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Research article

Histone deacetylation inhibitors affect *in vitro* oocyte maturation and somatic cell nuclear transfer in pigs

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Abstract

Histone deacetylase inhibitors (HDACi), such as Trichostatin A (TSA) and Valproic acid (VPA), are known treatment strategies to improve somatic cell nuclear transfer (SCNT) efficiency in various species, including porcine. However, their supportive roles in oocyte maturation and preimplantation embryonic development come with a prolonged meiotic division during *in vitro* maturation. This study examined the effects of TSA, VPA, and combined TSA and VPA treatments on porcine cumulus-oocyte complexes (COCs), as well as on parthenogenetic activation (PA)- and SCNT-derived embryos. Nuclear maturation of porcine oocytes *in vitro* was assessed through aceto-orcein staining, while the developmental rates of PA- and SCNT-derived embryos were also observed. Results revealed that TSA treatment significantly inhibited nuclear maturation in porcine oocytes, whereas VPA and TSA+VPA treatments did not differ significantly from the control. There were also no notable differences in blastocyst rates among PA-derived embryos. However, VPA treatment led to a higher blastocyst rate in porcine SCNT embryos. The combination of TSA and VPA appeared to have a synergistic effect on embryo development, with VPA supporting TSA's effects. Overall, VPA treatment resulted in fewer inhibitory effects on oocyte maturation and improved embryo development more effectively than TSA. These findings contribute to our present understanding of the role of histone deacetylation in porcine oocyte and SCNT-derived embryo development during *in vitro* production.

Keywords: Histone deacetylation, Oocyte maturation, Somatic cell nuclear transfer, Trichostatin A, Valproic acid

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INTRODUCTION

The potential of histone deacetylase inhibitors (HDACi) has been explored in various treatments, including *in vitro* maturation (IVM) culture of cumulus-oocyte complexes (COCs), embryo culture, and/or donor cell treatments in somatic cell nuclear transfer (SCNT) production (Endo et al., 2005; Jeseta et al., 2008; Srirattana et al., 2012; Gao et al., 2014; Jin et al., 2014; Luo et al., 2015). Among these HDAC inhibitors are trichostatin A (TSA) and valproic acid (VPA), which improve epigenetic reprogramming by restoring normal DNA methylation, increasing histone acetylation, reducing repressive histone methylation, and remodeling chromatin, thus promoting early development of cloned embryos (Bui et al., 2010; Srirattana et al., 2022). Cloning success rates from SCNT remain low due to issues from aberrant epigenetic reprogramming (Wang et al., 2021; Srirattana et al., 2022). To resolve these challenges in cloning efficiency, TSA, a commonly used HDACi, has been supplemented into embryo culture media (Costa-Borges et al., 2010; Lee et al., 2011; Oh et al., 2012; Srirattana et al., 2012; Inoue et al., 2015). On the other hand, VPA, which specifically inhibits HDAC class I, has also been explored as a promising, low-toxicity alternative to SCNT treatments by promoting histone acetylation (Göttlicher et al., 2001; Chen et al., 2011). Beyond SCNT, HDACi treatment has also been utilized during IVM to improve the quality of mature oocytes for other assisted reproductive technology applications like *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Jeseta et al., 2008; Jin et al., 2014).

