



# Reducing the cytoplasmic volume during hand-made cloning adversely affects the developmental competence and quality, and alters relative abundance of mRNA transcripts and epigenetic status of buffalo (*Bubalus bubalis*) embryos



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## ABSTRACT

Hand-made cloning (HMC) is a method of choice for somatic cell nuclear transfer (SCNT). There is 20% to 50% of cytoplasm lost during manual enucleation of oocytes with HMC. To compensate, two enucleated demicytoplasts, instead of one, are fused with each donor cell, which leads to cytoplasm pooling from two different demicytoplasts. In this study, effects of using one, instead of two demicytoplasts (controls) was examined, for production of embryos using HMC. Use of one demicytoplast decreased blastocyst development ( $12.7 \pm 1.98\%$  compared with  $47.6 \pm 3.49\%$ ,  $P < 0.001$ ), total cell number (TCN,  $167.6 \pm 14.66$  compared with  $335.9 \pm 58.96$ ,  $P < 0.01$ ), apoptotic index ( $2.11 \pm 0.38$  compared with  $3.43 \pm 0.38$ ,  $P < 0.05$ ) but did not significantly alter inner cell mass:trophectoderm cell number ratio ( $0.17 \pm 0.01$  compared with  $0.19 \pm 0.02$ ) and the global content of H3K9ac and H3K27me3 of blastocysts, compared to controls. There were gene expression alterations in pluripotency- (*SOX2* and *NANOG* but not *OCT4*), epigenetic- (*DNMT1* but not *DNMT3a* and *HDAC1*), apoptosis- (*CASPASE3* but not *BCL-2* and *BAX*), trophoctoderm- (*CDX2*), development- (*G6PD* but not *GLUT1*) and cell cycle check point control-related related genes (*P53*) compared with controls. Transfer of cloned blastocysts from one demicytoplast ( $n = 8$ ) to recipients resulted in a live calf birth that after 12 days died whereas, with transfer of control blastocysts ( $n = 14$ ) there was birth of a healthy calf. In conclusion, use of one, instead of two demicytoplasts for HMC, compromises *in vitro* developmental competence, and alters expression of several important genes affecting embryo development.

## 1. Introduction

Cloning by somatic cell nuclear transfer (SCNT) is conducted by transferring a differentiated somatic cell to an oocyte from which the nuclear material has been removed. The cytoplasm of the enucleated oocyte converts the differentiated somatic cell to the totipotent state through a process called reprogramming. Cloning with use of SCNT is a pivotal aspect with use of modern reproductive technologies because with this technique there is a means for rapid multiplication of elite animals and also there can be application of a diverse array of other reproductive technologies such as production of transgenic animals, use of farm animals as

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models for human diseases, xenotransplantation, conservation and restoration of endangered species etc. (Niemann and Lucas-Hahn, 2012).

Two different types of procedures for SCNT have been used in mammalian species for the production of cloned embryos. The first method (*i.e.*, the micromanipulation-based cloning), which was used for the production of the first cloned mammal 'Dolly' (Wilmut et al., 1997) with use of SCNT has been used extensively with 23 animal species having been successfully cloned using this method (Keefer, 2015). With use of this method, there are several disadvantages such as requirement for expensive micromanipulators and skilled personnel to operate the equipment resulting in the use of this technique in a few laboratories where the equipment and skilled personnel can be economically justified. The second method, termed Hand-made cloning (HMC), is rapid, requires less expertise and time than the micromanipulation-based method and does not have the disadvantages as compared with the use of micromanipulation-based cloning (Vajta et al., 2001). Furthermore, production efficiency with use of HMC and embryo quality of HMC-derived embryos are relatively greater than with use of micromanipulation-based cloning in terms of pregnancy rates and live births (Tecirlioglu et al., 2005). All these advantages make HMC suitable for large-scale SCNT programs, and it has been successfully used for production of cloned offspring in many farm animal species such as cattle, pigs, sheep and goats (Verma et al., 2015). There has been application of a modified HMC for production of cloned buffalo embryos and offspring from a wide variety of donor cells (Selokar et al., 2018; Saini et al., 2018).

The use of HMC involves *in vitro* maturation (IVM) of oocytes, removal of cumulus mass and zona pellucida by enzymatic treatment followed by manual bisection of zona-free oocytes for removal of nuclear material. There, however, is 20% to 50% of the cytoplasmic volume lost during protrusion cone-guided manual bisection of oocytes. Because the capacity of the cytoplasmic contents to induce reprogramming of the differentiated somatic cell to the totipotent state is dependent upon both the quality and quantity of cytoplasm, with loss of a substantial proportion of cytoplasm during enucleation there are adverse effects on the developmental competence of embryos produced using HMC (Panda et al., 2011). To compensate for this loss of cytoplasmic volume, two enucleated demicytoplasts, instead of one demicytoplast, are fused with the somatic cell for production of reconstructed embryos. Because the two demicytoplasts are from two different animals, use of this approach results in introduction of mitochondrial heteroplasmy in the reconstructed embryos. On an average,  $1.74$  to  $9.5 \times 10^5$  mitochondrial DNA (mtDNA) copies are present in mature oocytes in different mammalian species whereas, the copy number of mtDNA in somatic cells is only a few hundred to several thousand (Bowles et al., 2007). The clones produced using micromanipulation-based SCNT, in which one donor cell is fused with one enucleated oocyte, are expected to be heteroplasmic due to contribution of mtDNA from two sources and on equal replication of the mitochondrial genomes. Indeed, percentages of heteroplasmy ranging from 0% to 40% have been reported in SCNT-derived fetuses and offspring (Takeda et al., 2003; Burgstaller et al., 2007). The extent of mitochondrial heteroplasmy would be even greater with use of HMC than with micromanipulation-based SCNT because with HMC, there are three sources of mtDNA compared with micromanipulation-based SCNT in which mtDNA comes from only two sources. Mitochondrial heteroplasmy affects the development of cloned embryos (Schatten et al., 2005; Takeda, 2013). The sequence of nucleo-mitochondrial interaction is disrupted after nuclear transfer because the nuclear-encoded mtDNA transcription and replication factors persist in SCNT- but not IVF-derived embryos (Lloyd et al., 2006). The nuclear DNA-mtDNA interactions affect *in utero* developmental capacity, phenotype and cellular metabolism of cloned fetuses (Hiendleder et al., 2004). The aim of the present study was to reduce the mitochondrial heteroplasmy in cloned embryos produced using HMC by utilising one demicytoplast instead of the two typically used in production of HMC-derived embryos.

