The effect of valproic acid on bovine oocyte maturation and early embryonic development in vitro

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Received: 21 January 2013 / Accepted: 8 June 2013 / Published online: 10 July 2013
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Abstract Our objective is to investigate the effect of valproic acid (VPA), a histone deacetylase inhibitor, on early embryonic development. We studied the effect of VPA on the in vitro maturation of bovine oocytes, and on the development of bovine embryos derived from in vitro fertilization (IVF) or parthenogenesis. Germinal vesicle stage bovine oocytes were cultured with different concentrations of VPA for 24 h; low dose VPA treatment (0.03 and 0.3 mM) had no effect on oocyte maturation, but 3 and 6 mM VPA significantly decreased maturation rate; when used for IVF or parthenogenesis, VPA-treated oocytes generated significantly lowered blastocyst rate. Oocytes matured in vitro were fertilized or underwent parthenogenetic activation; 6 h later, they were exposed to VPA for 48 h, and then the cleavage rate, blastocyst rate and mRNA expression levels of transcription factors (*Oct4*, *Nanog*, and *Cdx2*) were assessed. For embryos cultured in 0.3 mM VPA, there was no remarkable change in cleavage rate or blastocyst rate, but the expression of *Oct4* and *Nanog* in blastocysts was significantly increased. For embryos treated with 3.0 mM VPA, the cleavage rate and blastocyst rate were significantly decreased. In conclusion, low dose

VPA has no effect on oocyte maturation but affects subsequent embryonic development. Low dose VPA administration to IVF embryos had no effect on embryonic development, but the expression of several important transcription factors was increased. Treatment of IVF embryos with low dose VPA may improve their development potential.

Keywords Valproic acid · Oocyte maturation · Parthenogenesis · In vitro fertilization

Introduction

Since 1997, when a cloned sheep named Dolly was created by somatic cell nuclear transfer, various other cloned animals have been produced by using the same method (Lorthongpanich et al. 2010). This technique is of significant importance in basic research and biomedicine; however, there are still some limitations, such as the low developmental rate of cloned embryos, heteroplasia of placentas, high rates of miscarriage and the occurrence of congenital birth defects. It is widely recognized that the main reason for these defects is the insufficient epigenetic reprogramming during early embryogenesis, the underlying mechanisms of which are not yet clear. In recent years, it has been found that some small molecules possess epigenetically regulatory activities that can improve cloning efficiency and the quality of cloned embryos (Wu et al. 2008). The proper screening and characterization of these

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small molecules to determine their function will provide an important basis for improving the somatic cell nuclear transfer technique.

Valproic acid (VPA), a synthetic short chain fatty acid composed of eight carbon atoms, has been used for the treatment of epilepsy since the nineteenth century (Phiel et al. 2001). Mechanistic studies have revealed that VPA is a histone deacetylase inhibitor (HDACi), with the specific inhibiting activity against histone deacetylases (HDACs), thereby promoting the acetylation of cellular histones with little toxicity or side effects (Chen et al. 2011). In support of this, it was found that VPA can be used to treat certain cancers via its inhibitory activity of HDACs (Wang et al. 2011). Furthermore, it was also found that VPA treatment leads to the up-regulation of *p21WAF1/CIP1* and down-regulation of *Cdk2*, with a resultant inhibitory effect on the cell cycle (Ocker and Schneider-Stock 2007). With respect to nerve cell repair, VPA was found to have a protective effect towards damaged nerve cells, being able to increase the survival rate of retinal ganglion cells in rats (Penas et al. 2011). VPA can directly reprogram human fibroblasts into induced pluripotent stem cells without transfection of transcription factors, such as *Klf4* and *c-myc* (Huangfu et al. 2008). Furthermore, it has been found that VPA treatment can increase the expression of pluripotency genes in murine myocytes (Teng et al. 2010) and bovine adipose stem cells (Addison et al. 2011), which indicates that VPA promotes the maintenance of a pluripotent state. VerMilyea et al. found that VPA did not affect the developmental rate of cloned embryos, but delayed embryonic development in mice (VerMilyea et al. 2009); however, Miyoshi et al. (Miyoshi et al. 2010) found that VPA could improve the blastocyst rate in their study on cloning miniature pigs.

