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Pig oocyte activation utilizing a Zn²⁺ chelator, TPEN

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Abstract

Artificial oocyte activation is a critical step during somatic cell nuclear transfer (SCNT). Most of current activation protocols focus on inducing an increase in the intracellular free Ca²⁺ concentration of the oocyte. Here, we have utilized a zinc chelator, TPEN, to enhance the efficiency of oocyte activation during SCNT. TPEN treatment of matured pig oocytes resulted in the reduction of available Zn²⁺ in pig oocytes; however, the cytosolic Ca²⁺ concentration in the oocytes was not affected by the TPEN treatment. When various concentrations (100 – 250 μM) and incubation durations (45 min – 2.5 hours) of TPEN were used to activate oocytes, the efficiency of oocyte activation was not different from conventional activation methods. When oocytes that were activated by conventional activation methods were incubated with a lower concentration of TPEN (5 – 10 μM), a significant increase in embryos developing to the blastocyst stage was observed. In addition, when oocytes receiving a small Ca²⁺ stimulus were further activated by higher concentration of TPEN (100 – 200 μM), a significant increase in the frequency of blastocyst formation was observed, compared to a conventional activation method. This result indicated that TPEN can be a main reagent in oocyte activation. No increase in the cytosolic Ca²⁺ level was detected when oocytes were exposed to various concentrations of TPEN, indicating the ability of TPEN to induce oocyte activation is independent of an intracellular Ca²⁺ increase. We were able to produce clones through SCNT using the TPEN-assisted activation procedure, and the piglets produced through the process did not show any signs of abnormality. In this study, we have

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Conflict of Interest: The University of Missouri has filed a patent on using the above procedures to activate oocytes.

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developed an efficient way to utilize TPEN to increase the developmental potential of cloned embryos.

1. Introduction

Somatic cell nuclear transfer (SCNT), i.e. cloning, can be used to generate genetically engineered animals, preserve endangered species, and produce animals with a select genetic background. For example, SCNT has been used to generate genetically engineered pigs that can be used for agriculture [1, 2] and biomedicine [3, 4]. Development of SCNT has dramatically expanded the application of pigs. However, current SCNT technology is inefficient as only one percent of SCNT generated pig embryos are able to develop to term. One of the factors that contribute to the poor efficiency is the ineffectiveness of current artificial oocyte activation procedures. Under physiological conditions, fusion of the sperm and oocyte plasma membranes leads to repetitive increases in the intracellular free calcium (Ca^{2+}) concentration in the oocyte cytoplasm [5, 6]. These transient increases (termed oscillations) result from the release of phospholipase C- ζ (PLC-zeta) from the sperm head [7]. The Ca^{2+} oscillations activate Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) [8] and the CaMKII phosphorylates early mitotic inhibitor 2 (EMI2, a.k.a. FBXO43) thus relieving the anaphase-promoting complex/cyclosome (APC/C) from FBXO43-mediated inhibition [9-11]. These events lead to the degradation of cyclin B [12, 13], a subunit of the M-phase promoting factor (MPF). These signaling pathways demonstrate that Ca^{2+} signaling is essential in the process of oocyte activation and in fact, disruption of Ca^{2+} signaling during oocyte activation can cause developmental defects [14].

Most artificial activation methods induce an increase in the intracellular free Ca^{2+} levels in the oocyte that mimic sperm-induced Ca^{2+} signaling. However, most methods are able to induce only a single Ca^{2+} rise in the ooplasm [15]. Compared to the repetitive Ca^{2+} increases observed after sperm-induced oocytes activation, a single Ca^{2+} spike is a relatively poor activator of oocytes. Different approaches have been attempted to increase the efficiency of oocyte activation. For example, it is possible to incubate activated oocytes with inhibitors in order to reduce the level of MPF [16, 17]; however, these inhibitors are not very specific toward MPF, and while they do degrade MPF, they also have a number of side effects.

Zinc (Zn^{2+}) is important to maintain oocytes arrested at the MII stage. In mice there is a 50% increase in the intracellular amount of Zn^{2+} as oocytes develop from the germinal vesicle to MII stage [18]. Zn^{2+} is necessary in activating MPF as Zn^{2+} regulates the activity of CDC25 [19], a phosphatase that can dephosphorylate and thus activate cyclin-dependent kinase 1, a component of MPF. In addition, EMI2 (official gene symbol, FBXO43), a zinc-binding protein, is required to maintain high MPF activity during the MII arrest [12], and the increase in total intracellular Zn^{2+} during oocyte maturation directly controls FBXO43 activity [20]. A previous report demonstrated that removal of Zn^{2+} from MII stage oocytes can successfully induce oocyte activation, and thus permit oocytes to exit the MII stage [21]. This was the first report demonstrating that full-term development can occur without Ca^{2+} signaling. Although the efficiency of full term development was lower, the study demonstrated that the Ca^{2+} signal is not required for full-term development in mice.

Zn²⁺ is released from oocytes after fertilization indicating that removal of Zn²⁺ is a natural part of oocyte activation [22]. TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl) ethane-1,2-diamine), known to have a high specificity toward Zn²⁺, was used to lower the level of available Zn²⁺ to activate mouse oocytes [21]. In this study, we developed an efficient method to activate pig oocytes by reducing the intracellular level of Zn²⁺ using TPEN. We found that a combination of proper Ca²⁺ signal and TPEN treatment can increase the developmental potential of activated oocytes. We also demonstrated that TPEN can be used to efficiently activate oocytes during SCNT. This approach might be used to increase application of SCNT in pigs.

