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Original Research Article

Cloned buffalo (*Bubalus bubalis*) embryos from adult cumulus cells and cytoplasts prepared by demecolcine-assisted enucleation of meiotically matured oocytes

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Abstract

We report a simplified chemically-enhanced enucleation of in vitro matured buffalo oocytes for generating cytoplasts for producing nuclear transfer (NT) embryo production. Oocytes aspirated from the ovaries from abattoir were subjected to in vitro maturation for 22h. In the first experiment, the in vitro matured (IVM) oocytes (22h) were denuded and treated with demecolcine (0.50 µg/ml IVM medium) for additional 2h. Oocytes exhibiting characteristic extrusion cones were enucleated using traditional or standard micromanipulator-guided enucleation. In the second experiment, the IVM (22h) oocytes were denuded and treated with demecolcine (0.50 µg/ml IVM medium) for 2h. The zona pellucidae of denuded oocytes were then removed using prteinase K (2µg/ml in M-199 with 0.4% FBS). Cytoplasts were prepared by micromanipulator-free or manual bisection of extrusion cones bearing zona-free oocytes. Chemically-assisted cytoplasts formation efficiency was found to be 59.99% using micromanipulator-guided enucleation (experiment-1), and 68.23% in zona-free IVM oocytes (experiment-2). The demecolcine-derived cytoplasts formation efficiency was higher (P<0.5) in zona-free oocytes compared to the micromanipulator-guided enucleation of zona-included in vitro matured oocytes. The cytoplasts generated were viable, and found suitable for reconstituting cytoplast-somatic cell couplets from prolonged cultured, serum starved adult cumulus cells, and supported the development of pre-implantation NT embryos to different stages of developments. Of the 36 (24 zona-included and 12 zona-free) NT embryos, 23 morulae (20 zona-included and 3 zona-free), and 4 hatched zona-included blastocysts were obtained at day 8 of the in vitro culture. Blastomere counts of the NT- and iv vitro fertilization (IVF)-derived embryos were comparable. The results demonstrate that demecolcine-induced enucleation would greatly facilitate the process of producing NT cloned quality embryos in water buffaloes.

Keywords: *Nuclear transfer; Induced enucleation; Demecolcine; Water buffalo*

1. Introduction

Water buffalo (*Bubalus bubalis*) is an economically important multipurpose livestock species and is the mainstay of Indian livestock agriculture. In view of the inherent reproductive problems (weak/ silent estrus signs, seasonal anestrous, a long post-partum anestrous, delayed age of puberty and low conception rates) (Nandi et al., 2002), and significantly limited adoption of the reproductive technologies namely, superovulation and embryo transfer (Madan *et al*., 1996), there is an increasing demand of large-scale production of buffalo embryos for faster dissemination, genetic improvement and conservation of valuable germplasm. Buffaloes have been subjected to various advanced reproductive biotechnological applications including production of cloned embryos (Meena and Das, 2006; Simon *et al*., 2006; Muenthaisong *et al*., 2007), derivation of embryonic stem cells (Verma *et al*., 2007) for producing transgenic embryos and studying the developmental molecular biology of the species. Nuclear transfer technology could be of high significance in buffalo for faster multiplication of valuable sires and elite dams, not only for enhanced productivity, but also for their applications in various nutritional and immunological investigations, and repopulations of indigenous buffalo breeds that are being endangered.

One of the initial and most critical steps in mammalian NT process is the generation of viable cytoplasts by removing the nuclear genetic material from IVM oocytes (Bordignon and Smith 1998), and using these cytoplasts

