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# Valproic acid improved *in vitro* development of pig cloning embryos but did not improve survival of cloned pigs to adulthood

Jin-Dan Kang, Suo Li, Yue Lu, Wei Wang, Shuang Liang, Xi Liu, Jun-Xue Jin, Yu Hong, Chang-Guo Yan, Xi-Jun Yin*

*Department of Animal Science, College of Agriculture, Yanbian University, Yanji, China*

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ABSTRACT

The objective was to examine the effects of valproic acid (VPA), a histone deacetylase inhibitor, on *in vitro* and *in vivo* development of Wuzhishan miniature pig somatic cell nuclear transfer (SCNT) embryos. Experiment 1 compared *in vitro* developmental competence of nuclear transfer embryos treated with various concentrations of VPA for 24 h. Embryos treated with 2 mM VPA for 24 h had a greater rate of blastocyst formation compared with control or embryos treated with 4 or 8 mM VPA (21.5% vs. 10.5%, 12.6%, and 17.2%, $P < 0.05$). Experiment 2 examined the *in vitro* developmental competence of nuclear transfer embryos treated with 2 mM VPA for various intervals after chemical activation. Embryos treated for 24 h had higher rates of blastocyst formation than the control or those treated for 4 or 48 h (20.7% vs. 9.2%, 12.1%, and 9.1%, $P < 0.05$). In Experiment 3, an average of 207 (range, 192–216) nuclear transfer embryos from the VPA-treated group were transferred to surrogate mothers, resulting in three pregnancies. Two of the surrogates delivered a total of 11 live piglets. However, for unknown reasons, nine of 11 piglets in the VPA-treated group died within 1 to 5 d after birth. Untreated control embryos (average, 205; range, 179–225) transferred to four surrogate mothers resulted in three pregnancies, two of which delivered a total of 12 live offspring, although four of 12 piglets in the VPA-untreated group died (cause unknown) within 1 to 3 d, whereas eight of the 12 piglets in the VPA-untreated group survived more than 3 or 4 mo. The average birth weight of the two litters from the VPA-treated group tended ($P < 0.05$) to be lower than that from the control groups (551.6 g vs. 675.2 g). In conclusion, VPA treatment increased the blastocyst formation rate of SCNT porcine embryos; both VPA-treated and the untreated clones developed to term, but offspring from VPA-treated embryos had a lower survival to adulthood than those from control embryos (18.2% vs. 67.0%; $P < 0.05$).



## 1. Introduction

Miniature pigs are optimal experimental animal models for biomedical research, because their anatomy and physiologic characteristics are similar to those of humans, and their size makes them easier to handle and less expensive to maintain than larger common domestic pigs [1]. The Wuzhishan inbred miniature pig, a Chinese variety with a high inbreeding coefficient, is well suited to biomedical research. By combining homologous recombination in somatic cells with that of SCNT, it is possible to introduce specific modifications into the pig genome [2–5]. Although several pig breeds have been successfully cloned, remodeling and reprogramming of differentiated somatic nuclei into a totipotent embryonic state by SCNT is not efficient, and the mechanism underlying the remodeling process is not known. In most mammalian species studied thus far, the survival rate to birth for cloned blastocysts is only

* Corresponding author. Tel.: +86 0433 2435623; fax: +86 0433 2435622.
*E-mail address:* yinxj33@msn.com (X.-J. Yin).

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approximately 1% to 5%, compared with a 30% to 60% birth rate for *in vitro* fertilization (IVF) blastocysts [6]. The low cloning efficiency associated with poor development of SCNT embryos to term is a major obstacle to widespread use of this technology for many animal science and biomedical applications. Therefore, increasing the developmental ability of SCNT embryos before transfer into recipient females is necessary to overcome low cloning efficiency.

