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Original article

Development of swamp buffalo (*Bubalus bubalis*) embryos after parthenogenetic activation and nuclear transfer using serum fed or starved fetal fibroblasts

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Abstract – The knowledge of oocyte activation and somatic cell nuclear transfer in the swamp buffalo (*Bubalus bubalis*) is extremely rare. The objectives of this study were the following: (1) to investigate the various activation treatments on the parthenogenetic development of buffalo oocytes, (2) to examine the events of nuclear remodeling and in the in vitro development of cloned buffalo embryos reconstructed with serum fed or starved fetal fibroblats, and (3) to investigate the in vivo development of cloned embryos derived from serum fed or starved cells after transfer into the recipients. The rates of cleavage and blastocyst development were found to be significantly higher ($P < 0.05$) when the oocytes were activated by the combination treatment of calcium ionophore (A23187) or ethanol followed by 6-DMAP than those activated by electrical pulses and 6-DMAP or other single treatments. Flow cytometric analysis revealed that the percentage in the G0/G1 phase in serum starved cells was significantly ($P < 0.05$) higher than that in serum fed cells (88.8 ± 6.2 vs. 68.2 ± 2.6). At 1 h post fusion (hpf), most of the transferred nuclei (71%) from serum fed cells did not change in size, and the nuclear envelope remained intact, whereas 29% underwent NEBD and PCC. When serum starved cells were used, 83% of the transferred nuclei underwent NEBD and PCC whereas 17% remained intact. The nuclear swelling and pronucleus (PN) formation were observed at 2–4 and 12 h post activation (hpa), respectively. The remodeled nuclei underwent mitotic division and developed to the 2-cell stage within 18–24 hpa. Fifty-five percent of oocytes reconstructed with serum fed cells were 2PN and 45% were 1PN, whereas 79% of the embryos reconstructed from starved cells were 1PN and 21% were 2PN. The percentage of blastocyst development of the embryos derived from starved cells was higher than that from the serum fed cells (35% vs. 21%, $P < 0.05$). Pregnancy was detected after the transfer of cloned blastocysts into the recipients but no recipients supported the development to term. The results of this work can be used to establish effective activation protocols for buffalo oocytes which can be used during nuclear transfer experiments.

buffalo / oocyte activation / nuclear transfer / nuclear remodeling / somatic cell

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1. INTRODUCTION

Somatic cell nuclear transfer (NT) has been successfully used to produce cloned offspring in several mammalian species including sheep [1], cattle [2], mice [3], goats [4], pigs [5], cats [6] and recently, rabbits [7]. However, the efficiency of cloning in all species has been extremely low, and the factors for the low efficiency are not fully understood. Previous experiments using embryonic blastomeres as donor nuclei showed that inefficient oocyte activation of the recipient cytoplast is a major factor in the low efficiency of cloning [8]. Currently, the common methods to activate MII oocytes are a physical stimuli such as an electrical pulse [9], chemical stimuli such as ethanol [10], calcium ionophore [11], ionomycin [12] and inositol 1,4,5-triphosphate [13]. These stimuli were used alone or combined with a protein phosphorylation inhibitor, 6-dimethylaminopurine (6-DMAP) or a protein synthesis inhibitor, cycloheximide. Electrical stimulation is more commonly used for the activation of NT embryos for pig and rabbit cloning whereas ethanol, ionomycin and calcium ionophore are chemical activators for cloning in cattle. A sequential treatment of electrical or chemical activated oocyte with 6-DMAP has been shown to enhance the activation rates and parthenogenetic development in mice [14]. Bovine oocytes activated with ethanol or calcium ionophore followed by the 6-DMAP treatment result in blastocyst development at a rate comparable to that of in vitro fertilized (IVF) embryos [10]. However, an effective activation method for buffalo oocytes has not yet been reported.

One factor that may affect the successful development of NT embryos is the cell cycle phase of a donor nucleus at the time of NT. Wilmut et al. [1] was the first who reported the production of cloned sheep from mammary cells. These cells were synchronized into the presumptive G0 phase (quiescent phase) by serum starvation. This result suggested that the chromatin at the quiescent phase is amenable to nuclear reprogramming [15]. Subsequent studies using serum starved donor cells have been reported in cattle [16], pigs [5] and goats [4]. In contrast, donor nuclei from non-quiescent or cycling cells could be successfully used to produce cloned cattle as well [2, 17] indicating that synchronization in the G0 phase is not a prerequisite for somatic cell cloning.

