitro* matured oocytes are partly removed by a pipette to enhance sperm penetration and ensure a higher fertilization rate.

To ensure good quality sperm cells for IVF, several tests can be employed to assess sperm quality parameters. The computer-assisted sperm analysis can be used to assess sperm motility, the eosin-nigrosine staining technique to assess the livability of the sperm cells, the chlortetracycline (CTC) fluorescence assay, and Pisum sativum agglutinin (FITCPSA) [92,96] or trypan blue/Giemsa [97] staining techniques to check capacitation and acrosome reaction, and the hypo-osmotic swelling tests to check the functional integrity of the plasma membrane which is also associated with sperm fertility [98].

The duration of sperm-oocyte co-culture for *in vitro* fertilization is carried out for 6 to 18 hours, depending on the IVF medium used and its composition. It is important to examine the appropriate duration of sperm-oocyte co-culture as differences exist depending on the IVF medium formulation, sperm concentration, and bull used [99].

In ICSI, chemical activation of oocytes is considered a key factor in water buffalo as, without chemical activations, none of the oocytes could be fertilized [84]. Ethanol (EtOH) in combination with cycloheximide (CHX) (EtOH + CHX) was found efficient as an activation medium with 62% second polar body extrusion. Activation protocol of Ionophore + 6-dimethylaminopurine (6-DMAP) and EtOH + CHX also resulted in blastocyst formation rates. Buffalo ICSI oocytes are effectively activated by combination treatment of Ionophore with 6-DMAP and EtOH with CHX, resulting in the highest cleavage and blastocyst formation rates [84,87]. Additional activation treatment is necessary for water buffalo oocytes subjected to ICSI for meiosis completion, pronuclear formation, and embryo development.

Aside from the use of sex-sorted sperm cells, one application of ICSI is the fertilization of cryopreserved-thawed oocytes. Cryoprotectants used for cryopreservation induced zona hardening and reduced fertilization rate in sperm-oocyte co-culture for IVF [100]. Using ICSI, this zona hardening is overcome in humans [101] and bovines [102].

Sperm sexing provides the opportunity to produce offspring of pre-determined sex. This was made possible by the creation and development of a flow cytometer. Proof of sorting efficacy has been demonstrated in many species, and numerous applications in a variety of species are anticipated, including endangered species and zoo and aquarium animals [103]. In water buffalo, successful results on sperm sexing were also achieved [11,104].

In most species, the accuracy of sperm sexing is around 90% making this technology rather expensive due to the sexing efficiency. So far, there were only about 20 million sperm cells sorted in an hour which is the sperm concentration required for 1 dose for artificial insemination (AI) in most livestock species, including the water buffalo. Furthermore, the process of sorting affects the fertility of sexed sperm, making it lower than the fertility of unsexed controls [105]. Two advances are made to improve the efficiency of sperm sorting: increasing the number of sperm sexed accurately per unit time and optimizing the pressure in the flow cytometer to make the process less damaging to the sperm cells [106].

While still expensive and invasive, sperm sexing offers an obvious benefit in animal breeding programs. Choosing a male or female calf in AI or IVF by using X- or Y- sorted sperm is an advantage. While studies and applications in water buffalo are limited, its commercialization in the United States showed a significant positive impact on the dairy and beef cattle industry by nearly doubling its productivity [103]. In water buffaloes, sperm sorting and the use of sex-sorted sperm cells in AI have resulted in the birth of sex pre-determined calves [104]. The same authors reported a 3.8% difference in DNA contents between buffalo X- and Y-chromosome-bearing spermatozoa. The number of sorted spermatozoa per hour has currently attained larger figures than those reported a decade ago (50–100 million compared to 350,000). This development implies that fewer sperm doses can be used for AI, reducing conventional breeding applications. The sperm sorting technology, however, is very promising and provides opportunities for producing sex-predetermined offspring and for sex selection of IVEP-embryos [105,107], surpassing the need for sex diagnosis of the embryos [107,108].

In buffaloes, Lu *et al*. [104] found that deposition of sexed semen in the body of the uterus resulted in higher pregnancy rates (45.5%) than when the deposition was done in the uterine horn (32.3%). The same authors found that conception rates did not differ between cow and heifer buffaloes, but a difference between bulls was observed [104]. The use of sexed semen for AI was done after an estrus synchronization protocol [109-112].