2. Materials and Methods

2.1 Animals

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Missouri.

2.2 Chemicals

All chemicals in the experiments were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) unless indicated otherwise.

2.3 Oocyte maturation

The oocytes used in the experiments were either obtained from pre-pubertal gilt ovaries collected at an abattoir (Farmland Foods Inc., Milan, MO) or were sow-derived oocytes purchased from A.R.T. (Monona, WI). Immature oocytes from gilt ovaries were aspirated from medium size (3–6 mm) follicles using an 18-gauge hypodermic needle attached to a 10-mL syringe. Oocytes with evenly dark cytoplasm and several layers of cumulus cells were then selected for maturation. Around 50 cumulus-oocyte complexes were placed in a well containing 500 mL of maturation medium, TCM 199 (Invitrogen, Grand Island, NY) with 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 0.5 mg/mL luteinizing hormone (LH), 0.5 mg/mL follicle stimulating hormone (FSH), 10 ng/mL gentamicin (APP Pharm, Schaumburg, IL), and 0.1% polyvinyl alcohol (PVA) for 42–44 h at 38.5°C, 5% CO₂, in humidified air. The oocytes from sows were shipped overnight in maturation medium (TCM199 with 2.9 mM HEPES, 5 mg/mL insulin, 10 ng/mL EGF, 0.5 mg/mL p-FSH, 0.91 mM pyruvate, 0.5 mM cysteine, 10% porcine follicular fluid, 25 ng/mL gentamicin) and transferred into fresh medium at 24 hr. At the end of the maturation, the surrounding cumulus cells were removed from the oocytes by vortexing for 3 min in the presence of 0.03% hyaluronidase. Oocytes with a visible polar body were selected in manipulation medium (TCM199 with 0.6 mM NaHCO₃, 2.9 mM HEPES, 30 mM NaCl, 10 ng/mL gentamicin, and 3 mg/mL bovine serum albumin [BSA]; osmolarity 305 mol/L) and then used for the experiments.

2.4 Oocyte activation

For parthenogenetic activation of pig oocytes, prepubertal gilt-derived oocytes were used. Different oocyte activation methods were used in the experiments. As conventional activation methods, either electrical or chemical activation was used. For electrical

activation, mature oocytes were electroporated in activation medium (0.3 M mannitol, 1.0 mM CaCl₂, 0.1 mM MgCl₂, and 0.5 mM Hepes) by two direct-current (DC) pulses (1-sec interval) at 1.2 kV/cm for 30 μsec (using BTX Electro Cell Manipulator, Harvard Apparatus, Holliston, MA). For chemical activation, mature oocytes were incubated in Hepes-buffered Tyrode's lactate (TL-Hepes) medium in the presence of 200 μM thimerosal for 10 min in the dark followed by 8 mM dithiothreitol for 30 min. For TPEN-mediated activation, mature oocytes were incubated in TL-Hepes medium with various concentrations of TPEN (100 – 250 μM) for different durations. Two different approaches were used to activate oocytes with a combination of Ca²⁺ signaling and TPEN. First, mature oocytes were activated by conventional methods, either electrical or chemical, then incubated in TL-Hepes with 5 – 10 μM of TPEN. The other method was to provide a Ca²⁺ signal to mature oocytes by giving two direct-current (DC) pulses (1-sec interval) at 1.2 kV/cm for 30 μsec in a low Ca²⁺ media (0.3 M mannitol, 0.1 mM CaCl₂, 0.1 mM MgCl₂, and 0.5 mM Hepes), and then incubating the oocytes in TL-Hepes containing different concentrations of TPEN (100 – 200 μM) for various durations. Activated oocytes were washed three times in PZM3-MU1 [23, 24] then incubated at 38.5°C, 5% CO₂ until the embryos are examined on either Day 6 or 7. At the end of the culture, where applicable, the frequency of cleaved embryos and blastocysts, and total cell number in the blastocysts were recorded. Hoechst 33342 (1.2 mg/mL) was used to stain nuclei and the embryos were then evaluated by epi-fluorescence microscopy.

2.5 Somatic cell nuclear transfer

Sow-derived mature oocytes were transferred into manipulation medium (TL-Hepes supplemented with 7.0 mg/mL cytochalasin B). By using micropipettes the polar body along with a portion of the adjacent cytoplasm was removed, and a donor cell was placed in the perivitelline space [25]. Genetically engineered cells, carrying a mutation on a gene, were used as donor cells for the *in vitro* study. For the *in vivo* study, either the same cell line used for the *in vitro* study or fetal fibroblast cells from Ossabaw miniature pigs served as donors. The reconstructed oocytes were then fused in a fusion medium (0.3 M mannitol, 0.1 mM CaCl₂, 0.1 mM MgCl₂, and 0.5 mM Hepes) by two DC pulses (1-sec interval) at 1.2 kV/cm for 30 μsec. After fusion, fused oocytes were fully activated using 200 μM thimerosal for 10 min in the dark followed by 8 mM dithiothreitol for 30 min [15]. Then a group of embryos were treated with either 5 μM TPEN for 30 min or 10 μM TPEN for 10 min. If necessary, embryos were then incubated in PZM3-MU1 with 0.5 mM Scriptaid, a histone deacetylase inhibitor, for 14–16 h. The embryos were then washed three times in PZM3-MU1 and incubated at 38.5°, under 5% CO₂ in air until further evaluation. At the end of the culture, SCNT-derived embryos were collected, and the frequency of blastocyst formation along with the total number of nuclei in the blastocysts was recorded.

