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Anomalous mRNA levels of chromatin remodeling genes in swamp buffalo (*Bubalus bubalis*) cloned embryos

T. Suteevun [a,b], S.L. Smith [b], S. Muenthaisong [a], X. Yang [b], R. Parnpai [a], X.C. Tian [c,*]

[a] *Embryo Technology and Stem Cell Research Center and School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*

[b] *Department of Animal Science and Center for Regenerative Biology, University of Connecticut, 1392 Storrs Road, Unit 4243, Storrs, CT 06269, USA*

[c] *University of Connecticut, 1392 Storrs Road, Unit 4243, Storrs, CT 06269, USA*

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Abstract

The swamp buffalo (*Bubalus bubalis*) is a multi-purpose animal in agriculture that is challenged by extinction due to low reproductive efficiency. Nuclear transfer (NT) has been used to preserve special breeds of buffalo, as well as to increase the number of animals. However, cloned buffalo embryos have impaired development, as in other species. To understand the chromatin remodeling activities in cloned embryos and to improve NT technology, we examined the expression profiles of five genes involved in DNA and histone modifications, *DNMT1*, *DNMT3A*, *DNMT3B*, *HAT1* and *HDAC1*, in single swamp buffalo metaphase II oocytes, NT and in vitro fertilized (IVF) embryos from the two-cell to the blastocyst stage, by quantitative real time RT-PCR. We observed similar expression dynamics for all genes studied in the NT and IVF embryos: relatively constant levels of expression for all genes were found from the MII oocyte up to the eight-cell stage; the levels of mRNA for *HAT1* and *DNMT3B* continued to be stably expressed up to the blastocyst stage; while dramatic increases were seen for *DNMT3A* and *HDAC1*. Alternatively, the levels of *DNMT1* started to decrease at the eight-cell stage. Despite the similarity in the dynamics of gene expression, dramatic differences in the relative levels of these genes between NT and IVF embryos were observed. The expression levels of all DNA modifying genes were higher in the NT embryos than in the IVF

* Corresponding author. Fax: +1 860 486 8809.
E-mail address: xiuchun.tian@uconn.edu (X.C. Tian).

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embryos at the eight-cell and blastocyst stages. The genes *HDAC1* and *HAT1* were also expressed significantly higher at the blastocyst stage in the NT embryos. Our results suggested differences in chromatin remodeling between NT and IVF embryos and that lower levels of DNA passive demethylation and higher levels of DNA de novo methylation occurred in the NT embryos. These observations are novel in the species of buffalo, and may be associated with developmental failure of cloned buffalo embryos due to the transcriptional repression effect of most genes studied here.
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1. Introduction

In cloned animals, characteristic anomalies such as increased birth weight, placental abnormalities and immune deficiency have been observed in many species [1–5]. Furthermore, abortion rates and neonatal deaths are consistently higher in animals generated by nuclear transfer (NT) than those by in vitro fertilization (IVF) or artificial insemination (AI). Incomplete epigenetic regulation is suspected to be the cause of the abnormalities and the low efficiency associated with NT [6–8].

Epigenetic modification involves altering gene expression without changing the DNA sequence. DNA methylation and histone acetylation are the key mechanisms of this process. DNA methylation has been shown to cause transcriptional silence; thus, the alteration of the methylation status could affect the expression of genes under epigenetic regulation. It has been reported that the DNA of cloned bovine embryos was aberrantly hypermethylated [9,10]. Kang et al. [11] also demonstrated that cloned bovine embryos display hypermethylated repetitive elements in their genome. Additionally, aberrant methylation patterns were found in cloned aborted fetuses, animals that died soon after birth and in adult animals [12]. In preimplantation embryos, the parental genomes are actively and passively demethylated after fertilization. This demethylation is followed by de novo methylation at the 8-cell to 16-cell stage [10]. The DNA methylation mechanism relies on the catalytic activity of DNA methyltransferases (*DNMT*s). The gene *DNMT1* maintains the methylation pattern during replication; whereas, *DNMT3A* and *3B* are responsible for de novo methylation. The DNA methyltransferases *3A* and *3B* work together with many related proteins, such as histone deacetylases in silencing gene expression. Another widely studied form of epigenetic modification is the acetylation of the lysine residues at the amino-terminus of histones. This is achieved by histone acetylases (*HAT*s) that cause the acetylation and neutralization of the positive charges on the amino-terminus of the histones, leading to chromatin decondensation and activation of gene transcription. Conversely, histones can be deacetylated by histone deacetylases (*HDAC*s), which cause chromatin condensation and gene repression [13]. Enright et al. [14] and Santos et al. [15] observed hyperacetylation in preimplantation cloned cattle embryos. In bovine IVF embryos, both *HDAC1* and *HAT1* genes were detected at the eight-cell stage and displayed the highest level of expression in blastocysts [16]. For *DNMT*s, the lowest level of *DNMT1* was observed at the blastocyst stage. *DNMT3A*/*B* have only been analyzed and detected in bovine blastocysts [17,18].