TSA and VPA, as HDACi, enhance both *in vitro* and in vivo development of SCNT-reconstructed embryos across various species (Shi et al., 2008; Costa-Borges et al., 2010; Kim et al., 2011; Oh et al., 2012; Qin et al., 2012; Srirattana et al., 2012; Kang et al., 2013). For instance, in mouse SCNT embryos treated with TSA or VPA, improvements in cloning efficiency, blastocyst rates, inner cell mass (ICM) numbers, histone H3K14 acetylation, and successful development of cloned offspring were observed (Costa-Borges et al., 2010). In porcine SCNT embryos, VPA treatment resulted in higher blastocyst formation and ICM numbers compared to the control group, with TSA treatment showing no significant differences (Kim et al., 2011). In vivo, VPA-treated porcine embryos showed improvements in blastocyst rates, but cloned offspring exhibited low survival rate to adulthood following embryo transfer (Kang et al., 2013). In bovine SCNT embryos, TSA treatment improved *in vitro* embryonic development to the blastocyst stage by enhancing blastocyst formation, upregulating pluripotent genes such as *Nanog* and *Oct4*, and downregulating DNA methylation in blastocysts (Oh et al., 2012). Moreover, the transfer of TSA-treated SCNT embryos led to increased pregnancy rates and the development of full-term bovine cloned calves (Srirattana et al., 2012). Furthermore, the interspecies SCNT (iSCNT) of gaur and bovine demonstrated that TSA treatment had no effect on blastocyst formation. Interestingly, only the TSA-untreated group resulted in a full-term developed gaur-bovine iSCNT calf (Srirattana et al., 2012). Conversely, Shi et al. (2008) demonstrated that TSA treatment enhanced blastocyst formation in rabbit SCNT embryos but did not have the same effect on human-rabbit iSCNT embryos. In a macaque-pig iSCNT study, TSA treatment improved the blastocyst rate, while VPA treatment did not increase the *in vitro* developmental potential of macaque-pig iSCNT embryos (Qin et al., 2012). These findings suggest that the effectiveness of HDACi treatment in rescuing somatic cell reprogramming may vary depending on different factors such as genetic background, species specificity, and laboratory conditions.

In mammals, oocyte maturation begins at the germinal vesicle (GV) stage, where *in vitro* meiotic division occurs. At this stage, nuclear histone deacetylases are isolated from cytoplasmic histone deacetylases and other cofactors by the nuclear envelope. Immature oocytes in the GV stage exhibit high levels of nuclear histone acetylation (HAT), while nuclear HDAC remain inactive (Endo et al., 2005). As oocytes progress through meiotic maturation, a global histone deacetylation

process triggers the transition to the mature stage by increasing HDAC activity after germinal vesicle breakdown (GVBD) (Endo et al., 2008).

In this study, we investigated the effects of different HDACi, including TSA, VPA, and their combination, on porcine *in vitro* oocyte maturation and embryonic development of parthenogenetic activated (PA) and SCNT-derived embryos. Our objective was to examine the role of histone deacetylation in porcine oocyte nuclear maturation and embryo development during *in vitro* production.

MATERIALS AND METHODS

Oocytes collection and *in vitro* maturation

Porcine oocytes were collected from local slaughterhouses and transported to the laboratory in phosphate-buffered saline (PBS) maintained at 25-37°C within 2-3 hours. The COCs were aspirated from 3-8 mm follicles using an 18-gauge needle attached to a 10-mL disposable syringe. Only COCs with homogenous cytoplasm and more than three layers of cumulus cells were selected for *in vitro* maturation culture. Approximately 50 COCs were cultured in 200 µL of *in vitro* maturation medium 1 (IVM1), covered with mineral oil in 4-well dishes, for an initial period of 20-22 hours. The COCs were then transferred to *in vitro* maturation medium 2 (IVM2) to complete the 42-44 hours maturation time. Incubation was carried out at 38.5°C in a controlled 5% CO_2 environment. After the culture time, mature COCs were denuded by gentle pipetting in 0.1% hyaluronidase to remove the cumulus cells. Oocytes that reached the metaphase II (MII) stage, indicated by the presence of the first polar body, were selected for subsequent PA and SCNT experiments.

