Comparison of in vitro fertilization and nuclear transfer techniques in the production of buffalo pre-implantation embryos

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ABSTRACT: This study was conducted to evaluate the timing of cleavage and blastocoel formation of in vitro fertilized and nuclear transfer embryos and to compare the efficiency of in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT) techniques in the production of buffalo embryos under the same culture system. Cumulus oocyte complexes (COCs) were matured in vitro for 22 and 24 hr for SCNT and IVF, respectively. For IVF, COCs were inseminated with frozen-thawed semen and cultured in modified synthetic oviductal fluid supplemented with amino acids for 7 days. For SCNT, ear skin fibroblasts were inserted individually into enucleated oocytes, fused electrically, and activated with ethanol followed by cycloheximide treatment for 6 hr before culture in the same condition as IVF embryos. The results showed that SCNT embryos cleaved and formed blastocoel earlier than IVF embryos. The cleavage rate is significantly higher in SCNT embryos; while the blastocyst formation rate of IVF embryos is significantly higher (P<0.01) than SCNT embryos. The shorter time taken from cleavage to blastocoel formation by NT embryos as compared to in vitro fertilized ones suggests a difference in their developmental kinetics. The difference in the developmental competence between NT and IVF embryos warrants further investigation.

Keyword: Buffalo, Embryo, Nuclear transfer, Somatic cell

INTRODUCTION - The carabao, our indigenous water buffalo, is an indispensable livestock commodity because of the paramount role it plays in agriculture primarily as draft animal and secondarily as source of meat and milk. Realizing the economic and social importance of water buffaloes in general, the government has been supportive to various works and research initiatives for buffalo development that are aimed not only at increasing the country's carabao population but also towards improving its genetic potentials for meat and milk. It is generally known however, that the water buffaloes have poor reproductive performance compared to cattle. The application, therefore, of assisted reproductive technologies (ART) has become the major tool in harnessing the potential of superior animals to bring about the desired genetic improvement in water buffaloes. Initial activities of carabao upgrading in the country have been carried out through Artificial insemination (AI)
and up until now it remains to be the widely used technique which maximizes the potential of superior males for buffalo reproduction. During the past several years, the Philippine Carabao Center has also refined other reproductive techniques such as Multiple Ovulation and Embryo Transfer (MOET), and In Vitro Fertilization (IVF) and other related techniques such as embryo cryopreservation in order to exploit the genetic potential of superior female animals. Several live calves had been successfully produced out of these techniques. Among the most recent advances in buffalo reproduction adopted by the Center is cloning by Somatic Cell Nuclear Transfer (SCNT), which is primarily aimed at enhancing the propagation of genetically superior dairy buffaloes to increase milk productivity. Following the birth of Dolly the sheep [Wilmut et al., 1997], clone animals had been produced thru SCNT in other animal species such as mice [Wakayama et al., 1998], cattle [Kato et al., 1998], goats [Baguisi et al., 1999], pigs [Polejaeva et al., 2000], and recently in buffalo [Shi et al., 2006].

At present, the two main reproductive methods being used in many laboratories to produce buffalo embryos in vitro are IVF and SCNT techniques. Although numerous studies had already been conducted involving each separate technique, the production of buffalo by IVF and SCNT under the same cultural environment has hardly been evaluated, thus only very limited information along this line is available to date. Determining the developmental capacity of buffalo embryos using the same culture system would elucidate important physiological mechanisms underlying some reproductive processes taking place under each technique. The present study therefore was conducted to evaluate the developmental kinetics, mainly the timing of cleavage and blastocoel formation in the in vitro fertilized and nuclear transfer embryos, and to compare the efficiency of IVF and SCNT techniques in terms of production of buffalo embryos under the same in vitro culture system. Aside from the advancement on our understanding on these modern reproductive biotechnologies, more importantly, the present research undertaking will provide significant information that are critical in defining the suitable in vitro culture conditions for each embryo production system to increase their efficiency and finally make them practical tools for genetic improvement in water buffaloes in the country.

