—Technical Note—

Birth of Piglets through the Non-Surgical Transfer of Blastocysts Produced *In Vitro*

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Abstract. In this study, we attempted to produce piglets by non-surgically transferring blastocysts produced *in vitro*, using a flexible catheter as the transfer instrument. Cumulus-oocyte complexes (COCs) were aspirated from the follicles of ovaries obtained at a local slaughterhouse. They were then matured in modified North Carolina State University (NCSU)-37 medium for 44–46 h and fertilized in porcine gamete medium (PGM). Ten hours after *in vitro* fertilization (IVF), presumptive zygotes were removed from the cumulus cells and cultured in porcine zygote medium (PZM)-5. Blastocysts were cultured for five days after IVF and, using a catheter for deep intrauterine insemination without sedation, they were transcervically transferred into the uterine horn of six recipients (45–50 blastocysts/recipients) whose estrous cycles were synchronized, at 5 days after human chorionic gonadotropin (hCG) injection. Of the six recipients, one sow became pregnant and farrowed seven piglets (four live piglets) 119 days after hCG injection. The body weight at birth of the newborns ranged from 0.8 to 1.4 kg. These results indicate that it is possible to obtain piglets by transcervically transferring blastocysts produced by IVF and *in vitro* cultures in chemically defined media.

Key words: *In vitro*-produced blastocysts, Non-surgical embryo transfer, Production of piglets

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Porcine embryo transfer could contribute to the controlling mechanisms of animal reproduction, including transgenesis and cloning [1]. In pigs, embryo transfer techniques are important not only for conducting genetic improvements and maintaining superior genotypes but also for the clearance of chronic infectious diseases, such as Aujeszky's disease, porcine reproductive and respiratory syndrome, and mycoplasmal pneumonia of swine. It is difficult to collect and transfer pig embryos using non-surgical techniques because of the anatomical complexity of the cervix and uterus. Thus, pig embryos are generally collected through surgery or after the slaughter of a donor, and transferred surgically into recipients [2].

Recently, Yoshioka *et al.* developed a porcine *in vitro* production (IVP) system using chemically defined media [3, 4]. Blastocysts produced with this system can effectively develop to full-term after surgical transfer. Non-surgical embryo transfer has the advantage that surgical facilities and techniques are not required. There are some reports that document the production of piglets by transfer of embryos derived *in vivo* into the uterine body or uterine horn using a catheter for artificial insemination or a specially designed flexible instrument [5–11]. However, there are currently no reports that document the production of piglets by non-surgical transfer of IVP embryos. This study was designed to examine the developmental competence of IVP embryos after transcervical transfer without surgical procedures or anesthesia using a catheter for deep intrauterine insemination

Fig. 1. Diagram showing the set-up of the transferred embryos in the extension tube. Embryos were placed into the end of an extension tube attached to a 10-ml syringe. The tube and syringe were filled with approximately 6 ml of TALP-Hepes as a transfer medium.

Fig. 2. Catheters used for non-surgical pig embryo transfer. The AI catheter (A) and the flexible catheter (B) were used as a guide to the external uterine orifice, and for deep intrauterine embryo transfer, respectively.

as the transfer instrument.

Materials and Methods

IVP of porcine embryos

In vitro maturation (IVM) and *in vitro* fertilization (IVF) were conducted as described previously [3]. Briefly, the COCs were aspirated from medium sized follicles (3–6 mm in diameter) of ovaries collected from prepubertal gilts in a slaughterhouse. The COCs were then washed twice with Hepes-buffered Tyrode medium (TALP-Hepes), and twice with modified North Carolina State University (NCSSU)-37 medium supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyl cAMP (dbcAMP), 10 IU/ml hCG (Gonotropin; Teikoku Hormone MFG., Tokyo, Japan) 10 IU/ml eCG (Serotropin; Teikoku Hormone MFG.), and 50 µg/ml gentamicin sulfate. They were then cultured for 20–22 h in the same medium and for a further 24 h in the same medium without the hormone or dbcAMP.