In Vitro Culture for Embryo Development

Like any other livestock species, the *in vitro* culture (IVC) of water buffalo embryos requires the appropriate medium and environment to undergo cleavage and be able to reach the preimplantation stage of development. In this respect, several culture media have been tested, and all resulted in the development of blastocysts with varying degrees of success: TCM199 [2-4], CR1aa, CR2aa, MEM, RPMI-1640, and mSOF media [15,93,113]. The efficiency and effectiveness of each medium formulation depend mainly on supplementing the appropriate combination of antioxidants, co-culture, growth factors, and gas phase. The methods used in bovine for IVC of embryos by co-culture with cumulus cells [114] and the sequential media system containing pyruvate and lactate and different concentrations of serum and presence of glucose [115] were adopted in water buffaloes. These resulted in full-term development and the birth of live-healthy calves after ET [2-4].

The success of IVF is usually assessed by the cleavage rate on the second day of IVC [23,62]. At this time, unfertilized oocytes are removed, and the cleaved oocytes are further cultured to reach blastocyst development. While monitoring of embryo development can be done daily, or the culture medium renewed every 2 days depending on the culture system, keeping the culture dish inside the incubator left undisturbed until the 6th or 7th day of IVC is beneficial [15,23]. This promotes the development of better-quality embryos with blastomeres exhibiting equal sizes and consistency. Synchronous cell division is generally maintained to the 16-cell stage if cell block signals are avoided. Cell division becomes asynchronous with a conducive culture environment, and the individual cells possess their own cell cycle. The cells inside an embryo are termed blastomeres and are easily identified as spherical cells at the 16-cell stage. After the 32-cell stage, which is the morula stage, embryos undergo compaction. This results in individual cells in the embryo becoming difficult to discern. During compaction, the most obvious morphological manifestation is the loss of a concise cellular outline. After compaction, the blastocyst formation characterized by dynamic cell differentiation begins with two distinct cell types; the inner cell mass and the trophectoderm cells. These two cell types formed and differ in their morphology, biochemistry, developmental potential, and eventual expression. At this stage, the embryos are in the preimplantation stage and can be used for ET or for cryopreservation for future transfer activities.

Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT) is biotechnology that provides the opportunity to produce embryos from animals that are desired to conserve and propagate, especially those at extinction. It is a form of cloning and enables the production of offspring of pre-determined sex. It involves the use of a somatic cell nucleus from the animal desired to be conserved and a recipient cytoplast from either the same species (intraspecific) or from a relative or distant species (interspecific). Interspecific and intraspecific SCNT has been tried in water buffalo resulting in the development of preimplantation stage embryos and even birth of live calves of a limited number [8,12,20]. This provides an alternative tool for the preservation of endangered species like the tamaraw (Bubalus mindorensis) of the Philippines. Attempts of interspecies somatic cell nuclear transfer (iSCNT) in water buffalo used buffalo as a donor of somatic cells and cattle oocytes as recipient cytoplasts [116]. This successfully produced blastocysts stage embryos, but none developed to full-term [116,117]. The iSCNT trials of river buffalo donor cells transferred to enucleated swamp buffalo oocytes using a micromanipulation system resulted in the birth of a live river-swamp buffalo calf [70]. By hand-made cloning (a nuclear transfer method without using a micromanipulation system), a 38 to 50% blastocyst stage development rate was obtained from a wild buffalo (Bubalus arnee) skin fibroblast as donor cell and using oocytes from river buffalo (*Bubalus bubalis*) as a recipient cytoplast of [118,119]. Buffalo oocytes were found to be able to reprogram the cattle and goat somatic cells, which resulted in the development of preimplantation stage embryos [120]. However, the success of SCNT is still far from commercial application in water buffalo. The mitochondrial genomes and nuclear incompatibility, the failure of embryonic genome activation by the recipient oocyte of the donor nucleus, mtDNA heteroplasmy, and the availability and readiness of recipient animals need to be solved.

Grading of *In Vitro* Produced Embryos

Water buffalo embryos morphologically look like bovine embryos. The overall diameter is 150–180 µ, including a zona pellucida thickness of 12–15 µ [121]. While preimplantation stage embryos can be observed on a culture dish, not all of them can be cryopreserved or transferred. Embryos are classified and graded to determine which are suitable for cryopreservation (Rank A embryos) and which are suited for immediate embryo transfer (B embryos). Embryos graded as Rank A are excellent embryos with no visible imperfections and with consistent cellular mass. Rank B embryos are those with few recognizable imperfections, such as poor consistency and few extruded cells, with variation in the size of the blastomeres. Embryos that show more disruption, such as irregular shape and with a small embryonic mass, many extruded cells with signs of cellular degeneration and disintegrated cytoplasm, or their development is delayed by one or two days; are discarded and not used for ET or cryopreservation.