To date, research on VPA has mainly focused on the influence of treating donor cells with VPA on the development of cloned embryos and the dynamic change of histone acetylation, with less emphasis on the effect of VPA treated oocytes and embryos in vitro. In this study, using oocyte in vitro maturation, in vitro fertilization (IVF) and parthenogenetic activation, we aimed to investigate the effect of VPA, on bovine oocyte maturation and early embryonic development. The results of this study provide an improved understanding of how VPA influences early embryonic development, with potential applications in the creation of cloned bovine embryos.

Materials and methods

Reagents

Oocyte maturation medium (TCM199) was purchased from Gibco-BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China). RNAiso Reagent, RT-PCR kit and SYBR green master mix were from TaKaRa (Dalian, China). Caffeine, D-Glucose, D-Lactose, Na-Citrate·2H₂O, Na₂HPO₄, K₂HPO₄, NaHCO₃, NaCl and KCl were purchased from Wako (Osaka, Japan). Unless otherwise mentioned, all other reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

In vitro oocyte collection and maturation

Fresh bovine ovaries were collected from abattoir, placed into sterilized normal saline (35 °C) and brought to our laboratory within 3 h. A 10-mL syringe with an 18 gauge needle was used to aspirate follicles (2–8 mm) from the ovary surface. Cumulus oocyte complexes (COCs) were gathered from follicular fluid using a stereomicroscope and were transferred into TCM199 (with 0.38 mmol/L sodium pyruvate, 10 µg/mL FSH, 5 µg/mL LH, 1 µg/mL 17-β estradiol, 50 U/mL penicillin, 100 µg/mL streptomycin and 10 % FBS) after washing three to four times. 80–100 COCs were placed into 1 mL pre-equilibrated TCM199 overlaid with mineral oil in a well of a 4-well plate. Oocyte maturation was conducted under the conditions of 38.5 °C, 5 % CO₂ and saturated humidity.

In vitro fertilization

Frozen breeding bull semen (Livestock Improving Station, Inner Mongolia, China) was thawed in a 37 °C water bath, and washed twice by centrifugation (5 min at 3,500 rpm) using BO liquid containing 10 mM caffeine. After washing two times by centrifugation, floating sperm were carefully aspirated and incubated in an equal volume of BO with 3 mg/mL BSA and 8 µL/mL heparin. Sperm was then counted using a microscope. The sperm suspension was aliquoted into 100 µL droplets, one of which was added into cultured droplets containing 15–20 mature oocytes. Six to seven hours after fertilization, cumulus

cells were removed, and the fertilized eggs were placed into 40 μ L droplets of maturation medium (CR1 plus 0.8 % FBS). Cleavage and blastocyst rate was determined on the 2nd and 6th to 8th day of culture, respectively.

Parthenogenetic activation

COCs matured in vitro for 22 h were digested with 0.1 % hyaluronidase to remove cumulus cells. After washing three times with maturation medium, oocytes that had extruded first polar bodies and had even cytoplasm and good morphology were collected for further use. Selected oocytes were placed into the development medium containing 5 μ M ionomycin for 5 min, and they were then incubated for 6 h with CR1 medium containing 10 μ g/mL 6-dimethylaminopurine, at 38.5 °C, in 5 % CO₂ and saturated humidity. Lastly, activated oocytes were washed three times with CR1 medium, and then transferred into 40 μ L droplets of CR1 medium covered with mineral oil and cultured at 38.5 °C, in 5 % CO₂ with saturated humidity. The cleavage and blastocyst rates were examined on the 3rd and 6th to 8th day of culture, respectively.

Feeder layer preparation

Bovine cumulus cells were used as feeder layers. After in vitro maturation, COCs were digested with 0.1 % hyaluronidase to release cumulus cells. The cumulus cells were collected, washed three times with DMEM medium by centrifugation, and then cultured in DMEM medium containing 10 % FBS. Cells at passage 7–8 were collected by trypsinization and suspended in 10 % FBS-DMEM medium to a density of 1.0×10^4 /mL. Aliquots of 40 μ L cell suspension were placed under mineral oil in 60 mm culture dishes. Before embryo culture, the DMEM medium was replaced with CR1 medium.