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as recipients for competent donor somatic cells. Using the position of first polar body (PB1) in IVM or metaphase-II oocytes is one possibility for determining the orientation of chromatin material in oocytes, generally employed in traditional cloning (TC). Here, the maternal chromosomes are removed invasively by cutting the zona pellucida of oocytes and aspirating chromatin materials underlying the PB1 followed by staining with bisbenzimide stain Hoechst 33342. Ultraviolet (UV) irradiation required necessarily in the process with Hoechst 33342 has been found to induce alterations of the membrane integrity and intracellular molecular components of the bovine oocytes matured in vitro (Smith 1993). Oocyte damage is further aggravated by the concomitant removal of oocyte cytoplasm believed to contain molecular components (mRNAs, proteins and other precursors) which are critical for early embryonic development events until entire genome activation (Barnes and Eyestone, 1990). Alternatively, to minimize the detrimental effects of invasive enucleation, fluorescent stains and UV irradiations, non invasive methods for generation of cytoplasts have been emphasized. Among these methods, chemically-induced enucleation has been in use in different experimental and livestock animals (Fulka and Moor, 1993; Gasparrini *et al*, 2003, 2004; Vajta *et al*., 2005; Hou *et al*., 2006; Tani *et al*., 2006). Demecolcine (N-Deacetyl-N-methylcolchicine), a microtubule depolarizing agent is most widely used chemical enucleating agent in mammalian NT studies presently.

The present investigation was carried out to optimize and validate demecolcine-assisted enucleation for micromanipulator-guided enucleation of the IVM buffalo oocytes, and using the cytoplasts to reconstitute the embryos from prolonged cultured cumulus cells and support their development in vitro.

2. Materials and methods

2.1 Materials

Unless otherwise indicated, chemicals, culture media and supplements were cell-culture/ embryo culture-tested media from Sigma Chemical Co. (St Louis, MO, USA). The cell culture plastic wares were from Nalgene (Genetix Biotech Asia Pvt. Ltd.). Disposable plastic syringes were from HenkeSaas Wolf GmBH, Tuttingen, Germany, and membrane filters were from Millipore Corporation, Bedford, MA, USA. Wherever required the culture media were reconstituted as per manufacturer's instructions and passed through 0.2µm membrane filters prior to use.

2.2 Oocytes collection and maturation in vitro

Buffalo ovaries were collected from nearby abattoirs, held in an isotonic sterile normal saline (32-37˚C) supplemented with gentamicin (5 µg/ml). The ovaries were transported to the laboratory within 3-4h and processed immediately.

Surface-visible (8-10 mm in diameter) as well as embedded follicles were aspirated in aspiration medium (M-199, bovine serum albumin, BSA 3%, w/v and gentamicin 50 µg/ml) using a 19G hypodermic needle attached to a 10 ml disposable syringe. Cumulus oocyte-complexes (COCs) with an unexpanded multilayered cumulus cells and homogeneous ooplasmic granulation (A-grade) were selected as per criteria already in use in our laboratory (Singla et al., 1997; Chauhan et al., 1998 and Verma et al., 2007).

2.2 In vitro maturation of oocytes

A-grade COCs were washed 6-8 times in washing medium (M-199, fetal bovine serum, FBS 10% v/v, sodium pyruvate 0.5 mM and gentamicin sulphate 50 µg/ml), and then twice with IVM medium (M-199, FBS 10% v/v, porcine FSH 5µg/ ml, sodium pyruvate 0.81 mM, and buffalo follicular fluid 5% v/v, prepared in the lab).

15-20 COCs were transferred into every 50 µl droplets of IVM medium, overlaid with 400 µl pre-warmed and CO_2-equilibrated mineral oil in 35 mm cell culture dishes. The COCs were cultured for 22h in a humidified CO_2 incubator (5% CO_2 v/v in air) at 38 ± 0.5˚C. The COCs with uniformly expanded and surface anchoring cumulus monolayer were considered to be matured in vitro and used further.

2.3 Demecolcine treatment and enucleation of IVM oocytes

In experiments 1 and 2 (described below), after maturation, the COCs were transferred into IVM medium supplemented with demecolcine (0.50 µg/ml), and incubated for 2h. In each experiment, IVM COCs without demecolcine treatment were processed and used as controls.

Cumulus cells of the IVM COCs were removed by treating the COCs with hyaluronidase (0.5 mg/ml Ca^{2+} and Mg^{2+} free DPBS) for 2 min. followed by gentle pipetting. Completely denuded oocytes were separated by unopettes or fine glass Pasteur pipettes.

Enucleated oocytes including control as well as treated groups were stained with Hoechst 33342 (6.0 µg/ml M-199 with 0.4% FBS) in dark (5-7min.) and then examined under an inverted epifluorescence microscope (Nikon, Japan, Model TMD). The oocytes with no fluorescence were treated as the cytoplasts. Oocytes exhibiting any fluorescence were discarded and not processed further.