Parthenogenetic activation (PA)

Porcine PA was performed using a BTX electrofusion, ECM 830 (BTX, Holliston, MA, USA). A single direct current pulse of 84 V/mm was used for 100 µs in an activation medium containing 0.3 M mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.5 mM HEPES (pH adjusted to 7.0–7.4). Activated oocytes were then incubated in porcine zygote medium-3 (PZM-3) medium supplemented with 10 µg/mL cycloheximide (CHX) and 5 µg/mL cytochalasin B (CB) for 3-4 hours. After rinsing, PA-reconstructed embryos were cultured in PZM-3 medium at 38.5°C in a humidified atmosphere of 5% CO_2 with maximum humidity. Cleavage, morula, and blastocyst formation rates were evaluated at 48-, 96-, and 168-hours post-culture, respectively. Embryos were classified as blastocysts based on the prominence of a blastocoele while those exhibiting compacted blastomeres without a visible blastocoele were identified as morula.

Somatic cell nuclear transfer and *in vitro* embryo culture

The MII oocytes were enucleated by aspirating the first polar body and adjacent cytoplasmic protrusion using a micromanipulator system in a manipulation medium supplemented with 5µg/mL of CB. Then, a single porcine fibroblast cell was injected into the perivitelline space of each enucleated recipient cytoplast. Simultaneous fusion and activation were accomplished using the BTX electrofusion, ECM 830 (BTX, Holliston, MA, USA). A single direct current pulse of 84 V/mm was used for 100 µs in a fusion/activation medium composed of 0.3 M mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.5 mM HEPES (pH adjusted to 7.0–7.4). Post-activation, the reconstructed embryos were incubated in PZM-3 supplemented with 10 µg/mL CHX and 5 µg/mL CB for 3-4 hours. After three washes with PZM-3, only successfully fused embryos were cultured in PZM-3 at 38.5 °C and 5% CO_2 with maximum humidity. Cleavage, morula, and blastocyst formation rates were evaluated at 48-, 96-, and 168-hours post-culture,

respectively. The classification criteria for stage progression in SCNT embryos were similar to those used for PA embryos.

Histone deacetylase inhibitors (HDACi) treatment

The final concentrations of HDACi in the treatment groups were 5 nM TSA, 2 mM VPA (Costa-Borges et al., 2010), or a combination of 5 nM TSA + 2 mM VPA, whereas the control group received fresh culture media. For oocyte maturation, TSA, VPA, and TSA+VPA were added to the IVM media for a 42-44 hours culture period. For the PA and SCNT embryos, TSA, VPA, and TSA+VPA were supplemented in PZM-3 media for 24 hours. After treatment, embryos were washed and cultured in fresh PZM-3 medium without TSA or VPA.

Aceto-orcein staining

To visualize the meiotic stages, 5-7 denuded oocytes were fixed on slides in a 1:3 acetic acid-ethanol solution. After fixation for 7–10 days, the slides were stained with orcein solution (1% orcein in 60% acetic acid) for 30 minutes. Excess stains were removed by washing the slides in a destaining solution of glycerol, water, and acetic acid (1:3:1 ratio). Slides were sealed with white petroleum jelly and meiotic stages were observed under an inverted microscope (Phung et al., 2018).

Statistical analysis

The results between the control and the HDACi treatment groups were expressed as percentages. Datasets were analyzed using the Chi-squared test or Fisher's exact test in SPSS Statistics Version 17. A probability of < 0.05 was considered statistically significant.

RESULTS

The effect of HDACi on nuclear maturation of porcine oocytes

Porcine COCs were cultured in IVM media supplemented with TSA, VPA, or TSA+VPA. After 42-44 hours of treatment, oocyte nuclear maturation was observed through aceto-orcein staining, and maturation rates at GV, GVBD, MI, and MII stage were noted. The nuclear development reached maturity at the MII stage, thereby statistical analyses were conducted only at this stage between the treated groups and the control. Results indicated that TSA-treated oocytes exhibited a significantly lower rate of maturation to MII stage compared to the control group (32.14% vs. 53.85%, respectively). In contrast, no significant differences were observed in the maturation rates of the VPA- and TSA+VPA-treated oocytes and the control group (42.22% vs. 36.59% vs. 53.85%, respectively) (Figure 1).

