

Research Article

Effect of Hormone-treatment Duration in *in vitro* Maturation of Immature Oocytes on Pig Cloning Efficiency

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Abstract

This study aims to analyze the influence of hormone-treatment duration in *in vitro* maturation of porcine immature oocytes on the pig cloning efficiency. Although there were no significant differences in the maturation rate of immature oocytes and cleavage rate of somatic cell nuclear transfer embryos according to the hormone-treatment period (22, 27, 32, 37 and 42 h), the blastocyst rates of somatic cell nuclear transfer embryos derived from oocytes cultivated for 22, 27 and 32 h (19.0%, 21.5% and 22.4%, respectively) were significantly ($P<0.05$) higher than those for 37 and 42 h (13.2% and 12.4%, respectively). In particular, there were significant ($P<0.05$) increase in the hatching blastocyst rates of 27 and 32 h cultivated groups (13.7% and 16.5%, respectively) compared to 22, 37, 42h cultivated groups (8.2%, 5.6% and 6.7%, respectively). Moreover, the cloning efficiency and the delivery rates of normal lives of 27 (1.4% and 1.2%, respectively) and 32h (1.5% and 1.3%, respectively) cultivated groups were significantly ($P<0.05$) higher than those of 22h cultivated group (0.9% and 0.6%, respectively). To sum up, this study shows that the increase of hormone-treatment duration in *in vitro* maturation of porcine immature oocytes to a certain extent has a positive impact on the cloning efficiency.

Keywords

In vitro Maturation, Hormone, Nuclear Transfer, Cloning, Pig

1. Introduction

Somatic cell nuclear transfer (SCNT) is one of the most feasible reproductive tool which helps to preserve and propagate genetic resources by reproducing progenitors with good traits [1-5] and establish the system of breeding superior pedigree stocks which is important in increasing livestock production [6-14]. It has generally been known that one of the many important factors that have unignorable impact on the success rate of cloning is the quality of recipient oocytes

[15-24].

During *in vitro* maturation (IVM) substances required for following embryo development, especially for reprogramming of somatic cell nucleus, are accumulated within oocytes showing that the establishment of optimal *in vitro* maturation system including the medium composition and the culture condition is one of the critical factors that influence the control of oocytes meiosis [25-31]. Generally, hormones,

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steroids, serum, or follicular fluid contained in maturation medium provide nutrients for the growth and maturation of oocytes [32, 33]. In particular, hypophysial gonadotrophins and steroids which play an important role in regulating the oocyte maturation are added in *in vitro* maturation medium to induce the cytoplasm maturation and expansion of cumulus cells [34-39].

Growing porcine oocytes from follicles neither mature nor complete meiosis I, even under appropriate culture conditions in which fully grown oocytes mature to MII. In pig follicles, whilst supporting the oocyte, which is essential for oocyte growth, granulosa cells proliferate and differentiate in response to FSH stimulation, and they increase meiotic and developmental competence of oocytes. In the ovary, mammalian oocytes are arrested in the initial stage of meiotic division (germinal vesicle) until the release of the pre-ovulatory gonadotrophin that stimulates the resumption of meiosis [40]. During the final stage of maturation, immature oocytes develop from germinal vesicle (prophase I) to metaphase II (MII).

The *in vitro* maturation culture process of porcine immature oocytes consists of 2 stages. Firstly, they are cultured in the medium containing gonadotrophin for 20-24 h and subsequently, in the medium without gonadotrophin for 18-22 h. Eventually, most of the researchers claim that the oocytes collected from the medium follicle (3-6mm)-derived oocytes should be cultured for 22-24 h with hormones as the primary culture followed by 18-20 h of the secondary culture (total 40-44 h). Since it is an important guarantee to provide good-quality and enough numbers of oocyte in the porcine nuclear transfer process, some researchers have attempted to improve the quality of oocytes by adding some useful substances in the process of culture for oocytes maturation, so they eventually increased the maturation rate up to 60-90% by culturing oocytes for 32-48 h [41, 42]. Especially, the hormone-treatment period plays an important role in inducing the synchronization of *in vitro* maturation of oocytes and allowing oocytes to fully synthesize substances required for their late development [43].