Swamp buffalos (*Bubalus bubalis*) are multi-purpose animals important for agriculture. Many countries depend heavily on buffalos' production for meat and milk, in addition to their value for labor. However, the buffalo population in many countries has markedly decreased due to low reproduction efficiency, such as a long gestation period and late onset of puberty. Nuclear transfer is a potential strategy for preserving endangered buffalo breeds through the cryopreservation of buffalo donor cells. To achieve cloning of the buffalo, swamp buffalo NT was attempted and blastocyst development was obtained in 2001 [19]. However, the pregnancy rate after embryo transfer for NT embryos was much lower than that of IVF [20]. In the case of NT, fetal loss is commonly found throughout gestation, suggesting that NT embryos have intrinsic abnormalities that are manifested after transfer, even though they can reach the blastocyst stage [21]. Our own studies, examining DNA methylation and histone acetylation by confocal microscopy, revealed aberrant DNA demethylation and histone acetylation patterns in cloned buffalo embryos as compared to IVF embryos during the preimplantation period. Exploring the underlying mechanism for the abnormal methylation by studying genes encoding chromatin-modifying enzymes will help to elucidate whether this is due to abnormal DNA demethylation or de novo DNA re-methylation. This may provide insight into the causes of the low efficiency in swamp buffalo NT and enable the modification of existing protocols for greater success. In the present study, we examined the levels of mRNA for five chromatin remodeling genes: *DNMT1*, *DNMT3A*, *DNMT3B*, *HAT1* and *HDAC1*, all in single metaphase II oocytes, NT and IVF embryos at various stages of preimplantation development by quantitative real time RT-PCR. We found that the NT embryos contained significantly higher mRNA levels of DNA methylation genes as well as *HDAC1*, all of which have been shown to repress gene transcription.

2. Materials and methods

2.1. Donor cell preparation

Ear skin was biopsied from a single swamp buffalo in order to minimize genetic variation. The biopsy was kept in modified Dulbeccco's phosphate buffer saline at 4 °C during transportation to the laboratory. Skin tissues were removed from cartilage and cut into small pieces (about 1 mm^2) before being placed in 60 mm culture dish (Nunc GmbH & Co. KG, Wiesbaden, Germany) and covered with a glass slide. Five milliliters of alpha modified Eagle's medium (αMEM, Sigma, St. Louis, MO, USA) plus 10% fetal bovine serum (FBS) was added to the dish and the tissue was cultured under a humidified atmosphere of 5% CO_2 in air at 37.0 °C. The fibroblast cell outgrowth from the ear skin tissue was harvested and seeded in 25 cm^2 culture flask (Nunc) in αMEM plus 10% FBS. To preserve these cells, ear fibroblasts were frozen with 10% DMSO in αMEM plus 20% FBS at the third cell culture passage and stored in liquid nitrogen. For NT, frozen-thawed fibroblasts were cultured four to eight passages in αMEM plus 10% FBS and used as nuclear donor cells. No serum starvation was performed and the sub-confluence donor cells were re-suspended in Emcare medium (ICP bio, Pets-Inc., Texas, USA) before injection.

