FULL PAPER

Theriogenology

Cytoplasmic volume of recipient oocytes affects the nucleus reprogramming and the developmental competence of HMC buffalo (*Bubalus bubalis*) embryos

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ABSTRACT. The present study was undertaken to examine the effects of cytoplasmic volume on nucleus reprogramming and developmental competence of buffalo handmade cloning (HMC) embryos. We found that both HMC embryos derived from ~150% cytoplasm or ~225% cytoplasm resulted in a higher blastocyst rate and total cell number of blastocyst in comparison with those from ~75% cytoplasm (25.4 ± 2.0, 27.9 ± 1.6% vs. 17.9 ± 3.1%; 150 ± 10, 169 ± 12 vs. 85 ± 6, $P<0.05$). Meanwhile, the proportions of nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) were also increased in the embryos derived from ~150 or ~225% enucleated cytoplasm compared to those from ~75% cytoplasm. Moreover, HMC embryos derived from ~225% cytoplasm showed a decrease of global DNA methylation from the 2-cell to the 4-cell stage in comparison with those of ~75% cytoplasm ($P<0.05$). Furthermore, the expression of embryonic genome activation (EGA) relative genes (*eIF1A* and *U2AF*) in HMC embryos derived from ~225% cytoplasm at the 8-cell stages was also found to be enhanced compared with that of the ~75% cytoplasm. Two of seven recipients were confirmed to be pregnant following transfer of blastocysts derived from ~225% cytoplasm, and one healthy cloned calf was delivered at the end of the gestation period, whereas no recipients were pregnant after the transfer of blastocysts derived from ~75% cytoplasm. These results indicate that the cytoplasmic volume of recipient oocytes affects donor nucleus reprogramming, and then further accounted for the developmental ability of the reconstructed embryos.

KEY WORDS: buffalo, cytoplasmic volume, embryo developmental competence, handmade cloning

Somatic cell nuclear transfer (SCNT) is the most efficient and viable technique to propagate highly valued endangered and extinct domestic animals [15]. Following the birth of first cloned sheep, "Dolly", numerous endangered species and elite domestic animals were generated by SCNT via micromanipulation-based enucleation and nuclear transfer. More than 99% of embryos or offspring dealing with SCNT that have been reported were produced via a micromanipulation-based approach [37]. However, the complicated micromanipulation procedure and expensive micromanipulators hamper the advancement of SCNT in domestic animals. Thus, one of the major necessities in traditional cloning was to reduce the costs without compromising with the efficiency [39]. Handmade cloning (HMC) is an advanced procedure of enucleation of zona-free mammalian oocytes by hand bisection that is based on SCNT and was first reported by Vajta *et al*. in 2001 [38]. The requirement of expensive micromanipulators and skilled expertise was eliminated in the HMC technique, proving that it was a major revolution in the field of embryology [42]. With the improvement in the enucleation of zona-free oocytes [17, 32, 33] and the culture system [1, 36, 40], HMC, as a more efficient and economical technique in comparison to the micromanipulator-based approach, was successfully used to produce cloned offspring in several livestock species such as cattle [22], buffalo [11], sheep [50], pigs [9] and horses [14]. Although HMC has achieved certain success and offers a new route for SCNT, the problems that restrict the success of traditional SCNT, including incomplete nucleus reprogramming, chromosome remodeling failure, embryonic genomic activation delay, and lower *in vivo* developmental competence, still need to be resolved.

In normally fertilized embryos, the epigenetic modification pattern of sperm and oocyte nucleus can be reprogrammed to a totipotent state by oocyte cytoplasm. In order to have successful reprogramming in SCNT, the donor cells should be completely erased to switch off the expression of tissue-specific gene and reprogrammed to switch on gene expression in embryos [21]. The quality and quantity of reprogramming factors in the oocyte cytoplasm are considered to be the deciding factors of the overall reprogramming efficiency in SCNT [23]. Previous reports showed that the blastocyst development rate and the total number of blastomeres decreased remarkably in micromanipulation-based cloned embryos when the cytoplasmic volume of the recipient oocyte was sufficiently reduced [12, 49]. Compared to the process of *in vitro* fertilization (IVF), both micromanipulator-assisted and HMC enucleation resulted decrease in cytoplasm volume, which is considered to contain reprogramming factors. While micromanipulator-assisted enucleation results in the removal of 5–50% of the cytoplasm [44], HMC also results in almost a 15–50% loss of cytoplasmic volume [23]. Therefore, increasing the cytoplasmic volume should be an effective way to improve the efficiency of HMC embryo development, and accumulated data have proven that it is feasible. It had been reported that increasing cytoplasmic volume either by fusion or aggregation, had a positive effect on the *in vitro* development of HMC embryos and the establishment of pregnancies [23, 26]. However, there is little information available that is related to the mechanism of how cytoplasmic volume can affect the developmental capacity of HMC embryos. Therefore, further investigation is required to explore the molecular mechanism of recipient oocyte cytoplasm and its association with nucleus reprogramming and embryonic development.

