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—Original Article—

Effects of Trichostatin A on *In Vitro* Development and DNA Methylation Level of the Satellite I Region of Swamp Buffalo (*Bubalus bubalis*) Cloned Embryos

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Abstract. Trichostatin A (TSA), a histone deacetylase inhibitor, has been widely used to improve the cloning efficiency in several species. This brings our attention to investigation of the effects of TSA on developmental potential of swamp buffalo cloned embryos. Swamp buffalo cloned embryos were produced by electrical pulse fusion of male swamp buffalo fibroblasts with swamp buffalo enucleated oocytes. After fusion, reconstructed oocytes were treated with 0, 25 or 50 nM TSA for 10 h. The results showed that there was no significant difference in the rates of fusion (82–85%), cleavage (79–84%) and development to the 8-cell stage (59–65%) among treatment groups. The highest developmental rates to the morula and blastocyst stages of embryos were found in the 25 nM TSA-treated group (42.7 and 30.1%, respectively). We also analyzed the DNA methylation level in the satellite I region of donor cells and in *in vitro* fertilized (IVF) and cloned embryos using the bisulfite DNA sequencing method. The results indicated that the DNA methylation levels in cloned embryos were significantly higher than those of IVF embryos but approximately similar to those of donor cells. Moreover, there was no significant difference in the methylation level among TSA-treated and untreated cloned embryos. Thus, TSA treatments at 25 nM for 10 h could enhance the *in vitro* developmental potential of swamp buffalo cloned embryos, but no beneficial effect on the DNA methylation level was observed.

Key words: Cloning, DNA methylation, Embryo development, Swamp buffalo, Trichostatin A

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Although somatic cell nuclear transfer (SCNT) in the buffalo has been attempted throughout the last decade, the success rate of producing buffalo cloned offspring is still low when compared with the bovine. From our previous report, the developmental potential to the blastocyst stage of swamp buffalo cloned embryos was lower than that of bovine cloned embryos [1]. Increasing the blastocyst rate in swamp buffalo cloned embryos enhances the possibility to obtain viable clones. Multiple factors have been shown to influence the development of cloned embryos. One of the key factors is the incomplete reprogramming involved in abnormal epigenetic remodeling such as DNA methylation and histone modifications [2–4]. In theory, assisted reprogramming of the donor nucleus for SCNT should improve embryo development. Trichostatin A (TSA), a histone deacetylase inhibitor, has been widely used to improve the cloning efficiency of several species such as the mouse [5, 6], bovine [7–9], pig [10–14] and rabbit [15]. However, TSA treatment in swamp buffalo cloned embryos has not yet been reported. This work was carried out to investigate the effects of TSA on the developmental potential of swamp buffalo cloned embryos.

One of the epigenetic modifications in embryos is involved in DNA methylation at cytosine residues in CpG dinucleotides [16]. Hypermethylation in the satellite I region has been shown to influence the developmental potential of bovine and sheep cloned embryos by altering gene expression [17, 18]. Wee *et al*. (2007) reported that the DNA methylation level in the satellite I region of TSA-treated bovine cells was significantly lower than that of untreated cells [19]. When using TSA-treated bovine cells as donor cells, the DNA methylation level of blastocysts derived from TSA-treated cells was significantly lower than those of untreated cells. An increased developmental rate to the blastocyst stage of cloned embryos derived from TSA-treated cells was observed. TSA can reactivate a silenced transgene in transgenic pig fibroblast cells by inducing DNA hypomethylation and histone hyperacetylation of the promoter region [20]. Therefore, based on previous reports, TSA can not only induce histone hyperacetylation but can also induce DNA demethylation.

To the best of our knowledge, there has been no report on the methylation status of the satellite I region in swamp buffalo *in vitro* fertilized or cloned embryos. The relation between TSA treatment and the change in DNA methylation is not known in buffaloes. Therefore,

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this is the first study to examine the effect of TSA treatment on DNA methylation levels of swamp buffalo cloned embryos. Study of the methylation status of satellite I should provide useful information concerning the molecular events in epigenetic reprogramming during development of swamp buffalo cloned embryos.

