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Zinc supplementation during *in vitro* maturation increases the production efficiency of cloned pigs

Yubyeol JEON[1, 4), Junchul David YOON[1), Lian CAI[1), Seon-Ung HWANG[1), Eunhye KIM[1), Eunsong LEE[2), Eui Bae JEUNG[3), Sang-Hwan HYUN[1) and Woo Suk HWANG[4)

[1)*Laboratory of Veterinary Embryology and Biotechnology (VETEMBIO), Veterinary Medical Center and College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Chungbuk, Republic of Korea*
[2)*Laboratory of Theriogenology, College of Veterinary Medicine, Kangwon National University, Chuncheon 24341, Kangwon, Republic of Korea*
[3)*Laboratory of Veterinary Biochemistry and Molecular Biology, College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Chungbuk, Republic of Korea*
[4)*Sooam Biotech Research Foundation, Guro-gu 08359, Seoul, Republic of Korea*

Abstract. Zinc supplementation (0.8 µg/ml) in *in vitro* maturation (IVM) medium significantly enhances oocyte quality. In this study, we compared the development of somatic cell nuclear transfer (SCNT) embryos produced from conventional IVM (control) and zinc-supplemented IVM oocytes. A total of 1206 and 890 SCNT embryos were produced using control and zinc-supplemented oocytes, respectively, and then were transferred to 11 and 8 recipients, respectively. Five control recipients and three zinc-supplemented recipients became pregnant. Two live piglets and eight mummies were born from two control recipients, and ten live piglets and six stillborn piglets were born from three zinc-supplemented recipients. The production efficiency significantly increased in the zinc-supplemented group (0.33% *vs*. 3.02%). This report suggests that zinc supplementation in IVM medium improved the production efficiency of cloned pigs.
Keywords: Cloning efficiency, *In vitro* maturation (IVM), Porcine, Somatic cell nuclear transfer (SCNT), Zinc

Transgenic (TG) cloned pigs have recently become essential in biomedical research [1]. For example, TG cloned pigs have been used in xenotransplantation to generate organ grafts [2, 3], as human disease models to study etiology and therapy [4, 5], and as living bioreactors to produce valuable proteins for medical applications [6].

Production of TG cloned animals requires advanced reproductive and genetic engineering technologies. However, until recently porcine embryology was one of the least developed and most frustrating fields in reproductive research involving domestic animals [7]. Despite significant improvements in *in vitro* embryo production for porcine embryology, the development of *in vitro*-produced embryos remains suboptimal [8–10]. Porcine oocytes and embryos are sensitive to physical and chemical factors used *in vitro* [11]. Therefore, modified *in vitro* production systems that more closely mimic the *in vivo* microenvironment are required to improve the development of porcine embryos. In a previous report, zinc supplementation at a normal body fluid concentration (0.8 µg/ml) during *in vitro* maturation (IVM) was shown to have beneficial effects on the embryonic development of parthenogenetic and *in vitro*-fertilized

(IVF) embryos [12]. Zinc supplementation during IVM may also be beneficial for the embryonic development of embryos produced by somatic cell nuclear transfer (SCNT) and the production of cloned pigs. Thus, in this report, we attempted to produce cloned pigs using a modified protocol that included zinc supplementation during IVM to improve the developmental capacity of SCNT embryos *in vivo*.

A total of 485 cloned embryos were produced to evaluate the effect of zinc supplementation during IVM on SCNT embryo development *in vitro*. There were no significant differences in the cleavage and blastocyst formation rates between the conventional IVM group (control) and the zinc-supplemented group (Table 1).

A total of 1,206 and 890 cloned embryos were produced using control and zinc-supplemented oocytes, respectively; these were then transferred to 11 and 8 recipients, respectively. Five recipients in the control group became pregnant; three aborted and two delivered two live piglets and eight mummies. Three recipients in the zinc-supplemented group became pregnant; all three delivered to produce ten live piglets and six stillborn piglets (Tables 2 and 3). The pregnancy rate and pregnancy status were not significantly different; however, zinc supplementation during IVM had a tendency (P = 0.09) to affect pregnancy status (Table 2). As shown in Table 3, the production efficiency significantly increased in the zinc-supplemented group.

The low efficiency of cloned pig production has been attributed to multiple factors, including oocyte quality, the use of inadequate culture and manipulation media during nuclear transfer, the lack of an efficient activation method, difficulty in achieving genetic modification, and the requirement for a minimum number of fetuses to

Table 1. Effects of zinc supplementation during IVM on *in vitro* porcine SCNT embryonic development

Group	No. embryo cultured*	No. cleaved embryos (%) at day 2	No. blastocyst (%) at day 7
Control	252	205 (80.6 ± 4.4)	56 (22.9 ± 4.0)
Zinc	233	180 (75.3 ± 4.8)	59 (23.7 ± 4.7)

* Eight replicates.