IVF was performed using the same batch of frozen-thawed ejaculated semen. The IVF medium was porcine gamete medium containing 2.5 mM theophylline, 1 µM adenosine and 0.2 µM cysteine (PGM-tac [3]). Frozen-thawed semen layered on Percoll gradient (90% and 45%) was centrifuged at 700 × g for 20 min and then washed with PGM-tac

by centrifugation at 500 × g for 5 min. After IVM, the COCs were co-incubated for 10 h with the spermatozoa at a concentration of 2×10^6 spermatozoa/ml in 100 µl droplets of PGM-tac. Mean (± SD) proportions of oocytes penetrated and fertilized normally in the present study were $75.2 \pm 16.8\%$ and $47.4 \pm 13.8\%$, respectively. They were then transferred to a 15-ml conical tube with 1 ml TALP-Hepes and the cumulus cells were removed from the oocytes by vortexing for 4 min. Presumptive zygotes were washed three times with TALP-Hepes and once with porcine zygote medium (PZM)-5 [12], in which the glutamine concentration was modified from 1 mM to 2 mM in PZM-4 [4]. Presumptive zygotes were cultured in PZM-5 for five days. IVM, IVF and IVC were performed at 39 °C in a humidified atmosphere containing 5% CO₂, 5% O₂, and 90% N₂.

Non-surgical embryo transfer

Six sows (Large White or Landrace × Large White; 12–15 months old; 153–187 kg body weight) were used as the recipients for non-surgical embryo transfer. All animal-related procedures followed in this study were approved by the Institutional Care and Use Committee for Laboratory Animals of the National Institute of Animal Health (protocol no. 398). Luteolysis of the recipients was induced by intramuscular injections of prostaglandin (PG) F_{2α} (Panacelan Hi, Daiichi Pharmaceutical Co. Ltd.,

Tokyo, Japan) as 15 mg of dinoprost twice daily for 3 days during the functional luteal phase. Ovulation was induced with intramuscular injections of 1500 IU hCG 72 h after the fifth PGF_{2α} treatment [3]. Blastocysts cultured for 5 days in PZM-5 were washed twice with TALP-Hepes and placed (45–50 embryos) in the end of a polyvinyl chloride tube (SAFEED® Extension Tube, SF-ET2527, Terumo, Tokyo, Japan), as shown in Fig. 1. The tube including the embryos was transferred to the pig house in a heat-insulating box at 37 °C. Embryo transfer was performed within 1 h on each sow without the use of tranquilizers or anesthesia. A flexible catheter (FIREFLEX®, Magapor, Zaragoza, Spain) was used for the embryo transfer. The perineal area of each recipient was disinfected with benzalkonium chloride and the vulva was cleaned with alcohol cotton. A commercial artificial insemination (AI) catheter covered with a vinyl sheath was introduced into the cervical opening through the vagina using a colposcope. The vinyl sheath was removed after reaching the cervical opening. The AI catheter was then inserted into the cervix by turning it counter-clockwise until it was firmly locked in place. The flexible catheter was inserted 100–120 cm through the AI catheter after the inside of the flexible catheter was rinsed with 2–3 ml TALP-Hepes. The tube including the embryos was attached to the end of this flexible catheter and, therefore, along with the 6 ml TALP-Hepes the embryos were infused into the uterus (45–50 embryos/animal).

Pregnancy diagnosis was carried out by ultrasonography 21 days after hCG injection. Animals that were diagnosed as pregnant were allowed to term and the gestation length, litter size

and body weight of the piglets at birth were examined.

Result and Discussion

A total of 948 presumptive zygotes were cultured in PZM-5 following IVF. Two hundred eighty-seven embryos (30.2%) developed to the blastocyst stage on Day 5. Of a total of 287 blastocysts, 284 embryos were transferred into six recipients. The result of embryo transfer for each recipient is presented in Table 1. Of the six recipients, only one achieved pregnancy and farrowed seven piglets; of these four were alive. No clinical abnormalities were observed in the piglets that were born. The gestation length and body weight of the piglets were within the normal range.