Materials and Methods

Chemicals and media

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Embryo production by somatic cell nuclear transfer

Donor cell and oocyte preparation, the somatic cell nuclear transfer procedure and *in vitro* embryo culture were performed as describe previously [1]. Briefly, buffalo oocytes were obtained from local abattoir-derived ovaries and cultured in *in vitro* maturation (IVM; TCM199 supplemented 10% FBS, 50 IU/ml hCG (Intervet, Boxmeer, Netherlands), 0.02 AU/ml FSH (Antrin®, Kyoritsu Seiyaku, Tokyo, Japan) and 1 μg/ml 17β-estradiol) medium at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 23 h. Then, cumulus cells were removed from cumulus oocytes complexes (COC) by repeat pipetting in 0.2% hyaluronidase. Metaphase-II oocytes with a first polar body (1st PB) were incubated in 5 μg/ml cytochalasin B for 5 min. The zona pellucida above the 1st PB was cut using a glass needle, and the 1st PB and a small amount of cytoplasm were squeezed out through the slit of the zona pellucida. Successful enucleation was performed by staining the squeezed out cytoplasts with 10 μg/ml Hoechst 33342 for 10 min and visualization of the metaphase plate under ultraviolet light. A male buffalo fibroblast donor cell was inserted into the perivitelline space of the enucleated oocyte. The donor cell-cytoplast couplet (DCCC) was fused with electric pulses (26 V, 17 μsec, 2 times) in Zimmermann fusion medium [21] and washed in Syngro® holding medium (Bioniche, Belleville, ON, Canada). At 1 h post fusion, successfully fused DCCCs were activated in 7% ethanol (Carlo Erba Reagents, Val de Reuil, France) in Syngro® holding medium for 5 min at room temperature and cultured in mSOFaa medium [22] supplemented with 1.25 μg/ml cytochalasin D and 10 μg/ml cycloheximide at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 5 h. Then the embryos were cultured in mSOFaa medium at 38.5 C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 2 days. Eight-cell stage embryos were selected and co-cultured with bovine oviductal epithelial cells in mSOFaa medium at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 5 days.

TSA treatment

TSA treatment was performed as previously reported in the mouse [5] and bovine [9]; both reports showed that TSA treatment significantly increased the blastocyst formation rate, resulting in viable cloned offspring. Briefly, DCCCs were separated into three groups after fusion: an untreated group and 25 and 50 nM TSA groups (treated groups). For the TSA-treated groups, DCCCs were placed in Syngro® holding medium supplemented with 25 or 50 nM TSA for 1 h. After that, the fused DCCCs were activated and cultured in medium supplemented with 25 or 50 nM TSA for another 9 h [5, 9]. For the control group, fused DCCCs were activated and cultured under the same conditions except without TSA supplementation.

Embryo production by in vitro fertilization

Sperm preparation and the *in vitro* fertilization procedure were performed as describe previously [23]. Briefly, frozen spermatozoa were thawed at 37 C for 30 sec. The thawed spermatozoa were loaded into the bottom of a 5 ml culture tube containing 2 ml of modified Tyrode's albumin lactate pyruvate (TALP) [24] supplemented with 2.5 mM caffeine, 50 μg/ml heparin, 20 mM penicillamine, 10 mM hypotaurine and 20 mM epinephrine and then incubated at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 30 min. The 1.5 ml of supernatant was taken out from the culture tube and centrifuged at 500 × *g* for 5 min. The pellet was resuspended in TALP at 4 × 10^6 sperms/ml. After 23 h post IVM, 10 COCs were transferred into 100 ml droplets of sperm supernatant and co-incubated at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 24 h. After that, embryos were washed and cultured in mSOFaa medium at 38.5 C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 1 day. Eight-cell stage embryos were selected and co-cultured with bovine oviductal epithelial cells in mSOFaa medium at 38.5 C under a humidified atmosphere of 5% CO_2 in air for 5 days.