Real-time quantitative PCR

Total RNA from embryos in different treatment groups were extracted using TaKaRa RNAiso reagent. In brief, the embryos were lysed in RNAiso reagent and total RNA was extracted by chloroform, which was then washed using 75 % alcohol and dissolved in

nuclease-free water. The concentration and quality of total extracted RNA was then calculated by ultraviolet spectrophotometry. Isolated total RNA was reverse-transcribed into cDNA, which was subsequently used for real-time quantitative PCR. The reference gene *Gapdh* was used to normalize the relative expression levels of the transcription factors, *Oct4*, *Nanog*, and *Cdx2*. The respective threshold value for each gene was determined and the relative transcriptional level between groups was calculated using the $2^{-\Delta\Delta Ct}$ method. The PCR cycling conditions were as follows: 40 cycles of 4 °C for 30 s, 94 °C for 5 s, and 60 °C for 31 s. A total reaction volume of 20 μ L was used for all PCR reactions. All primer sequences are shown in Table 1. Primers were designed using the bovine mRNA sequences of *Oct4*, *Nanog* and *Cdx2* obtained from GenBank, using Primer Premier 3.0 software. Primers were synthesized by Dalian TaKaRa Biotechnology Co. Ltd.

Experimental design

Experiment one: germinal vesicle (GV) stage bovine oocytes were treated with different concentrations (0, 0.03, 3 and 6 mM) of VPA for 24 h to determine the effect of VPA on the maturation of bovine oocytes. This initial experiment was used to determine what dose of VPA to be used for follow-up studies. Bovine oocytes treated with VPA were subsequently used for studies on its effect the development of embryos produced by IVF or parthenogenetic activation in vitro.

Experiment two: Oocytes matured in vitro were used for IVF. After 6 h of fertilization, granular cells were removed and the IVF embryos were put into CR1 droplets with different concentrations of VPA and incubated for 48 h. Then, after washing 3 times in normal CR1 medium, the embryos were cultured in normal CR1 medium containing a feeder layer for a further 6 days for the purpose of determining blastocyst rate.

Experiment three: Oocytes matured in vitro were used for parthenogenetic activation. After 6 h of activation, the parthenogenetic embryos were placed into CR1 droplets with different concentrations of VPA and incubated for 48 h. Then, after washing 3 times in normal CR1 medium, they were cultured in normal CR1 medium containing a feeder layer for 6 days for the purpose of detecting the blastocyst rate.

Table 1 Primer sequences

Gene	Primers	Length (bp)
Oct4-F	AGTAGGTTGGGTAGAGGGGTATTAG	184
Oct4-R	AAATTAATAAAATCTCTATAAAAAA	
Nanog-F	ATAATGGTTTTGGTGAGATTGGTAG	
Nanog-R	ATAAACTCAACCATACTTAACCCC	161
CDX2-F	AAGACAAATACCGGGTCGTG	
CDX2-R	CTGCGGTTCTGAAACCAAAT	
GAPDH	5'-TTCAACGGCACAGTCAAGG-3'	119
GAPDH	5'-ACATACTCAGCACCAGCATCAC-3'	

Results

Effect of VPA treatment on bovine oocyte maturation in vitro and early embryonic development

Bovine GV oocytes were in vitro matured in medium containing 0, 0.03, 0.3, 3 and 6 mM VPA. We found that the maturation rate decreased after 24 h treatment with increasing concentrations of VPA. Low doses of VPA (0.03 and 0.3 mM) had no significant effect on the maturation rate; however, high doses of VPA (3 mM and 6 mM) significantly decreased the maturation rate ($p < 0.01$, Table 2). Treatment of oocyte with 6 mM VPA also greatly reduced cleavage rate and abolished the development to blastocyst after IVF, indicating its toxic effect on bovine oocytes at this concentration (Table 3). Furthermore, the majority of bovine oocytes died after the application of 12 mM VPA. Based on the above results, follow-up experiments were conducted using 0.3 and 3 mM VPA.

Effect of VPA treatment of bovine oocytes on early embryonic development

The oocytes in vitro-matured in the presence of 0, 0.3 and 3 mM VPA were subjected to IVF. VPA treatment significantly lowered the cleavage and blastocyst rates. Specifically, the cleavage rate of the 0.3 mM VPA-treated group was significantly lower than that of control group ($p < 0.05$), as was the blastocyst rate ($p < 0.01$). Both the cleavage rate and blastocyst rate of 3 mM VPA-treated oocytes were significantly lower than those of control group ($p < 0.01$, Table 4).