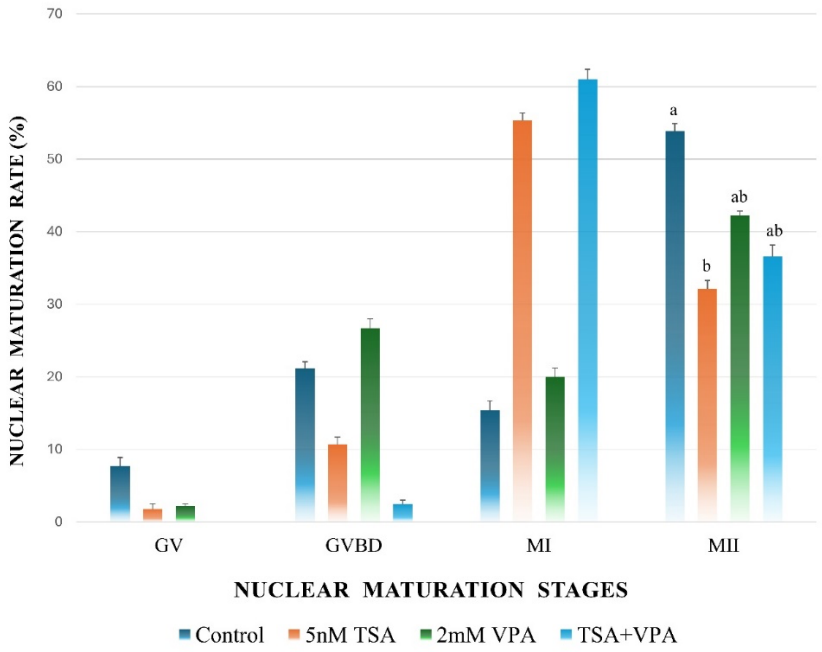

Figure 1 Comparison of nuclear maturation rates (%) of porcine oocytes treated with different histone deacetylase inhibitors. Porcine oocytes were cultured *in vitro* with trichostatin A, valproic acid, and a combination of both. Significant differences between the MII groups are denoted by different letters (a, b) ($P < 0.05$). The statistical test was performed only in the maturation stage. (GV: Germinal vesicle, GVBD: Germinal vesicle breakdown, MI: Metaphase I, MII: Metaphase II)

The effect of HDACi on porcine PA embryo development

Porcine PA embryos were incubated in media supplemented with each HDACi for 24 hours, and the number of cleaved embryos, morulae, and blastocysts were recorded at 48-, 96-, and 168-hours post culture, respectively. Results revealed no significant differences between the treatment and control groups ($P>0.05$) (Figure 2). However, PA embryos treated with 2 mM VPA (Figure 3a) exhibited a developmental trend towards higher percentages of cleavage, morula, and blastocyst stages (93.75%, 81.25%, and 37.50%, respectively) compared to other treatment groups (Figure 2). Although not statistically significant, this trend implies a potential positive effect of VPA on early porcine embryo development.

