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# Effects of Scriptaid on the Histone Acetylation and *in vitro* Development of Porcine Somatic Cell Nuclear Transfer Embryos

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**Abstract:** Effects of Scriptaid (SCR), low toxicity Histone Deacetylase inhibitors (HDACi) on the histone acetylation and development of porcine Somatic Cell Nuclear Transfer (SCNT) embryos were examined in this study. Treatment of activated reconstructed SCNT embryos with 500 nM SCR for 12 h resulted in a significantly increase in the blastocyst development compared to untreated group (21.0 vs 9.7%, $p<0.05$). Further, SCR treatment resulted in SCNT embryos at the 2, 4-cells and blastocyst stage with higher acetylation level of H3K18 in comparison with untreated group and similar to fertilized counterparts. Measurement of four selected developmentally important genes (*HDAC2*, *HAT1*, *OCT4* and Phosphoglycerate Kinase 1 (*PGK1*)) in blastocysts showed that treatment of SCNT embryos with SCR resulted in an increase expression in HAT1 and decrease expression in HDAC2 and PGK1. A similar expression profile of these genes was found in blastocysts developed from SCR treated SCNT embryos and *In Vitro* Fertilization (IVF) embryos. These results suggest that SCR treatment can improve the development competence of porcine SCNT embryos and result in a similar expression pattern of genes related to histone acetylation and acetylation level of histone compared to IVF embryos.

**Key words:** Development, histone acetylation, porcine, scriptaid, somatic cell nuclear transfer

## INTRODUCTION

Somatic Cell Nuclear Transfer (SCNT) is a promising technology with potential applications in agriculture and regenerative medicine (Lee *et al*., 2005; Watanabe *et al*., 2005; Lai *et al*., 2006; Li *et al*., 2006). Although, cloned animals have been successful in many species, the efficiency of cloning was still very low, birth rate of cloned animals from blastocysts is only about 1-5% in contrast to a 30-60% of *In Vitro* Fertilized (IVF) blastocysts. Moreover, the cloned animals have a high rate of abnormalities such as high mortality, short life span and obesity (Tamashiro *et al*., 2007) which may be associated with their earlier reprogramming dysfunction (Boiani *et al*., 2002; Santos *et al*., 2003; Yang *et al*., 2007). Abnormal epigenetic reprogramming may lead to errors or trigger differences in gene expression, especially trigger some defects of gene expression associated with the developmental competence of the SCNT embryos (Boiani *et al*., 2002; Santos *et al*., 2003; Beaujean *et al*., 2004).

It has been reported that treatment of cattle SCNT embryos with Histone Deacetylase inhibitors (HDACi) such as sodium butyrate and trichostatin A (TSA) or DNA methylation inhibitor (5-aza-2-deoxycytidine and S-adenosyl homocysteine) can increase the formation rate of blastocyst. Meanwhile, treatment with TSA can also increase the development of mice cloned embryos (Kishigami *et al*., 2006; Rybouchkin *et al*., 2006) and pig SCNT embryos (Zhang *et al*., 2007; Li *et al*., 2008). Recently, a low toxicity histone deacetylase inhibitor SCR was employed to improve the epigenetic status of donor cells and SCNT embryos (Tsuji *et al*., 2009).

Treatment of mini pig cloned embryos with SCR resulted in a significantly increase in the blastocyst yields from 9-21% (Zhao *et al*., 2009). These reports clearly showed that epigenetic factors are important for improving the developmental competence of SCNT embryos. However, it is not clear how SCR enhances the epigenetic reprogramming ability of donor cells and gene expression in embryos. In the process of the histone modification, acetylation and deacetylation is regulated

