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Comparison Between the Effects of Valproic Acid and Trichostatin A on *in vitro* Development of Sheep Somatic Cell Nuclear Transfer Embryos

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Abstract: The present study was carried out to assess and compare the effects of Valproic Acid (VPA) and Trichostatin A (TSA) on *in vitro* development of sheep Somatic Cell Nuclear Transfer (SCNT) embryos. The results showed that treatment of cloned sheep embryos with 4 mM VPA or 50 nM TSA for 24 h after activation could significantly improve blastocyst rate compared to the control (30.7 vs. 23.3 vs. 16.7%, respectively $p<0.05$). VPA treatment resulted in a significant higher blastocyst rate than that of TSA-treated group ($p<0.05$). Moreover, VPA treatment significantly increased ($p<0.05$) total cell number per blastocyst compared with the TSA treatment and control groups (78.8±9.3 vs. 69.6±9.7 vs. 64.1±8.6, respectively). Furthermore, VPA treatment increased expression of the development-related genes OCT4 and SOX2 in SCNT blastocysts. These results demonstrate that VPA may be more potent than TSA in supporting developmental competence of cloned embryos.

Key words: Valproic acid, Trichostatin A, sheep, SCNT embryo, development-related genes

INTRODUCTION

Although, generation of transgenic animal by Somatic Cell Nuclear Transfer (SCNT) has great potential applications in agriculture and regenerative medicine, there is a high incidence of abnormalities in SCNT clones. In livestock, cloning by SCNT is inefficient with <5% of blastocysts developing through to term, the losses being mostly due to placental deficiency (Yang *et al*., 2007). These problems may be attributed to incomplete reprogramming of transferred donor nuclei which cause some errors at epigenetic level (Kang *et al*., 2001, 2003; Santos *et al*., 2003). The nuclear reprogramming process mainly involves various epigenetic modifications such as DNA and histone modifications. Therefore, an intervention to assist reprogramming of the donor nuclei may enhance the cloning success rate in livestock animals.

Recently, some epigenetic remodeling drugs such as the Histone Deacetylase inhibitors (HDACi) Trichostatin A (TSA) (Kishigami *et al*., 2006; Zhang *et al*., 2007; Maalouf *et al*., 2009; Ono *et al*., 2010) have been used to improve development of cloned embryos. However, not all studies found beneficial effects by treatment of SCNT embryos with DNA methylation inhibitor: 5-aza-2-deoxycytidine (Enright *et al*., 2003; Shi *et al*., 2003) or the histone deacetylase inhibitor TSA (Shi *et al*., 2003).

Valproic Acid (VPA) an HDAC inhibitor with low toxicity that induce reprogramming of mouse fibroblasts with only three transcription factors with a considerably higher efficiency than TSA (Huangfu *et al*., 2008). VPA has been showed to improve the *in vitro* and full-term development of B6CBAF1 SCNT embryos at a similar level as TSA (Costa-Borges *et al*., 2010). VPA has been reported to enhance development of miniature pig SCNT embryos into blastocysts (Miyoshi *et al*., 2010). However, until now it is not yet known whether HDACi can also improve the development of SCNT embryos in sheep. Consequently, the present study was designed to examine and compare the effect of TSA and VPA treatment on *in vitro* developmental competence of sheep cloned embryos.

MATERIALS AND METHODS

Somatic cell culture: Sheep fibroblast cells were isolated from 40 days old fetuses as previously described (Hu *et al*., 2011). The skin tissue pieces were implanted into the 25 mL tissue culture flask in Dulbacco's Modified Eagels Medium (DMEM) (HyClone) with 10% Foetal Bovine Serum (FBS) (Invitrogen) in CO_2 incubator at 37°C. For SCNT, the cells were allowed to grow to confluency and continued to culture for an additional 5-6 days without a change of medium. A suspension of single cell

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was prepared by trypsinization of the cultured cell followed by resuspension in manipulation medium before SCNT.