2.6 Embryo transfer

For the *in vivo* study, at Day 1, the SCNT-derived embryos were surgically transferred into the ampullary-isthmic junction of surrogate gilts at 0 or 1 days after observed estrus. At the end of the gestation periods, the piglets were recovered through C-section.

2.7 Intracellular Ca²⁺ measurement

Matured oocytes were loaded with the Ca²⁺ indicator dye fura-2. For this purpose, they were incubated in the presence of 2 mM of the acetoxymethyl ester form of the dye and 0.02% pluronic F-127 (both from Invitrogen) for 40–50 min. The oocytes were transferred into a chamber with a cover-glass bottom; the chamber was then placed on the heated stage of an inverted microscope and changes in the intracellular free Ca²⁺ concentration in response to various TPEN treatments were recorded using InCyt Im2, a dual-wavelength fluorescence imaging system (Intracellular Imaging, Inc.; Cincinnati, OH). During measurements the emitted fluorescence was detected at 510 nm after exciting the dye alternately at 340 and 380 nm. The ratio of the two emitted fluorescence intensities was calculated and the data are presented as normalized fluorescence ratio values. In each treatment group, the measurements were repeated at least 5 times using different oocytes.

2.8 Intracellular Zn²⁺ measurement

After maturation, denuded oocytes were incubated in TL-Hepes medium in the presence of 2 μM FluoZinTM-3, AM (Invitrogen) for 1 hour. Embryos were then washed in TL-Hepes medium and placed on an inverted fluorescent scope and viewed through a FITC filter (Nikon). Images of individual oocytes were taken at a constant exposure (3 seconds). Intensity of fluorescence from the images was quantified using the Image J software (NIH). The average level of Zn²⁺ in oocytes from each treatment was calculated from the measurement of eight oocytes.

2.9 Statistical analysis

Differences in the frequency of blastocyst formation were determined after analysis of variance (ANOVA) using the PROC MIX procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA) or Chi-square test. Percentage data were arcsin transformed prior to the ANOVA analysis. Average total numbers of nuclei in blastocysts and the level of Zn²⁺ in oocytes were compared by using the Student's T-test. Differences with P<0.05 were considered significant.

2.10 Experimental design

2.10.1 Effect of TPEN on intracellular Ca²⁺ and Zn²⁺ in oocytes—Since intracellular Ca²⁺ is a key element of oocyte activation and TPEN is known to lower the concentration of Zn²⁺, the level of intracellular Ca²⁺ and Zn²⁺ in mature oocytes after TPEN treatment was measured. These experiments will show if the target of TPEN is specific to Zn²⁺. In addition, in a separate experiment exogenous Zn²⁺ was added to the medium containing TPEN to identify whether the target of TPEN is Zn²⁺ specific. If the target of TPEN is specific to Zn²⁺, the effect of TPEN should be attenuated by the presence of exogenous Zn²⁺. Pronuclear formation of embryos after TPEN treatment with or without exogenous Zn²⁺ was monitored. Pronuclear formation was observed at 14 hours post TPEN treatment by staining the embryos with Hoechst 33342 (1.2 mg/mL) and viewing the epifluorescence.

2.10.2 Activation of oocytes with TPEN alone—To test if TPEN alone can successfully activate pig oocytes, various concentrations (100 – 250 μM) and incubation durations (45 min – 2.5 hours) of TPEN were used and subsequent development to blastocyst was recorded.

2.10.3 Treatment of activated oocytes with TPEN—To investigate whether TPEN treatment can enhance development of activated oocytes, various concentrations of TPEN (0.5 – 200 μM) were combined with conventional activation approaches used in SCNT. Optimum conditions identified from preliminary screening was used on activated oocytes and SCNT embryos, then subsequent development of the embryos was monitored.

2.10.4 TPEN as the main activating reagent—To investigate whether TPEN can be a main reagent of oocyte activation, oocytes which received the minimum amount of Ca^{2+} signaling through electroporation were incubated with different concentrations and durations of TPEN. Subsequent development was monitored to observe the effectiveness of the method.

2.10.5 *In vivo* development of SCNT embryos treated with TPEN—To test *in vivo* competency of SCNT embryos treated with TPEN, conventionally activated SCNT embryos were incubated with either 5 μM TPEN for 30 min or 10 μM TPEN for 10 min. A total of six embryo transfers were conducted; three groups were treated with Scriptaid, a histone deacetylase inhibitor.

3. Results

3.1 TPEN does not induce a Ca^{2+} increase in pig oocytes

To demonstrate that TPEN treatment stimulates signaling pathways independent of Ca^{2+} signaling, oocytes were exposed to various concentrations of TPEN and the changes in the intracellular Ca^{2+} levels were measured. As expected, no Ca^{2+} increase was detected when oocytes were exposed to 5, 10, or 200 μM TPEN. On the other hand, the oocytes incubated in the presence of thimerosal (which served as a positive control) showed massive Ca^{2+} increases (Figure 1). This indicates that the benefit of TPEN observed during oocyte activation is independent of Ca^{2+} signals.