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by Histone Acetyltransferase (HAT) and Histone Deacetyltransferases (HDACs) (Lee *et al*., 1993; Smith, 2008). Inhibition of histone deacetyltransferases will increase the acetylation level of histone and promote the expression of genes (Smith, 2008; Cervera *et al*., 2009). In mammalian cells, the 18th lysine is a main acetylation site of histone H3 (Kelly *et al*., 2000). Earlier studies showed that DNA demethylation and 18 lysine acetylation of histone H3 is an appropriate marker of nuclear reprogramming which is related to the development potential of SCNT embryos (Santos *et al*., 2003; Li *et al*., 2008). Thus, increasing histone acetylation level of donor cells or SCNT embryos with deacetylase inhibitor may improve the embryonic development competence (Enright *et al*., 2003; Li *et al*., 2008). Moreover, unlike IVF embryos, the AcH4K5 level remarkably reduced at the 8-cells stage in cloned bovine embryos, indicating that the abnormal H4K5 acetylation may led to abnormal imprinting gene expression and low developmental competence in cloned embryos (Wee *et al*., 2006). Therefore, in this study, effects of scriptaid, a low toxicity inhibitor of HDAC on the embryonic development of porcine SCNT embryos were examined. Meanwhile, the histone acetylation level and expression of genes related to pluripotency (*OCT4*) histone acetylation (*HDAC2* and *HAT1*) and metabolism (Phosphoglycerate Kinase 1, *PGK1*) were also determined.

## MATERIALS AND METHODS

**Chemicals:** Unless otherwise stated, chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

***In vitro* maturation of oocytes:** Ovaries of prepubertal gilt were collected from a local slaughterhouse and were transported to the laboratory in physiological saline within 3 h of slaughter. Cumulus-Oocyte Complexes (COCs) from 3-6 mm diameter follicles were aspirated using an 18-gauge needle attached to a 10 mL syringe. The COCs with at least three layers of cumulus cells were selected and washed three times with *In Vitro* Maturation (IVM) medium: bicarbonate-buffered TCM-199 supplemented with 25 mM $NaHCO_3$, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10% Fetal Bovine Serum (FBS), 10% Porcine Follicular Fluid (PFF), 50 ng $mL^{-1}$ Epidermal Growth Factor (EGF), 10 ng $mL^{-1}$ Insulin-like Growth Factors (IGF-1), 10 IU mL human Chorionic Gonadotrophin (hCG), 10 IU $mL^{-1}$ Pregnant Mare Serum Gonadotrophin (PMSG) and cultured in 4-wells culture plate. Each well was filled with 500 μL IVM medium and covered with mineral oil. About 100 COCs were cultured for 20-22 h under a humidified 5% $CO_2$ in air atmosphere at 38.5°C. After 20-22 h of culture, the COCs were transferred to IVM medium without hCG and PMSG and cultured for an additional 20-22 h under the same conditions.

***In vitro* fertilization:** Porcine semen was supplied by a local porcine artificial insemination station and stored at 17°C until use. The method of IVF was carried out as previously described (Silvestre *et al*., 2007). Briefly, the semen sample was suspended in 10 mL modified Tyrode's medium (mTALP) and centrifuged at 500 g for 5 min to eliminate foreign particles and dead cells. After the first wash, the clean fraction of semen was obtained and centrifuged again (500 g, 5 min). The washed spermatozoa pellet was resuspended in mTALP at a concentration of $5\times10^5$ $mL^{-1}$ for subsequent fertilization.

Then, approximately 50 COCs were transferred into 500 μL of mTALP containing spermatozoa for IVF. At 6 h after IVF, oocytes were gently washed to eliminate cumulus cells and sperm adhering to the zona pellucida and 15-20 oocytes were cultured in a 50 μL droplet of modified Porcine Zygote Medium (PZM-3).

**Cell preparation for SCNT:** Porcine fetuses (about 35-40 days) were obtained from a local slaughterhouse. Fetuses were transported the laboratory in physiological saline within 3 h after slaughter. The fetus was sterilized with 75% alcohol and then washed three times in PBS containing antibiotics (penicillin G 100U $mL^{-1}$ and streptomycin 100 μg $mL^{-1}$). After washing twice with PBS again and the skin tissue was minced with a surgical scissors in a 100 mm Petri dish. The minced tissues were dissociated in 0.2% trypsin for 30 min at 37°C. Trypsinized cell were terminated by DMEM containing 10% FBS (Hyclone) and then washed by centrifugation at 500 g for 3 min. Cells were resuspended in 1 mL DMEM and placed in culture in a 60 mm tissue culture dish under a humidified atmosphere of 5% $CO_2$ in air atmosphere at 37°C. After 7-10 dyas of culture, confluent fetal fibroblast monolayers were obtained and then routinely passaged 3-6 times using an enzymatic solution (0.2% trypsin and 0.05% EDTA) for 3 min. The fibroblasts were synchronized by contact inhibition for 3-5 days before using for SCNT.