Buffalo (*Bubalus bubalis*) is an important domestic animal that inhabits the tropical and subtropical region, and is characterized with a high content of fat and protein in milk. However, the milk yield of Chinese swamp buffalos is extremely low (normally less than 1,000 kg/year) so it is in urgent need of improvement. The cloning of buffalos through nuclear transfer is a potential alternative approach in the genetic improvement of buffalos [29]. Nowadays, HMC is a simple and inexpensive technique that is preferred over micromanipulation-based SCNT [35]. Previous studies have proven that the *in vitro* developmental potential of HMC embryos is equal to those produced through traditional SCNT. However, very few HMC buffalos have been reported [11, 23, 27], so the *in vivo* developmental potential of HMC embryos still needs to be evaluated.

Precise nucleus reprogramming of somatic cells is a prerequisite for the success of somatic cell nuclear transfer [23]. The quantity and quality of the cytoplasm play an important role in the process of nuclear reprogramming. It has been reported that the development of bovine [31], porcine [5], and ovine [25] SCNT embryos can be improved by treating donor cells with oocytes extracts. This evidence suggested that reprogramming factors in cytoplasm determine the overall reprogramming efficiency. Recently, accumulated data proved that HMC embryo development can be enhanced by increasing the cytoplasmic volume of reconstructed embryos [4, 23, 26]. The developmental potential of cloned embryos has been related to nucleus remodeling, epigenetic reprogramming, and embryonic genome activation. Up to now, the effects of cytoplasmic volume on the *in vivo* development of HMC embryos, important events involving nucleus remodeling, DNA methylation, and embryonic genome activation (EGA) of HMC embryos were still unclear.

The present study was undertaken to investigate the molecular mechanism of recipient oocyte cytoplasm and its association with the nucleus reprogramming and success of embryo development. The *in vitro* developmental competence of handmade cloned buffalo embryos; the molecular mechanism including the remodeling pattern of donor nucleus in reconstructed embryo; global DNA methylation of HMC embryos from the 2-cell stage to blastocyst; and the status of EGA; were examined. Finally, the *in vivo* developmental competence of the HMC embryos derived from ~75% cytoplasm and ~225% cytoplasm were evaluated.

MATERIALS AND METHODS

Ethics statements

This study was carried out in accordance with the guidelines for the care and use of animals of Guangxi University. All of the experiments and protocols were performed in strict accordance with the Guide for Care and Use of Laboratory Animals and explicitly approved by the Guangxi University Committee on Animal Research and Bioethics.

Reagents and media

All of the chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), with the exception of TCM 199 powder, which was purchased from Gibco BRL (Paisley, Scotland, U.K.), and fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Media (DMEM), which were bought from Invitrogen Co. (Carlsbad, CA, U.S.A.). The preparation of media used in this study, including *in vitro* maturation (IVM) medium and embryo culture medium (CM) was described by Shi *et al.* [29].

In vitro maturation of oocytes

In vitro maturation of oocytes was carried out as described previously [29]. Chinese swamp buffalo ovaries were obtained from a local abattoir. Ovaries were excised within 20 to 30 min after slaughter and were transported to the laboratory within 4 hr in a thermos containing PBS at 35 to 37°C. Buffalo cumulus-oocyte complexes (COCs) were recovered via the aspiration of follicles in diameter of 2–6 mm using a 10 m*l* disposable syringe with an 18-gauge needle. COCs with multi-layers of cumulus cells were selected for IVM. Then, COCs were washed twice in the IVM medium (TCM-199 supplemented with 26.2 mM NaHCO$_3$, 5 mM HEPES, 5% OCS and 0.1 μg/m*l* FSH) and cultured in a 30 mm glass dish containing 1.5 μl IVM medium for 22 hr under a humidified atmosphere of 5% CO$_2$ in air at 38.5°C.
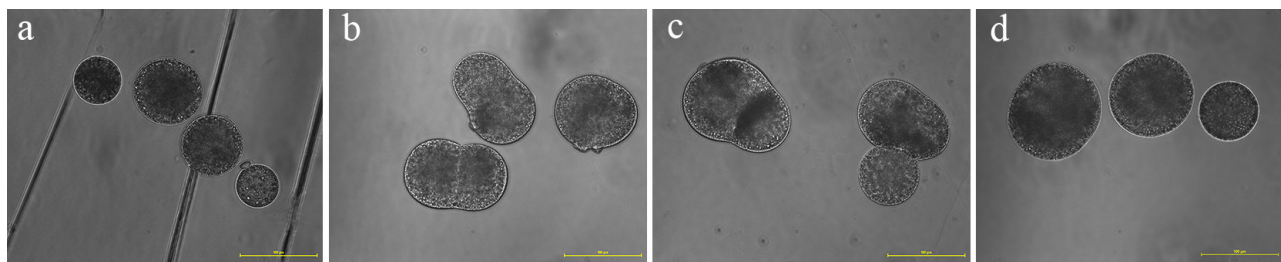
Fig. 1. Reconstruction of embryos with different cytoplasm. (a): Cytoplasm with different volume selected after bisection, (b) ~150% cytoplasm fusion processes, (c) ~225% cytoplasm fusion processes, and (d) embryos with different cytoplasm after fusion. Bar=100 μm