Oocyte collection and *in vitro* maturation: Ovaries were collected from a local abattoir and transported to the laboratory within 4 h after slaughter. Cumulus-Oocyte Complexes (COCs) were aspirated from 2-5 mm follicles with PBS (containing 5% FCS) by using a 5 mL syringe fitted with a 20 gauge needle. The COCs were cultured in maturation medium (TCM-199 containing 10% foetal bovine serum, 5, FSH, 5 LH, 1 μg mL^{-1} estradiol, 0.3 mM sodium pyruvate and 100 μM cysteamine) at 38.5°C in a humidified atmosphere for 22 h. Thereafter, cumulus cells were removed by 1 mg mL^{-1} hyaluronidase treatment. Oocytes with a first polar body were used for further experiments.

Production of SCNT embryos: Oocytes were enucleated manually in the presence of 7.5 μg mL^{-1} of cytochalasin B following treatment for 15 min in 10 μg mL^{-1} of Hoechst 33342 and 7.5 μg m^{-1} of Cytochalasin B. A single intact donor cell was injected into the perivitelline space and placed adjacent to the recipient cytoplasm. After injection, reconstructed embryos were transferred into an electrical fusion chamber overlaid with Zimmermann's fusion medium. Cell fusion was induced with two direct current pulses (1.0 kV cm^{-1}, 60 μ sec, 1 sec apart). All fused reconstructed embryos were further activated in 5 μM ionomycin for 4 min followed by exposure to 1.9 mM 6-dimethylaminopurine in Synthetic Oviduct Fluid with amino acids (SOFaa) for 4 h. Following activation, reconstructed embryos were transferred and cultured in SOFaa medium until the two-cell or blastocyst stages.

Experimental designs

Experiment 1: To optimize concentration of VPA treatment on preimplantation development of reconstructed embryos. Immediately following ionomycin treatment, SCNT embryos were incubated for 4 h in SOFaa medium with 1.9 mM 6-dimethylaminopurine containing 0, 2, 4, 6, 8 mM VPA. SCNT embryos were then incubated for another 20 h in SOFaa medium supplemented with 0, 2, 4, 6 and 8 mM VPA. After each treatment, the embryos were washed twice and then cultured in SOFaa medium until the two-cell or blastocyst stages.

Experiment 2: Comparison between the effects of VPA and TSA on *in vitro* development of sheep SCNT embryos. About 50 nM TSA treatment have been showed to significantly improve *in vitro* development of cloned embryos in mice (Kishigami *et al*., 2007) and pig (Zhang *et al*., 2007; Beebe *et al*., 2009). About 4 mM VPA and 50 nM TSA treatment was added to SOFaa after ionomycin treatment for 20 h. The cleavage rate, blastocyst formation and the total cell number per blastocyst were monitored and recorded after each treatment. SCNT embryos that were not exposed to TSA or VPS were used as control.

Experiment 3: The relative expression levels of development-related genes (*OCT4, Nanog* and *SOX2*) in blastocysts were compared between VPA treatment and control groups.

Blastocyst differential staining: The differential staining and counting of Inner Cell Mass (ICM) and Trophectoderm (TE) cells in cloned blastocysts were performed as described by Thouas *et al*. (2001). Briefly, blastocysts were first incubated in Solution I (SOFaa plus 1% Triton X and 100 μg mL^{-1} propidium iodide) until TE cells showed a visible change to the color red and became slightly shrunken. Embryos were then transferred to Solution II (100% absolute ethanol plus 25 μg mL^{-1} Hoechst 33342) and incubated at 4°C for 12 h. Fixed and stained blastocysts were then washed in a drop of glycerol and mounted on a glass microscope slide in a drop of glycerol and flattened with a coverslip. Under an inverted microscope equipped with fluorescence, the total cell number of blastocyst were counted.