**Nuclear transfer and SCR treatment:** After 40-44 h of IVM, the surrounding cumulus cells of COCs were removed by manual pipetting in the presence of hyaluronidase at 2 mg $mL^{-1}$ and oocytes with and extruded first polar body were selected for enucleation. The selected oocytes were placed in the manipulation

medium drop supplemented with 5 μg $mL^{-1}$ cytochalasin B for 5 min before micromanipulation. The first polar body and metaphase II plate were removed by aspiration with a beveled pipette (inner diameter, 20 μm) under the LEICA DMIRB inverted microscope equipped with a Narishige micromanipulator (Tokyo, Japan) and the Spindle View System. Exposing all the removed cytoplasm the Spindle View System and checking for the presence of the removed metaphase plate confirmed successful enucleation. A single donor cell was injected into the periviteline space of each oocyte and the fusion was induced by application of two direct current pulses of 1 KV/cm for 20 μ sec in fusion medium (280 mM of mannitol, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 mM Hepes and 0.1% BSA). The fused oocytes were cultured in PZM-3 medium covered with mineral oil under a humidified atmosphere of 5% $CO_2$ in air at 38.5°C. Reconstructed embryos were randomly divised into two groups. One is treatment with 500 mM SCR 12 h then cultured in PZ3-3 medium (NTS), the other is directly cultured in PZM-3 as control group (NT). Cleavage and blastocyst percentages were evaluated on days 2 and 7, respectively. And day 7 blastocysts were stained with 5 μg $mL^{-1}$ of HOECHST 33342 20 min for assessment of the total number blastomeres.

**Embryo collection and reverse transcription:** The method of reverse transcription and QRT-PCR was carried out as previously described (Li *et al*., 2006). Each five blastocysts of IVF, NT and NT treated with Scriptaid were collected and treated using a cells-to-cDNA$^{tm}$ kit (Ambion Co., Austin, TX) according to the manufacturer's instructions with minor modifications.

Briefly, blastocysts were washed three times with PBS and transferred into 10 μL of cell lysis buffer and incubated at 75°C for 10 min. The genomic DNA in the samples was digested using 1 μL DNase I with incubation at 37°C for 30 min. Finally, the DNase was inactivated at 75°C for 5 min. The Reverse Transcription (RT) reaction was performed using SuperScript TM II Reverse Transcriptase kit (invitrogen) according to the manufacturer's instructions.

The total reaction volume of 20 contained 2.5 μM $oligo_{18t}$ primers, 4 μL of reverse transcription buffer, 20 IU RNase inhibitor, 200 IU the M-MLV reverse transcriptase enzyme and 0.5 mM of each dNTP. Tubes were heated to 65°C for 5 min to denature the secondary RNA structure before adding the MLV.

The reaction mixture was then incubated at 25°C for 5 min 50°C for 60 min, followed by 95°C for 10 min to inactivate the RT enzyme. Afterward, sterile $H_2O$ was added to adjust the final volume of cDNA to 0.1 embryos per microliter.

**Quantitative real-time polymerase chain reaction:** Quantification of all transcripts was performed by real-time quantitative PCR using the ABI 7500 system (Applied Biosystems, Singapore). With ACTB as internal control gene, the expression pattern of HAT1, HADC2, OCT4 and PGK1 were assayed. Sequences and GenBank accession numbers of the primers used for amplification of the target genes are listed in Table 1. The PCR reaction mixture (20 μL) contained 10 μL of SYBR Premix EX TaqTM (2x) (Takara), 0.25 μM of each primer and 0.1-0.3 embryo in each tube. Thermal cycling conditions were 95°C for 5 min followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec followed extension stage at 72°C for 30 sec. All analyses were repeated with three replicates for each gene.