Table 2. Effect of zinc supplement during IVM on pregnancy of surrogates after transfer of porcine SCNT embryos

Group	No. surrogates	No. transferred embryos	No. (%) pregnant surrogates (Pregnancy rate)	Full-term pregnancy status (%) of surrogates [#]	
				Abortion	Parturition
Control	11	1206	5 (45.5)	3 (27.3)	2 (18.2)
Zinc	8	890	3 (37.5)	0 (0)	3 (37.5)

[#] $P = 0.09$.

Table 3. Effect of zinc supplement during IVM on production of cloned piglets after transfer of porcine SCNT embryos

Group	No. pregnant surrogates	No. transferred embryos	Average litter size	No. dead fetuses	No. live fetuses (Production efficiency) *
Control	5	570	2.00 ± 1.76	8	2 (0.33 ± 0.20)
Zinc	3	337	5.33 ± 0.67	6	10 (3.02 ± 0.90)

* $P < 0.05$.

maintain pregnancy in pigs [13]. To improve the efficiency of cloned pig production, this report focused on improving oocyte quality.

Oocyte maturation is an important step in cloned pig production. Recipient oocytes have a marked effect on blastocyst formation and the development of embryos to term [14]. Cloned pigs can be produced using both *in vivo*- [15] and *in vitro*- [2, 16, 17] derived oocytes; however, *in vivo*-derived oocytes enhance the probability of obtaining cloned offspring. According to Lai and Prather [18], incomplete cytoplasmic maturation of porcine oocytes influences both blastocyst formation and full-term development. Therefore, researchers have attempted to identify an alternative method for enhancing oocyte quality and the developmental ability of embryos produced by SCNT. Kun *et al*. [19] reported that leptin supplementation of IVM medium may be beneficial, not only for the meiotic maturation potential of oocytes, but also for the subsequent developmental competence of embryos produced by parthenogenetic activation and the cleavage of embryos derived by SCNT. In another study, supplementation with cysteamine and β-mercaptoethanol to improve SCNT embryo development was attempted; however, a stimulatory effect was not observed [20]. Further, L-carnitine treatment during IVM improved the developmental competence of SCNT embryos [21]. This was probably due to increased cytoplasmic maturation, and the stimulation of nuclear reprogramming via increased transcription factor expression. These studies suggest that improving oocyte quality can be beneficial for the developmental competence of SCNT embryos. However, this effect has been not confirmed by an assessment of *in vivo* viability.

In this report, zinc supplementation during IVM did not improve *in vitro* development of SCNT embryos, unlike the effect on PA and IVF embryos. Therefore, it is not clear why zinc supplementation

improved the production efficiency of cloned pigs. However, the production efficiency of cloned pigs increased in the group that received zinc supplementation during IVM. The beneficial effects of zinc supplementation during IVM on oocyte maturation and embryonic developmental competence were demonstrated in previous report [12]. Specifically, zinc supplementation during IVM increased intracellular GSH synthesis, reduced ROS levels, and improved transcription factor expression. These factors probably improved the developmental competence of SCNT embryos. Also, zinc is involved in cytoskeleton organization. Abnormal microfilament distributions in oocytes were observed under conditions of zinc insufficiency during IVM [22]. Abnormal microfilament distributions can also influence SCNT embryos. According to Cheng *et al*. [23], cytoskeleton-associated proteins may be key determinants of early clone development. This suggests a supporting role for cytoplasmic components of oocytes in nuclear reprogramming. Zinc is also an essential factor for normal growth and development [24]. Inadequate zinc supplementation during early development caused developmental arrest and abnormal development in early pregnancy. It is believed that zinc supplementation during IVM supports the development of cloned embryos and maintenance of the minimum number of fetuses required for pregnancy.

In conclusion, the present report shows that zinc supplementation during IVM improved the production efficiency of cloned pigs. However, although the production of cloned pigs was improved by zinc supplementation during IVM, the efficiency was still unsatisfactory. Further study to enable more efficient cloned pig production is needed.

Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Veterinary and Quarantine Service (Anyang, Korea). The protocol was approved by the Committee on the Ethics of Animal Experiments of Chungbuk National University (Cheongju, Korea) (Permit number: CBNUA-584-13-01). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Oocyte collection and IVM

Porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/v) NaCl solution that was supplemented with penicillin-G (100 IU/ml) and streptomycin sulfate (100 mg/l) at 30–35°C. Follicular fluid with oocytes was aspirated from antral follicles (3–6 mm in diameter) using an 18-gauge needle connected to a 10-ml disposable syringe and collected into a 15-ml centrifuge tube. Cumulus-oocyte complexes (COCs) were recovered under a stereomicroscope, and those with at least three layers of compact cumulus cells and a homogenous cytoplasm were selected for IVM. The selected COCs were washed three times in HEPES-buffered Tyrode's medium containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA), and transferred to 500 µl of tissue culture medium 199 (Invitrogen, Carlsbad, CA, USA) supplemented with 0.8 µg/ml zinc (zinc supplemented group only), 0.6 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/ml epidermal growth factor, 75 µg/ml kanamycin, 1 µg/ml insulin, and 10% (v/v) pig follicular fluid. The concentration of zinc was set at 0.8 µg/ml in accordance with previous report [12]. For maturation, the selected COCs were washed three times in the above described tissue culture medium 199 (IVM medium) containing hormone supplements (10 IU/ml equine chorionic gonadotropin and 10 IU/ml human chorionic gonadotropin [Intervet, Boxmeer, the Netherlands]), and approximately 50–60 oocytes were transferred to each well of a 4-well Nunc dish (Roskilde, Denmark) containing 500 µl of culture medium and equilibrated for at least 2 h with 5% CO_2 at 39°C in a humidified atmosphere. After 22 h of maturation with hormones, the COCs were washed twice and cultured in hormone-free IVM medium for an additional 18–20 h.

Preparation of donor cells

Fetal fibroblasts were derived and cultured as described previously [25]. Briefly, a pig fetus (Landrace × Duroc crossbreed) at embryonic day 40 was isolated from the uterus of a female pig. The tissues of the fetus were cut into 0.5-mm^2 pieces and cultured in a 100-mm culture dish. Cells were grown at 37°C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose (Gibco BRL, Grand Island, NY, USA) and supplemented with fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 µg/ml). The adherent cells were subcultured. The fetal fibroblasts were frozen and kept at –150°C until SCNT. Prior to SCNT, the cells were thawed and cultured in above described DMEM for 3–4 days until they reached 80% confluence. Adherent cells (passage 7~10) were treated with trypsin for ~1 min and used for SCNT.

Micromanipulation for SCNT, fusion, and activation

After 40–42 h of IVM, denuded oocytes were incubated for 5 min in manipulation medium (calcium-free TLH containing 0.2% bovine serum albumin [TLH-BSA]) containing 5 µg/ml Hoechst 33342, washed twice with fresh manipulation medium, and transferred to a drop of manipulation medium containing 5 µg/ml cytochalasin B. The oocytes were enucleated by aspirating the polar body and MII chromosomes using a 16-µm glass pipette (Humagen, Charlottesville, VA, USA). After enucleation, a trypsinized fetal fibroblast of 14–15 µm with a smooth cell surface was transferred to the perivitelline space of the enucleated oocyte. The couplets were equilibrated with 280 mM mannitol solution containing 0.001 mM $CaCl_2$ and 0.05 mM $MgSO_4$ for 2–3 min and then transferred to a fusion chamber containing two electrodes overlaid with 280 mM mannitol solution. Membrane fusion was induced by applying an alternating current field of 2 V cycling at 1 MHz for 2 sec, followed by two pulses of 160 V/mm direct current for 60 µsec using a cell fusion generator (LF201; Nepa Gene, Chiba, Japan). The activation was performed simultaneously with the fusion. After fusion, the couplets were washed 3–4 times with TLH-BSA. The oocytes were examined after 30 min, and fused, normally shaped oocytes were collected.

In vitro embryo culture (IVC) and embryo evaluation

The collected SCNT embryos were washed three times with IVC medium (PZM 3) and cultured in 30-µl microdrops of IVC medium. Embryos in culture medium were covered with pre-warmed mineral oil and incubated at 39°C for 7 days under a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2. The day when SCNT were performed was designated as day 0. The embryos were evaluated under a stereomicroscope for cleavage on day 2. Blastocyst formation was assessed on day 7.

Embryo transfer

At 4 h post-activation, SCNT embryos were transferred to naturally cycling Landrace × Duroc crossbreed gilts on the first day of standing estrus. A midventral laparotomy was performed under general anesthesia using isoflurane. The reproductive tract was exposed, and the SCNT embryos (100–120 embryos per recipient) were transferred into an oviduct at the ampullary isthmic junction. Pregnancy was diagnosed on day 30 and was checked every 2–4 weeks by ultrasonography. If fetal echoes did not correspond with the gestational age of the fetuses and if signs of fetal absorption, such as small vesicles without any detectable fetuses, were observed, an abortion was considered to have occurred. Each of the cloned piglets was delivered naturally.

Statistical analysis

Data related to the rates of cleavage, blastocyst formation, litter size, and production efficiency are shown as mean ± SEM and were compared by Student's t-test. Data related to pregnancy rate and pregnancy status were analyzed using chi-square tests. Statistical differences at P < 0.05 were considered significant.

Acknowledgements

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