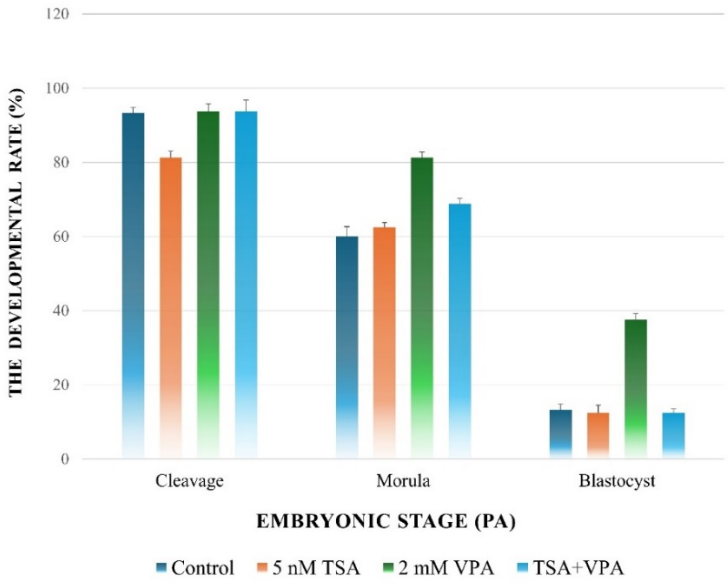

Figure 2 Comparison of cleavage, morula, and blastocyst rates (%) in porcine parthenogenetic activated embryos treated with various histone deacetylase inhibitors. The developmental progress of porcine parthenogenetic activated embryos treated trichostatin A, valproic acid, and a combination of both were shown. No significant differences were observed in the cleavage, morula, and blastocyst formation rates between the treatment and control groups following *in vitro* culture in porcine zygote medium-3 ($P > 0.05$).

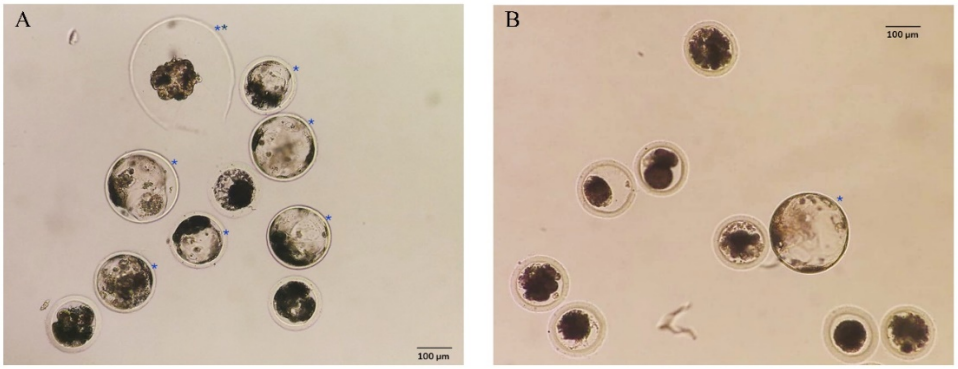

Figure 3 Embryonic development on day 7 post-activation. (A) Developmental records of porcine parthenogenetic activated embryos at the blastocyst stage (Asterisks (*) indicate blastocyst stage embryos characterized by the presence of the blastocoele, while double asterisks (**) show hatching blastocysts exhibiting signs of cell compaction and embryonic shrinkage; (B) Blastocyst stage embryos derived from porcine somatic cell nuclear transfer treated with valproic acid. (Asterisks (*) show blastocyst stage embryos characterized by the presence of the blastocoele).

The effect of HDACi on porcine somatic cell nuclear transfer (SCNT) embryo development

Reconstructed somatic cell nuclear transfer (SCNT) embryos were treated with HDACi for 24 hours, and the rate of cleavage, morula, and blastocyst formation were assessed at 48-, 96-, and 168-hours post-culture, respectively. While significant differences in cleavage rates were observed between the TSA-, VPA-, and TSA+VPA-treated embryos and the control group (50.00%, 40.74%, 43.48%

vs. 13.04%, respectively) (P<0.05), no significant differences existed between the HDACi treatment groups (P>0.05). In terms of morula stage development, the TSA-treated group showed a significantly higher rate than the control group (31.82% vs. 4.35%) (P<0.05), with no differences between the VPA- and TSA+VPA-treated groups and the other treatments (P>0.05). Notably, only the VPA-treated embryos were able to develop to the blastocyst stage (Figure 3b), reaching a 3.70% blastocyst rate (Figure 4).