The comparative $C_T$ method was used for relative quantification of target gene expression levels (ABI Prism Sequence Detection System, Applied Biosystems). The relative quantification was normalized according to the internal control gene *ACTB*. Within the log-linear phase region of the amplification curve each difference of one cycle is equivalent to a doubling of the amplified product of the PCR.

The $\Delta C_T$ value was determined by subtracting the ACTB $C_T$ value for each sample from the target gene $C_T$ value of the assayed samples. Calculation of $\Delta\Delta C_T$ involved using the highest sample $\Delta C_T$ value as an arbitrary constant to subtract from all other $\Delta C_T$ sample values. Fold changes in the relative mRNA expression of the target were determined by using the formula $2^{-\Delta\Delta C_T}$.

Table 1: Primers used for teal-time polymerase chain reaction analysis

| Genes | Primers | Sequence (5'-3') | Accession number |
|---|---|---|---|
| *ACTB* | Forward | TGCGGGACATCAAGGAGA | DQ452569 |
| | Reverse | CAGGAAGGAGGGCTGGAA | |
| *HDAC2* | Forward | AAATACAGTCCATGCCAAAGTAGT | AY556465.1 |
| | Reverse | TTTTAACAGAGCCAAATCAGAAC | |
| *OCT4* | Forward | GTGTTCAGCCAAACGACCAT | NM_001113060 |
| | Reverse | TTGCCTCTCACTCGGTTCTC | |
| *PGK1* | Forward | TCGGGCTAAGCAGATTGTATG | AY677198 |
| | Reverse | GGCTGACTTTATCCTCCGTGT | |
| *HAT1* | Forward | TTCAAGCCATTTGGAACCTT | AY805696 |
| | Reverse | GCCATCTTTCATCATCCACG | |

**Immunofluorescence analysis of histone acetylation:** An immunofluorescence analysis was performed to examine the histone acetylation level according to the method reported (Yamanaka *et al*., 2009). Briefly, IVF, NT and NTS embryos at the 2, 4-cells and blastocyst stage were fixed with 4% paraformaldehyde in PBS supplemented with Polyvinyl Alcohol (PVA) for 1 h at Room Temperature (RT) and then washed in PBS containing 0.1% Tween 20 for 1 h and permeabilized with 0.5% Triton X 100 in PBS for 30 min. Thereafter, the embryos were blocked using PBS-PVA supplemented with 1% BSA at 4°C overnight. Afterward, the samples were incubated with acetyl-histone H3 lysine 18 prmary antibody (rabbit polyclonal antibody against histone H3 acetyl lysine 18, 1:100 dilution; Upstate, Millipore USA) for 1 h at 37°C. After washing in PBS with PBS-BSA they were incubated with the secondary antibody labeled with Fluorescent Isothiocyanate (FITC) (goat anti-rabbit lgG FITC-conjugated antibody, 1:100 dilution, Upstate, Millipore USA) for 30 min at 37°C. Then, the embryos were mounted on glass slides with a drop of mounting medium and observed under the confocal microscopy (Zeiss LSM510 Meta). The histone acetylation of H3K18 were analyzed and quantified according to the emission intensities of FITC by manually outlining a limited area of each individual nucleus. Two embryos were analyzed in each replicate and 5 replicates were performed for each type of embryos. Appropriate controls for autofluorescence and nonspecific binding by thesecondary antibodies were used to subtract the background from the arbitrary fluorescence measurements.

**Statistical analysis:** Within each experiment, the difference between treatments in frequencies of oocytes undergoing cleavage and developing to the blastocyst stage was analysed by $\chi^2$-test. The difference in the cell number of blastocysts was analyzed by ANOVA using SPSS 17.0. Relative quantification of target gene expression is presented as fold-difference and the difference was analyzed using SPSS 17.0 Software One-way repeated-measures analysis of variance followed by multiple pairwise comparisons using the Student-Newman-Keuls Multiple Comparisons test. The $p<0.05$ were considered to be significant.

## RESULTS AND DISCUSSION

***In vitro* development of porcine SCNT embryos treated with SCR:** As shown in Table 2, treatment of activated reconstructed SCNT embryos with 500 nM SCR for 12 h resulted in a significantly increase in the blastocyst development compared to untreated group (21.0% vs. 9.7%, $p<0.05$), although there was no significant difference in cleavage rates and cell number of blastocysts between the SCR treatment group and control group (77.4 vs. 72.6%).