Production of handmade cloning embryos

HMC was performed as described previously [23] with some modifications. In brief, oocytes with an extruded first polar body were selected for enucleation. Denuded oocytes were stripped of their zona pellucida using 2 mg/ml pronase. Then oocytes with completely digested zona pellucida were transferred to TCM199 (TCM199 medium containing 20% FBS) and incubated at 38.5°C until a prominent protrusion cone was easily visible. Protrusion cone guided bisection was performed under a stereo zoom microscope (Nikon, Tokyo, Japan) using an ultra-sharp splitting blade (ESE020, Total Reproduction Pty. Ltd., Camperdown, Australia) in 50 μl TCM199 with 5 μg/ml cytochalasin-B. The larger enucleated cytoplasm (~75% of the original oocyte, Fig. 1a) without a protrusion cone were transferred to TCM199 and incubated for 30 min to regain the spherical shape, and then immersed in Phytohemagglutinin (0.5 mg/ml) for 5–10 sec and transferred to PVA (TCM199 with 1% polyvinyl alcohol) containing donor cells. Each enucleated cytoplasm in the PVA containing donor cells was then conjoined with a single, rounded fetal fibroblast, followed by conjoining one or two enucleated cytoplasm to the couplets. Then, the couplet was transferred to a droplet of 100 μl fusion medium (0.28 M mannitol, 0.1 mM CaCl$_2$, 0.1 mM MgSO$_4$, 5 mM Hepes and 0.1% BSA) overlaid with mineral oil, and then placed on the micromanipulator with two platinum needle electrodes (0.2 mm apart). The fusion was induced with two direct current pulses of 1 kv/cm for 10 μs using an ECM2001 Electrocell Manipulator (BTX Inc., San Diego, CA, U.S.A.). For the construction of embryos with decreased or increased cytoplasmic volume, either only one enucleated cytoplasm (~75% cytoplasm) or couplets with two (~150% cytoplasm, Fig. 1b) or three (~225% cytoplasm, Fig. 1c) enucleated cytoplasm were allowed to fuse, creating a different cytoplasmic volume (Fig. 1d). Then, the couplets were incubated in the TCM199 for 30 min at 38.5°C. The reconstructed embryos were activated previously [23]. Briefly, the reconstructed embryos were induced by exposure to 5 μM Ionomycin in CM for 5 min and subsequent incubation in 2 mM 6-dimethylamino-purine for 3 hr at 38.5°C and 5% CO$_2$ in air.

In vitro fertilization

In vitro fertilization was carried out as described previously [30]. Briefly, the frozen semen straw (0.25 ml/straw) was thawed in a 37°C water bath. The thawed semen was layered under fertilization medium (Tyrode's medium supplemented with 10 mM caffeine, 20 μg/ml heparin, and 20 mg/ml BSA) in a conical tube for the swim-up procedure. After incubation for 30 min at 38.5°C, the top of the medium containing the more motile sperm was collected and centrifuged to harvest the sperm. Then, the spermatozoa pellet was resuspended in fertilization medium at a concentration of 5.0×10^6 sperm/ml for fertilization. IVM oocytes were washed twice in fertilization medium and transferred into a 30 μl droplet of fertilization medium under sterile mineral oil (20 oocytes/drop). Then, 5 μl of semen was added to the droplet containing oocytes and incubated for 24 hr at 38.5°C under a humidified 5% CO$_2$ atmosphere.

In vitro culture of embryos

The *in vitro* culture of IVF embryos was also performed as previously described [30]. Briefly, embryos derived from IVF were placed into co-culture with granulosa cell monolayers in a 30 μl droplet of CM overlaid with mineral oil under a humidified atmosphere of 5% CO$_2$ in air at 38.5°C. Granulosa cell monolayers were established 48–72 hr before the introduction of embryos. After the introduction of the embryos, half of the medium was replaced with fresh medium every 24 hr. The cleavage of the reconstructed embryos was checked on Day 2 (Day 0 was the day of IVF), and the number of developed blastocysts was recorded within eight days of co-culture.

Embryos derived from HMC were placed in a Well of the Well (WOW) system and cultured in 400 μl embryo CM under a humidified atmosphere of 5% CO$_2$ in air at 38.5°C. The WOWs were prepared in a 4-well dish according to the method reported previously [40].

Estrous synchronization of recipients and embryo transfer

Estrous synchronization of recipients and embryo transfer was carried out as described previously [46]. Briefly, Non-pregnant buffalo with normal uterus were synchronized with 100 μg of a GnRH analogue given at Day 0, 500 μg PGF$_2\alpha$ analogue at Day 7, and another 100 μg GnRH analogue at Day 9. Estrus was observed on Days 10 to 13, and blastocysts were transferred non-surgically into the uterine horn ipsilateral to the ovary containing a palpable corpus luteum of recipient buffalos at Day 6 of estrous cycle. The pregnancy status was determined by rectal palpation 60 days after embryo transfer.
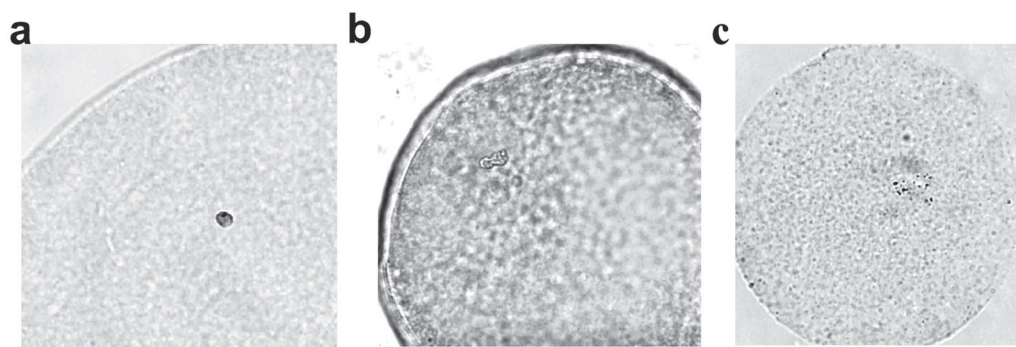
Fig. 2. The remodeling pattern of the donor nucleus in a reconstructed embryo (× 200). (a): The donor nucleus into the oocyte, (b): nuclear envelope breakdown, and (c): premature chromosome condensation.