There are several reports of the effects of cytoplasm on development and quality of nuclear transferred embryos using blastomeres as nuclear donors (Westhusin et al., 1996; Zakhartchenko et al., 1997; Peura et al., 1998). Very limited information, however, is available on the effects of cytoplasmic volume on SCNT-derived embryos produced using HMC. In a previous study, there was investigation of the effects of using one or two or three demicytoplasts for generation of reconstructed embryos on developmental competence (Panda et al., 2011). In this study, however, there was a limited number of cloned embryos, and several important variables in cloned embryos such as the extent of apoptosis, epigenetic status and expression of important genes, were not examined. In the present study, there was examination of the effects of using one demicytoplast, instead of two demicytoplasts, on the *in vitro* and *in vivo* developmental competence and quality of buffalo embryos produced using HMC. There was comparison of the global content of two important epigenetic markers (*i.e.*, acetylation of lysine 9 at histone 3 - H3K9ac) and trimethylation of lysine 27 at histone 3 (H3K27me3) in blastocysts produced using one or two demicytoplasts, using blastocysts produced utilising *in vitro* fertilization (IVF) procedures for production of control embryos. Furthermore, there was comparison of the relative abundance of mRNA transcripts for some important genes related to pluripotency, epigenetics, apoptosis, trophoblast, embryonic development and cell-cycle checkpoint control in these two types of blastocysts, using IVF-derived embryos as controls.

## 2. Materials and methods

All the chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA), the disposable plasticware was obtained from Nunc (Roskilde, Denmark) and the media were purchased from GIBCO (Grand Island, NY, USA) unless otherwise mentioned. Fetal bovine serum (FBS) was purchased from Hyclone, Logan, UT, USA. The  $0.22 \mu\text{m}$  filters were from Millipore Corp., Bedford, MA, USA and the French straws (0.25 ml) were from IMV, L'Aigle, France. The IVM, IVF and *in vitro* culture (IVC) of cloned and IVF embryos were conducted at  $38.5^\circ\text{C}$  in a  $\text{CO}_2$  incubator (5%  $\text{CO}_2$  in air, 90%–95% relative humidity). Somatic cells used as donor cells for HMC were also cultured using similar conditions.

## 2.1. Hand-made cloning

Somatic cells which had been isolated from the seminal plasma of cryopreserved semen of a progeny-tested bull (MU 4393) that had died more than 10 years before the present study was conducted, and which had been established in culture in a previous study (Selokar et al., 2014), were used as donor cells for the production of embryos using HMC in the present study. The stage of the cycle of all cells was synchronized in G1 by growing the cells in culture to full confluence for contact inhibition as described previously (Selokar et al., 2012). All the procedures of HMC (i.e., IVM, cumulus/zona removal, manual enucleation, fusion, activation and IVC) were performed as described previously (Selokar et al., 2014).

## 2.2. Assessment of extent of apoptosis

The total cell number (TCN) and the extent of apoptosis were determined using TUNEL staining with the *in situ* Cell Death Detection Kit, Fluorescein (11684795910, Roche, Germany). Briefly, the blastocysts were fixed with 4% paraformaldehyde for 1 h, permeabilized by incubating with 0.5% Triton X-100 for 1 h and then incubated with FITC-conjugated dUTP and terminal deoxynucleotidyl transferase (TdT) enzyme for 1 h at 37 °C in a darkened area. The treated blastocysts were incubated with RNase (50 µg/mL) for 20 min at 37 °C and then stained with Hoechst 33342 (5 µg/mL) for 5 min at 37 °C in a darkened area. For the negative control, the assay was conducted without addition of TdT enzyme. For the positive controls, there was treatment with DNase solution (100 U/mL) for 20 min at 37 °C before incubating the blastocysts with FITC-conjugated dUTP and TdT for 1 h. The stained blastocysts were washed with DPBS (Ca<sup>2+</sup> and Mg<sup>2+</sup>-free), mounted on glass slides in 3 µL droplets of antifade solution and flattened with a cover slip. Cell counting was performed from the digital images obtained on inverted Nikon fluorescence microscope. Each experiment was repeated at least three times. The apoptotic index was calculated using the equation: Apoptotic index = (number of TUNEL positive nuclei/total number of nuclei in the blastocyst) × 100.

