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Effect of a short-term *in vitro* exposure time on the production of *in vitro* produced piglets

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ABSTRACT

Although piglets have been delivered by embryo transfer (ET) with *in vitro* produced (IVP) embryos and blastocysts, a success rate has still remained lower level. Unlike mouse, human, and bovine, it is difficult to a production of piglets by *in vitro* fertilization (IVF) because of an inappropriate *in vitro* culture (IVC) system in pig. Therefore, the present study was conducted to investigate whether minimized exposure time in IVC can improve the pregnancy and delivery rates of piglets. Immediately after IVM, the oocytes were denuded and co-incubated with freshly ejaculated boar semen for 3.5 to 4 hours at 38.5 ˚C under 5% CO_2 in air. To avoid long-term exposure to *in vitro* state, we emitted IVC step after IVF. After that the presumptive zygotes were transferred into both oviducts of the surrogate on the same day or 1 day after the onset of estrus. Pregnancy was diagnosed on day 28 after ET and then was checked regularly every month by ultrasound examination. The 3 out of 4 surrogates were determined as pregnant (75%) and a total of 5 piglets (2 females and 3 males) were delivered at 118.3 ± 2.5 days of pregnancy period. In conclusion, a short-term exposure time may be an important factor in the production of IVP-derived piglets. It can be apply to the *in vitro* production system of transgenic pig by IVF, cloning, and pronuclear microinjection methods.

(Key words: inappropriate in vitro culture system, *in vitro* production, embryo transfer, piglet)

INTRODUCTION

Pigs are widely used as an attractive model host due to their high susceptibility and based on several practical considerations such as size and cost, make them considerably more convenient for research purposes. For those fascinating reasons, many researchers have focused on a production of piglets using *in vitro* production systems. Unfortunately, however, the success rate remains low in pig.

According to previous reports, *in vitro* maturation (IVM) system for pig oocytes is well established. The IVM rate was around 80 % regardless of one or two step culture method (Kobayashi et al., 2007; Moon et al., 2009; Shim et al., 2009). Despite recent highly improved progresses in an IVM and *in vitro* fertilization (IVF) in pig, a production of viable and good quality embryos exposed to *in vitro* system is lower than the results obtained *in vivo*. One of the main reasons for the inefficient outcomes was resulted from a poor quality of embryos produced by IVM–IVF (Kikuchi et al., 2002). Many research groups have tried to improve the quality of pig embryos produced *in vitro* during last few decades, but pig IVM-IVF system still remains to be optimized to show standardized results from different origins (Kikuchi et al., 1993; Abeydeera et al., 1998; Koo et al., 2005; Georgiou et al., 2011).

For the production of *in vitro* produced (IVP) offspring, embryos are kept for a short time in a synthetic medium before transfer into their recipient mothers. To improve an efficiency of *in vitro* culture (IVC) system in pig, several kinds of synthetic IVC medium have been developed for pig embryos such as Whitten's medium (Wright, 1977), North Carolina State University (NCSU)-23 medium (Petters and Wells, 1993), Beltsville embryo culture medium-3 (Dobrinsky et al., 1996), and porcine zygote medium (PZM)-3, PZM-4, and PZM-5 (Yoshioka et al., 2002). Recently the NCSU-23 medium and PZMs have been worldwidely used because of its ability of porcine embryo culture after IVF. One additional

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report described that the PZM-3 medium was more beneficial for embryonic development of pig parthenogenetic oocytes than NCSU-23 medium (Lee et al., 2012). Using one of these medium, the piglets originated from IVP embryos and blastocysts could be delivered (Misumi et al., 2014), but a delivery rate is still limited. Additionally, to improve the quality of IVP-derived embryo, many kinds of substrates were applied to culture medium such as horse serum (Hwang et al., 2012) and vitamin (Park et al., 2014)

Unlike human, mouse, and cattle, it is difficult to produce piglets from IVP embryos because of an inappropriate IVC condition. There has been reported that an exposure of an inappropriate IVC condition may affect to an abnormal fetal development such as overgrowth syndrome Beckwith–Wiedemann in human and large offspring syndrome in mouse and bovine (Fernández-Gonzalez et al., 2003; Chen et al., 2015) and epigenetics (Chen et al., 2013; Hidenobu and Ken, 2013). One simple method to improve the productivity of piglets may be a reducing exposure time to IVC condition. Therefore, the present study was conducted to investigate whether a minimized exposure time in IVC condition before ET can improve the pregnancy and delivery rates of piglets.