Early events of nuclear reprogramming of transplanted somatic cell nuclei have been demonstrated in a number of mammalian species. When donor nuclei are introduced into the enucleated oocytes, the nuclear envelope is broken down (NEBD), and the donor chromatins are prematurely condensed (premature chromosome condensation; PCC). Finally, to complete reprogramming, the transferred nucleus is swollen to form a pronucleus. However, the knowledge of cloning in this swamp buffalo is extremely rare. In the present study, we investigated the optimum activation protocol for buffalo oocytes to establish an efficient method for buffalo cloning. We also examined the events of nuclear remodeling and in vitro and in vivo development of NT embryos using serum fed or starved fetal fibroblasts as the donor nuclei.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals in this study were purchased from the Sigma Chemical Company (Sigma, St. Louis, MO) unless stated otherwise.

2.2. Oocyte collection and in vitro maturation

Buffalo oocytes were collected and matured in vitro (IVM) by the method previously described [18]. Cumulus oocyte complexes (COC) from abattoir ovaries were collected by aspiration of small antral

follicles (2 to 6 mm) using an 18-guage needle with a disposable syringe. The COC were morphologically assessed and only oocytes with compact and homogeneous cytoplasm were selected for IVM. All selected COC were cultured in 50 µL drops of maturation medium (TCM 199) (Gibco, Grand Island, NY) supplemented with 10% FCS (Hyclone Laboratories, Inc, Logan, UT), 0.2 mM pyruvate, 5 $\mu g \cdot mL^{-1}$ FSH, 5 $\mu g \cdot mL^{-1}$ LH and 1 $\mu g \cdot mL^{-1}$ estradiol in a humidified atmosphere of 5% CO_2 at 39 °C. After culturing for 22 h, mature oocytes were subjected to parthenogenetic activation, in vitro fertilization or somatic cell NT.

2.3. Parthenogenetic activation

After maturation culture for 22 h, the oocytes were manually denuded by pipetting in 0.2% hyaluronidase in TALP-Hepes. Oocytes with an extruded first polar body (MII) were assigned to the following treatments: stimulation by 2 DC electrical pulses of 2.1 kV/cm for 20 µs (1 s interval), exposure to 7% ethanol or 5 µM calcium ionophore A23187 for 5 min with or without subsequent treatment by 2 mM 6-DMAP for 4 h under a humidified atmosphere of 5% CO_2 at 39 °C. Parthenogenetically activated oocytes were cultured in TCM 199 supplemented with 10% FCS containing buffalo oviductal epithelial cells under a humidified atmosphere of 5% CO_2 at 39 °C. The percentages of cleavage and blastocyst development were evaluated on Days 2 and 7, respectively.

2.4. In vitro fertilization

In vitro production of buffalo embryos was based on the procedures reported in our previous study [18] with minor modifications. Frozen ejaculated semen was thawed at 37 °C and sperm was prepared by using the swim-up technique. A 10 µL aliquot ($5 \times 10^5 \cdot drop^{-1}$) of sperm after the swim up and 1 h of incubation was placed in a culture dish with 50 µL of glucose free-TALP containing 10 $\mu g \cdot mL^{-1}$ heparin to facilitate capacitation. Spermatozoa were incubated for 2 h under a humidified atmosphere of 5% CO_2 at 39 °C. Ten in vitro matured COC were added to each fertilization drop containing capacitated sperm. After 18 to 20 h of insemination, presumptive zygotes were cultured and evaluated as described above.

2.5. Somatic cell nuclear transfer

2.5.1. Recipient cytoplasm preparation

After 22 h in vitro maturation, buffalo oocytes were denuded and the oocytes with extruded first polar body (MII) were selected for enucleation and used as recipient cytoplasm in the NT procedure. The MII oocytes were placed in TALP-Hepes supplemented with 10% FCS and 7.5 $\mu g \cdot mL^{-1}$ cytochalacin B and were enucleated by aspirating the first polar body and the MII plate with a small volume of surrounding cytoplasm. Successful enucleation was confirmed by Hoechst 33342 staining and observed under ultraviolet light.

2.5.2. Donor cell preparation

Buffalo fetal fibroblasts were obtained from a 40-day-old fetus and were cultured as described previously [19]. The head and internal organs were removed and the remnant tissues were cut into small pieces. They were then minced and dispersed by 0.25% trypsin-EDTA for 15 min. The minced tissues were put into a 15-mL conical centrifuge tube containing 10 mL Dulbecco modified Eagles medium (DMEM) (Gibco) supplemented with 10% FCS (Hyclone). The cells were cultured under a humidified atmosphere of 5% CO_2 at 37 °C and used for NT between passages 2 and 8 of the culture. Cultured fetal fibroblasts were either cultured in DMEM supplemented with 0.5% FCS for 3–5 d (serum starved) or in DMEM supplemented with 10% FBS until

60–80% confluent (serum fed) prior used as nuclear donors.