3.2 Target of TPEN is Zn^{2+} specific

To demonstrate that TPEN can remove available Zn^{2+} in oocytes, mature pig oocytes were exposed to 200 μM of TPEN for 30 min then the level of intracellular Zn^{2+} was measured. As expected, TPEN treatment almost completely depleted available Zn^{2+} in the oocytes as we couldn't detect any fluorescent signal representing Zn^{2+} after the TPEN treatment (Figure 2A). When the level of fluorescence was quantified using Image J software, the difference between control and TPEN treated group was significant ($p < 0.05$, Figure 2B). When the oocytes were treated with TPEN (200 μM , 30 min) with or without the presence of exogenous Zn^{2+} , the presence of exogenous Zn^{2+} dramatically decreased the number of oocytes that formed a pronucleus at 14 hours post TPEN treatment; only one out of ten oocytes showed spontaneous pronuclear formation in the control group (Figure 2C).

3.3 Parthenogenetic activation of porcine oocytes by TPEN

We first tested if the known concentration/duration of TPEN (100 μ M, 45 min) in the previous mouse study [21] had any effect on pig oocytes. The protocol could activate pig oocytes; however, the development was inferior to conventional artificial activation methods. The frequency of forming blastocysts on day 7 was comparable to the conventional oocyte activation method used (13% vs. 15%, respectively) but the rate of blastocyst formation was slower; no blastocyst formation was observed on day 5 with the TPEN treatment whereas 10% of activated embryos reached blastocysts in the control group. In addition, no expanded blastocysts were derived from the TPEN activation group on day 7, indicating lower developmental competency of the embryos derived from the TPEN activation method.

A wide range of concentrations (100 μ M, 200 μ M, and 250 μ M) and durations (30 min to 2.5 hours) were tested to identify the optimum parameters for TPEN-induced oocyte activation (Table 1). Optimum embryo development was achieved by using 200 μ M TPEN for 30 min and the frequency of blastocyst formation using the condition tended to be higher than a conventional activation method ($p=0.1$). However, a lower total number of nuclei in blastocysts was observed in the embryos derived from the optimal TPEN activation group (200 μ M, 30 min), compared to the control (27.2 ± 1.92 vs. 32.6 ± 3.12 , respectively). When the oocytes were incubated in 250 μ M TPEN over 2.5 hours, a toxic effect of TPEN was observed. The 250 μ M TPEN treatment over 2.5 hours had a negative effect on embryo development as there was no blastocyst formation. Here we identified an optimum concentration of TPEN which successfully induced development of activated oocytes, but the total number of nuclei in these embryos was lower compared to the conventional activation method.

3.4 Treatment of activated oocytes with TPEN can increase developmental potential in vitro

Next, we examined whether treating activated oocytes with TPEN could increase the efficiency of oocyte activation. When chemically activated oocytes were incubated with TPEN (100 μ M, 45 min), no blastocyst formation was observed indicating the combination of the two methods is toxic to embryos. We hypothesized that the detrimental effect was due to excess stimuli from Ca^{2+} signaling and TPEN. Therefore, we introduced lower concentrations of TPEN after activating oocytes with a conventional method. Incubating activated oocytes with a lower concentration of TPEN increased the developmental potential of parthenogenetic embryos. Interestingly, when activated oocytes were incubated with a low concentration of TPEN (5-10 μ M), the TPEN-treated group showed higher developmental potential compared to the control group. Specifically, the average percent of blastocyst formation in TPEN-treated oocytes (5 μ M for 30 min) was $27.2 \pm 1.7\%$ but only $10.6 \pm 2.5\%$ developed to blastocyst in the control group (Table 2 and Figure 3). Moreover, the average number of nuclei in blastocysts was higher in TPEN-treated oocytes compared to the control group, which was activated by the thimerosal/DTT approach (Table 2). Next, the optimal concentration and duration of TPEN (5 μ M, 30 min) was used to artificially activate oocytes reconstructed by SCNT. There was an increase in the frequency of blastocyst formation when activated SCNT oocytes were treated with TPEN (7.9% vs.

18.6%, respectively) (Table 3). This increase in development was comparable to the effect of Scriptaid, a histone deacetylase (HDAC) inhibitor, known to increase developmental potential of SCNT embryos. Thus, lower concentrations of TPEN (5–10 μM) can enhance embryo development when applied after conventional oocyte activation.

3.4 TPEN can serve as the main activating reagent

Because we hypothesized that a proper combination of Ca^{2+} signal and TPEN may be required to increase development of the activated oocytes, we tested if TPEN can be the main activating reagent. First, oocytes received an intracellular Ca^{2+} increase through electroporation; although the concentration of Ca^{2+} (0.1 mM) in the electroporation medium was not sufficient to induce oocyte activation. Then the oocytes were incubated with various concentrations of TPEN for various durations. The combination of a smaller Ca^{2+} increase and lower concentration of TPEN (10 μM) was not effective in inducing oocyte activation as no day 6 blastocysts were observed in the group. Surprisingly, a smaller Ca^{2+} increase followed by TPEN incubation at higher concentrations (100 μM or above) could successfully activate oocytes, and some combinations resulted in a superior *in vitro* development compared to the oocytes activated by a conventional method (Table 4). We have obtained the highest frequency of blastocysts, both day 6 and 7, when oocytes were activated by a smaller Ca^{2+} signal followed by incubating in 200 μM TPEN for 30 min.