## 2.3. Production of IVF embryos

Cumulus-oocyte complexes collected from buffalo ovaries after slaughter in an abattoir were subjected to IVM and IVF using procedures that have been previously described (Sharma et al., 2011). For IVC, the presumed zygotes were washed several times with Research Vitro Cleave medium (K-RVCL-50, Cook®, Queensland, Australia) supplemented with 1% fatty acid-free BSA and were cultured in this medium for as long as 8 days post insemination in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> in air) at 38.5 °C.

## 2.4. Immunofluorescence staining for epigenetic markers

The global content of H3K9ac and H3K27me3 in the blastocysts was determined using immunofluorescence staining. Briefly, the blastocysts were fixed with 4% paraformaldehyde, washed three times with DPBS + 0.3% PVA (DPBS-P), permeabilized with 0.5% Triton X-100 and then blocked with 1.5% BSA. The cells were then incubated overnight at 4 °C with the respective primary antibody (anti-H3K9ac, 1:1000, Catalog No. H9286, Sigma and anti-H3K27me3, 1:1500, Catalog No. ABE44, Millipore) diluted in 1.5% BSA. After washing five times with DPBS + 0.3% PVA + 0.1% Triton X-100 solution (DPBS-PT), the blastocysts were incubated for 90 min with FITC-conjugated goat anti-rabbit secondary antibody (Sigma) diluted 1:1500 in DPBS-P and Alexa Fluor 594-conjugated donkey anti-mouse IgG (H + L) secondary antibody (A21203, Invitrogen, Carlsbad, CA, USA) for CDX2, diluted 1:1000 in DPBS. After washing five times with DPBS-PT, the nuclei were counterstained with Hoechst 33342 (10 µg/mL) and rinsed with DPBS-PT. The stained embryos were mounted on slides in mounting medium (glycerol with 2.5% DABCO, Sigma), and were observed using a Nikon fluorescence microscope. The NIS-element basic research image processing software (Nikon, Tokyo, Japan) equipped with the microscope was used for image acquisition and quantitative measurements of the mean pixel intensity emitted by each individual nucleus. At least 15 blastocysts (50 nuclei from each image) were analysed for each epigenetic marker.

## 2.5. Relative abundance of mRNA transcript analysis

Quantitative real-time PCR (qPCR) was performed as described earlier (Selokar et al., 2014) with some modifications. Briefly, RNA was isolated from blastocysts using the RNAqueous micro kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The genomic DNA contamination was removed by DNase treatment at 37 °C for 20 min. The RT reaction was conducted using the M-MLV RT provided in the superscript reverse transcriptase III kit (Invitrogen). The PCR primers were designed based on published nucleotide sequences available or were based on sequence homology analysis (using BioEdit software) between mouse, human, pig and cattle sequences obtained from NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and ENSEMBL genome browser for the respective genes. Primers were designed and verified using the web-based software Perl-primer designer. qPCR was performed using the optimized primer sets included in Supplementary Table 1 using a CFX96 real-time system CFX 96 I Cyclor (Bio-Rad, Hercules, CA, USA) with maxima@SYBR Green master mix (Fermentas, St. Leon-Rot, Germany) at the following thermal cycling conditions: 95 °C for 5 min, followed by 40 PCR cycles of 95 °C for 15 s, 58 °C or 60 °C for 30 s, and 72 °C for 30 s. Melting peaks were determined using melting curve analysis to ensure the specific amplification. Relative abundances of mRNA transcripts were determined using 2<sup>-DDCt</sup> method, where DCT = Ct (target gene) - Ct (internal reference), and DDCt = DCT sample - DCTcalibrator. GAPDH and β-actin were used as internal control genes for normalization of data. For comparison, the average relative abundance of mRNA transcript for each gene from IVF-derived blastocysts was set as 1. Three separate experiments were performed with three replicates for each gene.

## 2.6. Embryo transfer and detection of pregnancy

Oestrous cycling buffalo with a functional corpus luteum were treated with PGF<sub>2α</sub> analogue (Cloprostenol sodium, 500 mg) intramuscularly. Those expressing oestrus 72 h after the treatment were selected as recipients. Blastocysts at the Day 7/8 developmental stage, produced by using one or two demicytoplasts, were transferred into recipients with two blastocysts being transferred into each recipient. Pregnancies were examined using trans-rectal palpation procedures at about day 50 subsequent to the day of oestrous expression.