2.5.3. Cell cycle analysis by flow cytometry

Flow cytometric cell cycle analysis was performed as previously described [20]. Briefly, the cells were trypsinized and resuspended in DMEM with 10% FCS at a concentration of approximately 5×10^5 cells/tube. The cells were then pelleted and resuspended in 1 mL of cold (4 °C) GM saline (6.1 mM glucose, 137 mM NaCl, 5.4 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 7H_2O$, 0.9 mM KH_2PO_4, and 0.5 mM EDTA). The cells were fixed by slowly adding 4 °C ethanol and were incubated overnight at 4 °C. The fixed cells were then washed 2 times with PBS. The cell pellets were stained with 25 $\mu g \cdot mL^{-1}$ of propidium iodide for 1 h at 37 °C and were filtered through a 41 μm nylon mesh (Spectrum, Los Angeles, CA) before flow cytometry. The cells were analyzed on a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA) at a rate of 500 to 1000 cells$\cdot s^{-1}$ and 10 000 events for each sample were recorded for further analysis with WinMDI Version 2.8 (J. Trotter, http://facs.scripps.edu/software.html).

2.5.4. Nuclear transfer, fusion and activation

Nuclear transfer was performed by the method previously described [19, 21]. Immediately prior to NT, a single cell suspension was prepared from donor cells by standard trypsinization using 0.25% trypsin-EDTA. An individual donor cell was transferred into the perivitelline space of the enucleated oocytes. Nuclear transfer units were placed in a fusion chamber between two platinum electrodes 0.2 mm apart and overlaid with the fusion medium (0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4.7H_2O$, 0.5 mM Hepes and 0.05% fatty acid-free (FAF) BSA) for a 1 min Cell fusion that was induced by two DC electrical pulses of 2.1 $kV \cdot cm^{-1}$ for 20 μs (1 s interval). The fusion rate was determined after being cocultured with BRL cells in TCM 199 supplemented with 10% FCS for 2 h. Fused NT embryos were activated by exposure to 5 μM calcium ionophore for 5 min followed by incubation with 2 mM 6-DMAP in culture medium for 4 h under a humidified atmosphere of 5% CO_2 at 39 °C.

2.6. In vitro culture and nuclear remodeling evaluation

Activated NT units were cocultured with BRL cells in 50 μL of TCM 199 supplemented with 10% FCS under a humidified atmosphere of 5% CO_2 at 39 °C. To observe the events of nuclear remodeling of a transferred nucleus, the samples of oocytes reconstructed with serum fed or starved cells were collected to observe the nuclear envelope and condensation of donor cell chromosomes at 1 h post fusion (hpf), just prior to activation. To examine the progression of nuclear remodeling, some of the reconstructed oocytes were collected at 4, 12, 18, and 24 h post activation (hpa) and were stained with 10 $\mu g \cdot mL^{-1}$ Hoechst 33342. The remaining NT embryos were cultured and assessed for the percentage of cleavage and blastocyst formation as described above. Blastocysts were stained with Hoechst 33342 for 1 h and the total number of their nuclei was counted under a fluorescent microscope.

2.7. Embryo transfer and pregnancy monitoring

Cloned embryos derived from serum fed or starved cells that reached the blastocyst stage (Day 7) were transferred into recipient buffaloes approximately 7 days after estrus was detected. Two to three embryos per recipient were nonsurgically introduced into the uterine horn ipsilateral to the ovary containing a palpable corpus luteum. Pregnancy status was assessed by transrectal ultrasonography approximately

Table I. Parthenogenetic development of buffalo oocytes after various activation treatments.

Treatment	N	Cleavage *n* (%)	Blastocyst *n* (%)	Cell number (mean ± S.D.)
Electrical pulses	37	16 (43)^{a}	0^{a}	0^{a}
Ethanol	65	43 (66)^{ab}	1 (2)^{a}	36.0 ± 0.0^{b}
A23187	72	31 (43)^{a}	1 (1)^{a}	26.0 ± 0.0^{b}
DMAP	45	0^{c}	0^{a}	0^{a}
Electrical + DMAP	83	46 (55)^{a}	31(37)^{b}	79.3 ± 4.2^{c}
Ethanol + DMAP	86	71 (83)^{b}	48 (56)^{bc}	72.6 ± 9.6^{c}
A23187 + DMAP	85	65 (77)^{b}	50 (59)^{c}	81.3 ± 7.8^{c}
IVF	69	43 (62)^{a}	24 (35)^{b}	108.2 ± 8.3^{c}

Different superscripts within columns are significantly different ($P < 0.05$).