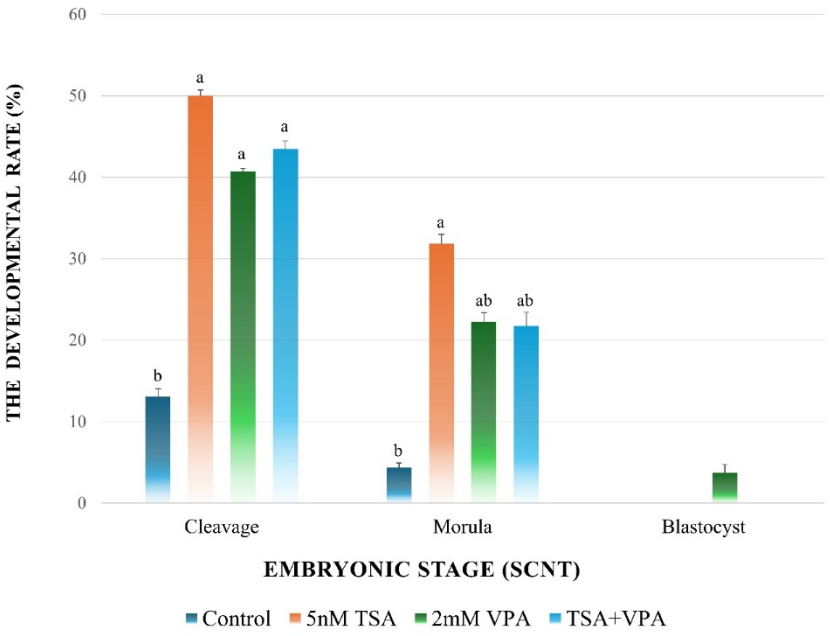

Figure 4 Comparison of cleavage, morula, and blastocyst rates (%) in porcine SCNT embryos treated with histone deacetylase inhibitors. Developmental rates (cleavage, morula, and blastocyst stages) of porcine somatic cell nuclear transfer embryos were depicted here after 24-hour treatment with trichostatin A, valproic acid, and their combination. Significant differences between the groups are indicated by different letters (a, b) (P < 0.05).

DISCUSSION

Our study underscores the differential effects of histone deacetylase inhibitors TSA and VPA on oocyte nuclear maturation and early embryonic development. By examining HDACi effects on these developmental processes, we highlight the significant roles of histone deacetylation, involving both HAT and HDAC, in the regulation of chromatin structure and gene function (Dsilva et al., 2023). During the GV stage, immature oocytes show high acetylation of nuclear histones, particularly H3 and H4, while nuclear and cytoplasmic HDACs remain separated by the nuclear envelope. As oocytes transition to the GVBD stage, cytoplasmic factors induce global HDAC activity, facilitating meiosis-specific deacetylation (Endo et al., 2008). During IVM, TSA-treated oocytes exhibited lower progression to the MII stage, whereas those treated with VPA showed comparable development to that of the control group. Our findings are consistent with earlier studies, indicating that TSA inhibits global HDAC action and prevents meiosis-specific deacetylation after GVBD by maintaining HDAC inactivity (Kim et al., 2003; Endo et al., 2005). While we observed reduced progression to maturity in TSA-treated oocytes after IVM culture, other works suggest that TSA may have a

reversible effect, delaying oocyte maturation instead of persistently inhibiting HDACs (Jin et al., 2014).