## 2.7. Experimental design and statistical analyses

Following IVM and enucleation of oocytes, the demicytoplasts were divided into two groups. In Group 1 (control), two demicytoplasts were fused with one somatic cell for producing reconstructed embryos whereas, in Group 2, one demicytoplast was fused with one somatic cell. For examining the effects of cytoplasmic volume on the developmental competence, there was comparison of the percentage of embryos in which cleavage and blastocyst development occurred of the two groups recorded on Day 8 of IVC. For examining the effect on the quality of cloned embryos, there was comparison of the values for three variables (*i.e.*, TCN, inner cell mass (ICM):trophectoderm (TE) cell number ratio and apoptotic index) of blastocysts of the two groups. The IVF-derived blastocysts were used as controls for comparing the ICM:TE cell number ratio. The TCN was determined by staining the blastocysts with Hoechst 33342 whereas, the number of TE cells was determined by staining the cells for detection of the CDX2 protein. The ICM cell number was calculated by subtracting the number of TE cells from the TCN. The extent of apoptosis was determined using the TUNEL assay. The *in vivo* developmental competence was examined following transfer of cloned embryos of the two groups to recipients. The global content of H3K9ac and H3K27me3 was compared between the blastocysts of the two groups using IVF-derived embryos as controls. Furthermore, there was comparison of the relative abundance of mRNA transcript of some important pluripotency- (*OCT4*, *SOX2* and *NANOG*), apoptosis- (*BCL-2*, *BAX* and *CASPASE3*), epigenetic- (*DNMT1*, *DNMT3a* and *HDAC1*), trophectoderm- (*CDX-2*), development- (*GLUT1* and *G6PD*) and cell cycle check point-related related genes (*P53*) among cloned blastocysts of the two groups, using IVF-derived embryos as controls.

Statistical analysis was performed using the GraphPad Prism 5 software for conducting the student's 't' test to compare the means of different groups. The datasets were analysed using a one-way analysis of variance (ANOVA) followed by use of the Tukey test. The percentage values were analysed after arcsine transformation prior to analysis. The differences were considered significant at  $P < 0.05$ . Data are presented as mean  $\pm$  SEM.

## 3. Results

### 3.1. Effect of cytoplasmic volume on *in vitro* developmental competence and quality

The cleavage and blastocyst rate was less ( $P < 0.001$ ) following use of one demicytoplast than when two demicytoplasts were used for HMC (Table 1). The TCN of blastocysts was less ( $P < 0.01$ ) when one demicytoplast was used than when two demicytoplasts were used for HMC (Table 2). The ICM:TE cell number ratio did not differ between the two groups of cloned embryos and was less ( $P < 0.05$ ) than in IVF-derived blastocysts. The apoptotic index was less ( $P < 0.05$ ) for the blastocysts produced using one demicytoplast than those produced using two demicytoplasts (Table 2).

### 3.2. Effect of cytoplasmic volume on *in vivo* developmental competence

Following transfer of blastocysts ( $n = 8$ ) produced using one demicytoplast, one recipient became pregnant and delivered a live calf which died after 12 days. After transfer of blastocysts produced using two demicytoplasts ( $n = 14$ ), one recipient conceived and delivered a live healthy calf (Fig. 1). The parentage of the cloned calf was confirmed by DNA microsatellite analysis (Supplementary Table 2).

**Table 1**  
Effect of cytoplasmic volume on the *in vitro* developmental competence of embryos produced by Hand-made cloning.

Number of demicytoplasts used for HMC	Reconstructs (n)	Cleavage rate n (%)	Blastocyst rate n (%)
One	516	457 (88.7 $\pm$ 1.37) <sup>a</sup>	63 (12.7 $\pm$ 1.98) <sup>a</sup>
Two	213	210 (98.8 $\pm$ 0.61) <sup>b</sup>	104 (47.6 $\pm$ 3.49) <sup>b</sup>

Data from 8 trials.

Values are Mean  $\pm$  SEM.

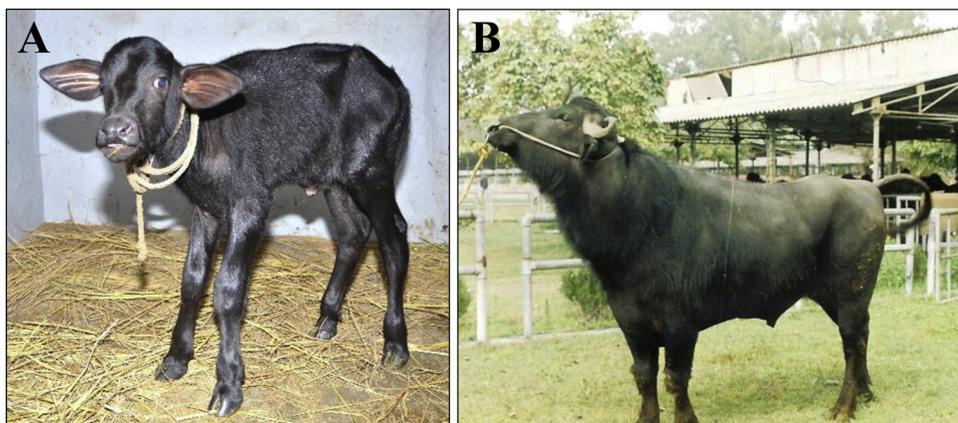
Values with different superscripts within the same column differ significantly ( $P < 0.001$ ).

**Table 2**

Effect of cytoplasmic volume on inner cell mass/trophectoderm cell number ratio and apoptotic index of embryos produced by Hand-made cloning.

Embryo Type	Number of demicytoplasts used	Blastocysts examined (n)	Total cell number	ICM:TE cell number ratio	Blastocysts examined (n)	Apoptotic index
IVF	–	20	163.9 ± 17.01 <sup>a</sup>	0.27 ± 0.02 <sup>c</sup>	–	–
Cloned	One	11	167.6 ± 14.66 <sup>a</sup>	0.17 ± 0.01 <sup>d</sup>	13	2.11 ± 0.38 <sup>c</sup>
	Two	12	335.9 ± 58.96 <sup>b</sup>	0.19 ± 0.02 <sup>d</sup>	18	3.43 ± 0.38 <sup>d</sup>

Values are Mean ± SEM.