2.4 Culturing and preparing the donor cells

Cumulus cell cultures were established from the ovaries from adult slaughtered females. Cell cultures were established from A-grade COCs and maintained in M-199 supplemented with 15% FBS. The confluent cumulus cells were passaged regularly. Following 8-9 passages, the representative aliquots of cumulus cells were frozen (by vitrification) and maintained in liquid nitrogen for

future use. The confluent monolayers established from frozen-thawed cumulus cell aliquots were prepared for use as nuclear donors by starving of serum (M-199 with 0.4% FBS v/v) for 48-60h before nuclear transfer.

2.5.1 Experiment 1

In experiment-1, enucleation of the control as well as demecolcine-treated denuded oocytes was performed with a blunt aspiration pipette and a cutting needle following the standard NT protocols. The cut oocyte was slightly pressed along one side for extruding the polar body along with minimum possible quantities of oocyte cytoplasm. The presumptive cytoplasts were confirmed using Hoechst 33342 staining. As much as possible oocytes were processed within 60-75 min. of initiating the micromanipulation process.

2.5.2 Experiment 2

Demecolcine-treated oocytes were incubated with proteinase-K (2 mg/ ml in M-199 with 0.4% FBS) for dissolution of their zona pellucidae. The zona-free oocytes were bisectioned near the extrusion cones using ultra sharp blades (Model MTB-05, Micromanipulator Microscope Co. Inc.) under a zoom stereomicroscope (Nikon, Japan, Model SMZ-2T). The bisected oocytes were transferred immediately into cell culture media in separate culture dishes for determining their viability. Viable cytoplasts (regaining the spherical shapes within 60-90 sec. after bisection) were separated from the non-viable cytoplasts. The cytoplasts were finally confirmed by staining with Hoechst 33342 and visualization under UV epifluorescence. The cytoplasts were washed immediately and held in TCM-199 with 4% FBS till further use.

2.6 Reconstituting NT embryos

The cytoplasts (Experiments 1 and 2) were used as recipients of donor somatic nuclei for producing cloned embryos. Working in groups of 20-25, the zona-included cytoplasts were transferred into an elongated (12-15mm) droplet (M-199 with FBS, 4%). A small rounded drop of the same culture medium was prepared adjacent to the elongated drop, for resting the donor cumulus cells. A 5-7 µl partially trypsinized (0.25% trypsin-EDTA) serum-starved donor cells-suspension was loaded into the spherical drop taking care that the cells settled down separately. The drops were overlaid with mineral oil.

The ooplast-cell couplets (experiment 1) were reconstructed by transplanting a single donor cell through cut zona pellucida into perivitelline space of the cytoplast. As much as possible couplets were constituted within 1.5h of initiating the process. Whenever needed, additional donor cells were added to the droplets to have non-adhering donor cells, as these cells were found anchoring to the cell culture dish if left undisturbed for more than 20-25 min.

The couplets/ triplets (experiment-2) were reconstructed by adhering and subsequently fusing the donor cells to zona-free cytoplasts. For this, the zona-free cytoplasts were picked up individually and transferred into a 20 µl droplets of phytohemagglutinin (500 µg/ ml M-199 with 2% FBS) for 30 sec. The cytoplast were then taken out of the droplets and carefully dropped over a cumulus cell for adhesion. Wherever desired the resulting cytoplast-somatic cell couplet was fused with another cytoplast to form cytoplast-cell-cytoplast triplet.

2.7 Electrofusion and activation of the NT embryos

Reconstituted couplets were equilibrated (in groups of 8-10) in a freshly prepared cell fusion buffer (5 min.) following the methods described by Wolfe and Kraemer (1992). The electrofusion chamber was filled up with 100-150 µl of cell fusion buffer, and the couplets were aligned between two electrodes. Alignment was also induced through alternating current (AC) 6V, for 10 sec., and electrofused through three pulses of direct current (DC) 15V for 50 µsec., delivered by a BTX Electrocell Manipulator 200 (Genetronics, San Diego, CA).