Assessment of nucleus remodeling pattern by acetic orcein staining

The nucleus remodeling pattern of reconstructed embryos was stained and evaluated as described [2]. Briefly, reconstructed embryos derived from different cytoplasmic volumes were collected respectively at 0, 1.5 and 3 hr post-fusion and fixed in ethanol: acetic acid (3:1, v:v) for 72 hr. Then, embryos were stained with acetoorcein (1% orcein in 45% acetic acid) for 6 hr and differentiated by gently running in differentiation solution (20% glycerol [v:v] and 20% acetic acid [v:v] in distilled water). The nucleus remodeling patterns of the reconstructed embryos were evaluated using phase-contrast microscopy and characterized as nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) (Fig. 2).

Immunohistochemistry

The dynamic pattern of DNA methylation during HMC embryonic development was examined using immunohistochemistry. IVF embryos were used as the control group. Embryos at different developmental stages (2-cell, 4-cell, and blastocyst) were washed in PBS and fixed in 3.7% paraformaldehyde for 30 min at room temperature. The fixed embryos were washed three times in phosphate-buffered solution (PBS) supplemented with 0.01% Triton X-100 and 0.3% BSA (TBP), followed by permeabilization with 1% Triton X-100 for 30 min at room temperature. Thereafter, the embryos were blocked by 1% BSA for 1 hr. After washing three times with TBP, the embryos were treated with 2 M HCl for 20 min, and neutralized with Tris-HCl (PH-8.0) for 10 min before incubation with the primary antibody (5-mC, 1:300 from mouse, Abcam). All of these samples were incubated overnight at 4°C. Thereafter, the embryos were washed three times in TBP and then incubated with fluorescein isothiocyanate (FITC)-conjugated second antibody (goat anti-mouse immunoglobulin G, 1:200, Millipore) for 1.5 hr at room temperature. Samples were mounted on slides with anti-fade solution (Fluoromount-GTM, SouthernBiotech, Birmingham, AL, U.S.A.) and analyzed with a confocal laser scanning microscope (Zeiss, Heidelberg, Germany). At least 10 embryos at different development stages were checked randomly, and fluorescence intensity was measured with Image J software (NIH, Bethesda, MD, U.S.A.).

Embryo collection and reverse transcription

Five embryos at each stage were collected and treated using a Cells-to-cDNA^{TM} II Kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the method reported previously [30]. In brief, the embryos were incubated with cell lysis II, digested with DNase I (Fermentas, Hanover, MD, U.S.A.) to remove genomic DNA, and then the DNase was inactivated with EDTA. The reverse transcription reaction system consisted of SuperScript™ II Reverse Transcriptase (Invitrogen), 4 μM random primer, 10 mM dNTPs mixture, RNase inhibitor (Takara, Dalian, China), 5 × First-Strand Buffer, and dithiothreitol (DTT). The reaction mixture was incubated at 42°C for 60 min and 95°C for 10 min. Finally, sterile free H_2O was added to adjust the final volume of cDNA to 0.2 μl per embryo.

Analysis of gene expression by quantitative real-time polymerase chain reaction

cDNA samples from embryos were analyzed via an ABI 7500 Real-Time System (Applied Biosystems, Foster City, CA, U.S.A.), and primers were designed by the Oligo 6.0 software (Table 1). The housekeeping gene β-*actin* was used as the reference gene, and reaction mixture in each well included 10 μl of SYBR Premix Ex TaqTM (Takara), 0.3 μl primer (10 nM), 0.4 μl of ROX Reference Dye II (50 ×), 1 μl of cDNA and 8.3 μl of H_2O (total volume of 20 μl). The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression of the target genes. All of the experiments were performed with at least three replicates.

Statistical analysis

The experiments were repeated at least three times. The HMC embryos that underwent cleavage and developed to the blastocyst stage were analyzed by one-way ANOVA least significant difference (LSD)*post-hoc* test using the SPSS 18.0 (IBM, Armonk, NY, U.S.A.) software. The global DNA methylation and expression profiles of the target genes between the different groups were analyzed by one-way repeated-measures analysis of variance (ANOVA). χ^2 test was used to analyze the nucleus remodeling data. Probability values of <0.05 were considered to be statistically significant.