30 days after embryo transfer. The pregnant recipients were checked for the presence of a conceptus by rectal palpation at 60, 90 and 150 days of gestation.

2.8. Statistical analysis

Data of parthenogenetic activation experiments were collected from eight replicates, whereas data of nuclear remodeling and in vitro development of NT embryos using serum fed or starved donor cells were collected from five and over ten replicates, respectively. The rate of cleavage and blastocyst development in parthenogenesis and NT experiments were analyzed by the chi-square test, whereas the cell cycle (six replicates) and blastocyst cell number data (mean ± S.D.) were analyzed using an ANOVA. The data were considered for significant differences at $P < 0.05$.

3. RESULTS

3.1. Parthenogenetic development of buffalo oocytes

To compare parthenogenetic development, oocytes were activated with electrical pulses, ethanol and calcium ionophore with or without subsequent administration of 6-DMAP. The cleavage rates of oocytes activated with ethanol or calcium ionophore followed by treatment with 6-DMAP were significantly higher ($P < 0.05$) than those activated by electrical pulses + 6-DMAP or IVF controls (83%, 77%, 55% and 62%, respectively, Tab. I). The rate of blastocyst development of oocytes treated with a calcium ionophore + 6-DMAP and ethanol + 6-DMAP was significantly higher ($P < 0.05$) than that activated with electrical pulses + 6-DMAP or IVF groups (59%, 56%, 37% and 35%, respectively, Tab. I). Only one blastocyst was obtained from the oocytes activated in the ethanol or calcium ionophore alone, whereas no blastocyst formation was obtained from the oocytes treated with electrical pulses alone. Total cell numbers of parthenote blastocysts derived from electric ($n = 19$), ethanol ($n = 25$), and calcium ionophore ($n = 28$), combined with 6-DMAP treatments were comparable to those of the IVF group ($n = 14$), and significantly higher ($P < 0.05$) than those activated by the ethanol or calcium ionophore alone. The morphology of parthenote and IVF blastocysts are shown in Figure 1.

3.2. Cell cycle analysis of donor cells

Flow cytometric analysis showed that the percentage of cells in G0/G1 was significantly ($P < 0.05$) higher in serum