2.2. Somatic cell nuclear transfer

Swamp buffalo oocytes derived from abattoir-collected ovaries were matured in in vitro maturation medium [22] for 22 h. The cumulus cells were mechanically removed by repeat pipetting using a fine-tip pipette in 0.2% hyaluronidase and were subsequently washed five times in Emcare medium. For enucleation, matured oocytes were placed in culture medium containing 5 μg/mL cytochalasin B (Sigma) for 15 min. The zonae pellucidae above the first polar body was cut with a glass needle and a small volume (about 5–10%) of cytoplasm was extruded. Complete enucleation was confirmed by staining with Hoechst 33342 (Sigma). To transfer the donor cells, individual fibroblasts were injected into the perivitelline space of the enucleated oocytes, and fusion was initiated by placing the couplets into Zimmerman fusion medium [23] followed by electrical stimulation using two dc pulses at 26 V, for 17 μs using SUT F-1 (Suranaree University of Technology). The reconstructed embryos were activated for 5 min in 7% ethanol and then cultured in modified synthetic oviductal fluid culture media (mSOF) [24] containing 3 mg/mL BSA (Sigma), 1.25 μg/mL cytochalasin D (Sigma) and 10 μg/mL cycloheximide (Sigma) for 5 h. The activated embryos were cultured in 100 μL droplets of mSOF under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C for 2 days. The eight-cell stage embryos were selected and co-cultured in 100 μL droplets of mSOF with bovine oviductal epithelial cells (BOEC) under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for another 5 days. Embryos at two-cell, four-cell, eight-cell, morula, and blastocyst stages were collected at 22, 28, 34, 108, and 132 h post-activation, respectively.

2.3. In vitro fertilization (IVF)

In order to minimize variation from genetic differences, buffalo semen from the same bull was used for the production of the IVF embryos. The frozen semen was thawed at 37 °C and washed twice by centrifugation at $500 \times g$ for 7 min with Brackett and Oliphant (BO) medium [25] without BSA but supplemented with 10 mM caffeine (caffeine-BO; Sigma). The pellet was re-suspended in caffeine-BO at the concentration of 8×10^6 sperm/mL and then diluted with an equal volume of BO medium supplemented with 20 mg/mL BSA and 20 μg/mL heparin (Sigma). The cumulus oocyte complexes (COC) after 22 h in in vitro maturation medium, were transferred into 100 μL droplets of sperm suspension under mineral oil (Sigma) and incubated under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C for 2 days. The eight-cell stage embryos were selected and co-cultured in 100 μL droplets of mSOF with BOEC under humidified atmosphere of 5% CO_2 in air at 38.5 °C for another 5 days. The two-cell, four-cell, eight-cell, morula, and blastocyst embryos were collected at approximately 28, 34, 40, 114, and 138 h in culture, post-insemination, respectively.

2.4. Embryo staining and cell count

To distinguish cells of the inner cell mass (ICM) and trophectoderm (TE), embryos were counter-stained. Briefly, the zonae pellucidae of blastocysts at Day 6.5 post-activation/

insemination were removed by 0.5% protease (Sigma) and washed in SOF medium. The zona-free blastocysts were incubated in 10% rabbit anti-buffalo spleenocyte antibodies generated in our laboratory [26] for 45 min, and subsequently transferred into a mixture of 10% guinea pig complement (Sigma), 75 μg/mL propidium iodide (PI; Sigma) and 100 μg/mL Hoechst 33258 (Bis-benzimide; Sigma) for 45 min. The ICM cells (blue) and TE cells (red) were counted under UV light fluorescence microscopy.

2.5. Real time reverse transcription (RT)-PCR

The sequences of the five genes of interest for buffalo were not available in GenBank; in order to design specific primers for the buffalo, we first amplified fragments of these genes from buffalo genomic DNA using primers designed from cattle sequences of the same genes. The PCR products were then sequenced and the new sequences were used to design buffalo primers (Table 1). Six MII oocytes, NT and IVF embryos in every stage of preimplantation development were analyzed individually in the study. Total RNA from single oocytes and embryos was extracted using TRIzol reagent (Invitrogen Life Technologies, CA, USA) with linear acrylamide (Ambion Inc., Austin, TX, USA) followed by RNase-free DNase treatment to remove any possible genomic DNA contamination. Reverse transcription was carried out at 50 °C for 1 h with Superscript III (Invitrogen). Individual cDNA samples were diluted based on the results of a pilot study that measured the cycle threshold (CT) for each target gene at each developmental stage. The total volume of 25 μL real time RT-PCR reaction mixture contained 5 μL of cDNA, 12.5 μL of SYBR green master mix (Applied Biosystems Inc., CA, USA) and 0.3 μM of forward and reverse primers. All oocytes and embryos were analyzed in triplicate for every gene. The specificity of the real time RT-PCR product was proven by melting curve analysis. To compare the relative levels of gene expression in NT and IVF embryos, the comparative cycle (CT) method, also known as the $2^{-\Delta\Delta CT}$ method [27], was applied using the following formula: the relative amount of mRNA for the target