The couplets were removed immediately after the electrofusion, and held in the droplets of M-199 with FBS 10% v/v for 1h (38$\pm$0.5°C and 5% CO_2) in a humidified environment. The fusion was ascertained through microscopic examination. Electrofused couplets were activated additionally by treating with cytochalasin-D (2.5 µg/ ml modified Charles-Rosenkrans medium-2 supplemented with amino acids, mCR2aa) and cycloheximide (10 µg/ ml mCR2aa) for 1h, and then with cycloheximide (10 µg/ ml mCR2aa) alone for 5h. The embryos were observed for cleavage, and dividing embryos were separated from the quiescent or non-viable embryos.

2.9 In vitro culture of NT embryos

NT embryos were transferred onto the cumulus cells monolayer in 50 µl droplets of mCR2aa containing FBS 10% v/v, overlaid with mineral oil. In vitro cultured buffalo motile oviductal epithelial cell-clumps were included into the embryo culture droplets to avoid adherence of the embryos to each other and/ or to the cumulus cell monolayer. The culture medium was changed after every 48h. The developmental competence of the embryos was observed till day 8 after initiating in vitro culture.

Embryo cells or blastomeres were counted by staining the embryos with Hoechst 33342. NT- as well as IVF-derived embryos were stained with Hoechst 33342 (10 µg/ ml DPBS) for 10 min. in dark, and transferred onto the glass slides and covered with fine cover slips. The cover slips were pressed slightly to rupture the zona of embryo and release the blastomeres.

3. Results

3.1 Experiment 1

Results of experiments 1 and 2 are shown in Tables 1 and 2 respectively. In experiment 1 (Table1), a total of 3536 oocytes were used. The replicates in total 36 trials reveal that a total of 594 oocytes were used as control groups to compare the efficiency of demecolcine as enucleating agent. Of the 594 IVM oocytes (control groups) enucleated, 104 (17.50% of the IVM oocytes used) cytoplasts were obtained with micromanipulator-guided enucleation. From a total of 2942 demecolcine-treated and enucleated oocytes, 1765 cytoplasts (59.99% of the total IVM oocytes) were obtained. The couplets formation efficiencies from the zona-included cytoplasts were 4.54% and 21.21% in control and treatment groups respectively.

3.2 Experiment 2

The cumulative data of the 12 trials (Table 2) reveal that of a total of 980 IVM oocytes, 215 oocytes were used as control. A total of 101 zona-free cytaoplasts (45.97% of total oocytes) were obtained. Of the 765 oocytes treated with demecolcine, a total of 522 (68.23% of total oocytes) cytoplasts were obtained. The couplets formation efficiencies were 14.41 % and 41.04% in control and treatment groups respectively.

3.3 NT embryos from the demecolcine-assisted cytoplasts

A total of 24 zona-included NT embryos were obtained. The embryos developed to different stages of development in vitro. Of the 20 morulae, a total of 4 NT embryos or blasocysts were found to reach hatched stage of development by day 8 of in vitro culture. A total of 314 zona-free couplets (41.04% of the total oocytes) were obtained, of which 12 zona-free NT embryos exhibited viability, and only 3 morulae could be obtained

Compared to the blastomeres counts ranging from 126-140 (130±6.08, mean ± SD) in IVF-derived buffalo embryos, 101-116 blastomeres (107±7.94, mean ± SD) were present in NT embryos at day 8 of in vitro culture. Only early morulae (day 3 of culture) were obtained from the zona-free couplets and their cells could not be counted. Further work on handmade or zona-free embryo cloning is in progress and data are being compiled separately.

4. Discussion

Buffaloes are becoming endangered due to culling of the females with reproductive problems and inefficiencies. This is of serious concern because countries like India depend largely on these animals and their products. Cloning is a potential technology for restoring of threatened, or even extinct species and populations (Lanza *et al*., 2000). Whereas the technology has successfully been exploited for producing clones of laboratory and livestock animals (reviewed by Tamada and Kikyo, 2004), cloning in buffalo has mainly succeeded in the creation of NT embryos (Meena and Das, 2006; Simon *et al*., 2006; Suteevun *et al*., 2006) in most of the laboratories.