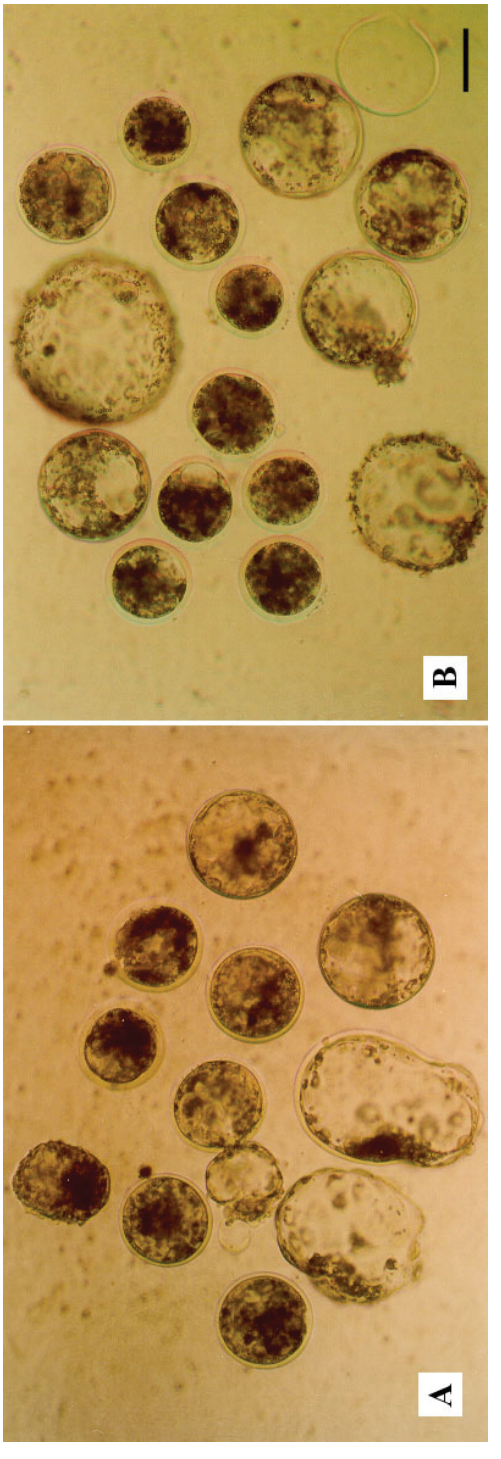

Figure 1. Blastocyst development of buffalo embryos derived from parthenogenetic activation or in vitro fertilization. (**A**) Blastocysts derived from oocytes activated with 5 µM calcium ionophore. (**B**) Blastocysts derived from in vitro – fertilized with frozen-thawed spermatozoa. Bar 100 µm.

starved cultures as compared to serum fed or cycling cultures (88.8 ± 6.2 vs. 68.2 ± 2.6). In contrast, the percentages of cells in both S and G2/M phases in serum starved cultures were lower ($P < 0.05$) than those in the serum fed cultures being 3.8 ± 1.2 vs. 12.1 ± 2.4 and 5.6 ± 2.7 vs. 19.4 ± 5.2, respectively.

3.3. Nuclear remodeling of transferred donor nuclei

When the nuclei of serum fed or starved cells were fused with enucleated oocytes, morphological changes during nuclear remodeling were observed (Tab. II and Fig. 2). At 1 hpf, 71% (40/56) of the transferred nuclei from serum fed donor cells did not change in size and the nuclear envelope remained intact (Fig. 2A), whereas 29% (16/56) underwent NEBD and PCC were observed (Fig. 2B). The nuclear enlargement (Fig. 2C), and swelling (Fig. 2D), were observed at 2–4 hpa. When serum starved cells were used, 83% (40/48) of the transferred nuclei underwent NEBD with PCC at 1 hpf, whereas 17% (8/48) of the nuclei remained intact. Nuclear swelling and the new nuclear envelope formation occurred at 2–4 hpa. Pronucleus formation of the transferred nuclei reconstructed from either serum fed or starved cells was seen within 12 hpa (Fig. 2E-F). The remodeled nuclei underwent mitotic division (Fig. 2G) and developed to the 2-cell stage (Fig. 2H) within 18 to 24 hpa. Two categories of PN formation were observed including 1PN (Fig.2E) and 2PN (Fig. 2F) without PB extrusion. Seventy-nine percent (33/42) of the embryos reconstructed from serum starved cells were 1PN and the other 21% (9/42) were 2PN. Fifty-five percent (38/69) of the oocytes reconstructed with serum fed donor cells were 2PN and the other 45% (31/69) were 1PN. At 24 hpa, 67% (32/48) and 83% (45/54) of the embryos reconstructed with serum fed or starved donors developed to the 2-cell stage, respectively.

3.4. In vitro development of NT embryos

In vitro development of NT embryos using serum fed or starved cells is presented in Table III. The percentages of fusion and cleavage of embryos reconstructed with serum fed or starved cells were not different. The nuclei of starved donors supported the development to blastocysts (Fig. 3) significantly higher ($P < 0.05$) than those of serum fed donors (35% vs. 21%). However, there were no significant differences in the numbers of Day 7 blastocyst nuclei derived from serum fed or starved donor cells (71.3 ± 5.4 vs. 87.6 ± 8.2).

3.5. Embryo transfer and pregnancy monitoring

Because of the silence of estrus in the buffalo, cloned embryos were non-surgically transferred into recipients that responded to estrus synchronization (12 out of 15). Forty percent (2/5) and 57% (4/7) of the recipients that received cloned blastocysts derived from serum fed and starved fibroblasts, respectively, were pregnant on Day 30 as revealed by ultrasonography. Pregnancy was confirmed on Day 60 in 1/5 (20%) and in 2/7 (29%) of the recipients receiving cloned blastocysts derived from serum fed and starved fibroblasts, respectively. Unfortunately, no recipient could carry the pregnancy beyond Day 90.

4. DISCUSSION

The present study has clearly shown that high efficiency of oocyte activation in the buffalo can be achieved by sequential treatment with either a calcium ionophore or ethanol followed by incubation in 6-DMAP. Our results were in agreement with the previous findings that a combination treatment of chemical agents, which increase intracellular calcium (calcium ionophore or ethanol), and a protein phosphorylation inhibitor,

Table II. Nuclear remodeling of the reconstructed oocytes after transfer of serum fed or starved fetal fibroblasts into enucleated oocytes.