Table 1
Swamp buffalo primer sequences used in real time RT-PCR

Gene	Accession number for cattle	Buffalo primer sequences 5′-3′	Product size (bp)	Melting temperature (°C)
DNMT1	AY173048	Forward- GAGGGCTACCTGGCTAAAGTC Reverse- CATTCGCTTCCCGACTGAAA	88	78
DNMT3A	AY271299	Forward- CGAGGTGTGTGAGGACTCCAT Reverse- ACGTCCCCGACGTACATGA	93	79
DNMT3B	AY224713	Forward- AGCATGAGGGCAACATCAAAT Reverse- CACCAATCACCAAGTCAAATG	98	77
HAT1	BT021536	Forward- CTTCAGACCTTTTTGATGTGGTTTATT Reverse- GCGTAGCTCCATCCTTATTATACTTCTC	112	85
HDAC1	AY504948	Forward- GCACTGGGCTGGAACATCTC Reverse- GGGATTGACGACGAGTCCTATG	98	79
H2A	M37585	Forward- TTTGTGGATGTGTGGAATGAC Reverse- TTCGTGGAGATGAAGAATTGG	95	76

gene = $2^{-\Delta\Delta CT}$, where CT is the threshold cycle for the target gene amplification, $\Delta CT = CT_{target\ gene} - CT_{endogenous\ reference}$, and $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{calibrator}$. The endogenous reference gene chosen for this study was histone H2A (*H2A*) because it was reported to have a constant expression level throughout preimplantation development [28]. The calibrator was a mixture of RNA from organs and blastocysts and was included in each real-time amplification. Validation of the amplification efficiency of target genes and the endogenous reference gene was completed before using the $2^{-\Delta\Delta CT}$ method for quantification. Briefly, different dilutions of cDNA were amplified by real time RT-PCR using the gene specific primers. The corresponding ΔCT values were plotted against the log of each cDNA amount and the data were plotted using linear regression analysis. The values of the slopes of the validation curves for each gene was less than 0.1, indicating that the same amplification efficiency was obtained for all samples with both high and low amounts of the cDNA. All validation and quantification methods were suggested by the manufacturer of the real time amplification kit (Applied Biosystems Inc.). The CT values were adjusted based on cDNA dilution and the 1/CT values were used to examine the temporal pattern of gene expression throughout preimplantation development.

2.6. Statistical analysis

Data analyses for the differences in embryonic development and relative mRNA levels for each gene were carried out by ANOVA using the general linear model (GLM) procedure in Statistical Analysis Systems (SAS, version 9.0, SAS Inc., Cary, NC, USA). For embryo development, the percentages of cleavage and blastocyst development were arc sine transformed and analyzed by one-way ANOVA with the main effect as the embryo type (IVF versus NT). For the comparison of the relative mRNA levels for each gene, two-way ANOVA was utilized with the main effects as embryo type and developmental stage; and the interaction term as the embryo type × developmental stages. The relative levels of gene expression in NT and IVF embryos were log 10 transformed and the Least Square Means method in GLM was used for multiple comparisons within developmental stages between each embryo type. A probability value of $P \leq 0.05$ was considered significant. To determine the correlation between the expression patterns of *HDAC1* and *DNMT3A*, Pearson correlation analysis was performed in SAS.

3. Results

3.1. Development of IVF and NT swamp buffalo embryos

The developmental rates of the swamp buffalo embryos derived from IVF and NT are summarized in Table 2. No significant differences were observed in development rates from cleavage to morula between NT and IVF embryos. However, a higher blastocyst rate ($P < 0.05$) was obtained for IVF embryos. Similar numbers of cells in ICM (~30 cells/embryo) and TE (~90 cells/embryo) were found in both NT and IVF blastocysts.

Table 2
Development of preimplantation swamp buffalo embryos derived from NT and IVF

Embryo type	Experiment (n)	Fertilized/fused embryos	Embryo development (mean ± S.E.M.)			No. cells in blastocysts (mean ± S.E.M.)		
			Cleavage	Morula	Blastocyst	ICM	TE	% ICM
NT	3	206	0.90 ± 0.06	0.74 ± 0.03	0.34 ± 0.03^{a}	25.6 ± 5.21	88.7 ± 10.8	20
IVF	3	199	0.76 ± 0.06	0.67 ± 0.04	0.50 ± 0.03^{b}	29.2 ± 2.09	97.3 ± 4.15	22

Within a column, values with the different superscripts (a, b) are different ($P \leq 0.05$). The cleavage rate was calculated based on the number of fertilized/fused embryos. The developmental rates to morula and blastocyst were calculated based on the number of cleaved embryos.