Quantitative RT-PCR: The cDNAs of single embryos were synthesized with qRT-PCR miRNA Detection kit (Ambion). Real-time PCR was performed using an Applied Biosystems 7500 sequence detection system. The primer sequences were as follows. For OCT4, 5'-CAA TTT GCC AAG CTC CTA AA-3' and 5'-TTG CCT CTC ACT TGG TTC TC-3'; for SOX2, 5'-CGG CAG CTA CAG CAT GAT-3' and 5'-AGA AGA GGT AAC CAC AGG G-3'; for Nanog 5'-TGA CAG ATT TCA GAG GCA G-3' and 5'-TCC AGG AGA GTT CAC CAA G-3'. Reactions were incubated in a 96 well optical plate at 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 52°C for 1 min. All experiments were done in triplicates and repeated three times. About 5S small RNA was used as an internal control to normalize miRNA input in the real-time RT-PCR assay.

RESULTS AND DISCUSSION

Experiment 1: To assess whether VPA treatment could improved *in vitro* development of sheep SCNT embryos, we treated SCNT embryos with different concentrations of VPA for 24 h and compared rate of cleavage and

blastocyst formation. There were no significant differences in the cleavage rates (69.3-75.1%) among the different concentrations of VPA (Table 1). However, the blastocyst rate of embryos treated with 2-6 mM VPA (24.5-29.3%) was significantly ($p<0.05$) higher than those of embryos treated with 0 or 10 mM VPA (17.3 and 18.1%). There were no significant differences among 2-6 mM VPA treatment but 4 mM VPA treatment supported the highest developmental competence of sheep SCNT embryos.

Experiment 2: Researchers compared the effects of TSA and VPA treatment on *in vitro* developmental competence of sheep SCNT embryos (Table 2). Reconstructed oocytes treated with 50 nM TSA and 4 mM VPA after oocyte activation showed similar cleavage rates (72.4 vs. 73.2%). However, VPA treatment (30.7%) resulted in a significant higher ($p<0.05$) blastocyst rate than TSA treatment (23.3%) and control groups (16.7%). Moreover, VPA treatment significantly increased ($p<0.05$) total cell number per blastocyst compared with the control (78.8±9.3 vs. 64.1±8.6) whereas no differences were observed between the TSA treatment and control groups (69.6±9.7 vs. 64.1±8.6).

Experiment 3: Relative expression levels of three development-related genes were analyzed in VPA-treated and control blastocysts using quantitative RT-PCR (Fig. 1). The expression levels of OCT4 and SOX2 were significantly higher in VPA-treated blastocyst than that in control blastocyst ($p<0.05$). There were no significant differences in the expression of Nanog between VPA-treated and control blastocysts.

Recently investigations have focused on the ability of histone deacetylase inhibitors to improve SCNT efficiency. SCNT embryos treated with the TSA have a remarkable and significant increase in the developmental competence of SCNT embryos in mouse, pig, cattle and rabbit. However, Li *et al.* (2008) reported that five of TSA-treated six cloned piglets died during delivery and the remaining one piglet survive for only 2 months. Meng *et al.* (2009) reported no differences in the cleavage and blastocyst rates or the blastocyst cell numbers between TSA-treated and untreated SCNT rabbit embryos. Considering the detrimental effects of TSA on the cloning procedure, VPA an HDAC inhibitor with low toxicity that might be a suitable an alternative to TSA for epigenetic reprogramming of nuclear donor cells in SCNT. Recently, Costa-Borges reported that VPA haved a similar effect than TSA on cloning efficiency but females produced from VPA-treated embryos survived to adulthood and showed more normal phenotype and reproductive ability. Hence, researchers examine whether VPA treatment can improve *in vitro* development of sheep SCNT embryos.