Table 1. Details of primers used for the real-time PCR analysis

Gene	Primer name	Sequences (5′-3′)	Fragment size (bp)	Accession No.
eIF1A	Forward	CTCCCAAGTGGCTGAGAAAG	163	FJ415608.1
	Reverse	TCACTCTCCTCCTCGCTCTC		
U2AF	Forward	GATGTCGAGATGCAGGAACA	155	FJ415609.1
	Reverse	TCTTCTTCACGGCGAAACTT		
β-actin	Forward	ACCGCAAATGCTTCTAGG	199	NM173979.3
	Reverse	ATCCAACCGACTGCTGTC		

Table 2. *In vitro* developmental competence of buffalo HMC embryos produced by distinct cytoplasmic volume

Cytoplasmic volume	NT embryos	Cleaved (%)	Blastocysts developed (%)	Blastocyst cell number
~75%	78	54 (69.2 ± 8.8)[b]	14 (17.9 ± 3.1)[b]	85 ± 6[b]
~150%	71	51 (71.8 ± 6.5)[b]	18 (25.4 ± 2.0)[a]	150 ± 10[a]
~225%	68	59 (86.8 ± 2.7)[a]	19 (27.9 ± 1.6)[a]	169 ± 12[a]

Data presented were from more than three replicates. Values within brackets are presented as mean ± standard error of the mean (SEM). a, b) Within a column, values with different superscripts are significantly different ($P<0.05$).

Table 3. Effects of cytoplasmic volume on nuclear remodeling pattern of buffalo HMC embryos

Cytoplasmic volume	1.5 hr after fusion		3 hr after fusion	
	NEBD	PCC	NEBD	PCC
~75%	18/51 (35.3%)[b]	6/51 (11.8%)[b]	18/18 (100%)	12/18 (66.7%)
~150%	33/45 (73.3%)[a]	30/45 (66.7%)[a]	27/27 (100%)	27/27 (100%)
~225%	27/27 (100%)[a]	27/27 (100%)[a]	21/21 (100%)	21/21 (100%)

Data presented were from more than three replicates. a,b) Within a column, values with different superscripts are significantly different ($P<0.05$).

RESULTS

Effect of cytoplasmic volume on in vitro development of HMC embryos

In order to identify whether recipient oocyte cytoplasm of HMC embryos could influence the development of cloned buffalo embryos, a single trypsinized donor cell was fused respectively with one, two, or three enucleated cytoplasm to produce HMC embryos with variable cytoplasmic volume. As shown in Table 2, reconstructed embryos with ~225% cytoplasm resulted in a higher cleavage rate (86.8 ± 2.7%, $P<0.05$) compared with the other groups. In addition, when the donor cells were fused with ~150 or ~225% cytoplasm, the blastocyst rates of the reconstructed embryos were increased in comparison with that of donor cells fused with ~75% cytoplasm ($P<0.05$). In particular, in the group of donor cells fused with ~225% cytoplasm, the blastocyst developmental rate reached 27.9%. The total cell number of blastocysts derived from ~150 or ~225% cytoplasm was evidently increased compared with those developed from ~75% cytoplasm ($P<0.05$). However, no significant difference in the blastocyst rates and total cell number of blastocysts were observed between the ~150% cytoplasm and ~225% cytoplasm groups ($P>0.05$).

Effect of cytoplasmic volume on nucleus remodeling pattern of HMC embryos

According to the above results, to explore the mechanism of recipient oocyte cytoplasm-related developmental potential of the HMC embryos, the nucleus remodeling pattern of the HMC embryos derived from distinct cytoplasmic volumes at different time points (0, 1.5, and 3 hr post-fusion) was examined. A minimal change in donor nucleus was observed after fusing with different recipient cytoplasmic volumes (Fig. 2a). NEBD occurred (Fig. 2b), and then PCC was assembled (Fig. 2c) at 1.5–3 hr post-fusion. As shown in Table 3, with the increase of recipient cytoplasmic volume, both the proportions of NEBD and PCC were increased. The proportions of HMC embryos with ~225% cytoplasm appearing NEBD and PCC was 100% (27/27) at 1.5 hr post-fusion, which was significantly higher than that of the cytoplasmic volume ~75% group cytoplasm (100 vs. 35.3% and 100 vs. 11.8%, respectively). However, no significant difference in the frequency of NEBD and PCC were observed between the ~150% cytoplasm and ~225% cytoplasm groups (73.3 vs. 100% and 66.7 vs. 100%, respectively). At 3 hr post-fusion, the proportions of NEBD and PCC were increased to 100 and 66.7%, respectively, in the ~75% cytoplasm group, while all (100%) of the HMC embryos from the ~150 and ~225% cytoplasm group completed the process of NEBD and PCC.