3.2. Levels of mRNA for chromatin remodeling genes in oocytes and preimplantation embryos

In the present study, mRNA levels of five chromatin remodeling genes were examined throughout preimplantation development. The endogenous reference gene, *H2A*, displayed a constant mRNA level from the oocyte up to the eight-cell stage. The mRNA levels of *H2A* increased with embryonic development after the eight-cell stage until reaching its highest level at the blastocyst stage (Fig. 1). Due to the variable expression of *H2A* during development, the $2^{-\Delta\Delta CT}$ method could not be used for comparisons across different stages of development within each embryo type. Therefore, we only compared statistically the relative levels of mRNA between embryo types at the same stage of development and temporal changes within each embryo type were evaluated based on 1/CT values.

The relative levels of mRNA for the passive DNA methylation gene, *DNMT1*, were approximately constant from the MII oocyte stage up to the four-cell stage. However, *DNMT1* started to decrease at the eight-cell stage and reached its lowest level at the blastocyst stage. Despite the similar trends in the levels of *DNMT1* in NT and IVF embryos, *DNMT1* appeared to always be higher in the NT embryos and this became significant at the eight-cell stage and nearly so at the blastocyst stage ($P = 0.06$; Fig. 2A). In contrast, the de novo DNA methylation genes, *DNMT3A* and *3B*, showed nearly the reverse change after cleavage development compared to that of *DNMT1*. The levels of mRNA for both genes

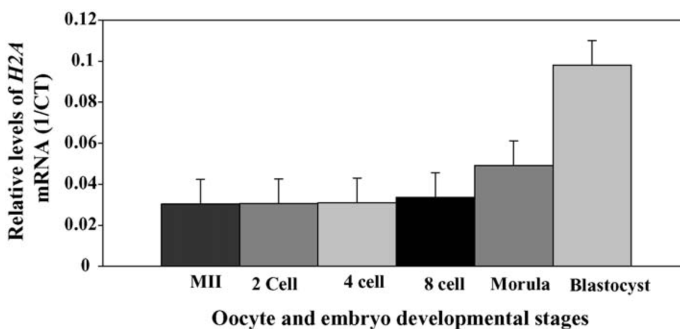

Fig. 1. The relative levels of *H2A* expression in oocytes and IVF embryos at different developmental stages.