In this study, we attempted to analyze and evaluate the influence of the hormone-treatment period in *in vitro* maturation on the cloning efficiency in pigs.

2. Materials and Methods

All chemicals and reagents in this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. Porcine follicular fluid (pFF) was extracted from 4.0-7.0mm follicles, filtered and stored at -20 °C. All procedures for animal care, SCNT, embryo transfer was approved by the Ethics Committee, the State Academy of Sciences, DPR Korea.

2.1. Oocyte Collection and *in vitro* Maturation

Abattoir-derived ovaries were transported to the laboratory at 30 to 35 °C within 2-3 h. Ovaries were washed several times with warm (35–37 °C) 0.9% NaCl solution and removed moisture with sterilized gauze. The cumulus-oocyte complexes (COCs) were aspirated with a 10 mL disposable syringe and 18-gauge needle from 3.0 to 6.0mm follicles located on the ovaries and COCs with at least three layers of compact cumulus cells and evenly granulated homogenous cytoplasm were selected under a stereoscope microscope for IVM. Next, COCs were washed more than six times with NCSU-23 HEPES buffered manipulation medium containing 0.4% bovine serum albumin (BSA, Hyclone, Logan, Utah, USA). Then, approximately 100 COCs were cultured in each well of a 24-well dish (Nunc, Roskilde, Denmark) containing 750 µL of NCSU-23 medium supplemented with 10 IU/mL Pregnant Mare's Serum Gonadotropin (PMSG), 10 IU/mL human Chorionic Gonadotrophin (hCG), 10 ng/mL epidermal growth factor (EGF), 25 µg/mL ascorbic acid, 10% (v/v) pFF, 0.36 µg/mL sodium pyruvate, 75 µg/mL penicillin G and 50 µg/mL streptomycin covered with 250 µL mineral oil for 22-42 h. Then, the COCs were cultured in above mentioned medium without PMSG and hCG for additional 20-0 h, so the total duration of IVM was 42 h. Experimental groups were divided as follows to evaluate effect of the hormone-treatment period during IVM on pig cloning efficiency.

Group 1 (M 22): hormone-treatment period 22h

Group 2 (M 27): hormone-treatment period 27h

Group 3 (M 32): hormone-treatment period 32h

Group 4 (M 37): hormone-treatment period 37h

Group 5 (M 42): hormone-treatment period 42h

In all cases, culture was performed at 38.5 °C in a humidified atmosphere of 5% CO₂ in air. At the end of defined periods of oocyte maturation, the cumulus cells were removed by repeated pipetting in NCSU-23 HEPES buffered manipulation medium supplemented with 0.1% hyaluronidase. Denuded oocytes were placed in droplets of NCSU23-HEPES buffered solution and then washed 6 times. Denuded oocytes with the first polar body and evenly granulated homogenous cytoplasm were evaluated as mature oocytes and used in SCNT experiments.

2.2. Preparation of Donor Cells

Cloned fetus derived from Large White Progenitor pig's ear fibroblasts was separated on day 35 gestation and, it was confirmed identical with the previous generation by genetic sequencing. Fetal fibroblasts were isolated from a cloned fetus and cultured for 3 to 8 passages in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) supplemented with 1% nonessential amino acids (GIBCO), 2mm L-glutamine (Sigma), 0.1mm β-mercaptoethanol, 0.1mm sodium pyruvate (Sigma-Aldrich), and 15% FBS (Hyclone, USA). These cells were stored in liquid nitrogen until used as donor cells for

SCNT in the present experiment. Donor cells were thawed and cultured for 2 to 3 passages in above mentioned medium, then further cultured at confluence for 3 d before nuclear transfer to synchronize the cell cycle at G0/G1 phase.