Concerning the initial technical constraints in buffalo NT studies, we have observed that nuclei in the IVM oocytes are compactly embedded into the lipid-enriched ooplasm and their visualization requires higher speed centrifugations (Verma *et al*., 2006). Also, the removal of nuclear chromatin from IVM oocytes is hindered by the oocyte lipid contents. In the present study we report improved chemically-induced enucleation of IVM buffalo oocytes, and using the cytoplasts for producing cloned embryos through traditional cloning. Additionally, the experiments were also conducted for preparing the zona-free cytoplasts, and using them as recipients for reconstituting handmade cloned buffalo embryos from the in vitro cultured somatic cells.

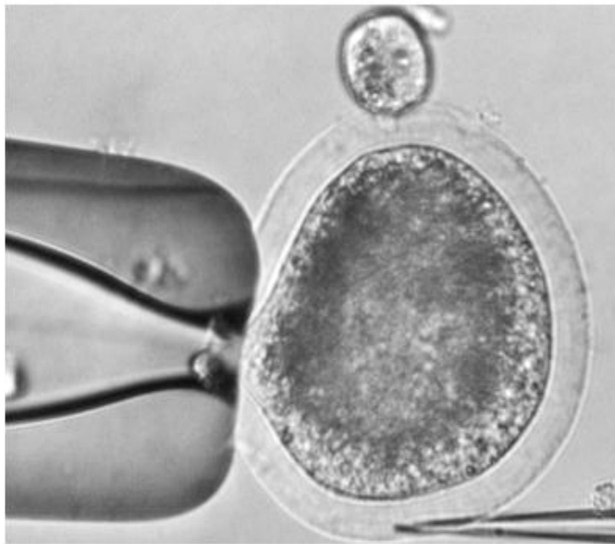
Derivation of viable cytoplasts through chemically-assisted enucleation is an accepted method for producing cloned embryos, and has been found applicable in farm animals like sheep (Hou *et al*., 2006), cattle (Russel *et al*., 2005; Vajta *et al*., 2005; Tani *et al*., 2006) and porcine (Li *et al*., 2006). It has also been emphasized that induced enucleation performed during meiosis-II could be achieved by a brief exposure of pre-activated oocytes to demecolcine (Baguisi and Overstrom, 2000; Fischer *et al*., 2002; Ibanez *et al*., 2003; Gasparrini *et al*., 2003). The recent approaches of chemically-induced enucleation are based on the phenomenon that treatment of M-II phase with some cytoskeleton-relaxing agents induces an extrusion cone over the surface of the oocyte cytoplasm, which facilitates the removal of genetic material by mechanical aspiration (Yin *et al*., 2002; Peura *et al*., 2003; Li *et al*., 2004) or manual bisection of zona-free oocytes.

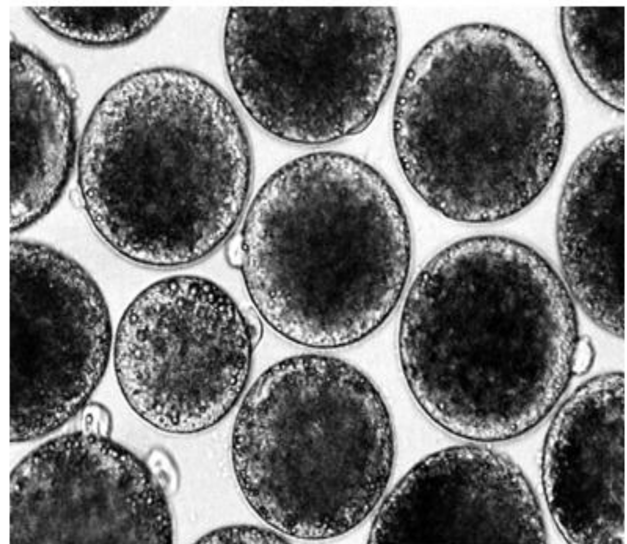
The demecolcine concentration reported here was optimized after a series of pre-experimental trials (data not shown). The cumulative results of 48 trials (experiments1 and 2) show that a total of 4516 oocytes were used in the present investigation. A total of 3707 oocytes were treated with demecolcine (0.50 µg/ml IVM medium), and manipulated for preparation of cytoplasts. Compared to control groups (enucleation efficiency, 17.50%), a total of 59.99% (1765/2942) cytolasts formation efficiency was observed in experiment 1 (Figure 1a). Using the same concentration of demecolcine, enucleation efficiencies were 45.47% (101/215) and 68.23% (522/765), in manually bisected control and treatment groups respectively. The demeicolcine-induced extrusion cones (Figure 1b) were more prominent and accessible for removal in most of the zona-included and zona-free oocytes. In some zona-free oocytes extrusion cones were found to be loosely associated with the oocyte cytoplasm (Figure 1c), and a simple gyratory shaking was effective for their detachment or dislocation, leaving behind almost 100% of the cytoplast. Consequently, in second experiment,