**MATERIALS AND METHODS -** Collection of ovaries and oocyte aspiration.

Swamp buffalo ovaries were obtained at local slaughterhouse and transported to the laboratory in normal saline solution (85% NaCl). Cumulus-oocyte complexes (COCs) were aspirated from 2- to 8-mm diameter follicles using an 18-gauge needle fitted to a 10 ml syringe. Collected COCs were held and washed 3 times with HEPES-buffered modified Tyrode’s medium [Bavister et al., 1983], supplemented with 3 mg/ml bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, MO, USA), 0.2 mM sodium pyruvate (Sigma) and 50 µg/ml gentamicin sulfate (Sigma). Only oocytes with compact layers of cumulus cells and evenly granulated cytoplasm were used for maturation in vitro.

**In Vitro maturation**

The in vitro maturation of buffalo oocytes was performed with HEPES-buffered TCM 199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% calf serum (CS, Gibco), 0.2 mM sodium pyruvate, 0.02 units/ml FSH (Sigma), 1µg/mm estradiol-17 β (Sigma), 10 ng/ml EGF (Sigma), and 50µg/ml gentamicin sulfate. Ten to twelve COC’s were cultured in the maturation medium for 22 to 24 hr at 38.5°C under 5% CO2.
**In Vitro fertilization**

In order to increase the percentage of live sperm for fertilization, dead and alive spermatozoa were separated using the discontinuous percoll gradient method. In brief, two 0.5-ml straws of frozen buffalo semen were thawed at 37°C for 1 min. The thawed semen was layered on top of the percoll gradients. The tube was then centrifuged at 2000 rpm for 20 min and the top layer was removed immediately after centrifugation with sterile micropipette. The washed sperm pellet was washed again with 6 ml Brackett and Oliphant Defined Medium (BODM) and centrifuged at 1000 rpm for 5 min. Supernatant was removed until 200 µl of sperm pellet was left in the tube. Semen concentration was determined using a hemocytometer and adjusted to $4 \times 10^6$ sperm/ml concentration with BODM. Fifty- (50) µl of the semen suspension was added to pre-equilibrated fertilization drop consisting of BODM supplemented with 3 mg/ml BSA and 2.5 mM theophylline to have a final concentration of $2 \times 10^6$ sperm/ml. Ten to twelve *in vitro* matured oocytes was transferred to the fertilization drop after 3 times of washing in sperm free BODM-Theophylline BSA drops. The spermatozoa and oocytes were co-incubated for 16 to 18 hr at 38.5°C under 5% CO₂.

**Embryo culture In Vitro**

For IVF studies, after 16 to 18 hr of sperm-oocyte co-incubation, the presumptive zygotes were stripped of cumulus cells by repeated pipetting, washed several times with 50 µl of TCM 199 + 10% FCS and transferred into 50µl drop culture medium: a modified synthetic oviductal fluid (mSOFaa) containing 20 amino acids, 10 µg/ml insulin (Sigma), 5 mM glycine (Sigma), 5 mM taurine (Sigma), 1 mM glucose (Sigma), 1mM L-glutamine (Sigma), and 3 mg/ml fatty acid-free BSA with cumulus cell co-culture. Embryos in culture dishes were cultured for 7-9 days and examined under the microscope for subsequent development. Culture medium was replaced at 48 hr interval during culture *in vitro*. The rates of cleavage and subsequent development to the blastocyst stage (Figure 1e) were determined during *in vitro* culture. The number of cell per blastocyst was determined by staining the expanded blastocysts with fluorescent dye, Hoechst 33342 stain and examined under the epi-fluorescence microscope (Eclipse TE 300, Nikon; Figure 1f).