Analysis of DNA methylation level in the satellite I region

The DNA methylation levels of IVF and cloned embryos and the fibroblasts used as donor cell were analyzed by the bisulfite method. Genomic DNA was extracted from IVF and cloned embryos (1-cell, 8-cell and blastocyst stages) and from donor cells by conventional DNA extraction. Three embryos were pooled and used for DNA extraction. Bisulfite treatment reactions were performed using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The satellite I region in bisulfite-treated DNA was amplified by polymerase chain reaction (PCR). The 20 μl reaction mixture consisted of 100 ng DNA sample, 1x PCR buffer (Promega, Madison, WI, USA), 2 mM $MgCl_2$ (Promega), 0.2 mM dNTPs mix (Fermentas, Burlington, ON, Canada), 0.5 μM of 5'-GTATTTTTTTTGGAGTTTTTTGAG-3' and 5'-ACCATAAAAACCTAACTTCCCTAAC-3' and 0.625 units Taq DNA polymerase (Promega). The amplification conditions were as follows: 95 C for 2 min followed by 35 cycles of 95 C for 30 sec, 50 C for 30 sec, 72 C for 30 sec. The final elongation step was performed at 72 C for 5 min. PCR products were ligated with pGEM® T-easy vector (Promega). The ligation mixture was transformed into the competent *E. coli* strain DH5α by electroporation. Positive clones were selected by blue-white selection on an LB agar plate containing 100 μg/ml ampicillin, 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). White colonies were cultured in LB broth with the above antibiotics. Plasmid preparation was performed with PureLink® Quick Plasmid Miniprep Kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. DNA sequencing was performed by Macrogen Korea (Seoul, Republic of Korea). DNA methylation analysis was performed with the Quantification tool for Methylation Analysis (http://quma.cdb.riken.jp/top/index.html) [25].

Table 1. *In vitro* development of swamp buffalo cloned embryos after TSA treatment for 10 h

Treatment	No. of replications	Fused (%)	Cultured	Cleaved (%)	No. (%)* embryos developed to		
					8-cells	Morula	Blastocyst
0 nM	11	120/140 (85.1 ± 6.4)	120	96 (81.9 ±13.2)	60 (62.9 ± 15.0)	33 (34.8 ± 8.1)^{b}	22 (23.4 ± 6.0)^{b}
25 nM	12	117/143 (81.6 ± 5.3)	117	91 (78.6 ± 19.1)	58 (65.4 ± 13.9)	38 (42.7 ± 9.6)^{a}	26 (30.1 ± 8.1)^{a}
50 nM	10	121/146 (82.9 ± 2.2)	118	96 (84.1 ± 11.8)	57 (59.2 ± 11.4)	33 (37.8 ± 4.6)^{b}	25 (25.4 ± 4.0)^{ab}

* Percentages calculated from the number of cleaved embryos and that developed to each stage. Values are means ± SD. Different superscripts within a column indicate significant differences (P < 0.05; Duncan's multiple range test).

Statistical analysis

All values for cloned embryo development and DNA methylation levels in IVF and cloned embryos and donor cells are presented as the mean ± standard deviation (SD). The proportional data were subjected to arcsine transformation before statistical analysis. Transformed values were evaluated using the Statistical Analysis System software (SAS Institute, Cary, NC, USA) with a Completely Randomized Design, and comparison of data means was carried out with Duncan's multiple range test. A p-value of 0.05 or less than (P<0.05) were considered significant.

Results

Development potential of cloned buffalo embryos after TSA treatment

After fusion, DCCCs were separated into three groups: two TSA-treated groups, the 25 and 50 nM TSA groups, and the untreated group. There was no significant difference in fusion rate (82–85%) after TSA treatment. The cleavage rate (25 nM *vs*. 50 nM: 79% *vs.* 84%) and rate of development to the 8-cell stage (65% *vs.* 59%) were not significantly different among the treatment groups. However, the rate of development to the morula stage of embryos treated with 25 nM TSA (42.7%) was significantly higher than those of the 50 nM TSA-treated (37.8%) and untreated embryos (34.8%). Moreover, the highest blastocyst formation rate was found in the 25 nM TSA-treated embryos (30.1%) when compared with the other groups, and the difference was significant when compared with the untreated embryos (23.4%, P<0.05) (Table 1).

DNA methylation analysis in IVF and cloned embryos

The DNA methylation levels of the satellite I region in the IVF and cloned embryos and in the swamp buffalo fibroblasts used as donor cells were analyzed. The methylation level of the donor cells was 73.9% (Fig. 1). In the cloned embryos, the DNA methylation levels of the untreated embryos (0 nM TSA) at the 1-cell (75.0%), 8-cell (76.5%) and blastocyst stages (67.2%) were not significantly different when compared with that of the donor cells. Moreover, no significant differences were found in the methylation levels of the 25 nM and 50 nM TSA-treated cloned embryos at the 1-cell (75.7 and 73.2%, respectively), 8-cell (66.8 and 71.3%, respectively) and blastocyst stages (64.4 and 61.4%, respectively) when compared with those of the untreated cloned embryos and donor cells. On the other hand, the methylation levels of the IVF embryos were significantly lower compared with those of the TSA-treated and untreated cloned embryos at the 1-cell, 8-cell and blastocyst stages (P<0.01, Fig. 2).