Timing (hpf/hpa)	No. of oocyte	No. n (%) of reconstructed oocyte with							
		Intact nucleus	NEBD/PCC	Swollen	Pronucleus (PN)			Mitosis	2-cell
					1PN	2PN	Total		
Serum fed									
1 hpf	56	40 (71)	16 (29)	–	–	–	–	–	–
4 hpa	60	–	18 (30)	42 (70)					
12 hpa	68	–	–	–	21	29	50 (74)	18 (26)	–
18 hpa	52	–	–	–	8	4	12 (23)	18 (35)	22 (42)
24 hpa	48	–	–	–	2	5	7 (14)	9 (19)	32 (67)
Serum starved									
1 hpf	48	8 (17)	40 (83)	–	–	–	–	–	–
4 hpa	40	–	–	40 (100)					
12 hpa	56	–	–	–	32	8	40 (71)	16 (29)	–
18 hpa	60	–	–	–	1	1	2 (3)	10 (17)	48 (80)
24 hpa	54	–	–	–	–	–	–	9 (17)	45 (83)

hpf = hour post fusion; hpa = hour post activation; NE = nuclear envelope; NEBD = nuclear envelope breakdown; PCC = premature chromosome condensation.

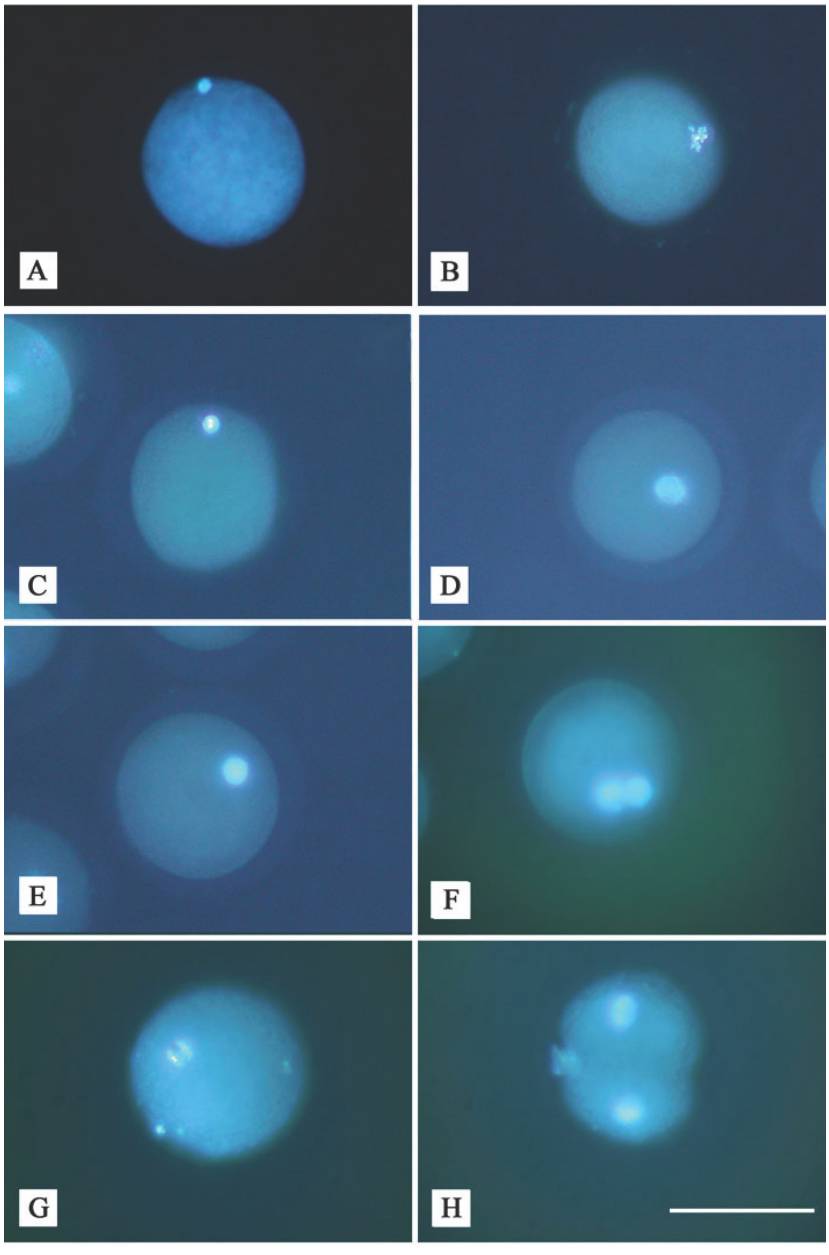

Figure 2. Nuclear remodeling of the transferred fetal fibroblasts in enucleated oocytes observed under fluorescent microscope after staining with Hoechst 33342. **(A)** Transferred nucleus with nuclear envelope intact at 1–2 h post fusion (hpf). **(B)** NEBD with condensed chromosomes at 1–2 h hpf. **(C)** Nuclear enlargement at 2–4 h post activation (hpa). **(D)** Nuclear swelling and reformed nuclear envelope at 2–4 hpa. **(E)** Reconstructed oocyte with 1PN without PB extrusion. **(F)** Reconstructed oocyte with 2PN without PB extrusion. **(G)** Reconstructed oocyte entered the first mitotic division (anaphase) at 18 hpa. **(H)** A 2-cell stage embryo at 24 hpa. Bar 100 µm.

Table III. In vitro development of cloned buffalo embryos reconstructed with serum fed or starved fetal fibroblasts.

Donor cell	NT	Fused *n* (%)	Cleavage *n* (%)	Blastocyst *n* (%)	Cell number (mean ± S.D.)
Serum fed	274	205 (75)	149 (73)	43 (21)[a]	71.3 ± 5.4
Serum starved	289	228 79)	187 (82)	80 (35)[b]	87.6 ± 8.2

Different superscripts within columns are significantly different ($P < 0.05$).

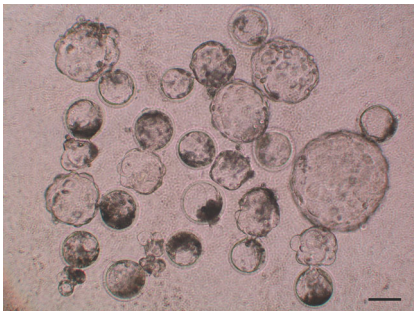

Figure 3. Representative of Day 7 cloned buffalo blastocysts derived from serum starved fetal fibroblasts. Bar 100 µm.

6-DMAP, are more effective in inducing activation and the subsequent development of bovine oocytes than any single treatment [10]. The efficiency of oocyte activation appears to vary among species as well as with the activation procedure. In goats, a combination treatment of MII oocytes by ethanol and 6-DMAP results in 58% cleavage [22] whereas oocytes reconstructed with somatic cells induce a 56% cleavage [4]. In rats, the percentages of activated oocytes with PN that developed to the 2-cell, 8-cell and blastocyst stages when electrical stimulation was followed