3.5 In vivo developmental competence of SCNT embryos following TPEN activation

To address *in vivo* competency of SCNT embryos whose development was stimulated by utilizing TPEN, a series of embryo transfers was performed. The embryos were transferred into six surrogates, two of which carried their pregnancy to term, each giving birth to two healthy piglets (Table 5). One of the term developments was from SCNT embryos that were not treated with a HDAC inhibitor, Scriptaid (Figure 4). Thus, TPEN treatment is compatible with term development after SCNT.

4. Discussion

Artificial activation of oocytes is an essential process during SCNT. Unfortunately, current artificial activation methods used during SCNT are suboptimal as they cannot emulate the natural signaling pathway used by the fertilizing sperm. Most current artificial activation methods are able to induce only a single elevation in the intracellular free Ca^{2+} concentration of the oocyte [15, 26]; however, this single Ca^{2+} increase is thought to be less effective in degrading MPF compared to repetitive Ca^{2+} increases observed during fertilization [27]. This is believed to be a major reason for the low developmental potential of SCNT embryos [28]. Different strategies have been used to increase the efficiency of artificial oocyte activation; one of the most popular approaches being the reduction of MPF levels in the ooplasm using various inhibitors [16, 29]. However, due to potential toxicity of the chemicals applied, they are not widely used in cloned pig production.

Here we developed a system to increase the efficiency of oocyte activation by utilizing a membrane-permeable Zn^{2+} chelator, TPEN. The amount of Zn^{2+} in the oocyte increases during maturation and can regulate the timing of meiotic exit in mammalian oocytes [18].

This increase in the level of Zn^{2+} during oocyte maturation has also been reported in pigs [30]. It was suggested that the release of Zn^{2+} from FBXO43, a cytosolic factor with a Zn^{2+} binding domain, relieves APC/C from FBXO43-mediated inhibition, thus initiating the degradation of MPF [20]. Release of Zn^{2+} is observed during fertilization [22], suggesting that this is a natural process. By utilizing the Zn^{2+} -specific chelator TPEN, we could stimulate the natural process of Zn^{2+} depletion seen during physiological oocyte activation, and thus increase the developmental potential of the activated oocytes.

The absence of cytosolic Ca^{2+} increase after a TPEN treatment indicates that the activation induced by the chelator does not utilize Ca^{2+} as a second messenger. Development of artificial oocyte activation procedures generally focused on inducing an increase in intracellular Ca^{2+} levels. Our strategy does not involve a Ca^{2+} increase but instead targets the signaling pathway downstream of the Ca^{2+} signal as the reduction in Zn^{2+} can directly affect the activity of APC/C thus by-passing the need of activating CaMKII. Treatment of oocytes with TPEN only reduced the level of Zn^{2+} but not Ca^{2+} indicating that the effect of TPEN is independent from Ca^{2+} signaling as previously described [21]. In addition, decrease in the number of embryos reaching the pronuclear stage when oocytes were treated with in the presence of exogenous Zn^{2+} indicates that the effect of TPEN is specific to Zn^{2+} . This is similar to previous reports in pig oocytes [31].

Incubating mature porcine oocytes with TPEN could stimulate activation; however, the frequency of blastocyst formation was not superior to conventional Ca^{2+} -mediated oocyte activation protocols. In fact, we observed fewer nuclei in blastocysts derived from the TPEN activation method. This is consistent with previous reports where mouse oocytes were activated by TPEN, and the efficiency was lower compared to natural sperm-induced activation [21] indicating that reducing the level of intercellular Zn^{2+} alone may not be enough to successfully induce oocyte activation. In the mouse study, a combination of Ca^{2+} increase and TPEN incubation had a deleterious effect on embryo development [21]. However, in those experiments $SrCl_2$ was used to activate oocytes, a compound known to cause repetitive Ca^{2+} oscillations in mice [32]. TPEN was reported to disrupt Ca^{2+} signaling [18, 33]; therefore, we assumed that the detrimental effect of TPEN was due to its disruption of the oscillatory Ca^{2+} signal induced by $SrCl_2$. We hypothesized that TPEN might be used in combination with an activation protocol which causes only a single Ca^{2+} increase. As we expected, proper concentration of TPEN (5 – 10 μ M) could increase the developmental potential of the activated oocytes. Similarly, when we used TPEN as the main activating reagent, we also saw a significant increase in embryo development. This indicates that proper combinations of Ca^{2+} increase and Zn^{2+} depletion are effective in inducing meiotic exit of oocytes.

TPEN is known to be toxic and induces apoptosis in a concentration- and duration-dependent manner [34, 35]. Here, a similar effect was observed: long exposure to TPEN led to lower embryo development. This suggests that although a reduction in the intracellular Zn^{2+} concentration is important for oocyte activation, maintenance of a basal Zn^{2+} level is required for cellular survival. Our *in vivo* study indicates that we could produce viable clones using activation protocols that incorporate TPEN. The approach also allowed us to produce cloned piglets without treating SCNT embryos with Scriptaid, a HDAC inhibitor

that is frequently used in SCNT protocols to increase developmental potential of SCNT embryos [36-39]. This full term development suggests that TPEN, at the concentration used in our experiments, is not toxic to the SCNT embryos. None of the animals displayed any health-related problems at birth as often seen in cloned animals. Moreover, they have already produced live progeny.