Effects of cytoplasmic volume on DNA methylation levels of buffalo HMC embryos

To investigate the mechanism of recipient oocyte cytoplasm and its association with the nucleus reprogramming and
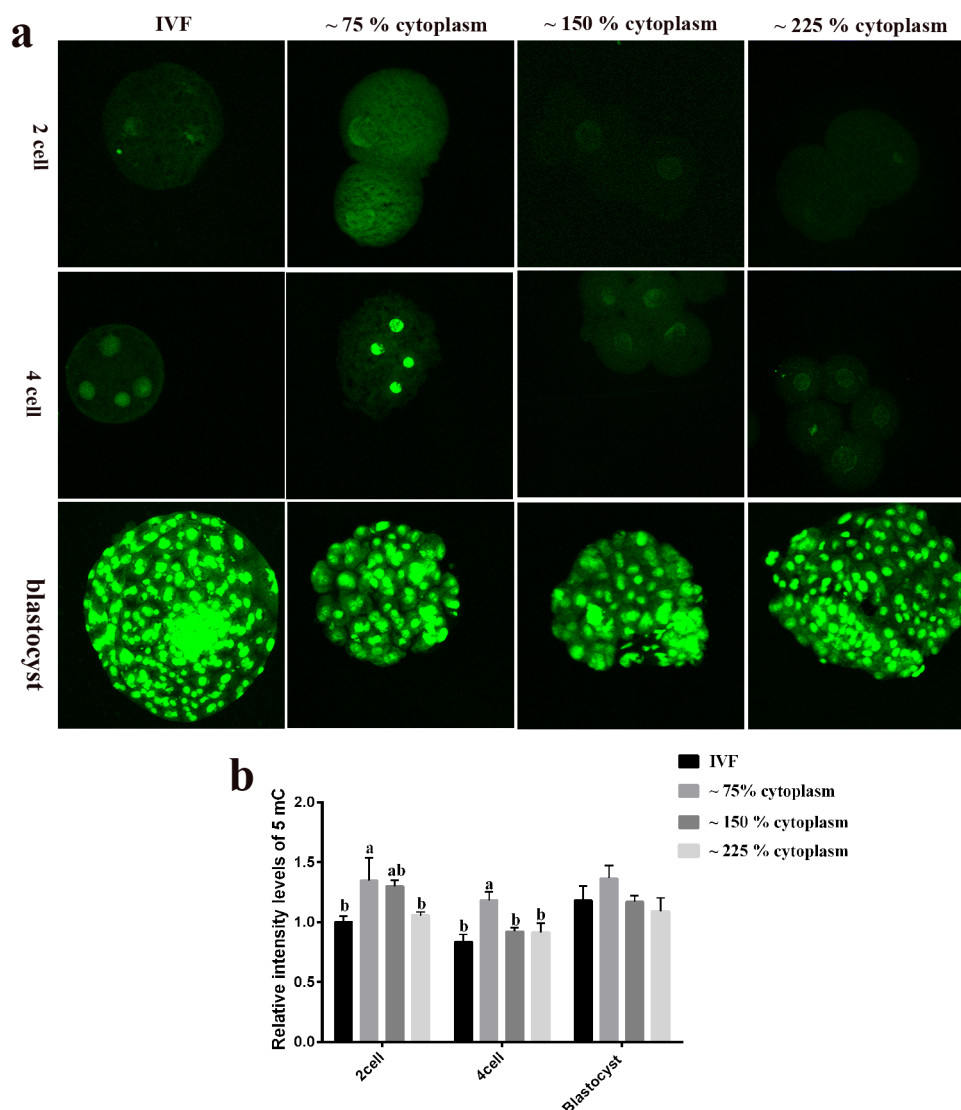
Fig. 3. The DNA methylation of embryos derived from IVF, ~75% cytoplasm, ~150% cytoplasm or ~225% HMC cytoplasm embryos during pre-implantation development. (a) Images of IVF, ~75% cytoplasm, ~150% cytoplasm or ~225% cytoplasm HMC embryos stained for 5 mC from the 2-cell to the blastocyst stages. (b) Relative levels of 5 mC in IVF, ~75% cytoplasm, ~150% cytoplasm or ~225% cytoplasm HMC embryos. Each bar represents the relative fold change across the developmental stages within each embryo type. Values are presented as mean ± SEM, and values with different superscripts (a, b) within groups are significantly different ($P<0.05$).

developmental potential of HMC embryos, the global change of DNA methylation in HMC and IVF embryos at the 2-cell, 4-cell and blastocyst stages was measured respectively by immunostaining (Fig. 3a). In comparison with the control group, decreasing recipient oocyte cytoplasm significantly increased the methylation level of HMC embryos from the 2-cell to the 4-cell stages (Fig. 3b). Interestingly, the ~225% cytoplasm embryos resulted in a decrease in the relative levels of global DNA methylation compared with that of the cytoplasmic volume ~75% cytoplasm group ($P<0.05$) but similar to the IVF counterparts ($P>0.05$).