Mammalian HDACs include 18 isoforms categorized into four classes (Li et al., 2020; Park and Kim, 2020). In porcine oocytes, HDAC 1, a member of class I, is predominantly located in the nucleus rather than the cytoplasm (Endo et al., 2006; Wang et al., 2006). Throughout meiotic maturation, total HDAC activity remains consistent. During the GV stage, nuclear histone acetylation occurs while nuclear HDAC class I remain inactive until GVBD initiation. In our study, VPA-treated oocytes reached the MII stage similar to the non-treated group, suggesting that VPA's effect was insufficient to inhibit global HDAC activity. VPA is recognized as a specific class I HDAC inhibitor (Göttlicher et al., 2001) capable of targeting nuclear HDAC in porcine oocytes. Endo et al. (2008) observed that during GVBD, nuclear HDACs were not involved in global histone deacetylation; instead, this process was governed by cytoplasmic HDACs during meiosis. Thus, the IVM of porcine oocytes to maturity depends on the activity of other cytoplasmic HDAC classes, excluding nuclear HDACs and HDAC class I (Endo et al., 2008). When VPA specifically prevented nuclear HDACs, other cytoplasmic HDAC classes regulate oocyte maturation. However, high doses of VPA can alter global HDAC activity during oocyte maturation, consequently reducing the blastocyst rate in IVF and PA embryos (Gao et al., 2014). In addition, HDACi can modulate post-translational histone modification and reactivate silenced genes without altering DNA methylation. HDACi treatments have been shown to improve early embryonic development by increasing histone acetylation at lysine 8 on histone H4 (H4K8ac) and promoting chromosome decondensation in cloned embryos (Bui et al., 2010; Zhao et al., 2010).

In this present work, the effect of TSA, VPA, and TSA+VPA on the development of PA embryos showed no statistically significant differences compared to the control group. While low-dose VPA treatment did not exert impacts on embryonic development, it significantly increased the expression of pluripotent transcription factors such as *Oct4* and *Nanog* in bovine IVF embryos (Gao et al., 2014). Notably, the VPA-treated porcine PA embryos in our study exhibited higher percentages of cleavage, morula, and blastocyst stages, corroborating with prior findings suggesting that VPA may enhance porcine PA embryo development (Huang et al., 2015).

In SCNT embryos treated with TSA, VPA, and TSA+VPA, significant differences in development were observed at the cleavage, morula, and blastocyst stages compared to the control group. This result indicates a potential synergistic interaction with combined TSA and VPA treatment, with VPA possibly enhancing the effects of TSA. Moreover, our study found that the TSA treatment of SCNT embryos led to higher cleavage and morula developmental rates compared to other treatment groups. A comparative analysis of the effects of scriptaid and TSA on the *in vitro* development of cloned embryos reported similar findings, with TSA affecting blastocyst formation at higher concentrations (Zhao et al., 2010; Al-Ghadi et al., 2020).

Development of the blastocyst stage in SCNT embryos was observed only in the VPA-treated group. This suggests a positive effect of VPA on HAT activity during early embryonic development in both *in vitro* and in vivo SCNT embryos in species such as mice, pigs, and cattle (Costa-Borges et al., 2010; Kim et al., 2011; Kang et al., 2013; Song et al., 2014). Additionally, VPA treatment may reduce endoplasmic reticulum (ER) stress and enhance the developmental competence of SCNT embryos when supplemented in *in vitro* embryo culture (Song et al., 2014).

In conclusion, TSA treatment effectively inhibits global HDAC activity in porcine oocytes, leading to a delay in oocyte maturation to MII stage. On the other hand, VPA treatment did not affect meiotic division, likely due to its specific inhibition of nuclear HDAC, which does not participate in the global deacetylation process during *in vitro* oogenesis. Moreover, VPA treatment was more beneficial in supporting *in vitro* PA- and SCNT-derived embryo development compared to TSA.

However, several limitations in this study, including chemical concentrations and sample size, should be addressed in future investigations. Further exploration of the molecular mechanisms behind HDACi effects on embryonic development is crucial to provide better understanding of the precise roles of these inhibitors in oocyte and embryo maturation.

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AUTHOR CONTRIBUTIONS

Peachanika Pankammoon: Methodology, Investigation, Writing–original draft, Writing–review and editing. Chaimongkol Potiruean and Prakon Boontawee: Investigation, Formal Analysis, and Writing–review and editing. Prapas Patchanee: Supervision, Methodology, Formal Analysis, and Writing–review and editing. Anucha Sathanawongs: Project administration, Conceptualization, Supervision, Methodology, Funding acquisition, Validation, and Writing–review and editing.

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