A recent study confirmed that TPEN can activate pig oocytes [31]. These authors demonstrated that events of oocyte activation such as cortical granule release and reduction in MPF occur when mature oocytes are incubated with TPEN. These events are a part of oocyte activation [40, 41] indicating that the depletion of Zn^{2+} by TPEN is sufficient to stimulate the machinery that is normally associated with meiotic resumption. However, the concentration of TPEN applied in the above-mentioned study was not effective in inducing embryo development as no blastocyst formation was observed. This is consistent with our findings as only higher concentrations of TPEN (higher than 100 μ M) could activate pig oocytes and promote subsequent development.

In this study, the overall frequency of blastocysts from conventional activation or SCNT tended to be lower compared to our previous report [38]. This could be seasonal observation; however, we believe this is due to the difference in the source of oocytes. The majority of oocytes used in this study were collected from prepubertal gilts and it is known that such oocytes show poor development in pigs [3, 42]. Interestingly, the use of TPEN allowed us to observe a frequency of blastocysts in activated oocytes as high as 40%, indicating that TPEN activation can be particularly beneficial if oocytes with low developmental competency are used in the experiments.

In summary, our results demonstrate that TPEN, a Zn^{2+} chelator, can be utilized to increase the developmental potential of activated oocytes. When used in combination with electroporation after SCNT, the novel activation approach led to the production of live offspring and was also effective in enhancing overall proficiency of the technology.

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Highlights

- A Zn²⁺ chelator, TPEN, can activate porcine oocytes
- TPEN can enhance development of activated oocytes and SCNT embryos
- TPEN can be a main oocyte activating reagent
- TPEN does not induce a cytosolic Ca²⁺ increase in oocytes
- TPEN-assisted oocyte activation method can be used to produce live clones

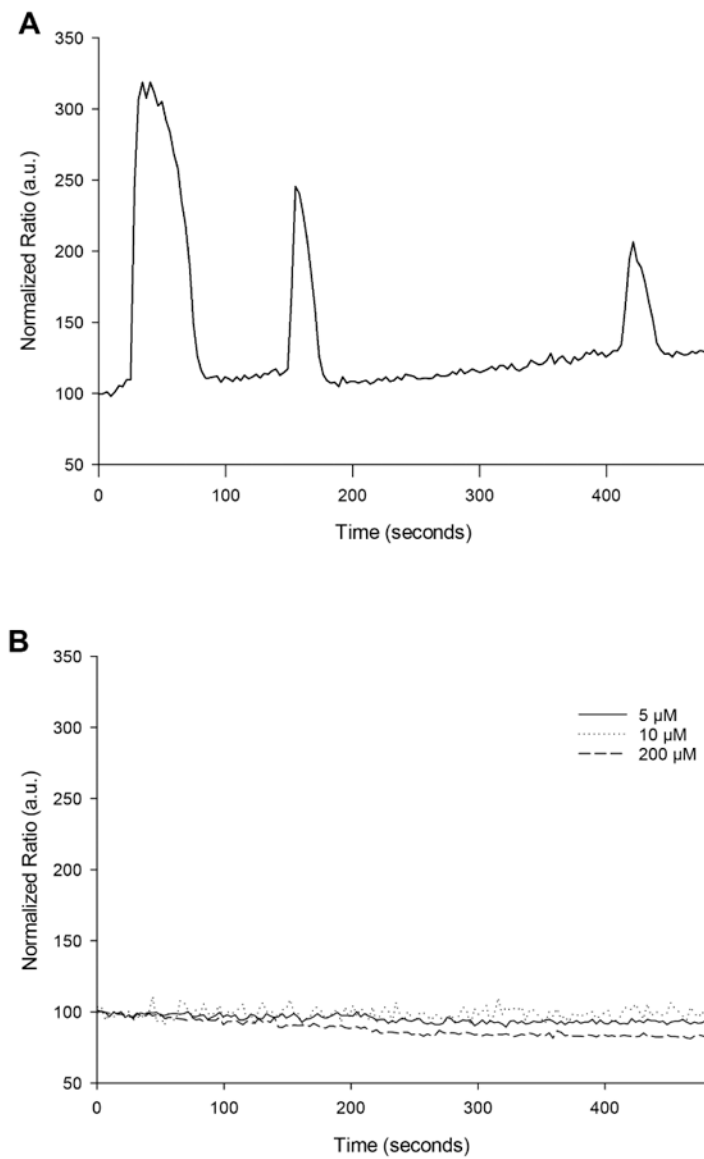


Figure 1. TPEN does not induce Ca^{2+} increase in MM oocytes. (A) Ca^{2+} increase after treating MII stage oocytes with thimerosal, a sulfhydryl-modifying agent. (B) Lack of Ca^{2+} increase after incubation in the presence of various concentrations of TPEN. The normalized fluorescence ratio on the Y-axis indicating Ca^{2+} changes is in arbitrary units.