The 0, 0.3 and 3 mM VPA-treated oocytes were also subjected to parthenogenetic activation, and the cleavage and blastocyst rates were examined.

Table 2 Effect of VPA treatment on the maturation rate of bovine oocytes—dose response analysis

Concentration (mM)	No. of oocytes	No. of matured oocytes	Maturation rate (%)
0.00	435	327	75.17 % $\pm$ 0.005 ^a
0.03	323	240	74.23 % $\pm$ 0.004 ^a
0.30	243	180	74.15 % $\pm$ 0.004 ^a
3.00	361	210	58.17 % $\pm$ 0.007 ^b
6.00	338	152	45.00 % $\pm$ 0.048 ^b

Values with different superscripts in the same column differ significantly ($p < 0.05$). Data are means from three individual experiments

Table 3 Effect of treating GV oocytes with 6.00 mM VPA for 24 h on cleavage rate of IVF embryos

Treatment	No. of oocytes	No. of cleavage	Cleavage rate (%)
0 mM 24 h	415	263	63.21 % $\pm$ 0.010 ^a
6 mM 24 h	445	124	28.41 % $\pm$ 0.083 ^b

Values with different superscripts in the same column differ significantly ($p < 0.05$). Data are means from three individual experiments

Compared with that of control group, the cleavage rate of the 0.3 mM VPA-treated group was not significantly changed, but the blastocyst rate was significantly decreased ($p < 0.01$). Both the cleavage rate and the blastocyst rate of the 3 mM VPA-treated group were significantly lower than those of control group ($p < 0.01$, Table 5).

Effect of 48 h VPA treatment on bovine early embryonic development

Bovine embryos derived from IVF were treated by VPA for 48 h, and then cleavage and blastocyst rates

Table 4 Effect of treating GV oocytes with VPA for 24 h on embryonic development after in vitro fertilization

Concentration (mM)	No. of oocytes	No. of cleavage	Cleavage rate (%)	No. of blastocysts	Blastocyst rate (%)
0.00	505	325	64.36 % $\pm$ 0.010 ^a	112	34.46 % $\pm$ 0.005 ^a
0.30	440	255	57.95 % $\pm$ 0.011 ^b	34	13.33 % $\pm$ 0.006 ^b
3.00	488	182	37.30 % $\pm$ 0.021 ^b	13	7.20 % $\pm$ 0.004 ^b

Values with different superscripts in the same column differ significantly ($p < 0.05$). Data are means from three individual experiments

Table 5 Effect of treating GV oocytes with VPA for 24 h on embryonic development after parthenogenetic activation

Concentration (mM)	No. of oocytes	No. of cleavage	Cleavage rate (%)	No. of blastocysts	Blastocyst rate (%)
0.00	244	223	91.39 % $\pm$ 0.027 ^a	85	38.12 % $\pm$ 0.013 ^a
0.30	154	132	85.71 % $\pm$ 0.016 ^a	20	15.15 % $\pm$ 0.002 ^b
3.00	275	149	54.44 % $\pm$ 0.011 ^b	16	5.56 % $\pm$ 0.02 ^b

Values with different superscripts in the same column differ significantly ($p < 0.05$). Data are means from three individual experiments

Table 6 Effect of VPA treatment after in vitro fertilization on embryonic development

Concentration (mM)	No. of oocytes	No. of cleavage	Cleavage rate (%)	No. of blastocysts	Blastocyst rate (%)
0.00	210	164	78.10 % $\pm$ 0.006 ^a	84	51.21 % $\pm$ 0.003 ^a
0.30	273	206	75.46 % $\pm$ 0.007 ^a	105	50.97 % $\pm$ 0.017 ^a
3.00	257	146	56.81 % $\pm$ 0.008 ^b	26	17.81 % $\pm$ 0.005 ^b

Values with different superscripts in the same column differ significantly ($p < 0.05$). Data are means from three individual experiments

were assessed. 0.3 mM VPA treatment had little effect on the cleavage rate and blastocyst rate; however, 3 mM VPA treatment significantly decreased cleavage rate and blastocyst rate ($p < 0.01$, Table 6).

Bovine embryos obtained by parthenogenetic activation were also treated by VPA for 48 h, and then cleavage and blastocyst rates were assessed. 0.3 mM VPA treatment resulted in a lower cleavage rate and a slightly higher blastocyst rate than control group. 3 mM VPA treatment generated a significantly negative effect on both cleavage rate and blastocyst rate ($p < 0.01$, Table 7).