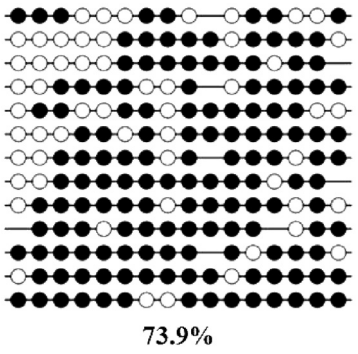

Fig. 1. DNA methylation level of the satellite I region in buffalo fibroblasts used as donor cells for somatic cell nuclear transfer. Values are means ± SD.

Discussion

TSA is one of the chromatin remodeling agents that enhances histone acetylation and DNA demethylation [26]. In this study, the reconstructed embryos were continuously exposed to TSA for 10 h beginning just minutes after fusion. The TSA concentration of 25 nM was shown to be effective for improving the morula and blastocyst formation rates in swamp buffalo cloned embryos. This is the first report of direct TSA treatment in swamp buffalo cloned embryos. According to previous reports, treatment with 5 nM TSA for 20 h [7] or 14 h [8] and with 50 nM TSA for 10 h [9] in bovine cloned embryos could increase the rate of development to the blastocyst stage. However, the effects of TSA treatment on bovine cloning efficiency have been controversial. Some previous reports have shown that TSA treatment did not improve *in vitro* development of bovine cloned embryos [27, 28]. Recently, Luo *et al*. (2013) found that treatment of donor cells with 150 or 300 μM TSA increased cleavage and blastocyst formation rates of swamp buffalo cloned embryos [29]. Treatment with 500–1000 nM scriptaid, another histone deacetylase inhibitor, has been shown to improve the developmental potential of