Values with different superscripts within the same column differ significantly. ab: ( $P < 0.01$ ), cd: ( $P < 0.05$ ).

**Fig. 1.** (A) One live healthy cloned calf was produced as a result of transfer of blastocysts produced using two demicytoplasts ( $n = 14$ ) to recipients; (B) the progeny-tested bull (MU4393), semen-derived somatic cells of which were used as donor cells.

### 3.3. Effect of cytoplasmic volume on epigenetic status

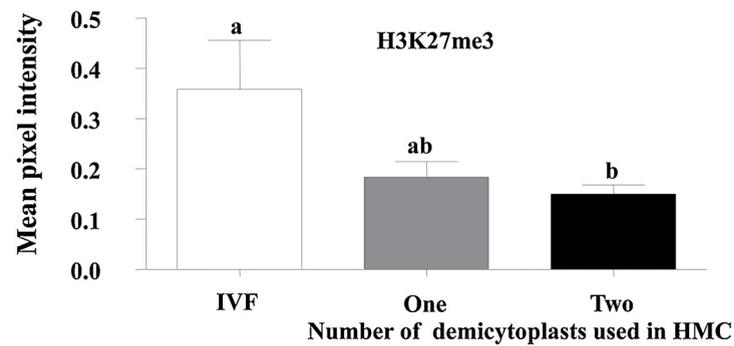
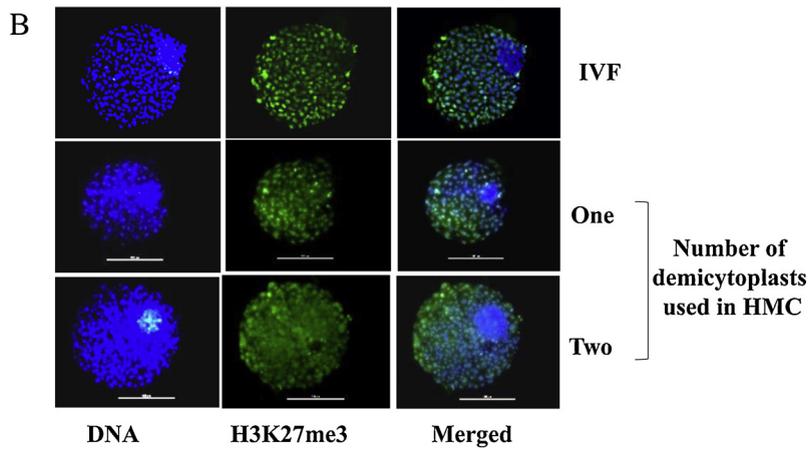
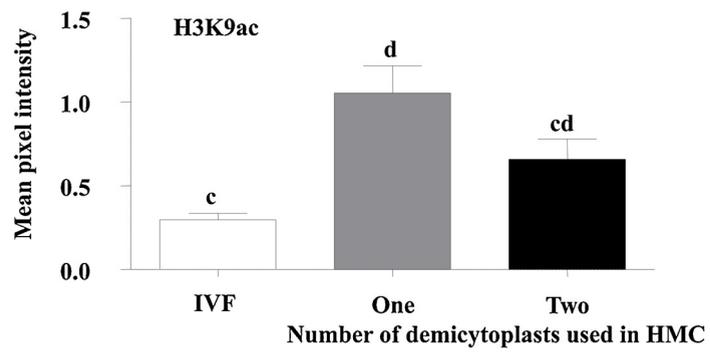
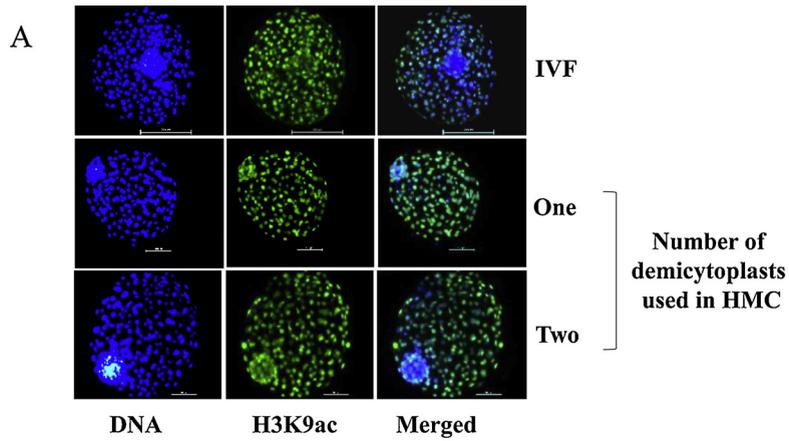
The global content of H3K9ac was greater ( $P < 0.01$ ) in blastocysts produced using one demicytoplast than in IVF blastocysts whereas, the content in blastocysts produced using two demicytoplasts, was not different from the two groups (Fig. 2A). The global content of H3K27me3 was greater ( $P < 0.05$ ) in IVF-derived blastocysts than in blastocysts produced using two demicytoplasts whereas, the content in blastocysts produced using one demicytoplast, was not different from the two groups (Fig. 2B).