Effects of cytoplasmic volume on embryonic genome activation of buffalo HMC embryos

To further understand how the mechanism of increasing recipient cytoplasmic volume enhanced the developmental potential of the HMC embryos, both the EGA marker genes (*eIF1A* and *U2AF*) in IVF and HMC embryos at the 2-cell, 4-cell, 8-cell and blastocyst stages were analyzed. The expression of *eIF1A* in the HMC embryos derived from ~225% cytoplasm was significantly elevated at the 4-cell and 8-cell stages ($P<0.05$) compared with that derived from ~75% cytoplasm (Fig. 4a). However, no significant differences were noted in the 2-cell or blastocyst stages ($P>0.05$). As shown in Fig. 4b, the expression of *U2AF* in the HMC embryos derived from ~225% cytoplasm at the 8-cell stage was increased when compared to that derived from ~75% cytoplasm and ~150% cytoplasm ($P<0.05$).
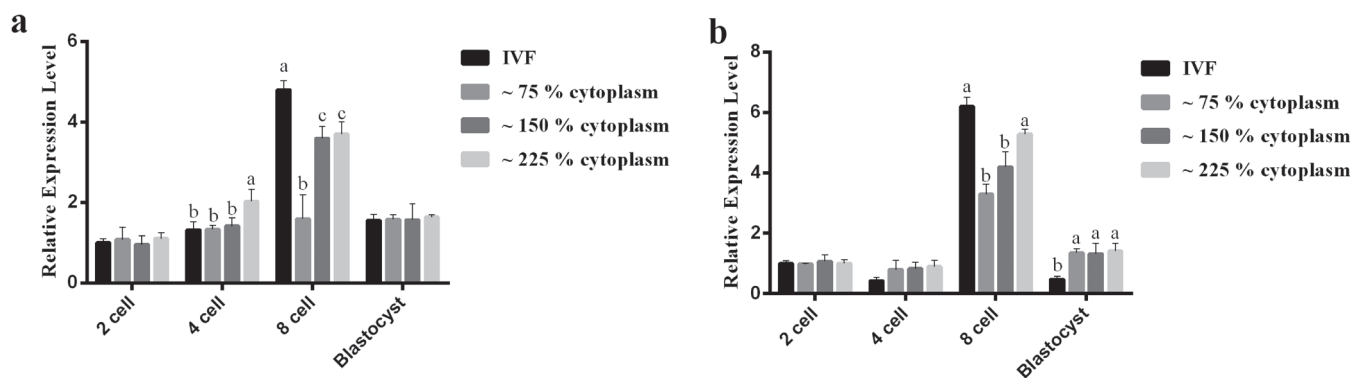
Fig. 4. Relative expression levels of EGA marker genes in embryos derived from IVF, ~75% cytoplasm, ~150% cytoplasm or ~225% cytoplasm HMC embryos. (a) Fold change of *eIF1A* and (b) fold change of *U2AF*. Each bar represents the relative fold change between the different embryos. Data shown in the figure are from three replicates (n=3) and values are presented as mean ± SEM. Values with different superscripts (a, b) within groups are significantly different (*P*<0.05).

Table 4. Pregnancy and calf birth following transfer of HMC embryos produced by distinct cytoplasmic volume

Methods	Embryos transferred	Recipients	No. pregnant recipients day 60 (%)	No. embryos developed to term (%)
HMC embryos with ~75% cytoplasm	12	6	0	0
HMC embryos with ~225% cytoplasm	19	7	2 (28.6%)	1 (14.3%)

In vivo developmental competence of HMC embryos

To evaluate the *in vivo* developmental competence of the HMC embryos reconstructed with ~75% cytoplasm or ~225% cytoplasm, blastocysts were transferred non-surgically into seven recipients. The pregnancy status of the recipients was examined by rectal palpation at 60 days after the embryo transfer. Two of the seven recipients were confirmed to be pregnant following the transfer of blastocysts derived from ~225% cytoplasm, whereas no recipients were pregnant after transfer of blastocysts derived from ~75% cytoplasm (Table 4). Unfortunately, one recipient died of illness on Day 200 of gestation and the HMC buffalo fetus was lost (Fig. 5). The remaining recipient maintained its pregnancy to term and delivered one health calf (Fig. 6).

DISCUSSION

The vast majority of data has shown that the larger the cytoplasm that was removed during enucleation, fewer embryonic cells were present at the morula or blastocyst stage, which could weaken the later developmental competence [12]. Previous studies have shown that the more cytoplasm in the reconstructed embryos, the higher the capacity of these embryos to develop further [4, 23, 26], which was further confirmed by our study. In this study, we found that increasing the recipient cytoplasmic volume resulted in a higher cleavage and blastocyst rate of reconstructed embryos, which was consistent with previous reports [4, 23]. A higher development of bovine HMC embryos was also achieved by the aggregation of reconstructed embryos with hemi-embryos [26], and with the increase of aggregated embryos, the cleavage, blastocyst rate, and total cell number of blastocysts were also increased. Our results showed that the blastocyst rates and total cell number of blastocysts developed from ~150% cytoplasm or ~225% cytoplasm were evidently increased compared with those developed from ~75% cytoplasm. These results confirmed that increasing cytoplasmic volume could enhance the *in vitro* development of buffalo HMC embryos. However, hemi-cytoplasts with cytoplasmic volume (~85% vs. 2 × 50%) showed no effect on the fusion rates after embryo reconstruction in goats [24]. In mice, embryonic aggregation did not improve the cloned embryo development to the blastocyst stage, but it increased the cell density in blastocysts and promoted eight-fold higher *in vivo* development than the controls [3]. This discrepancy might be attributed to the species specificity; the mechanism involved in this phenomenon still need to be further investigated.