Table 2: Effect of scriptaid treatment on *in vitro* developmental competence of porcine nuclear transfer embryos

| Groups | No. of embryos cultured | Replications | Cleavage (%) | Blastocyst (%) | No. of cells in blastocyst |
|---|---|---|---|---|---|
| NT | 113 | 3 | 82 (72.6)[a] | 11 (9.7)[a] | 45.3±2.6[a] |
| NTS | 124 | 3 | 96 (77.4)[a] | 26 (21.0)[b] | 49.0±4.2[a] |

NT represents no Scriptaid supplementation in culture medium whereas NTS represents 500 nM Scriptaid treatment for 12 h. Values with different superscripts in the same column are significantly different ($p<0.05$)

**Effects of SCR treatment on the histone acetylation level of SCNT embryos:** To understand the mechanism of SCR treatment in improving development competence of porcine SCNT embryos, researchers measured the level of histone acetylation H3K18 site of SCNT, SCR treated SCNT and *In Vitro* Fertilized (IVF) embryos at different developmental stages. As show in Fig. 1 and 2, treatment of SCNT embryos with 500 nM SCR for 12 h resulted in SCNT embryos at the 2 and 4-cells and blastocyst stage with higher acetylation level of H3K18 in comparison with untreated group and similar to fertilized counterparts as evidenced by Immunofluorenscence analysis.

**Effects of SCR on the expression of developmentally important genes in SCNT blastocysts:** To determine whether the beneficial effect of SCR on embryonic development is related to the expression pattern of developmentally important genes, the relative expression levels of *HAT1*, *HADC2*, *OCT4* and *PGK1* genes in blastocysts were measured using real-time PCR. As showed in Fig. 3, treatment of SCNT embryos with SCR resulted in an increase expression ($p<0.05$) in HAT1 and decrease expression ($p<0.05$) in HDAC2 and PGK1. A similar expression profile of these genes was found in blastocysts developed from SCR treated SCNT embryos and IVF embryos. There was no significant difference in the expression level of OCT4 among blastocysts developed from IVF, SCNT and SCR treated SCNT embryos.

The reprogramming events occur at the epigenetic level and one of the important epigenetic pathways is the global level of acetylation of the donor nuclei (Panda *et al*., 2012). Histone deacetylase inhibitors have been successfully used for treating the reconstructed SCNT embryos which could decrease the acetylation level and facilitate the reprogramming of the donor nuclear. It was reported that treatment of SCNT embryos with TSA, a HADCi can improve their subsequent developmental