2.3. Somatic Cell Nuclear Transfer and Fusion/Activation of Couplets

Denuded oocytes were enucleated by aspirating the first polar body and the adjacent cytoplasm with an enucleation/injection pipette (inner-diameter, 15 to 20 μ m) and a single donor cell was then injected into the perivitelline space of an enucleated oocytes in NCSU-23 HEPES buffered manipulation medium supplemented with 7.5 μ g/mL cytochalasin B (CB). After the manipulation, reconstructed couplets were transferred into NCSU-23 HEPES buffered medium drops and equilibrated for 30 min at 38.5 $^{\circ}$ C in 100% humidity. Fusion/activation was achieved simultaneously with two DC pulses of 1.6 KV/cm for 30 μ s in a chamber consisting of platinum wire electrodes 1mm apart containing fusion medium using a BTX-Electro-Cell Manipulator. After the fusion/activation, reconstructed couplets were transferred into NCSU-23 HEPES buffered medium drops and equilibrated for 40 min at 38.5 $^{\circ}$ C in 100% humidity. Fused/Activated oocytes were then cultured in NCSU-23 embryo culture medium supplemented with 7.5 μ g/mL CB for 2 h under mineral oil at 38.5 $^{\circ}$ C in a humidified atmosphere of 5% CO₂ in air to prevent the extrusion of second polar body and were transferred into CB-free embryo culture medium drops and cultured under the same condition.

2.4. Embryo Culture, Embryo Transfer, Pregnancy Diagnosis and Delivery of Piglets

Then, approximately 50 reconstructed couplets were cultured in each well of a 24-well dish containing 750 μ L of NCSU-23 embryo culture medium supplemented with 0.4% BSA covered with 250 μ L mineral oil at 38.5 $^{\circ}$ C in a humidified air of 5% CO₂. The rates of cleavage and blastocyst formation were evaluated on Day 2 and 7, respectively and the rates of hatched blastocyst formation of reconstructed couplets were evaluated on Day 8 finally. Naturally cycling preovulatory gilts on the first day of estrus were selected as

recipients and about 150-200 of cloned embryos cultured for 1 day were surgically transferred into both oviducts of each recipient. Pregnancies were monitored by ultrasound scanning on Day 25 after embryo transfer. All cloned piglets were delivered by naturally and cesarean section on Day 114-117.

2.5. Statistical Analysis

Chi-square test was carried out using the non-parametric test program of the SPSS Software to test difference of experimental group on the *in vitro* maturation rate, cleavage rate, blastocyst rate and cloning efficiency. The litter size was expressed as the means $\pm$ SEM and the significance of the differences between the means was tested using the Student's *t*-test. Statistical significance was determined when a *P* value was less than 0.05.

3. Results

3.1. The Influence of Hormone-treatment Duration on Development Rate of SCNT Embryos

As shown in Table 1, there were no significant differences in the maturation rate of immature oocytes and cleavage rate of SCNT embryos according to the hormone treatment period (22, 27, 32, 37 and 42 h), whereas the blastocyst rates of SCNT embryos derived from oocytes cultivated for 22, 27 and 32 h (19.0%, 21.5% and 22.4%, respectively) were significantly ($P < 0.05$) higher than those for 37 and 42 h (13.2% and 12.4%, respectively). Moreover, the hatching blastocyst rates of 27 and 32 h cultivated groups (13.7% and 16.5%, respectively) were significantly ($P < 0.05$) higher than that of 22, 37, 42 h cultivated groups (8.2%, 5.6% and 6.7%, respectively). As shown in Figure 1, although, in the hormone-treatment period, the cumulus cells in 32 h cultivated group were slightly more expanded compared to those of 22 h cultivated group, in the second maturation there were slightly less expansion of cumulus cells in 32 h cultivated group compared to those in 22 h cultivated group.

Table 1. Effect of hormone-treatment duration in *in vitro* maturation culture on *in vitro* development rate of SCNT embryos.