Effect of VPA treatment on Oct4, Nanog and Cdx2 mRNA expression level

The expression levels of *Oct4*, *Nanog* and *Cdx2* mRNA were assessed by real-time quantitative PCR in blastocyst after IVF embryos were treated with VPA for 48 h (Fig. 1). Both 0.3 and 3 mM VPA treatment

significantly increased the expression levels of *Oct4* and *Nanog*. 3 mM VPA treatment also significantly increased the expression level of *Cdx2* ($p < 0.05$).

Discussion

The effect of VPA on epigenetic reprogramming has attracted interest amongst researchers who specialize in cloning animals. It has been shown that the cloning efficiency in pigs could be improved by VPA treatment (Huang et al. 2011; Miyoshi et al. 2010); however, experiments in cloned mouse embryos did not show the same beneficial effect (Ono et al. 2010; Chen et al. 2011). The low cloning efficiency associated with somatic cell nuclear transfer might be related to the low efficiency of in vitro oocyte maturation and embryonic development. This study aimed to explore the effect of VPA on oocyte maturation and the early

Table 7 Effect of VPA treatment after parthenogenetic activation on embryonic development

Concentration (mM)	No. of oocytes	No. of cleavage	Cleavage rate (%)	No. of blastocysts	Blastocyst rate (%)
0.00	291	267	91.75 % $\pm$ 0.016 ^a	152	56.93 % $\pm$ 0.007 ^a
0.30	261	230	88.12 % $\pm$ 0.019 ^a	133	57.83 % $\pm$ 0.022 ^a
3.00	291	229	78.70 % $\pm$ 0.016 ^b	40	17.47 % $\pm$ 0.015 ^b

Values with different superscripts in the same column differ significantly ($p < 0.05$). Data are means from three individual experiments

Fig. 1 Effect of treating IVF embryos with VPA on the mRNA expression levels of *Oct4* (a), *Nanog* (b) and *Cdx2* (c) in bovine blastocysts

development of embryos obtained by IVF and parthenogenetic activation.

Low dose (0.3 mM) VPA had no effect on the maturation of bovine oocytes, while 3 mM VPA decreased the maturation rate but did not influence oocyte survival. The higher doses of VPA we tested were found to have toxic effects on bovine oocytes and were considered unsuitable for further investigation. The toxic effect of VPA at a higher dose (6 mM) is in agreement with the findings of Wang et al. (2006), who found that a high level of histone acetylation inhibits GV breakdown, and that ordered histone deacetylation is necessary for bovine oocyte to resume meiosis. This same effect might also explain why 3 mM VPA decreased the oocyte maturation rate. These initial investigations established that bovine oocytes can be safely treated with 0.3 mM VPA, and to a slightly lesser extent, with 3 mM VPA. However, exposure of embryos obtained by IVF or parthenogenetic activation to VPA indicated that this compound could negatively affect cleavage rate and blastocyst rate even at concentrations of 0.3 and 3 mM. Our data indicate that low dose (0.3 mM) VPA treatment of the bovine embryos does not affect their development; however high dose (3 mM) of VPA did have a negative effect. Interestingly, the effect of VPA treatment was more pronounced when maturing

bovine oocytes were exposed to this compound, whereas fertilized oocytes exposed to VPA were relatively less affected.

With regards to the toxic effects of VPA, it has been reported that VPA can delay the cell cycle (Phiel et al. 2001) and embryonic development of mice (VerMil-yea et al. 2009), which might explain the effect of 6 mM VPA on bovine oocytes. The death of granular cells could also lead to the delayed development of oocytes. Our data clearly established that exposure to a high concentration of VPA has a strong negative effect on oocyte maturation. Moreover, the negative effect of VPA on bovine oocytes extended to further development. These data indicate that VPA has a long-lasting effect, which does not dissipate after the withdrawal of the compound.

Previous studies have used different doses of VPA. Miyoshi et al. (2010) reported that 4 mM VPA could improve blastocyst rate in cloned miniature pigs, while 1 mM VPA was effective in increasing blastocyst rate and embryonic developmental rate in mice (Costa-Borges et al. 2010). Moreover, Xu et al. (2012) showed that 4 mM VPA treatment could significantly improve both cleavage and blastocyst rates of bovine somatic cell nuclear transfer embryos, whereas Huang et al. increased cloning efficiency of Landrace pigs with a dose of 1 mM. In our study, a dose of 0.3 mM

VPA was safely used, thus it seems that VPA acts in a relatively wide concentration range and may have different optimal concentrations in different species.