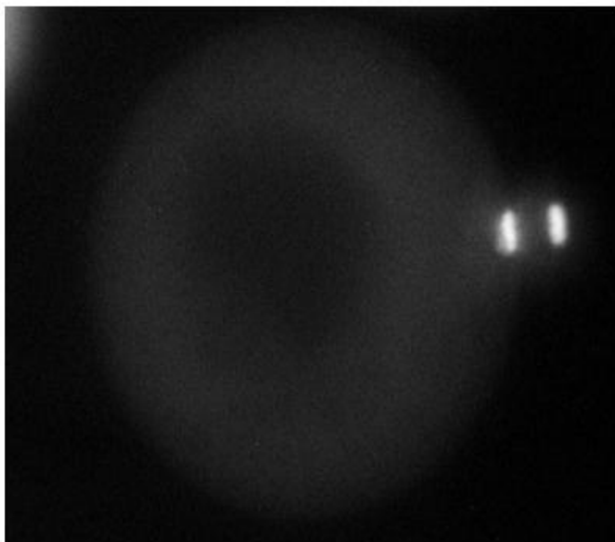
Table-1. In vitro developmental competence of the NT embryos prepared from zona-included cytoplasts and adult cumulus cells

Total IVM oocytes		Denuded IVM oocytes n (%)	Cytoplasts derived n (%)	Couplets formed n (%)	Dividing embryos n (%)	Stage of development (no. of cells)	
						Morulae n (%)	Blastocysts n (%)
Control	594	433 (72.79) [a]	104 (17.50) [a]	27 (4.54) [a]	2 (0.33) [a]	2 (30) [a]	0 [a]
Demecolcine treatment	2942	2088 (70.97) [a]	1765 (59.99) [b]	624 (21.21) [b]	24 (0.81) [b]	20 (30) [b]	4 (109) [b]

Data is from 36 trials ,Percent values are Mean±SEM ,Values within the same column with different superscripts are different significantly ($P<0.05$).







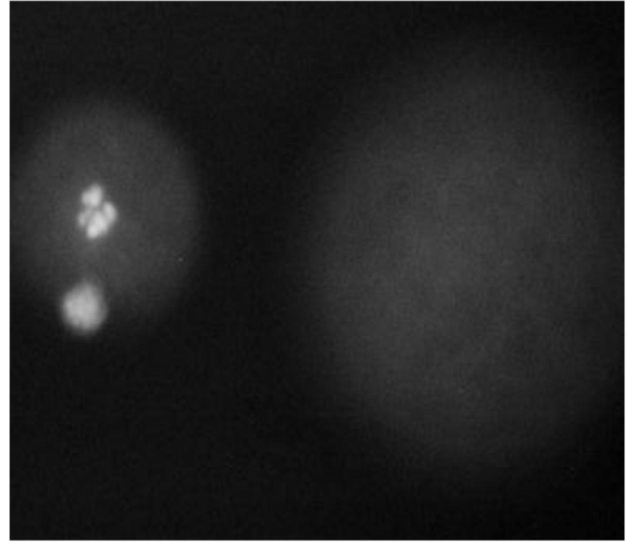


Figure1a. Micromanipulator-guided enucleation of demecolcine-treated buffalo oocytes
Figure1b. Extrusion cones in demecolcine-treated oocytes. Zona has been removed to clearly show the extrusion cones
Figure1c. Hoechst 33342 stained oocyte with extrusion cone containing nuclear chromatin
Figure 1d. Bisected extrusion cone (with chromatin material and PB1) and the cytoplast

cytoplast formation rates were higher, and time required was significantly less.