Somatic cell nuclear transfer. Donor cell preparation

Ear skin sample was biopsied from an outstanding dairy buffalo cow and kept in normal saline solution, maintained in crushed ice pack and brought to the Reproductive Biotechnology Laboratory of PCC for processing. The cartilage and muscle were subsequently removed and after final washing with saline, ear skin sample was cut into small pieces (about 1.0 mm²) in a 60 mm culture dish (Falcon, Primiria). The pieces of skin tissues were covered with a cover slip, pressed slightly and placed inside the CO₂ incubator at 38.5°C for 20 min to dry up. Thereafter, 2 ml of Dulbecco’s Modified Eagles Medium: Ham’s F12 nutrient mixture (DMEM/F12, Gibco) supplemented with 10% FCS and gentamycin stock (cell culture medium) was added into each dish and cultured in 5% CO₂ in air at 38.5°C. Medium change was done every 48 hr during a maximum of 15 days culture period. The tissue explants were removed after adequate proliferation of cells (about 5-6 days) during the primary culture period. After the cultured cell has reached the sub confluent stage, the skin fibroblasts were cryopreserved after 3 to 4 passages in DMEM/F12 with 10% DMSO and 20% FCS and stored in liquid nitrogen until use. One week before nuclear transfer,
frozen-thawed fibroblasts were cultured in the cell culture medium for 3 days and then in DMEM/F12 supplemented with 0.5% FCS for 4 days for serum starvation and used as donor cells. The serum-starved cells were trypsinized and re-suspend in the cell culture medium before injection as nuclear donor (Figure 1c).

**Nuclear transfer**

Removal of the chromatin materials of the recipient cytoplast was performed at 22 to 24 hr after IVM by aspirating the 1st polar body with 10 to 20% of underlying cytoplasm (Figure 1a and 1b) [Atabay et al., 2001]. Trypsinized ear skin fibroblast was inserted individually into the perivitelline space of the enucleated oocytes. The couplets were placed and manually aligned between two wire electrodes (1 mm apart) overlaid with 0.3 M mannitol (Kanto Kagaku) solution containing 0.1 mM CaCl₂ (Kanto Kagaku) and 0.1 mM MgCl₂ (Kanto Kagaku). The couplets of donor cells and enucleated oocytes were fused with 2 direct current pulses 200V/mm for 20 µsec, 1 sec apart using and Electro Cell Fusion (LF-100, Life Tec Co., Tokyo, Japan). Activation of reconstructed embryos were performed at 1 to 2 hr after fusion by incubation in 7% ethanol at room temperature for 5 min followed by culture in 10 µg/ml cycloheximide for 5 hr at 38.5°C under 5% CO₂.

**In Vitro culture of nuclear transfer embryos**

Reconstructed embryos were cultured in vitro for subsequent embryonic development following the procedure or culture system described for IVF embryos. Similarly, the rates of cleavage and development to the blastocysts stage were determined.

**Developmental kinetics**

The kinetics of the first cleavage of buffalo embryos produced in vitro by nuclear transfer and the conventional in vitro fertilization were compared. In vitro fertilized and NT embryos were examined for cleavage at 16, 24, 36 and 48 hr post insemination (hpi) and post fusion (hpf), respectively. The kinetics of blastocoel formation was determined at 120, 130, 140, 150, and 160 hpi/hpf.

**Statistical analysis**

Statistical differences between two means were analyzed by Student’s t-test using Stat View software (Abacus Concepts Inc. Berkeley, CA).

**RESULTS AND DISCUSSION** - The results of the present study revealed that the majority of nuclear transfer (NT) embryos reached the cleavage (2-cell stage) at 36 hr post fusion (hpf), while most of the in vitro fertilized (IVF) embryos reached the cleavage stage at 48 hr post insemination (hpi) (Figure 2a). About 12% of the NT embryos having 2 or more blastomeres were observed as early as 16 hpi. The cleavage kinetics data showed a time lag of 12 hr for in vitro fertilized embryos compared with nuclear transfer embryos. The present result is in agreement with previous observations in cattle [Mohamed Nour et al., 2000]. The delay in cleavage of in vitro fertilized embryos could be due to the time required for sperm penetration and for the initiation of DNA synthesis during early embryonic development. DNA synthesis of the first embryonic cell cycle of the in vitro fertilized embryos was reported to start at 14-15 hpi (Laurincik et al., 1998) when the sperm penetration occurs at
4 hpi. In vivo, 2-cell stage buffalo embryos were recovered in the oviduct 48 hpi or post mating [Sharifuddin and Jainudeen, 1987], a condition that is similar to that of IVF embryos. In addition, the difference in the percentages of IVF and NT embryos that cleaved at 36 hpf/hpi can also be attributed to the asynchronous oocyte population used in IVF.