In order to further understand the mechanisms underlying the effect of VPA, we studied the expression level of three important embryonic transcription factors, *Oct4*, *Nanog* and *Cdx2*. 0.3 and 3 mM VPA treatment was able to increase the expression levels of both *Oct4* and *Nanog* in blastocysts obtained by IVF. These data are in agreement with the effect of VPA on *Oct4* and *Nanog* expression in bovine adipose stem cells (Huangfu et al. 2008), and *Oct4* expression in murine myocytes (Teng et al. 2010). *Oct4* is a member of the POU transcription factor family and is known to be an important regulatory factor during early embryonic development, particularly in the pre-implantation embryos (Ovitt and Scholer 1998). Following *Oct4* knock-down, embryonic stem cells differentiate to trophectoderm-like cells (Hay et al. 2004). Similarly, *Oct4*^{-/-} embryos do not form any normal blastocysts (Rosner et al. 1990). Niwa et al. (Niwa et al. 2000) stated that the effect of *Oct4* depended on its concentration within nucleus, as such the VPA-induced increase in *Oct4* expression we observed could be one reason for the improved quality of VPA-exposed embryos. *Nanog* is a transcription factor expressed in the inner cell mass (ICM), archaeocytes and embryonic stem cells (Mitsui et al. 2003). It has been shown that an ICM lacking *Nanog* does not generate any ectoderm, but only endoderm-like cells (Mitsui et al. 2003). When lacking *Nanog* expression, embryonic stem cells lost their pluripotent capacity and differentiated into extraembryonic endoderm, leading to the conclusion that *Nanog* plays a key role in maintaining embryonic stem cell pluripotency within the ICM (Dietrich and Hiiragi 2007). We observed a VPA-induced increase in *Nanog* expression, which we interpret to indicate that VPA treatment can improve the quality of blastocysts.

The third gene we studied was *Cdx2* (Caudal type homeobox transcription factor 2), which is a nuclear transcription factor specifically expressed in the trophectoderm of blastocysts. Heterozygous *Cdx2* mutant mice have homeotic transformation defects, while homozygous *Cdx2* mutant mice die before implantation, demonstrating that *Cdx2* plays an essential role in early embryonic development (Chawengsaksophak et al. 2004). In this study, in embryos derived from IVF, 0.3 mM VPA had a marginal effect

on *Cdx2* expression whereas it was significantly increased by 3 mM VPA. Given the importance of *Cdx2* in early embryonic development, the inductive effect of VPA on *Cdx2* expression may be beneficial to the embryonic development after implantation. It can be seen that treatment of IVF embryos with 0.3 and 3 mM VPA both significantly increased the expression of the ICM marker genes *Oct4* and *Nanog*, and 3 mM VPA treatment also significantly increased the expression of the trophectoderm marker gene *Cdx2*. We thus hypothesize that treatment of IVF embryos with 0.3 and 3 mM VPA might be beneficial for the development of ICM at blastocyst stage, and 3 mM VPA treatment also beneficial for the growth of trophectoderm cells.

Our data suggest that VPA treatment has a dose-dependent effect on bovine oocyte maturation, with high doses of VPA seriously inhibiting the development of embryos derived from IVF and parthenogenetic activation. Differently, treatment of IVF and parthenogenetic embryos with VPA did not improve their developmental rates, but increased the expression of transcription factors important for early embryogenesis. To date, VPA has been shown to have beneficial effects in the cloning process for pigs and mice. Our data from bovine embryos indicate that VPA treatment can improve the expression of transcription factors important for early embryogenesis, suggesting that VPA may have a beneficial effect in this species. This study provides an important foundation for future studies using VPA treatment to facilitate the creation of cloned bovine embryos.

Acknowledgments This work was supported by a grant from Major Program of the Inner Mongolia Natural Science Foundation (No. 2012ZD04), a grant from the National Key Basic Research Program of China (No. 2010CB134410) and a grant from National Natural Science Foundation of China (No. 30860185) to Haiquan Yu.

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