In this study, researchers demonstrated that VPA can also improve the *in vitro* developmental potential and the blastocyst quality of sheep SCNT embryos. In Experiment 1, we defined the most effective concentration of VPA treatment for improving the development of sheep SCNT embryos. As shown in Table 1, 2-6 mM VPA treatment significantly increased blastocyst rate of SCNT embryos but blastocyst development was reduced at the 8 and 10 mM VPA treatment. It is possible that a high concentration of VPA treatment may cause negative effect on development of SCNT embryos as previously described in TSA treatment (Tsuji *et al.*, 2009). In Experiment 2, we compared the effects of 4 mM VPA with that of 50 nM TSA on *in vitro* development of sheep SCNT embryos. As shown in Table 2, treatment of SCNT

Table 1: Effect of different concentration of VPA on the in vitro development of cloned sheep embryos

Treatments (mM)	No. of reconstructed	No. of cleaved embryos (%)	No. of blastocysts (%)
0	190	132 (69.3±2.4)[a]	39 (17.3±2.8)[a]
2	201	145 (72.5±2.1)[a]	53 (25.7±2.5)[b]
4	175	129 (73.5±3.1)[a]	51 (29.3±2.4)[b]
6	183	136 (73.8±2.7)[a]	45 (24.5±2.2)[b]
8	187	139 (74.3±2.6)[a]	38 (20.7±1.9)[a]
10	194	146 (75.1±2.2)[a]	35 (18.1±1.7)[a]

Table 2: Effects of VPA and TSA treatment on *in vitro* development of cloned sheep embryos

Treatments	No. of reconstructed	No. of cleaved embryos (%)	No. of blastocysts (%)	Total cell No. in blastocysts
Control	184	126 (68.9±2.4)[a]	31 (16.7±2.8)[a]	64.1±8.6[a]
TSA	176	128 (72.4±3.1)[a]	41 (23.3±2.4)[b]	69.6±9.7[a]
VPA	189	139 (73.2±3.1)[a]	59 (30.7±2.4)[c]	78.8±9.3[b]

Three replicate experiments were performed per treatment. Numbers in parentheses represent development rates (mean±SEM%). Values with different superscripts within the same column differ significantly between treatments ($p<0.05$)

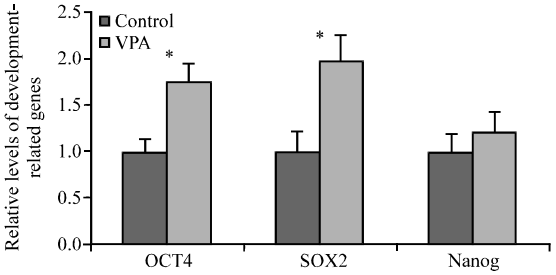

Fig. 1: Relative expression levels of development-related genes. Relative levels of development-related genes (*OCT4*, *SOX2* and *Nanog*) in VPA-treated blastocysts (gray bars) and control (open bars). *Statistically significant difference ($p<0.05$) with control

embryos with VPA (1.8 fold) or TSA (1.4 fold) could significantly improve blastocyst rate compared to the control ($p<0.05$). In addition, VPA treatment significantly improved the total cell number per blastocyst compared with the TSA and control groups ($p<0.05$).

OCT4, SOX2 and Nanog have prominent roles during embryo development, pluripotency and self-renewal of ES cells (Niwa *et al*., 1998; Avilion *et al*., 2003; Chambers *et al*., 2003). Here, we measured the relative expression levels of OCT4, Nanog and SOX2 and found that VPA treatment promoted expression of OCT4 and SOX2 in SCNT blastocysts. Both development-related genes *OCT4* and *SOX2* are important for maintaining embryo fate. Therefore, the up-regulated expression of these two genes in the treated blastocysts may be associated with the higher total cell number per blastocyst.

CONCLUSION

In this study, the results firstly demonstrate that VPA can improve *in vitro* development of sheep SCNT embryos may be more potent than TSA in supporting developmental competence of cloned embryos. These results may represent an strategy for enhancing the *in vitro* development of sheep SCNT embryos.

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