The time lag observed in the cleavage kinetics of IVF embryos is further reflected on the percentages of embryos that manifested blastocoeal formation at a certain time point during the succeeding culture period. Blastocoeal formation was observed in 30% of NT embryos as early as 130 hpf, whereas only 10% was achieved by IVF embryos around this time. The present finding is in agreement with the NT embryos constructed with swamp buffalo fetal fibroblasts and fresh swamp buffalo oocytes [Parnpai et al., 1999] and NT embryos constructed with ear skin fibroblasts and vitrified bovine oocytes [Atabay et al., 2004]. Majority of the NT embryos (78%) showed blastocoeal formation at 140 hpf, while the IVF embryos reached the same percentage (77%) of blastocoeal at 150 hpi which is about ten hours later compared with the NT embryos (Figure 2b). This result is similar to the in vivo derived embryos that reached the early blastocyst and mid blastocyst stages at 141 and 156 to 176 h after estrus, respectively [Anwar and Ullah, 1998]. Concerning the developmental competence of in vitro produced embryos, it is interesting to note that the proportion of SCNT embryos that developed to 2-cell stage was significantly higher than that of IVF embryos; however, their final development to the blastocysts stage was lower than the IVF ones (Table 1). The high cleavage rate initially obtained by SCNT embryos could be attributed to the use of matured oocytes with clearly defined first polar body as recipient cytoplast and by their being able to pass the fertilization process. The present result conforms to the recent reports on buffalo cloning [Parnpai et al., 2006; Saikhun et al., 2006], wherein the cleavage rate of SCNT embryos is higher than that of IVF ones, however, their subsequent development to blastocysts has been compromised. The remodeling and reprogramming of the transplanted nuclei and the gene expression during embryonic development could have influenced the preimplantation development of NT embryos. The complex mechanism underlying nuclear remodeling and reprogramming in somatic cell cloning needs serious and intense investigation.

The blastocyst cell numbers obtained from in vitro fertilized and somatic cell nuclear transfer embryos in the present study were not significantly different (Table 1). This finding is similar to the previous cloning reports [Parnpai et al., 2006; Saikhun et al., 2006]. It is important to note that the NT embryos exhibited superior competence over the IVF emb-

### Table 1. Development of buffalo pre-implantation embryos produced through in vitro fertilization (IVF) or somatic cell nuclear transfer (SCNT).

| Techniques | No. of oocytes cultured | % of oocytes that developed to 2-cell stage | % of oocytes that developed to Blastocyst | Blastocyst Cell No. (n) |
|------------|------------------------|------------------------------------------|------------------------------------------|------------------------|
| IVF        | 329                    | 73.83±3.02a                              | 28.91±2.50a                              | 79.3±1.70 (10)         |
| SCNT       | 188                    | 89.29±2.89b                              | 20.76±0.93b                              | 81.38±2.20 (8)         |

a, b Values (means± SD of 5 replicates) with different superscript within a column differ significantly (P<0.01).
bryos during early embryonic development, which is suggestive of high rate of cell division during this period. The development of NT embryos however, was compromised during the late pre-implantation embryonic stage. The result of the present study therefore suggests that the *in vitro* culture condition especially during embryonic development that works for IVF embryos may not be suited for NT embryos, and this could have also contributed to the reduction of their developmental competence. Therefore, more research works must be pursued on *in vitro* cultural requirements of NT embryos along with other factors influencing success of SCNT in order to increase the yield of transferable buffalo clonal embryos for buffalo production.

Figure 1. Recipient and donor cell preparation for somatic cell nuclear transfer procedure in buffaloes: a) metaphase II oocytes with 1st polar body, b) removal of the 1st polar body and underlying cytoplasm e.i., enucleation c) serum-starved ear skin fibroblast and trypsinized cells, d) injection of donor cells into the enucleated oocyte, e) NT blastocysts, f) NT blastocyst stained with Hoechst 33342 stain.

Figure 2. a) Kinetics of cleavage and b) blastocoel formation in *in vitro* fertilized (●) and somatic cell nuclear transfer embryos (◇). Each point represents the percentages of embryos that cleaved and formed blastocoel.

![Image of embryos and culture conditions](image-url)
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