### 3.4. Effect of cytoplasmic volume on relative abundance of mRNA transcripts

Among pluripotency-related genes, the relative abundance of *OCT4* mRNA transcript was greater ( $P < 0.05$ ) in IVF blastocysts than in cloned blastocysts produced using one or two demicytoplasts (Fig. 3). The relative abundance of *SOX2* mRNA transcript, which was similar in IVF blastocysts and cloned blastocysts produced using one demicytoplast, was greater ( $P < 0.05$ ) than in blastocysts produced using two demicytoplasts. The relative abundance of *NANOG* mRNA transcript, which was similar in IVF blastocysts and cloned blastocysts produced using two demicytoplasts, was less ( $P < 0.05$ ) than in blastocysts produced using one demicytoplast. For trophectoderm-related gene, *CDX2*, the relative abundance of mRNA transcript was greater ( $P < 0.05$ ) in blastocysts produced using two demicytoplasts than in those produced using one demicytoplast whereas, the relative abundance was similar to that of the two groups in IVF-derived blastocysts.

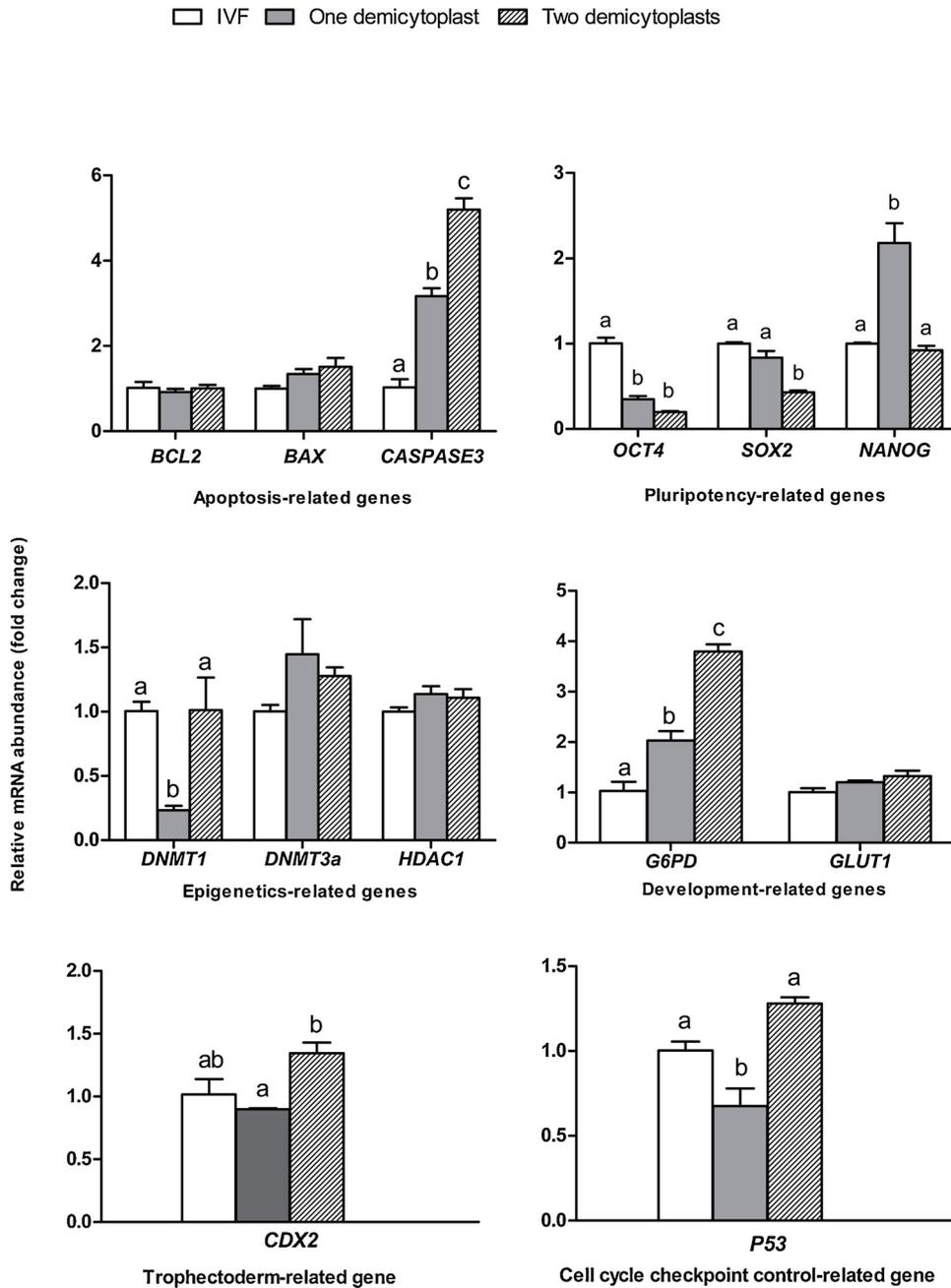
Among epigenetic-related genes, the relative abundance of *DNMT1* mRNA transcript, was similar in IVF and cloned blastocysts produced using two demicytoplasts, and was greater ( $P < 0.05$ ) than in blastocysts produced using one demicytoplast whereas, that of *DNMT3a* and *HDAC1*, was not different among the three groups. Among apoptosis-related genes, the relative abundance of *CASPASE3* mRNA transcript was greater ( $P < 0.05$ ) in blastocysts produced using two demicytoplasts than in those produced using one demicytoplast which, in turn, was greater ( $P < 0.05$ ) than in IVF-derived blastocysts. The relative abundance of *BCL-2* and *BAX* mRNA transcripts was similar in the three groups.