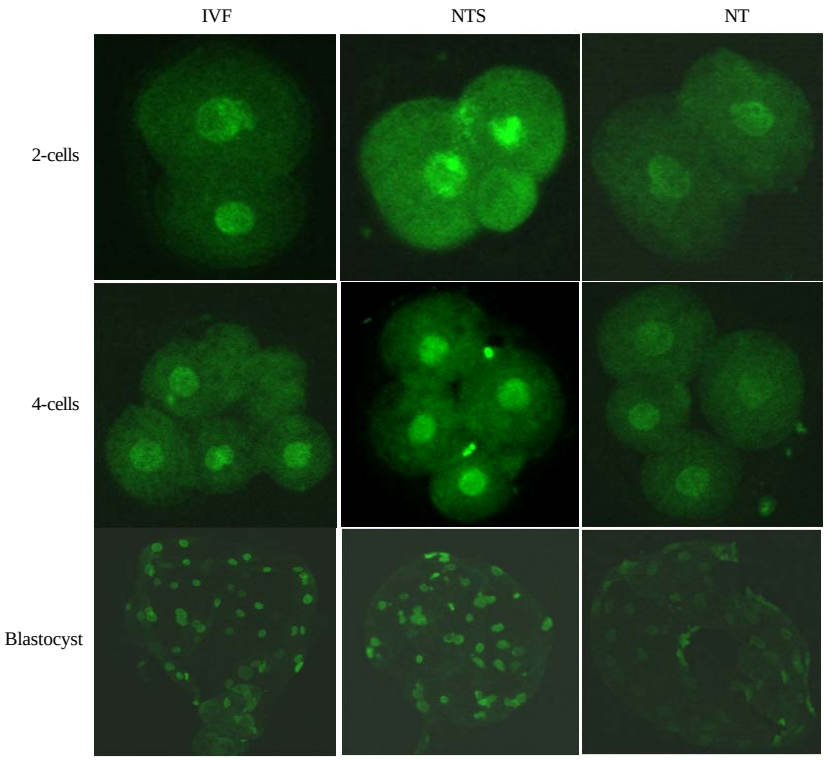


Fig. 1: Confocal images of IVF, NTS and NT embryos at different stages of development

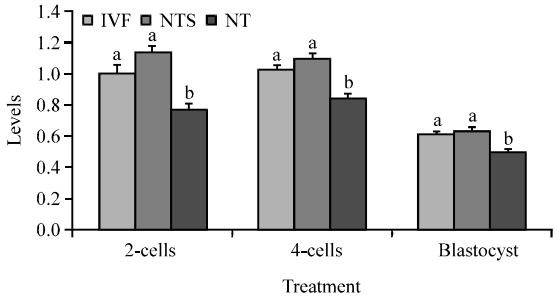


Fig. 2: Comparisons of ACH3K18-FITC intensity between IVF, NTS and NT embryos at different developmental stages

competence and animal cloning efficiency in mice, cattle, pigs and rabbits (Kishigami *et al*., 2006; Shi *et al*., 2008; Yamanaka *et al*., 2009; Akagi *et al*., 2011). However, the actions of TSA in a variety of other species remained contentious some reports indicated that treatment of reconstructed embryos with TSA could cause side-effects both *in vitro* and *in vivo* (Wu *et al*., 2008; Meng *et al*., 2009; Tsuji *et al*., 2009). Scriptaid is a novel lower toxicity HDACi and has been proved to improve the histone acetylation and gene transcription greatly in somatic cells (Su *et al*., 2000). Using pig fetus as donor for somatic cell nuclear transfer, treatment of porcine fetal fibroblasts with SCR resulted in a significant increase in the blastocyst formation after nuclear transfer (Zhao *et al*., 2009). A similar result was also achieved by treatment of donor cells with 5 nM SCR in bovine SCNT (Akagi *et al*., 2011). In the present study, researchers found that treatment of porcine reconstructed embryos with SCR for 12 h resulted in a significant, increase in the blastocyst yield and cell number of blastocysts developed, further demonstrating the beneficial action of SCR in promoting the reprogram of SCNT embryos.

Although, SCR has been used as HDACi to improve the development competence of SCNT embryos, the exact mechanism of scriptaid in improving cloning efficiency is still unclear. Previous studies indicated that the eighteenth lysine is a major acetylation site of histone H3 N-terminal (Thorne *et al*., 1990; Kelly *et al*., 2000) and H3K18 acetylation level can be served as a reasonable markers related to the developmental competence of SCNT embryos (Santos *et al*., 2003; Li *et al*., 2008). Yamanaka *et al*. (2009) found that treatment of miniature pig NT embryos with TSA resulted in an increase in the blastocyst yield and histone acetylation of NT embryos at the pronuclear and 2-cells stage and similar acetylation level to the IVF embryos. In the present study, researchers treated porcine SCNT embryos with 500 nM