Quality evaluation of embryos is predicted on several morphological features, which can be seen easily in a stereo microscope. An embryo that looks spherical and with blastomeres surrounded by a translucent gelatin-like shell called the zona pellucida could be a good marker for embryo classification. The important criteria in evaluating the standard quality and classification of embryos are (1) shape of the embryo and the blastomeres, (2) presence of a zona pellucida, (3) size, (4) color, (5) consistency of the blastomeres, (6) stage of development of the embryo, and (7) knowledge of the age of the embryo. Asiatic buffalo embryos are one-day ahead in their development compared to cattle [1]. Therefore it is suggested to harvest the embryos on Day 6 post insemination.

Considerations for a Successful *In Vitro* Embryo Production

The potential impact of the OPU and IVEP techniques combined with the cryopreservation of embryos on the diffusion of ET in water buffalo is highly recognized. This has been emphasized since the successful attempt was reported [122]. The success of the IVEP in water buffalo is affected by several factors: 1) The source of oocytes where the donors' age, body, and health condition are important. 2) Quality of the oocytes where the presence of the surrounding cumulus cells and the color and granulation of ooplasm need to be checked. 3) The component, pH, and osmolarity of the IVC medium, and 4) the IVC environment that includes the temperature, humidity, and gas concentration [15,23,113]. These factors could significantly affect the post-fertilization embryo development resulting in either good quality preimplantation stage embryos or embryos blocked at the cleavage stage. Juvenile donors have a high percentage of oocytes that lack developmental competence, while adult donors have oocytes with a high incidence of chromosome abnormalities. Animals that are sick or too fat or too thin will most likely provide oocytes with poor quality. Changes in temperature, pH, osmolarity, and contaminant factors that may affect viability must be guarded carefully. Oocytes, especially embryos, do not tolerate temperatures above body temperature (39°C) very well.

The holding, washing, and culture media for oocytes and embryos require a physiological pH ranging from 7.1 to 7.4. The Phosphate buffered saline supplemented with serum or Bovine Serum Albumin is commonly used as the change in pH is negligible under atmospheric conditions. Bicarbonate ($NaHCO_3$) buffered media like TCM 199, Ham's F-10, MEM, and RPMI can be used, but the speed of operation is needed as under atmospheric conditions, the high CO_2 would result in large shifts in pH (rising pH) at an extended time that highly compromise the quality of the oocytes and embryos [23].

The osmolarity or the concentration of salt in the medium also affects the viability of the oocytes and embryos. The osmolarity of the media below that of the *in vivo* environment would make the oocytes and embryos absorb water resulting in swelling to reach osmotic equilibrium. This could result in the rupture of the cell membrane, which highly compromises the developmental competence of the oocytes and embryos. Conversely, if the salt concentration is high, the oocytes and embryo will shrink due to dehydration, causing a reduction in metabolic activity. The osmolarity of PBS prepared from a powdered mixture should be checked to correct the volume of water that will be added. The osmolarity of the solution used for oocytes and embryos should follow the normal osmolarity of uterine fluid, which is 270-300 mOsm [121].

The exposure of embryos to ultraviolet rays should be avoided as this can cause cellular abnormality and death. An aseptic environment favorable for handling oocytes, sperm cells, and embryos must be maintained in the embryology room to ensure the viability of gametes and embryos. The use of insecticide sprays

inside the embryology room is detrimental and may cause chemical contamination. The ethylene oxide gas sterilization of supplies and materials requires sufficient aeration time since the residue is detrimental to the oocytes and embryos.

CONCLUSIONS

In vitro embryo production in water buffalo has been proven as working reproductive biotechnology that could be used as a tool to facilitate the water buffalo's genetic improvement, conservation of endangered species, and advancement of research in the developmental biology of buffalo. The success rate, however, remains a challenge that limits its commercial application in the water buffalo genetic improvement program. The inherently low follicular reserve of the water buffalo is a huge limiting factor in carrying out the ovum pick-up. However, the availability of ovaries from the slaughter of retired genetically superior females can be used as a source of female gametes in the Indian and European continents. Further studies are needed to increase the number of quality oocytes and improve the resultant embryo's developmental competence and viability.

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