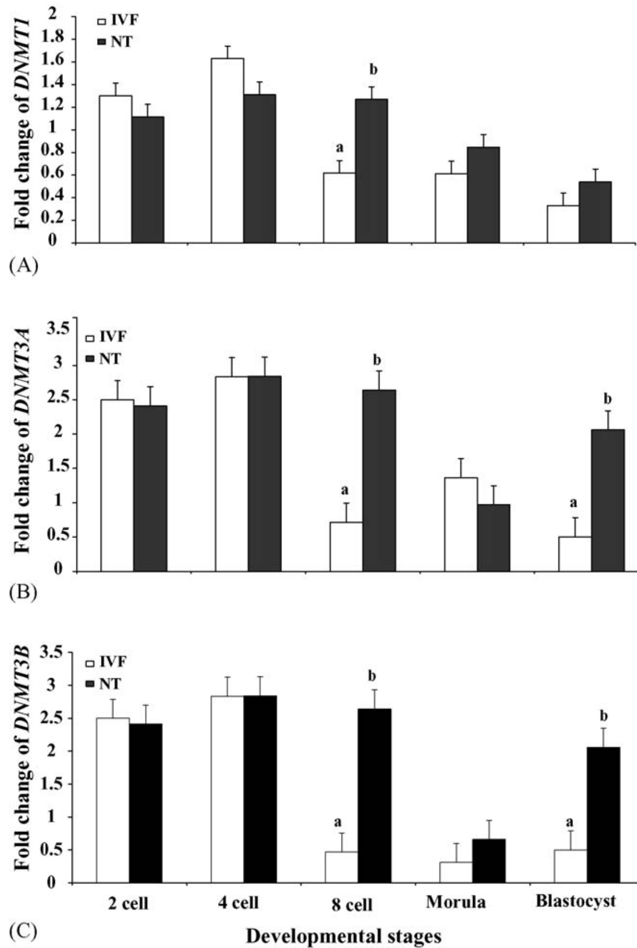

Fig. 2. Comparison of the fold change in the levels of mRNA for the DNA methyltransferase genes between IVF (white bars) and NT embryos (black bars) at different stages of embryo development: (A) *DNMT1*, (B) *DNMT3A*, and (C) *DNMT3B*. The fold change is the difference between the embryos and the calibrator. Within a particular stage of development, bars with different superscripts (a, b) are different ($P < 0.05$).

increased from the morula stage and reached the highest level at the blastocyst stage. Significantly higher levels of both genes were found at the eight-cell and blastocyst stages in the NT as compared to the IVF embryos ($P < 0.05$; Fig. 2B and C). Similar patterns of temporal expression were also observed in the mRNA levels of the histone modifying genes, *HAT1* and *HDAC1*, during cleavage development. They were relatively unchanged from the MII oocyte to the eight-cell stage in both IVF and NT embryos. Subsequently, both genes showed a dramatic increase in expression and reached their highest expression levels at the blastocyst stage. When gene expression levels were compared between the NT and IVF embryos, significantly higher levels for both genes ($P < 0.05$ and $P < 0.01$, respectively) were found in NT embryos at the blastocyst stage (Fig. 3A and B). We also

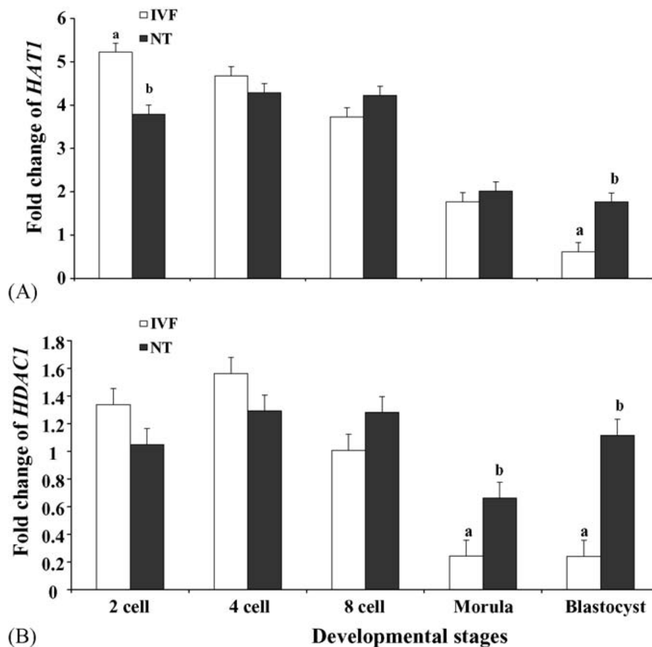

Fig. 3. Comparison of the fold change in the levels of mRNA for the histone modification genes between IVF (white bars) and NT embryos (black bars) at different stages of embryo development: (A) *HAT1* and (B) *HDAC1*. The fold change is the difference between the embryos and the calibrator. Within a particular stage of development, bars with different superscripts (a, b) are different ($P < 0.05$).

observed higher ($P < 0.05$) levels of *HDAC1* at the morula stage in the NT embryos. Interestingly, a high correlation ($r = 0.99$) was found in the expression levels between *HDAC1* and *DNMT3A* throughout the developmental stages examined.

4. Discussion

In the present study, we produced IVF and NT swamp buffalo embryos and documented for the first time, the dynamics of expression of five chromatin remodeling genes in these embryos. The efficiency of producing NT embryos and the cell numbers of NT and IVF embryos separated into the two lineages, ICM and TE, are comparable to or higher than those reported previously in the same species [21,29]. In our study, we found approximately 90 cells in the TE and an additional ~30 cells in the ICM. Although no data on the cell numbers for in vivo buffalo embryos are available for comparison, the number of cells in the ICM of the swamp buffalo NT and IVF embryos are low compared to those of cattle blastocysts [30], possibly due to inefficient culture conditions. The significantly lower developmental rate of NT embryos after cleavage development indicates that problems with nuclear reprogramming may have occurred. To understand the epigenetic changes during NT embryos' preimplantation development, we studied five genes responsible for DNA methylation and histone acetylation modifications.