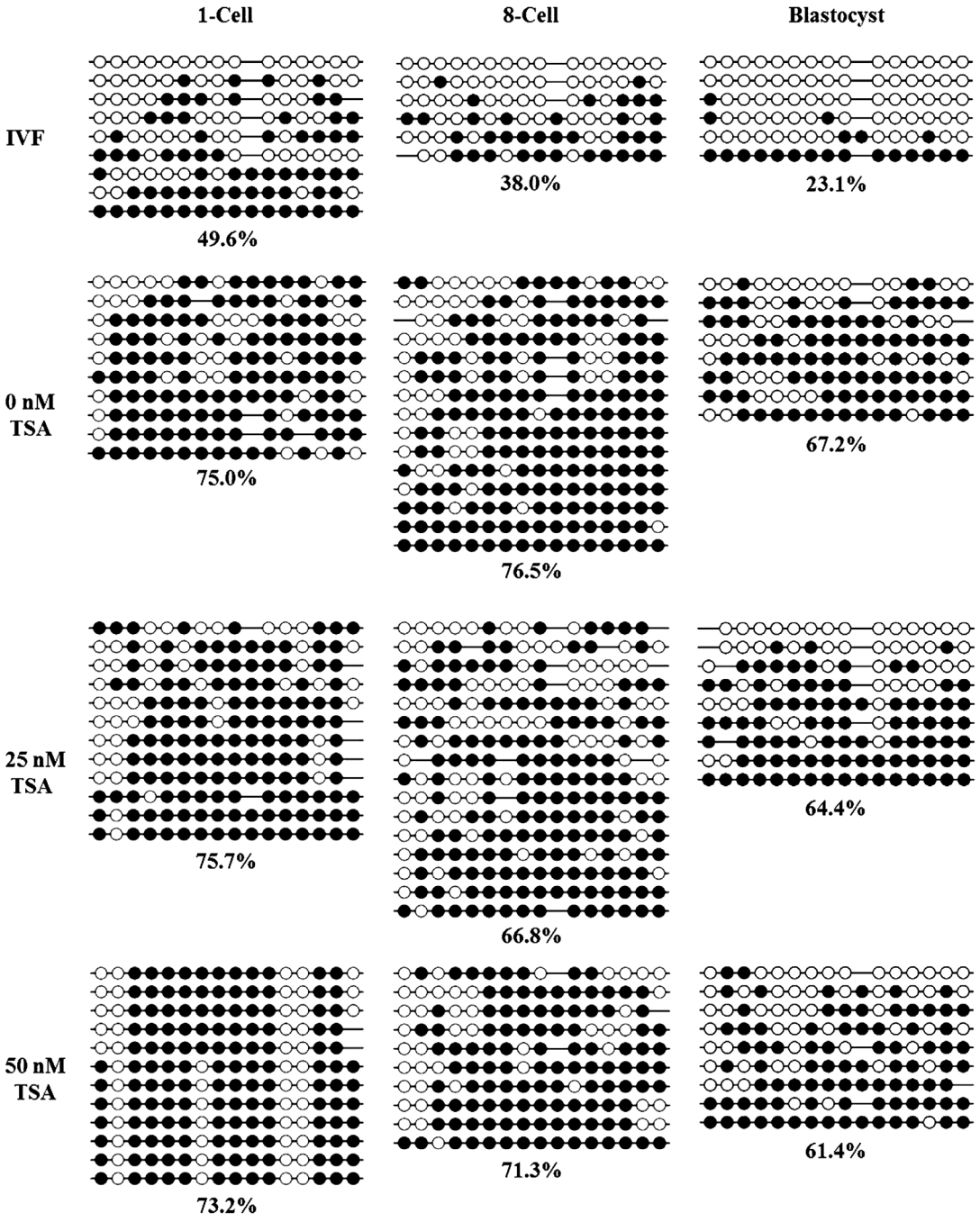

Fig. 2. DNA methylation level of the satellite I region in IVF and cloned embryos (0, 25 and 50 nM TSA) at the 1-cell, 8-cell and blastocyst stages. Values are means ± SD.

handmade river buffalo cloned embryos [30]. Moreover, treatment of pig cloned embryos with 50 nM TSA for 24 h after activation significantly improved the blastocyst formation rate when compared with untreated embryos, while treatment of donor cells under the same conditions resulted in increases in cleavage rates but did not improve the blastocyst rate when compared with an untreated group [31]. The conflicting results may be due to the differences in treatment method used (embryo or donor cell treatment), concentration of TSA, timing of TSA treatment, cloning protocol, conditions of embryo culture and species.

In this study, we focused on DNA methylation of the satellite I region because the difference in DNA methylation level in this region between bovine IVF and cloned embryos was significantly higher than for other satellite regions [32]. We found that the levels of DNA

methylation of the satellite I region in swamp buffalo cloned embryos were significantly higher than those of IVF embryos at every stage analyzed (1-cell, 8-cell and blastocyst stages) and similar to those of fibroblasts used as donor cells. This finding was similar to previous reports in bovines. Yamanaka *et al*. (2011) reported that high DNA methylation levels were found for the satellite I region in bovine fibroblasts used as donor cells [33]. Also, bovine cloned embryos at the blastocyst stage showed significantly higher DNA methylation levels than those of IVF embryos. Similarly, bovine donor cells were highly methylated in the satellite I region, resulting bovine cloned blastocysts with significantly more methylation than IVF blastocysts [34]. Moreover, Sawai *et al.* (2010) found that the DNA methylation level of the satellite I region in 50 nM TSA-treated bovine cloned embryos at the blastocyst stage was higher than in IVF embryos. However, there was no significant difference in methylation levels between TSA-treated and untreated cloned embryos [8]. A previous report suggested that DNA hypermethylation of the satellite I region in cloned embryos was caused by cloning procedures, not the *in vitro* embryo culture procedure [33]. The higher methylation level of the satellite I region in cloned embryos compared with IVF embryos suggests that reprogramming of donor nuclei was uncompleted, and this likely contributed to low cloning efficiency. These findings are important for understanding methylation changes of genomic satellite sequences during swamp buffalo preimplantation development.

In conclusion, treatment with 25 nM for 10 h after fusion could increase the blastocyst formation rate of swamp buffalo cloned embryos; however, no beneficial effect on the DNA methylation status of the satellite I region was found in our experiment. This is the first report to examine the effects of TSA treatment on *in vitro* development and DNA methylation level of the satellite I region of swamp buffalo cloned embryos.

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