by 6-DMAP treatment were significantly higher than the other combined treatments [23]. The rates of 1PN and 1PB formation in buffalo oocytes treated with electric, ethanol or calcium ionophore followed by 6-DMAP were very high (80–100%) whereas the oocytes treated with these agents alone were very low activated (0–20%, unpublished data). It has been reported that sequential treatment with ionomycin and 6-DMAP leads to 1PN + 1PB formation in bovine oocytes [12]. This finding indicates that 6-DMAP treatments induce diploid activation by preventing chromosomal separation and second polar body extrusion.

The percentages of cleavage and blastocyst development in both calcium and ethanol-activated oocytes were significantly higher than those of electrically-activated and IVF controls. The low percentages of cleavage and blastocyst development from IVF oocytes may be due to the low efficiency of the IVP system [24]. Since the appropriate IVP system in the buffalo has not been well established, the inefficiencies of IVP may be attributed to many factors including the quality of the oocytes and frozen semen [25], sperm preparation [26, 27], and the IVF and IVC medium [28]. In our laboratory, the ability of cultured buffalo oocytes to reach nuclear maturation is only about 50–60% and the percentage of cleavage in IVF buffalo embryos was significantly lower than that of IVF in cattle (62% vs. 85%). On the contrary, mouse oocytes activated with ethanol resulted in the development to the blastocyst stage similar to that of the IVF control [14]. In cattle, the percentage of blastocyst development obtained from oocytes that were activated by the ethanol or calcium ionophore followed by 6-DMAP was also similar to that of the IVF controls [10]. However, bovine parthenotes activated by a combined treatment of ionomycin and 6-DMAP had a lower total blastocyst cell number than that

of IVF embryos [29]. However, activation by a combination of ethanol and cytochalasin D resulted in blastocyst development with the total cell number comparable to the IVF embryos [30]. Moreover, there were no differences observed in the mean total cell number or the ratio of inner cell mass to the trophoblast cell of Day 8 IVF control blastocysts and those from oocytes activated by the calcium ionophore followed by exposure to 6-DMAP [31]. Our results in the buffalo also demonstrate the insignificant differences in the cell number of Day 7 blastocysts derived from oocytes activated by electrical pulses, calcium ionophore or ethanol followed by treatment with the 6-DMAP and IVF control.