IVM group	No. of used oocytes	No. of matured oocytes (%)	No. of SCNT embryos	Developmental rates (%)		
				Cleavage	Blastocyst	Hatched blastocyst
M 22	227	166 (73.1)	158	124 (78.5)	30 (19.0) ^a	13 (8.2) ^b
M 27	285	212 (74.4)	205	162 (79.0)	44 (21.5) ^a	28 (13.7) ^a
M 32	248	179 (72.2)	170	135 (79.4)	38 (22.4) ^a	28 (16.5) ^a

IVM group	No. of used oocytes	No. of matured oocytes (%)	No. of SCNT embryos	Developmental rates (%)		
				Cleavage	Blastocyst	Hatched blastocyst
M 37	226	150 (66.4)	144	109 (75.7)	19 (13.2) ^b	8 (5.6) ^b
M 42	278	185 (66.5)	178	134 (75.3)	22 (12.4) ^b	12 (6.7) ^b

^{a, b} Different subscripts in the same column indicate significantly differences ($P < 0.05$).

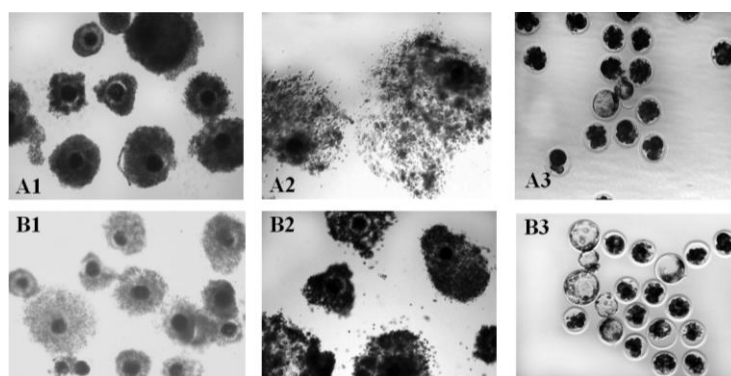


Figure 1. Morphology of oocytes after the hormone-treatment period (A1) and no-hormone-treatment period (A2) and blastocysts (A3) in the M 22 group. Morphology of oocytes after the hormone-treatment period (B1) and no-hormone-treatment period (B2) and blastocysts (B3) in the M 32 group. Magnification: 100× (A1-A3, B1-B3).

3.2. The Influence of Hormone-treatment Duration in *in vitro* Maturation Culture on Pig Cloning Efficiency

As shown in Table 2, the cloning efficiency and the delivery rates of normal lives of 27 (1.4% and 1.2%, respectively) and 32 h (1.5% and 1.3%, respectively) cultivated groups were significantly ($P < 0.05$) higher than those of 22h cultivated group (0.9% and 0.6%, respectively).

Table 2. Effect of hormone-treatment duration in *in vitro* maturation culture on pig cloning efficiency.

IVM group	No. of recipient	No. of transferred embryos	No. of pregnancy recipients (%)	No. of delivery recipients (%)	No. of cloned piglets (%)	No. of normal cloned piglets (%)	Litter size (Mean ± SEM)
M 22	8	1270	4 (50.0)	3 (37.5)	11 (0.9) ^b	8 (0.6) ^b	3.7 ± 1.2
M 27	12	1782	9 (75.0)	7 (58.3)	25 (1.4) ^a	22 (1.2) ^a	3.6 ± 2.2
M 32	6	1004	4 (66.7)	4 (66.7)	15 (1.5) ^a	13 (1.3) ^a	3.8 ± 2.9

Pregnancy rate = No. of pregnant recipients/No. of used recipients.

Delivery rate = No. of farrowed recipients/No. of used recipients.

Cloning efficiency = No. of cloned piglets / No. of cloned embryos received by all used recipients.

Normal cloning efficiency = No. of normal cloned piglets / No. of cloned embryos received by all used recipients.

Average litter size = No. of cloned piglets/No. of farrowed recipients.

^{a, b} Different subscripts in the same column indicate significantly differences ($P < 0.05$).