This is the first report to document the birth of piglets by non-surgical transfer of IVP blastocysts. Several studies have reported piglet production by surgical embryo transfer using porcine IVP blastocysts [3, 13, 14]. Yoshioka *et al.* [3] revealed that 25–30% of zygotes cultured in PZM-4 developed to blastocysts, and after surgical transfer of 90 blastocysts into 4 recipients, all were able to farrow a total of 21 piglets. Furthermore, in a previous study, the authors reported that PZM-5, in which the glutamine concentration was altered from 1 mM to 2 mM in PZM-4, improved blastocyst yield and the total number of cells in these blastocysts (31% and 42.9 cells, respectively) [12]. Although, in this study, the blastocysts were produced in a culture with PZM-5, only one recipient of the six sows impregnated farrowed piglets. To increase the conception rate with non-

Table 1. Result of transcervical embryo transfer in pigs

Recipient	No. of embryos transferred	Pregnancy	Gestation length (days) ^a	Total no. of piglets born (No. of live piglets)	Average body weight of piglets at birth ^b (of living piglets) (kg) [range]
A	47	no	—	—	—
B	47	yes	117	7 (4)	1.2 ± 0.2 (1.3 ± 0.1) [0.8–1.4 (1.1–1.4)]
C	50	no	—	—	—
D	50	no	—	—	—
E	45	no	—	—	—
F	45	no	—	—	—

^aDay 0=2 days after hCG injection.

^bMean ± SD.

surgical transfer of IVP embryos, further improvements in the catheter technique and handling of the embryos are needed.

Several reports have documented the success of non-surgical embryo transfer using porcine embryos developed *in vivo* [5–11]. In general, the farrowing rate of non-surgical embryo transfer (9–64%) [5–11] is lower than that of surgical embryo transfer (17–100%) [15]. In the reports on non-surgical embryo transfer, embryos were deposited in the uterine horn near the uterine body or the cervix. The location of the tip of catheter is important for the success of conception with embryo transfer. Wallenhorst *et al.* [16] revealed that by transferring embryos near the tip of the uterine horn they could achieve a high rate of embryo survival. In this study we attempted to transfer embryos deep into the uterine horn. The flexible catheter used in this study can be inserted deep into the uterine horn through the cervix for AI in estrous sows [17]. In contrast, there are some reports that claim it is not easy to insert a catheter into the uterine horn during the luteal phase because of cervix restriction [2, 6]. In some cases during the present study, the tip of the catheter might not have reached deep into the uterine horn. The location of the tip of the inserted catheter needs to be confirmed in future trials.

Transferring embryos with large amounts of medium might result in a backward flow of the embryos within the medium towards the vagina [18, 19]. In the early stages of pregnancy, substantial amounts of fluid are typically not found in the uterus, and these non-physiological conditions might affect the survival rate of the embryos [18, 19]. Yonemura *et al.* [20] reported that the pregnancy rate and number of fetuses transferred with 15 ml of medium using the AI catheter into the cervix, was higher than that of embryos transferred with 30 ml of medium. Although the embryos were transferred with 6–7

ml of medium in our study, only a minimal amount of medium should be used to flush the embryos from the catheter to increase the success of pregnancy after embryo transfer. Thus, the amount of medium used in the flexible catheter will need to be investigated in the future.

In porcine embryo transfer, when the estrous cycle of the recipients was asynchronous to that of the donors with a 2-day delay, the pregnancy rate appeared to be high [2]. In this study, Day-5 blastocysts were transferred after IVF into the recipients 5 days after hCG treatment, which is similar to the schedule used with 2-day delay, resulting in the birth of piglets. The development of IVP embryos is generally slower than that of embryos developed *in vivo*, and the cell number of IVP blastocysts (37–46 cells at 5 days after IVF [3, 12]) is smaller than that of blastocysts developed *in vivo* (62 cells at 4 days after ovulation [4]). To increase the farrowing rate, the effects of IVP embryo age and the estrous cycle of the recipients on conception after embryo transfer will also need to be examined.

In this study, piglets were born after non-surgical IVP embryo transfer. The production of piglets by non-surgical IVP embryo transfer is more economical than by the surgical transfer of embryos developed *in vivo*. To increase the farrowing rate and number of piglets born after non-surgical IVP embryo transfer, further methodological improvements are necessary.

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