It has been observed that when donor nuclei are transferred into MII oocytes, NEBD and PCC are observed [32], however, Dominko et al [33] did not observe NEBD and PCC when nuclei from fibroblasts presumably arrested at G0/G1 were fused with the MII cytoplasm. This discrepancy may be due to the MPF activity [34]. In the present study, both an intact nuclear envelope and NEBD with PCC were observed when either serum fed or starved donor cells were introduced into MII cytoplasts. The reason behind these results may be due to two electrical pulses used for the fusion of donor cells and the recipient cytoplasts in the present study may be sufficient to decrease the MPF activity and prevent PCC [33]. The results indicate that PCC is not a prerequisite for nuclear remodeling after transferring somatic cell nuclei into MII cytoplasts. However, most of the transferred nuclei derived from serum fed donors showed an intact nuclear envelope, whereas most of those derived from starved donors underwent NEBD with PCC. We also noticed that the events of nuclear remodeling such as nuclear swelling as well as subsequent cleavage of starved serum cells were more advanced than fed cells. The exact reason for the difference in nuclear remodeling between serum fed and starved donor cells is unclear. A possible explanation may be to the quiescence induced by serum starvation which makes the cells more amenable to nuclear reprogramming through NEBD and the condensation of the chromosome. This would allow cytoplasmic factor (s) existing in recipient cytoplasts to access easily to the DNA [35], and nuclei would undergo DNA replication and subsequent cleavage faster than in the case of serum fed donors. This may reflect the rapid reprogramming of serum starved nuclei after NT, which results in higher rates of blastocyst development.

We also observed that the percentage of 2PN embryos from serum fed donor cells was higher than that of serum starved cells (55% vs. 21%). The possible explanation for a high percentage of 2PN embryos from serum fed donor cells is that the donor cells used for NT are not homogeneous. Since cell sorting, which would increase the number of the G0/G1 stage of donor cells was not done, the presence of cells in the G2/M phase which contains replicated chromosomes (tetraploid chromosomes) might contribute to the formation of 2PN embryos [36]. Another possibility is that the transferred nuclei might undergo cell division just after fusion and separate into two masses of chromosomes and subsequent PN as reported previously [32]. Previous studies have suggested that the embryonic development of NT embryos is enhanced when the donor cells are in the G0 or G1 phase of the cell cycle [1,15]. The chromatin of quiescent nuclei has been reported to undergo a number of modifications, including a reduction in transcription and chromatin condensation [37]. Such modifications might be beneficial for complete reprogramming after NT. Our results were in agreement with the previous report in cattle that the nuclei of serum starved fibroblasts support the development of reconstructed embryos to the blastocyst stage significantly better than those of non-starved fibroblasts (39% vs. 20%) [38]. Serum starvation of fetal donor cells before NT was also found to significantly improve

NT blastocyst rates when compared with serum fed cells (43% vs. 12%) [39].

Live offspring were successfully produced by NT from serum starved donor cells in sheep [1], cattle [16], goats [4]) and pigs [5]. In contrast, viable cloned calves have been produced from nonstarved fetal fibroblasts [2, 37] and fetal muscle cells [40] indicating that the serum starvation of donor cells might not be necessary for nuclear reprogramming. In our study, no fetuses developed to term from transferring cloned embryos derived from both serum fed and starved donor nuclei into the recipients. The causes of abortion remain unknown but our finding implied that the differences in the cell cycle stage of donor cells may not correlate with the in vivo developmental competence of the cloned buffalo embryos and incomplete genetic modifications [41, 42] may contribute to high abortion rates after embryo transfer. Further experiments are needed to study the molecular mechanism(s) underlying the reprogramming such as DNA methylation as well as the expression of imprinted or nonimprinted genes in cloned embryos.

In conclusion, the efficient protocols of buffalo cloning using somatic cells were established in this study. The optimal activation of buffalo oocytes could be achieved by combining the treatment with a calcium ionophore or ethanol followed by 6-DMAP. Nuclear remodeling events of cloned embryos from serum starved cells were more advanced than serum fed donors resulting in faster cleavage and higher blastocyst development.

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