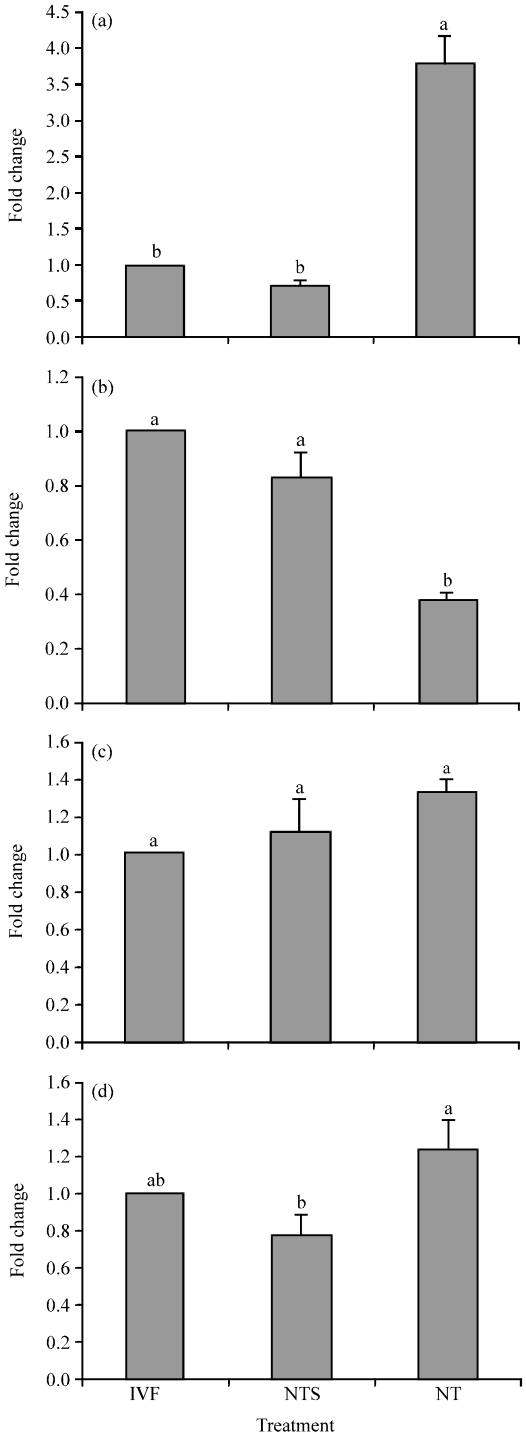


Fig. 3: Expression profiles of HDAC2, HAT1, OCT4, REX01 and PGK1 on IVF, NTS and NT embryos at the blastocyst stage. Quantification of gene expression levels is displayed as the mean±SEM. [a,b]Superscripts denote significant differences ($p<0.05$). a) HADC2; b) HAT1; c) OCT4 and d) PGK1

SCR for 12 h following activation and found that the H3K18 acetylation level of porcine SCNT embryos at the 2 and 4-cells and blastocyst stage was increased and similar to the IVF counterparts which was consistent with the report by Cervera *et al*. (2009). This result indicates that the beneficial action of SCR in facilitating the reprogram and development competence of SCNT embryos is mediated through increasing the H3K18 acetylation level.

The cells acetylation status of Histone terminals keep a dynamic balance under the regulation of HATs and HDACs which is associated with the chromatin structure and DNA transcription of the donor cells (Lee *et al*., 1993). Treating *in vitro* culture cells with HDACi resulted in an increase in their acetylation level and changes in their overall transcription pattern (Smith, 2008). It was reported that the expression pattern of genes implicated in epigenetic modification and pluripotency was alteration in the SCNT pig embryos in comparison with the *in vivo* or *in vitro* fertilized embryos (Kumar *et al*., 2007). Treatment of porcine SCNT embryos with TSA resulted in a decrease expression level of HDAC2 which was similar to the IVF embryos (Cervera *et al*., 2009). In the study, treating reconstructed porcine embryos with SCR resulted in high expression level of HDAC2 and low expression level of HAT1 in the blastocysts developed which was similar to the expression pattern of both genes in the IVF blastocysts.

*Phosphoglycerate kinase* is one of the X-chromosome linked genes which can catalyze 1,3-diphosphoglycerate into 3-phosphoglycerate and generate ATP for the energy metablism of living cells (Opperdoes, 1987). In the present study, researchers found that the PGK1 expression level of SCR treated SCNT blastocysts was significantly lower than that of the untreated SCNT blastocysts and similar to the IVF embryos indicating that the energy metablism during the early embryogenesis is relative lower compared to the somatic cells.

## CONCLUSION

The SCR treatment can improve the development competence of porcine SCNT embryos and result in a similar expression pattern of genes related to histone acetylation and acetylation level of histone compared to IVF embryos.

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