MATERIALS AND METHODS

1. General information

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

2. *In vitro* maturation

In vitro maturation protocol was described as follows (Hwang et al., 2016). Briefly, ovaries were obtained from prepubertal gilts at a local slaughterhouse (Nonghyup Moguchon, Gimje, Korea). and transported to the laboratory in saline at around 30 to 35°C within 1 h. Cumulus-oocyte complexes (COCs) were aspirated from follicles (3~6mm diameter) by 18 gauge needle attached to a 10 ml disposable syringe. The follicular fluid with COCs were collected into conical tubes and washed 3 times in tissue culture medium (TCM)-199 containing 0.1 % (w/v) polyvinyl alcohol (PVA). After sedimentation, the COCs consisting of several layers of compact cumulus cells were selected for IVM. Then, about 50 ~ 70 COCs were transferred into 500 μl of TCM-199 medium (Gibco BRL, USA) supplemented with 10 % porcine follicular fluid (pFF), 3.05 mM D-glucose, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 0.5 μg/ml FSH, 0.5 μg/ml LH, 10 ng/ml EGF, 75 μg/ml penicillin G and 50 μg/ml streptomycin in a four-well dish. The COCs were matured for 22 h with hormone and 22 h without hormone such as FSH, LH, and EGF at 39 °C under 5 % CO_2 in air. After 44h of IVM, the cumulus cells were removed from oocytes by treatment of 0.1 % hyaluronidase for 5 min. An extrusion of the first polar body was checked comprehensively and the oocytes with the first polar body were defined as matured and used for further experiments.

3. *In vitro* fertilization

Matured oocytes were washed three times in modified Tris-buffered medium (mTBM) consisting of 113.1 mM sodium chloride, 11.0 mM D-glucose, 2 mM caffeine sodium benzoate, 20 mM tris, 5.0 mM calcium chloride dehydrate, 7.5 mM D-sorbitol, and 4 mg/ml bovine serum albumin and transferred into 90 μl of mTBM in 6-well plate (Kitazato, Japan) covered by paraffin oil (Vitrolife, Sweden). About 20 to 25 oocytes were transferred into each well and kept until co-incubation with sperm. Freshly collected semen by globed-hand method was stored at 17 °C after quadruple dilution in BTS prior to IVF. The variation of fertilization results caused by sperm source, the semen was collected once a week from single Landrace boar. Briefly, the semen from 17 °C incubator was centrifuged for 5 min at 700 *g*. The sperm pellet was washed once in pre-warmed DPBS and once in pre-warmed mTBM for 5 min at 700 *g*. Then, the sperm was re-suspended in pre-warmed mTBM to yield a concentration of 2 x 10^6 sperm cells/ml. Twenty to 25 oocytes in the mTBM medium were co-incubated with the above sperm suspension at a final concentration of 2 x 10^5 sperm cells/ml for 3.5 to 4 h in a 100 μl microdrop at 38.5 °C under 5 % CO_2 in air.

4. Embryo transfer and delivery

After IVF, the presumptive zygotes were transferred into both oviducts of the surrogate (Landrace) on the same day or 1 day after the onset of estrus. Pregnancy was diagnosed firstly on day 28 after ET and then it was checked regularly every week by ultrasound examination. The IVF-derived piglets were delivered by natural parturition.

RESULTS

Developmental competence of IVM-IVF derived embryos is shown in Table 1. Among 105 of cleaved embryos after IVF, the 14 (around 11 %) embryos were developed to blastocyst at day 7. As shown in Table 2, all the 911 presumptive zygotes were transferred into both oviducts of the 4 surrogates on the same day or 1 day after the onset of estrus by surgical ET method (227.8 ± 20.8). Then, the pregnancy was firstly diagnosed on day 28 after ET and then it was checked regularly every month by ultrasound examination (Figure 1A). The 3 out of 4 surrogates were determined as pregnant (75%) and a total of 5 piglets (2 females and 3 males, Figure 1B) were delivered naturally after 118.3 ± 2.5 days of pregnancy period.

DISCUSSION

In vitro production technique for the production of piglets is expected to contribute to various areas of reproductive

Table 1. Developmental competence of IVM-IVF derived embryos

No. of oocytes fertilized	No. of embryos cleaved (%)	No. of blastocysts formed at (%)		
		Day 5	Day 6	Day 7
135	105 (77.8±4.5)	7 (4.2±1.3)	9 (6.7±0.5)	14 (10.9±1.3)

Data are represented as mean ± SEM of 4 replicates.

Table 2. Pregnancy and delivery of IVP embryos.

Surrogates	Exposure time after IVM*	No. of embryos transferred	Pregnancy**	No. of piglets delivered
#1	4 h	257	+	2 (♀1, ♂1)
#2	5 h	224	+	2 (♀1, ♂1)
#3	4 h	222	+	1 (♂1)
#4	5 h	208	-	-
Overall	Mean 4.5 h	911 (227.8 ± 20.8/n)	3/4 (75%)	5 (♀2, ♂3)

* In vitro matured oocytes were inseminated shortly and transferred immediately after IVF. ** The embryos were transferred into both oviducts of the surrogate on the same day or 1 day after the onset of estrus.