Among development-related genes, the relative abundance of *G6PD* mRNA transcript was greater ( $P < 0.05$ ) in blastocysts produced using two demicytoplasts than in those produced using one demicytoplast which, in turn, was greater ( $P < 0.05$ ) than in IVF-derived blastocysts whereas, that of *GLUT1*, was similar in the three groups. The relative abundance of cell cycle check point control-related gene, *P53*, mRNA transcript, was similar in IVF blastocysts and cloned blastocysts produced using two demicytoplasts, and was greater ( $P < 0.05$ ) than in blastocysts produced using one demicytoplast.



(caption on next page)

**Fig. 2.** Effect of cytoplasmic volume on the global content of (A) H3K9ac and (B) H3K27me3, examined using immunofluorescence staining in blastocyst-stage embryos produced by HMC or IVF; At least 15 blastocysts of each group (50 nuclei from each image) were analysed for each epigenetic marker; First column: nuclei of blastomeres labeled with Hoechst 33342 stain; Second column: blastomeres stained with anti-H3K9ac or anti-H3K27me3 antibody and then with FITC-labeled secondary antibody; Differences in the global content of H3K9ac and H3K27me3 indicated there were epigenetic differences among cloned blastocysts produced using one or two demicytoplasts and IVF-derived embryos; Bars with different superscripts differ (a,b:  $P < 0.05$ ; c,d:  $P < 0.01$ ).



**Fig. 3.** Effect of cytoplasmic volume on the relative abundance of mRNA transcripts for some important genes examined using real-time qPCR in blastocyst-stage embryos produced by HMC or IVF; Values are Mean  $\pm$  SEM; Bars with different letters differ ( $P < 0.05$ ); Relative abundance of mRNA transcript in IVF-derived embryos was considered to be 1.

#### 4. Discussion

In this study, there was examination of the effects of cytoplasm volume on SCNT-derived embryos produced using HMC. The use of one demicytoplast decreased the percentage of embryos developing to the blastocyst stage by approximately 75% of that obtained with use of two demicytoplasts (12.7% compared with 47.6%). These results are consistent with those from a previous study in which there was use of one, instead of two demicytoplasts for HMC, with the decrease in the percentage development to the blastocyst stage being 38% to 6.5% (Panda et al., 2011). Removal of large (30%–40%), medium (15–25%) and small (< 10%) amounts of cytoplasmic volume has been reported to result in a significant difference in development of embryos to the blastocyst stage (6.4%, 12.4% and 24.4%, respectively) of SCNT-derived cattle embryos (Hua et al., 2011). Similar results were reported when blastomeres were used as nuclear donors. The percentage of embryos developing to the blastocyst stage of nuclear transferred embryos decreased when the cytoplasmic volume of the recipient oocyte was sufficiently reduced (Zakharchenko et al., 1997) whereas, there was an increase, when embryos were reconstructed using double cytoplasts than with single cytoplasts, in which 100% and 50% of the original oocyte volume was used, respectively (Peura et al., 1998).

Using micromanipulation-based SCNT, Sayaka et al. (2008), generated ‘artificial giant’ (AG) mouse oocytes by the fusion of two enucleated oocytes and examined the reprogramming potential following microinjection of cumulus or embryonic stem (ES) cell nuclei into these cells. Compared to control oocytes in which each cumulus or ES somatic cell was microinjected into one enucleated oocyte, the development to the morula/blastocyst stage with use of AG oocytes was less for both cumulus (3% compared with 50%) and ES cells (0% compared with 11%). This decrease in developmental competence was attributed to impairment of the internal cell contents at the time of the first cell division of cloned embryos derived from AG cells even though these embryos had twice the amount of nuclear reprogramming factor(s). These findings indicate that it would be simplistic to attribute the decreased *in vitro* developmental competence following use of one demicytoplast, observed in the present study, solely to a lesser amount of nuclear reprogramming factor(s) due to loss of cytoplasmic volume during enucleation.

The quality of cloned embryos is one of the most important factors affecting the live birth rate. There was examination in the present study of the effect of using one demicytoplast, instead of two, on the quality of blastocyst-stage cloned embryos using three criteria (*i.e.*, TCN, level of apoptosis and ICM:TE cell number ratio). The TCN is considered to be an important criterion for evaluating embryo quality (Ushijima et al., 2008) because it is believed to affect the embryonic development after implantation in mammals (Yu et al., 2007). In the present study, use of one demicytoplast, instead of two, compromised the quality of blastocysts as indicated by a reduction in TCN as also observed in a previous study (Panda et al., 2011). Removal of cytoplasmic volume in a graded manner during micromanipulation-based SCNT of cattle embryos also decreased the TCN (Hua et al., 2011). Westhusin et al. (1996) reported that the cell numbers in the blastocysts were less when 50% of the cytoplasm was removed compared with 5% when blastomeres were used as nuclear donors. The extent of apoptosis in embryos is another important factor which affects the quality because it is believed to be related with a decrease in the TCN (Yu et al., 2007) and lesser percentage live birth rate with cloned embryos (Fahrudin et al., 2002). The extent of apoptosis is greater in cloned than in IVF-derived embryos (Cui et al., 2011; Selokar et al., 2013). In the present study, use of one, instead of two demicytoplasts, decreased the apoptotic index in cloned blastocysts. This is consistent with the lesser expression of the pro-apoptotic gene *CASPASE* observed with use of one as compared with two demicytoplasts although the extent of expression of two other apoptosis-related genes, *BCL-2* and *BAX*, was not affected by the reduction in cytoplasmic volume.