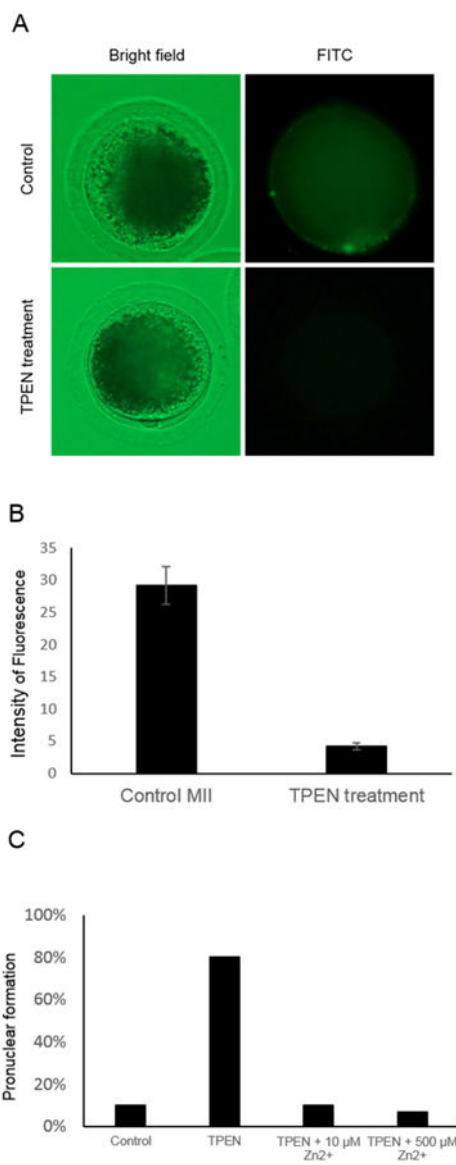


Figure 2.

Target of TPEN is Zn²⁺ specific. (A) Mature oocytes incubated with TPEN have a lower concentration of intracellular Zn²⁺ compared to the control group. (B) Quantification of the level of Zn²⁺ in mature oocytes treated with TPEN and the control. Intensity of fluorescence was measured by Image J (n=8 in each group). The difference was significant (p<0.05) (C) Presence of exogenous Zn²⁺ inhibits oocyte activation induced by TPEN. Pronuclear formation after TPEN treatment was dramatically reduced with the addition of Zn²⁺.

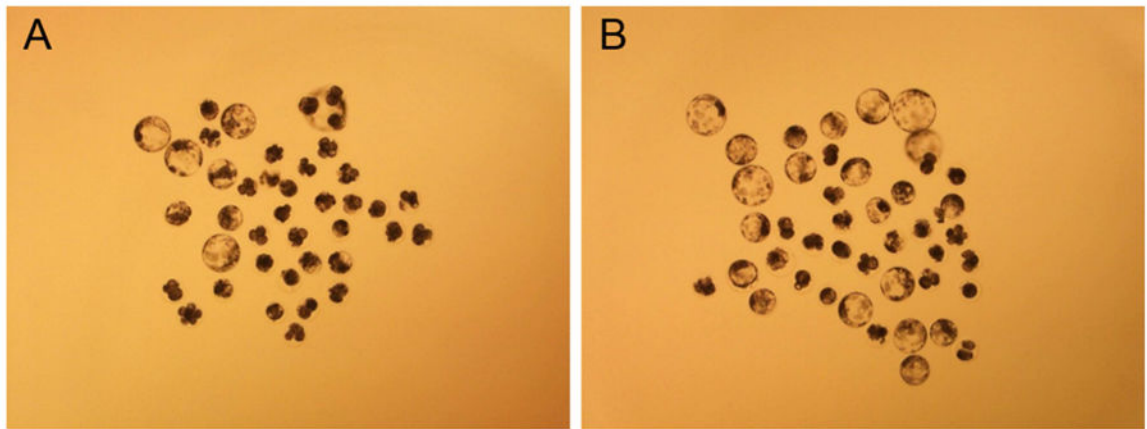


Figure 3. Image of parthenogenetic embryos generated through the TPEN assisted activation method. Oocytes were activated by thimerosal/DTT, then half were incubated with 5 μ M TPEN for 30 min. The pictures were taken on Day 6. (A) Control group. (B) Oocytes further activated with TPEN.