The early events in the nuclear reprogramming process during SCNT consist of the remodeling of the donor nucleus. Several significant morphological changes, such as NEBD and PCC, occur in the donor nucleus after being transferred into recipient cytoplasts. Abnormal nuclear remodeling was frequently observed after SCNT in association with low developmental efficiency. Previous studies suggested that complete reprogramming only occurred after remodeling of the donor nucleus [8], the PCC of the donor nucleus is important for subsequent embryonic development [7]. In mice [43] and pigs [13], a higher *in vitro* development rate was obtained by inducing PCC, suggesting that PCC might promote effective nuclear reprogramming of the donor cell and enhance the developmental competence of SCNT embryos. It has been reported that modulated oocyte meiotic maturation by treating oocytes or reconstructed embryos with MG132 could induce the PCC of donor cells and promote the pronucleus formation of SCNT embryos [16, 48]. Similarly, TSA treatment also caused an increase of PCC in 1-cell SCNT embryos that correlated with

Fig. 5. One HMC buffalo fetus was lost on Day 200 of gestation because the surrogate mother died of illness.

Fig. 6. Eight-month-old HMC buffalo calf (left) and her surrogate mother (right).

the improved rates of embryonic development at subsequent stages [6, 19]. These reports indicated that the early morphological changes of donor nucleus were closely related to the successful reprogramming. In this study, with the increase of recipient cytoplasmic volume, 100% of the HMC embryos derived from ~225% cytoplasm experienced NEBD and chromosomes condensed into PCC within 1.5 hr of injection into enucleated oocytes. Moreover, the proportions of NEBD and PCC were significantly higher than that derived from ~75% cytoplasm. Meanwhile, all of the HMC embryos with ~150% cytoplasm also completed NEBD and PCC after 3 hr of injection into enucleated oocytes. The different time points of completing nucleus remodeling may be the reason for resulting significantly higher cleavage rate in the ~225% cytoplasm group than that of the other two groups. Our results indicated that increasing recipient cytoplasmic volume promotes nucleus remodeling, which is beneficial to subsequent embryonic developmental potential.

Global epigenetic reprogramming has been reported as a major process that takes place following SCNT for normal development and successful cloning [47]. Many studies also found that the abnormal modification of histone acetylation and DNA methylation in donor cells might result in the failure of SCNT embryo development [51]. Methylation of cytosines in the mammalian genome represents a key epigenetic modification and global DNA demethylation is important for setting up pluripotent states in early embryos [45]. Currently, the accumulated data have shown that DNA methyltransferase activity was inhibited by chemical inhibitor treatment [20, 30] or reduced the DNA methyltransferase-related gene expression by RNA interference [28], which could induce a higher developmental competence of cloned embryos, indicating that the global change of the DNA methylation level is tightly correlated with normal embryonic development. Therefore, we analyzed the DNA methylation level of buffalo HMC embryos derived from different cytoplasmic volumes and found that increasing the recipient cytoplasmic volume resulted in a decrease of global DNA methylation. Conversely, decreasing the recipient cytoplasmic volume significantly elevated the methylation level of HMC embryos from the 2-cell to the 4-cell stages. Our results indicated that increasing the cytoplasm volume promotes the reprogramming of DNA methylation in the donor nucleus and that contributes to the enhancement of the subsequent development of cloned embryo.

EGA is the first major step toward the successful initiation of preimplantation embryonic development, which culminates in the formation of implantation-competent embryos [41]. Previous studies have found that EGA occurs in a species-specific manner: at the 2-cell stage in mice [18]; at the 8-cell to 16-cell stage in cattle [10], and the major EGA takes place between the 4-cell and 8-cell stages, with a minor activation phase between the 2-cell and 4-cell stages in buffalo [41]. The expression of EGA marker genes (*eIF1A* and *U2AF*) in HMC with different amounts of cytoplasm and IVF embryos were analyzed to document the embryonic transcription initiation events. We found that the expression of *eIF1A* in HMC embryos derived from ~225% cytoplasm at the 4-cell and 8-cell stages was higher than in the ~75% cytoplasm group. The expression profile of *U2AF* in the HMC embryos derived from ~225% cytoplasm was increased remarkably at the 8-cell stage compared with that of the ~75% cytoplasm and ~150% cytoplasm embryos. Our results partially confirmed that increasing the cytoplasmic volume benefits the onset of EGA.

A large-scale trial of HMC embryo transfer found that the pregnancy rate of HMC embryo transfers was significantly higher than those of fresh IVF or NT embryo transfers, but the overall outcome of cloned offspring did not differ [34]. The removal of the zona pellucida was considered to be the reason for the higher pregnancy rates following embryo transfer [34]. In our case, the pregnancy rate of the HMC embryos derived from ~225% cytoplasm was higher than those derived from ~75% cytoplasm. However, one HMC buffalo fetus was lost on Day 200 of gestation because the recipient was ill and died. Moreover, the embryo transfer data are too small to draw a reasonable conclusion. Therefore, the embryos reconstructed with ~150% cytoplasm or ~225% cytoplasm have increased cell density in blastocysts, but whether it results in improved embryo developmental potential or survival of the fetus still needs to be determined.

In summary, the cytoplasmic volume of recipient oocytes affects the processes of donor nucleus reprogramming and EGA, and then the related developmental competence of buffalo HMC embryos. However, further studies should be performed to elucidate the mechanism of cytoplasmic increase on full-term development of buffalo SCNT embryos.

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