The extrusion cones were detectable in 60-70% of the metaphase-II oocytes and cytoplasm surrounding it was found to contain PB1 and nuclear chromatin (Figure 1d). From the data it is clear that cytoplast formation efficiency is significantly higher (P<0.5) in case of zona-free enucleation compared to micromanipulator-based or zona-included enucleation. Through manual bisection of the demecolcine-treated oocytes 90-100% of total cytoplast per oocyte was recovered. This was advantageous in avoiding the necessity of fusing two cytoplasts for preparing zona-free triplets. Demecolcine-induced enucleation at this stage therefore, appears to be more promising for derivation of cytoplasts for producing NT embryos in buffalo.

Earlier studies also indicated that demecolcine could be used to derive viable cytoplasts to generate NT embryos from somatic cells (Baguisi and Overstrom, 2000; Fisher *et al*., 2002; Gasparini *et al*., 2003). Tani *et al*., (2006) demonstrated that demecolcine treatment for at least 30 min. induces a membrane protrusion in M-II stage bovine oocytes, facilitating the enucleation, though the demecolcine treatment did not increase the potential of NT oocytes to develop into blastocysts. Furthermore, one normal healthy calf was born from NT embryos produced from demecolcine-treated oocytes, indicating that chemically-assisted removal of chromosomes is effective for bovine cloning (Tani *et al*., 2006). Another study (Hou *et al*., 2006) in sheep reported a demecolcine-induced enucleation (58.1%) from treatment of sheep meiotically maturing oocytes with 0.4 µg/ml demecolcine for 20-22h. Combination treatment with demecolcine and cycloheximide or 6-dimethylaminopurine in the same study led to a single pronuclear formation rather that PB1 extrusion. On transfer of demecolcine-treated oocytes into demecolcine-free medium, PB1 extrusion cone was restored and a 72.1% induced enucleating efficiency was observed (Hou *et al*., 2006). Aimed at finding an efficient and reliable chemically-assisted procedure for enucleation in cattle, the time dependent manner of the development of extrusion cones, the efficiency (oriented bisection per oocyte; 94%), reliability (success per attempted enucleation; 98%) and the blastocyst per reconstructed embryo rates (48%) were measured in cattle (Vajta *et al*., 2005).

Demecolcine-derived cytoplasts in the present study were suitable to form couplets from the prolonged cultured adult cumulus cells and support in vitro development of the NT embryos. 24 zona-included dividing embryos were obtained, 4 of which remained quiescent after day 2 of culture, leaving 20 morulae which developed further to different stages of development. A total of 4 blastocysts were found to reach hatching stage of development by day 8 of in vitro culture. The NT embryos were healthy and no microscopically detectable abnormalities were observed. Furthermore, the cell counts (130±6.08, mean ± SD vs. 107±7.94, mean ± SD) in IVF-derived and NT embryos respectively, were comparable.

Preparation of zona-free cytoplasts in the present study appeared to be convenient and equally useful in reconstituting the cloned couplets from the cumulus cells, though only early stage NT morulae could be obtained. However, in view of ease of the process and enhanced enucleation efficiency, findings of the experiment-2 are viewed critically. The zona-free NT embryos were found to adhere to the cumulus monolayer and other embryos in a mixed co-culture system. Also, the zona-free embryos were fragile, and effort to separate them could not be successful as it damaged the clumped embryos. This could be the probable cause of low developmental competence or survival of the zona-free NT embryos. However, the studies are continuing on optimizing the culture media requirements, culture conditions including the role of a co-culture system, and associated factors for enhancing the yields of zona-free embryos.

Moreover, there are no reports on chemically-induced enucleation of buffalo IVM oocytes and formation of NT embryos, for comparison of the findings. Molecular studies addressing the problems associated with reprogramming of the genome of cloned embryos in buffalo, overcoming the species specific embryonic developmental blocks, and in vivo developmental competence of transplanted NT embryos are needed urgently.

In conclusion, the present report is a promising method for locating chromatin materials during the process of enucleation for traditional as well as handmade cloning in buffalo. Demecolcine-induced enucleation could be very important not only in obtaining an increased yield of cloned embryos for transfer into the recipients, but also for studying the developmental molecular dynamics, and establishing animal specific embryonic stem cell lines for genetic improvement and producing transgenic embryos in buffaloes.

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