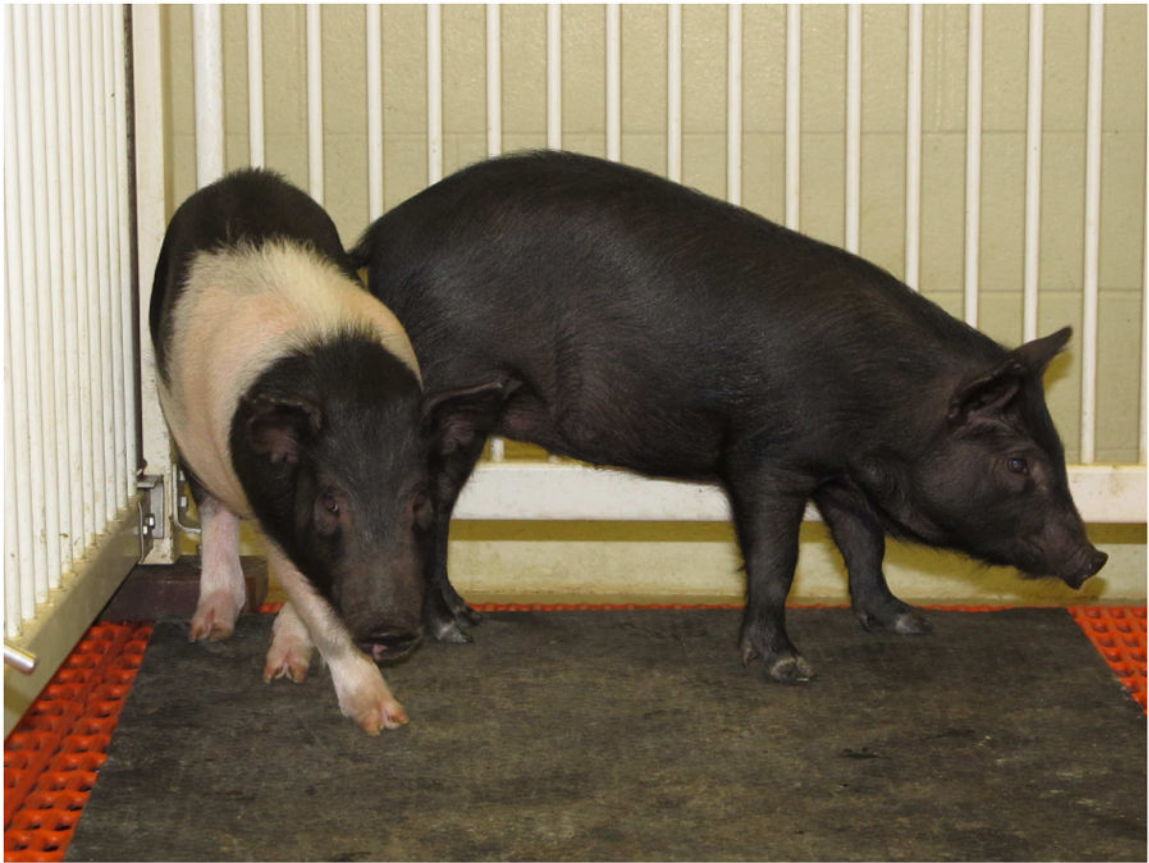


Figure 4. Images of cloned pigs produced by this method. The animals were healthy and did not show any developmental abnormality.

Table 1

Porcine oocytes activated with TPEN alone. When porcine oocytes were treated with TPEN, frequency of blastocyst formation was similar to conventional method; no beneficial effect was found. Longer exposure of oocytes to TPEN at high concentration was toxic. Frequency of blastocysts was recorded on Day 6.

Treatment	Total number of oocytes	% blastocysts
Electroporation	98	11.2%
100 μ M, 2 hr	65	12.0%
200 μ M, 30 min	25	23.0%
200 μ M, 1 hr	79	20.0%
250 μ M, 2 hr	30	10.0%
250 μ M, 2.5 hr	25	0%

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Table 2

Incubation of TPEN can increase development potential of activated oocytes. Incubating activated porcine oocytes with lower concentrations of TPEN (5-10 μ M) increased formation of blastocysts. Different letters indicate statistical difference ($p < 0.05$). Frequency of blastocysts was recorded on Day 6.

Treatment	Total number of embryos	% cleaved	% Blastocysts	Total cell number in blastocysts
THI/DTT	113	59.3 ^a	10.6 ^a	28.2 \pm 2.1 ^a
THI/DTT + 5 μ M TPEN, 30 min	114	74.6 ^b	27.2 ^b	33.1 \pm 2.6 ^b
THI/DTT + 10 μ M TPEN, 10 min	83	81.9 ^b	21.7 ^b	31.2 \pm 2.6 ^b

Table 3

In vitro development of SCNT embryos treated with Scriptaid or TPEN alone. SCNT embryos were initially activated using thimerosal/DTT method. Treatment of TPEN can increase developmental potential of SCNT embryos compared to the control and compatible to Scriptaid treated embryos. Different letters indicate statistical difference ($p < 0.05$). Frequency of blastocysts was recorded on Day 6.

Treatment	Number of embryos	% Blastocysts
SCNT (control)	76	7.9% ^a
SCNT + Scriptaid	194	22.7% ^b
SCNT + 5 μ M TPEN, 30 min	102	18.6% ^b

Table 4

Effect of TPEN as a main oocyte activating reagent. Different letters indicate statistical difference ($p < 0.05$).

Treatment	Number of embryos	Day 6 Blastocysts (%)	Day 7 Blastocysts (%)
Control - electroporation	80	13.75 ^a	17.5 ^a
10 μ M, 30 min	40	0 ^b	5 ^b
10 μ M, 1 hour	40	15 ^a	22.5 ^a
10 μ M, 2 hours	40	15 ^a	25 ^a
100 μ M, 10 min	40	15 ^a	15 ^a
100 μ M, 20 min	40	25 ^a	30 ^a
200 μ M, 30 min	81	37.04 ^c	41.98 ^c
200 μ M, 1 hour	80	18.75 ^a	27.5 ^a

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Table 5

Result of embryo transfer.

Treatment	Number of embryos transferred	Result
SCNT + 5 μ M TPEN, 30 min + Scriptaid	207	2 normal piglets were delivered
SCNT + 5 μ M TPEN, 30 min + Scriptaid	245	Cycled
SCNT + 10 μ M TPEN, 10 min + Scriptaid	180	Cycled
SCNT + 5 μ M TPEN, 30 min	280	2 normal piglets were delivered
SCNT + 5 μ M TPEN, 30 min	230	Cycled
SCNT + 5 μ M TPEN, 30 min	280	Cycled

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