4. Discussion

As the artificial reproduction technology (ARTs) develops rapidly, there has been a number of profound studies reported

to improve the cloning efficiency. Especially, the study to reduce the cost required to maintain good breeds by using nuclear transfer technology in pigs have been on the focus [5]. Here, one of the key factors is to provide good-quality

recipient oocytes [18, 22, 23].

Providing the best quality oocytes in the process of *in vitro* maturation is very important in improving the cloning efficiency and the key factor is to simulate the culture environment and condition just the same as those in *in vivo* [15, 17, 21, 27, 37]. With regard to the fact that the *in vitro* matured oocytes shows lower development rate than the one of *in vivo* matured oocytes and the oocytes collected from prepubertal sows shows lower *in vitro* development rate after *in vitro* fertilization and nuclear transfer compared to those of pubertal sows, there has been agreement among most of researchers [15, 36, 39].

In most of stock farms sows are slaughtered, there tends to be a little higher proportion of prepubertal ovaries that were not treated sufficiently by *in vivo* hormones. Therefore, in order to improve the quality of 3-6mm follicle-derived oocytes which are in the different developmental stages, we investigated the influence of hormone-treatment duration on the cloning efficiency. As a result, it has been demonstrated that extension of hormone-treatment period from 22 h to 27-32 h has positive impact on the *in vitro* and *in vivo* development of SCNT embryos. Also, it has been found that up to 47 h of maturation time is the most optimal in small sized follicles (2-4mm) while up to 43 h of maturation time is the most optimal in medium sized follicles (5-9mm) [44]. It has been reported that the parthenogenetic rate increases according to an increase in the maturation time but it results in the decrease in the efficiency of removing maternal chromosome in the nuclear transfer, negatively affecting the cloning efficiency [45].

The results obtained in our study are also in agreement with the previous study [44] that the extension of maturation duration in the *in vitro* maturation of small follicle-derived oocytes has improved the maturation rate and quality of oocytes. This suggests that hormone-treatment duration of *in vitro* maturation might be improve the oocytes maturation. Researcher demonstrated that maturation with hormonal supplementation would be necessary to improve nuclear maturation and with no hormonal supplementation would be necessary to improve cytoplasmic mature [46]. In our experiment shows that hormone-treatment duration improve the quality of IVM oocytes. It suggests the improvement of nuclear maturation gets of hormone-treatment duration.

When the hormone-treatment period and no-hormone-treatment period are set as 32 h and 10h, respectively, if the hormone-treatment period was finished this morning, the nuclear transfer should be carried out in the early dawn the day after tomorrow and if the hormone-treatment period was finished this afternoon the subculture should be carried out in the next evening giving experimenters much inconvenience. Therefore, it can be regarded that 27 h of hormone-treatment duration is proper not only for the improvement of cloning efficiency but also for giving experimenters less inconvenience. Therefore, more studies are needed to determine the role of these hormones in nuclear and cytoplasmic *in vitro* maturation of pig oocytes.

5. Conclusions

This study has explained the central importance of hormone-treatment duration in maturation of oocytes for cloning pig. The blastocyst rates of SCNT embryos were higher in the case of 27 and 32 hour groups. In addition, these groups showed higher cloning efficiency and delivery rate than the other groups. From the result, it can be concluded that extension of the hormone-treatment period up to 27-32 h is useful for the *in vitro* and *in vivo* development of SCNT embryos. These data will be valuable for increasing cloning efficiency of pig. However, more detailed and deep study is needed to investigate the correlative effect with the other parameters.

Abbreviations

SCNT	Somatic Cell Nuclear Transfer
IVM	<i>In vitro</i> Maturation
MII	Metaphase II
pFF	Porcine Follicular Fluid
COCs	Cumulus-Oocyte Complexes
PMSG	Pregnant Mare's Serum Gonadotropin
hCG	human Chorionic Gonadotrophin
BSA	Bovine Serum Albumin
EGF	Epidermal Growth Factor
DMEM	Dulbecco's Modified Eagle Medium
CB	Cytochalasin B
ARTs	Artificial Reproduction Technology

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Conflicts of Interest

The authors declare no conflicts of interest.

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