Valproic acid (VPA), a cell-permeable, a short-chain fatty acid that inhibits histone deacetylase, has been used for decades to treat epilepsy, and is also effective as a mood stabilizer and for treating migraine headaches and schizophrenia. Recently, VPA was identified for inducing reprogramming of differentiated cells; in that regard, it greatly improved the efficiency of reprogramming mouse embryonic fibroblasts by *Oct4*, *Sox2*, and *Klf4* [7]. The same group also reported that VPA enabled reprogramming of primary human fibroblasts, a differentiated cell type, to a pluripotent state using only *Oct4* and *Sox2* and without the oncogenes *c-Myc* or *Klf4* [8]. The addition of 4 mM VPA to embryo culture medium for 48 h after activation significantly increased the blastocyst formation rate of SCNT embryos compared with control, but did not affect their cleavage rate [9]. The proportion of SCNT embryos expressing enhanced green fluorescent protein gene on Day 5 of culture was not affected by the presence or absence of VPA. However, on Day 7 of culture, 4 mM VPA in the embryo culture medium for 48 h after activation significantly increased the rate of SCNT embryos expressing enhanced green fluorescent protein compared with control [9]. Oocytes reconstructed using fetal fibroblasts were cultured in porcine zygote medium without histone deacetylase inhibitor (HDACi) (control) or 5 mM VPA for 24 h, followed by an additional 5 d without HDACi. In that study, the frequency of blastocyst formation was significantly higher in embryos cultured with VPA than in those cultured without HDACi (125/306; 40.8% vs. 80/329; 23.4%). In addition, VPA significantly increased the number of inner cell mass (ICM) cells compared with that in the control ($15.6 \pm 1.7$ vs. $10.8 \pm 2.6$) [10]. Furthermore, it was recently reported that 1 mM VPA for 14 to 16 h after activation significantly increased the rate of blastocyst formation of porcine SCNT embryos constructed from Landrace fetal fibroblast cells compared with the control [11]. In that study, SCNT embryos were transferred to 38 surrogates, and the cloning efficiency in the treated group was significantly improved compared with the control group [11].

The objective of the present study was to examine and optimize the use of VPA to reprogram somatic nuclei after SCNT (using fibroblast cells derived from ears of Wuzhishan inbred miniature pigs as donor cells). Furthermore, to test the ability of VPA to enhance *in vivo* development of porcine SCNT embryos birth weight of cloned piglets and survival rate to adulthood were determined.

## 2. Materials and methods

This research was carried out in accordance with the Ethics Committee of Yanbian University.

### 2.1. Chemicals and reagents

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise indicated.

### 2.2. Isolation and culture of porcine somatic cells

Miniature pig fibroblasts were obtained from ear cells of one female Wuzhishan miniature pig (2 y of age). Ear tissues were cut into small pieces and cultured in a mixture of Dulbecco modified Eagle medium and Ham F-12 medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 10% (v:v) fetal calf serum in 5% $CO_2$ in air at 38 °C. After reaching confluence, cells were passaged several times. Cells between passages four and eight were used as donors for nuclear transfer. Cells were allowed to grow to confluence and cultured for an additional 5 to 6 d without a change of medium. A single cell suspension was prepared by standard trypsinization immediately before nuclear transfer.

### 2.3. In vitro maturation of oocytes

Ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory at 25 °C to 35 °C. Antral follicles (2–6 mm in diameter) were aspirated using an 18 ga needle. Aspirated oocytes with uniformly granulated cytoplasm and surrounded by at least three uniform layers of compact cumulus cells were selected and washed three times in HEPES-buffered NCSU-37 containing 0.1% polyvinyl alcohol. Oocytes were cultured in four-well plates (Nunc, Roskilde, Denmark) each containing 500 μL of NCSU-37 medium supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyryl cyclic adenosine monophosphate and 0.1 IU/mL human menopausal gonadotropin (Teikokuzoki, Tokyo, Japan) for 20 h, followed by culture without dibutyryl cyclic adenosine monophosphate and human menopausal gonadotropin for another 18 to 24 h, as reported [12].

### 2.4. Nuclear transfer

Nuclear transfer was performed as described by Yin et al. (2002) [12]. Briefly, mature eggs showing the first polar body were cultured in medium supplemented with 0.4 mg/mL demecolcine and 0.05 M sucrose for 1 h. Sucrose was used to enlarge the perivitelline space of the eggs. Treated eggs with a protruding membrane were moved to medium supplemented with 5 mg/mL cytochalasin B and 0.4 mg/mL demecolcine and the protrusion was removed with a beveled pipette. A single donor cell was injected into the perivitelline space of each egg and electrically fused using two direct current pulses of 150 V/mm for 50 μs in 0.28 M mannitol supplemented with 0.1 mM $MgSO_4$ and 0.01% polyvinyl alcohol. Fused eggs were cultured in NCSU-37 medium for 1 h before electroactivation and then cultured in 5 mg/mL of cytochalasin B-supplemented medium for 4 h. The reconstructed oocytes were activated by two direct current pulses of 100 V/mm for 20 μs in 0.28 M mannitol supplemented with 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$. Activated eggs were cultured in the medium for 6 days in an atmosphere of 5% $CO_2$ and 95% air