The blastocyst-stage embryos possess two distinct types of cell populations. The ICM, which forms the embryo, and the TE, which form the extra-embryonic tissues of the placenta. Presence of both cell populations in an appropriate ratio is necessary for normal development of embryos. A disrupted ratio of ICM:TE cell numbers in cloned embryos is believed to result in greater incidences of placental abnormalities and early embryonic mortality (Im et al., 2006). An abnormally large number of ICM cells and lesser number of TE cells in cloned embryos result in insufficient placental development (Koo et al., 2002). The ICM:TE cell number ratio, therefore, is considered to be an important criterion for evaluating blastocyst quality (Van Soom et al., 1996; Yu et al., 2007). In the present study, the ICM:TE cell number ratio was less in cloned blastocysts of both the groups than in IVF-derived blastocysts and that use of one demicytoplast could not restore ICM:TE values to that of IVF-derived blastocysts. Removal of cytoplasmic volume in a graded manner by micromanipulation decreased the ICM:TE ratio of SCNT-derived cattle blastocysts (Hua et al., 2011). Collectively, these results indicate the use of one demicytoplast instead of two, compromises embryo quality due to a significant decrease in TCN, and does not offer any advantage in terms of correcting the ICM:TE cell number ratio. There, however, is a decrease in the extent of apoptosis with use of one demicytoplast. Live birth is the ultimate desired outcome from transfer of cloned embryos. In the present study, there was transfer of a limited number of blastocysts to recipients for comparing the percentage birth rate of live calves when there was transfer of cloned embryos from the two groups. Following transfer of eight blastocysts, produced using one demicytoplast, one live calf was born, which died after 12 days. Transfer of 14 blastocysts, produced using two demicytoplasts, resulted in birth of a live healthy calf. The live birth rate that results from transfer of cloned embryos is very low. Only 0.9% to 6% of cloned embryos that are transferred develop into live offspring for all the mammalian species (Loi et al., 2011). The live birth rate that occurred as a result of the present study is similar to that of 9% in cattle (Panarace et al., 2007).

Covalent modifications of histones, especially acetylation and methylation of specific lysine residues, have an important function in regulation of gene expression, and in general, acetylation is associated with gene activation and methylation with inhibition of gene expression (Valls et al., 2005). Among many epigenetic markers associated with reprogramming of the somatic genome, there was focus on two important ones in the present study. The abundance of the H3K9ac marker is inversely correlated with the extent of DNA methylation (Kondo et al., 2004), and has been reported to be associated with gene expression and zygote genomic activation in mice (Stein et al., 1997). The H3K27me3 protein is an important epigenetic marker associated with gene repression. Demethylation

of H3K27me3 before and after embryonic genome activation is essential for normal embryonic development of IVF-derived cattle embryos (Canovas et al., 2012). Both acetylation and trimethylation of the H3K27 gene vary between SCNT-derived and embryos for which there has been no embryo manipulations (Breton et al., 2010; Zhou et al., 2014). In the present study, the use of one, instead of two demicytoplasts did not affect the global content of both H3K9ac and H3K27me3 in blastocysts.

The effect was assessed in the present study of cytoplasmic volume on relative abundance of mRNA transcripts for some important developmentally-regulated genes in cloned blastocysts using the IVF-derived embryos as controls. Normal embryonic development requires proper expression of pluripotency-associated genes such as *OCT4*, *NANOG* and *SOX2*, which are expressed in the ICM, and *CDX2*, which is expressed in the TE. Use of one demicytoplast instead of two, increased the extent of expression of the *SOX2* and *NANOG* genes, and decreased that of *CDX2* whereas, expression of the *OCT4* gene, was not affected. A group of enzymes termed the DNA methyltransferases (DNMTs) are responsible for methylation of DNA. The DNMT1 is responsible for maintenance of DNA methylation whereas, DNMT3a and DNMT3b function in establishment of *de novo* methylation during gametogenesis and early embryonic development (Bestor, 2000). In the present study, use of one demicytoplast decreased the expression of the *DNMT1* gene but not the *DNMT3a* gene as compared to when two demicytoplasts were used. Histone acetyl transferases, in conjunction with opposing histone deacetylases (HDACs) are responsible for maintaining the acetylation status of chromatin domains. In the present study, the expression of the *HDAC1* gene was not affected by the use of one, instead of two demicytoplasts. Use of one demicytoplast also decreased the expression of the development-related gene, *G6PD*, and cell cycle check point control-related gene, *P53*, but not that of the *GLUT1* gene compared to when two demicytoplasts were used.

## 5. Conclusion

Use of one demicytoplast for HMC decreases the percentage of embryos that develop to the blastocyst stage to nearly one-fourth of that obtained following use of two demicytoplasts. Although it decreases the extent of apoptosis, it compromises embryo quality due to a lesser TCN. Use of one demicytoplast instead of two does not provide any advantage in terms of correcting the ICM:TE cell number ratio. The use of this approach, however, alters the expression of several important genes compared to when two demicytoplasts are used.

## Declaration of Competing Interest

The authors do not have any conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.anireprosci.2019.106136>.

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