** Pregnancy was diagnosed on day 28 after ET and then was checked regularly every week by ultrasound examination.

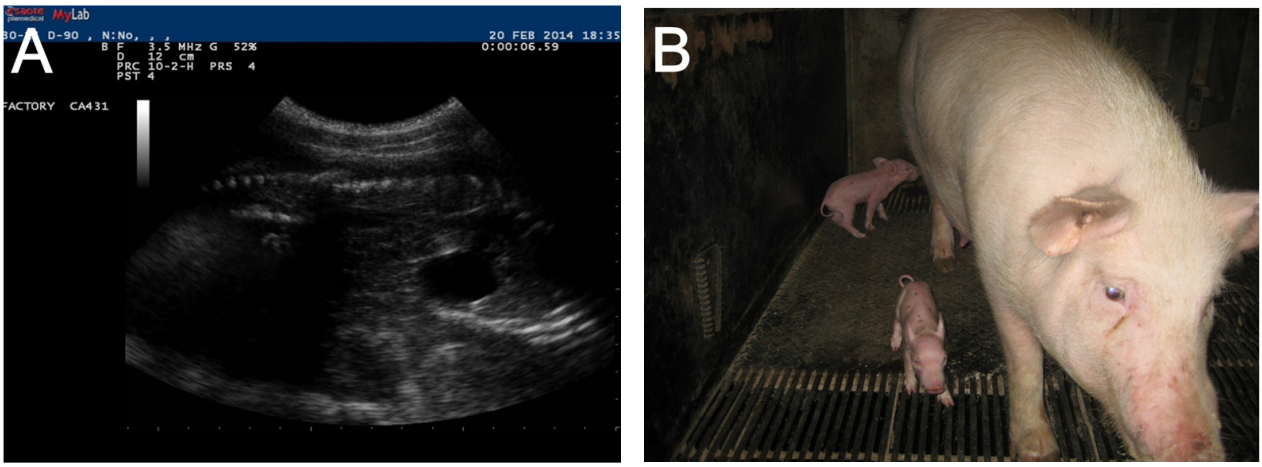

Figure 1. Pregnancy diagnosis by ultrasound examination (A) and IVF-derived piglets (B). Pregnancy was diagnosed on day 28 after embryo transfer and then was checked regularly every month by ultrasound examination.

technology for the production of pure, rare, and endangered breeds such as middle white pig (Misumi et al., 2014) and to contribute to generation of transgenic piglets for the study of medical and agricultural science (Park et al., 2006; Lee et al., 2008; 2009; Ahn et al., 2011). However, some evidences suggest that inappropriate IVC process may alter normal development and may produce specific abnormalities during fetal and postnatal development because of improper regulation of genomic imprinting (Bavister, 1995; Walker et al., 1996; McEvoy, 2003). Despite these potentially negative possibilities, it might be a useful method to the production and proliferation of transgenic piglets produced from IVP embryos, because the number of transgenic founders is very limited.

To improve the productivity of piglets originated from IVM-IVF embryos, we minimized the *in vitro* exposure time. For that, the presumptive zygotes were immediately transferred into surrogates. Although *in vitro* developmental competence of IVM-IVF derived embryos developed to blastocyst is around 11 %, the pregnancy rate was 75 % (3 out of 4 surrogates) and a total of 5 piglets were successfully delivered. Additionally, firstly diagnosed as pregnant surrogates were maintained their pregnancy and delivered successfully (100%).

In the present study, we reduced a fertilization time for less than 4 h replace to 8 h. Normally fertilization time is needed for overnight (around 17 h) in human and bovine, but 6 to 8 h is necessary for pig to reduce polyspermy. According to previous report (Misumi et al., 2014), the IVM oocytes were co-incubated only for 3 h with frozen-thawed spermatozoa. They also showed 9.9 % of *in vitro* developmental rate developed to blastocyst and 75 % (3/4 surrogates) of delivery rate.

In our previous report, none of the day 1 α-1,3 Galactosyltransferase knock-out with hCD46 (membrane cofactor protein) knocked-in (GT KO/hMCP KI) embryos were implanted at all (0/12 surrogates, unpublished data), but we produced GT KO/hMCP KI piglets with pregnancy (66.7 %, 8/12) and delivery (50 %, 6/12) rate using day 0 embryos. For that we transferred the GT KO/hMCP KI reconstructed embryos immediately after the confirmation of fusion into the surrogates (Hwang et al., 2013). It can be postulated that the shorter *in vitro* exposure time, the better production of IVP piglets.

In conclusion, we demonstrated that the minimized exposure time in *in vitro* state before ET could lead to improved production rates of piglets. And also it can be applied to the *in vitro* production of transgenic pig by IVF, NT, and pronuclear microinjection methods.

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