We found that NT embryos contained higher levels of mRNA for *DNMT1* (eight-cell embryos) and for *DNMT3A/B* (eight-cell embryos and blastocysts). Taken together, the increased levels of all DNA methylation genes studied here, suggest that the NT embryos have higher levels of enzymatic activities not only for maintaining existing methylation, but also for de novo DNA methylation. These data provide an underlying mechanism for the global hypermethylation in the buffalo NT embryos observed by our laboratory. While these findings are novel in the species of buffalo, they agree with observations in cattle NT embryos, which were found to be aberrantly hypermethylated and also to contain higher levels of mRNA for the *DNMT*s [9–11].

The enzyme *HDAC1* is responsible for deacetylating histones, which causes transcriptional repression. Interestingly, our results demonstrated a strong positive correlation ($r = 0.99$) between *HDAC1* and *DNMT3A*, due to a similarity of expression patterns for these two genes at all stages, suggesting a coordinated regulation of these two genes and a connection of their functions. This observation was consistent with the reports of Fuks et al. [31] and Datta et al. [32], who found that *DNMT3A* is associated with *HDAC1* and forms a protein complex involved in transcriptional repression. Because both *HDAC1* and *DNMT3A* act to inhibit transcription, the observation that the mRNA for these two genes was elevated in the NT embryos at the blastocyst stage, may indicate reduced transcription levels which could lead to further developmental abnormalities.

The gene *HAT1* is a type B histone acetylase. It functions to acetylate non-specific nascent histone targets and is involved in chromatin assembly [33,34]. To date, the expression of *HAT1* and *HDAC1* has only been studied in bovine IVF embryos [35], neither has been studied in NT embryos. Thus, our study represents the first characterization of histone acetylation capabilities of NT embryos in all species cloned. It has been reported that global levels of histone acetylation were higher in NT than IVF embryos in cattle [35]. It is not surprising that higher expression of *HAT1* was found in the buffalo NT embryos studied here.

We found that *H2A*, which was used as a reference gene in our study, had constant expression at early developmental stages, and this was followed by increased expression at the morula and blastocyst stages. This finding was inconsistent with the study of Robert et al. [28], who measured the expression of many housekeeping genes in bovine embryos. They found that *H2A* was the only gene which had relatively constant expression during preimplantation development; thus, identifying it as the best internal reference for early embryos. The discrepancy between our study and that of Robert et al. [28] could be due to their use of pooled embryos or because of species differences. Because *H2A* expression increased at the blastocyst stage in our study, we only used it when comparing NT and IVF embryos at the same developmental stage, hence all comparisons are valid. A housekeeping gene that can serve as an ideal internal control for gene expression studies across early preimplantation development has yet to be identified.

Another interesting finding of the present study may indicate the timing of maternal-zygotic transition (MZT), which is still unclear in buffalo embryos. The MZT is the period where maternal mRNA is replaced by embryonic mRNA. In this study, we found that the NT embryos had a lower blastocyst rate than the IVF embryos. However, this did not result from a lower cleavage rate of the NT embryos. In fact, the NT embryos had a higher, although not significant, cleavage rate than the IVF embryos. These data indicate that the development of NT embryos up to the eight-cell stage may be sufficiently supported by the maternal

components of the oocytes. Upon the depletion and degradation of maternal mRNAs, the NT embryos had lower further developmental rates, which may be due to an incomplete activation of the embryonic genome. We suggest that the eight-cell stage, as is the case for cattle, may be when MZT occurs in the swamp buffalo. Additionally, our real time RT-PCR results also support this assumption as critical changes, either increases or decreases, occurred in gene expression at the eight-cell stage for all genes studied. Future studies examining this critical event in swamp buffalo embryos are needed to confirm this hypothesis.

In summary, our results showed relatively constant levels of expression of all chromatin remodeling genes studied up to the eight-cell stage. Interestingly, higher expression levels of all genes at the blastocyst stage and all DNA modifying genes at the eight-cell stage were found in the NT embryos as compared to the IVF embryos. These data provide the underlying mechanism for the abnormal hypermethylation in cloned embryos, which could cause transcriptional repression and may be one of the reasons for the developmental failure of cloned embryos at later stages.

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