ESTABLISHMENT OF PRACTICAL EMBRYO TRANSFER FOR FRESH OR FROZEN EMBRYOS IN PIGS

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ABSTRACT

The most important benefit of embryo transfer (ET) in the swine industry is the ability to introduce new genetic stock as replacement herd animals with minimal risk of disease transmission and cost for transportation, in comparison with the transport of live animals. However, the commercial application of ET in the pig has been limited because of the need for surgical procedures for both the collection and transfer of the embryos. Recent advances in systems for in vitro production (IVP) of pig embryos, including in vitro oocyte maturation, fertilization and embryo culture, have enabled us to generate viable embryos that can develop to full term after transfer into recipients. In our previous studies, surgical transfer of 18–25 IVP blastocysts per recipient into 11 recipients has resulted in the pregnancy rate of 100%, the efficiency of piglet production of 25.5% (61 piglets for 239 transferred blastocysts) and the average litter size of 5.5 ± 2.5 (mean ± SD) piglets. We also have succeeded in producing piglets after surgical transfer of cryopreserved IVP blastocysts, which were vitrified using the Cryotop method. Now, IVP of pig embryos can provide viable embryos more efficiently in terms of cost and time when compared with the collection of in vivo derived embryos. In the 1990s to 2000s, instruments to deposit embryos in the pig uterus non-surgically have been developed, resulting in the birth of live piglets. The non-surgical ET technique using a deep intrauterine catheter for insertion through the cervix deep into a uterine horn is practicable on farms, because it does not need special facilities, such as surgical and anesthesia equipment. We previously developed a simple catheter made from polyethylene for non-surgical intrauterine transfer of pig embryos. Using this catheter, 17 of 68 recipients (25.0%) transferred IVP blastocysts farrowed and the litter size averaged 4.6 ± 2.7 piglets. The efficiency of piglet production was calculated to be 3.5%. Non-surgical ET of fresh in vivo derived embryos using a similar system into 47 recipients resulted in a farrowing rate of 34.0% and an average litter size of 6.9 ± 2.3 piglets, giving an efficiency of piglet production of 13.8% (110 piglets for 800 transferred embryos). Moreover, non-surgical transfer of vitrified in vivo embryos resulted in a farrowing rate of 29.0%. Although the farrowing rate and the efficiency of piglet production after the non-surgical ET are still inferior to those achieved by surgical ET and needed to be improved, it appears that IVP of embryos together with non-surgical ET and embryo storage technology could be applied at a practical level in swine industry.

Keywords**:** *In vitro* Embryo Production, Non-Surgical Embryo Transfer, Pig, Cryopreservation

INTRODUCTION

The benefits of embryo transfer (ET) technologies to the livestock industry include the rapid introduction of greater value genetics to replacement herd animals (Martinez *et al.* 2005). From the viewpoint of disease risk, movement of embryos is undoubtedly much safer for trading than moving animals or semen. ET is also essential for the application of other reproductive biotechnologies, including transgenesis and cloning (Day 2000). In cattle, ET technique has now become a routine procedure on many countries and more than 500,000 *in vivo* derived and 380,000 *in vitro* produced embryos were transferred to recipients worldwide in 2012. The non-surgical techniques for embryo collection and transfer have accelerated the growth of ET technology in cattle. However, the commercial application of ET in the pork industry has been limited because of the requirement for surgical procedures for both the collection and transfer of the embryos.

Although the first success of pregnancy through non-surgical ET in the pig was demonstrated over 40 years ago (Polge and Day 1968), procedures for non-surgical ET were considered inefficient techniques for many years. In the 1990s to 2000s, new instruments to deposit embryos in the pig uterus non-surgically have been developed, resulting in the birth of live piglets (reviewed in Cameron *et al*. 2006). In particular, a technique for non-surgical deep intrauterine ET using a flexible catheter with a stainless-steel coil between the inner and outer tubes could achieve an acceptable reproductive performance (60%–71% farrowing rate and 6.3–6.9 piglets born) with fresh embryos produced *in vivo* (Martinez *et al.* 2004; Nakazawa *et al.* 2008), although having such complicated materials in the catheter increase production costs.

Remarkable progress has also been made since the 1990s in the refinement of procedures for *in vitro* production (IVP) and cryopreservation of bovine embryos and the non-invasive techniques of ultrasonography for oocyte aspiration from live animals. A standard IVP system for preimplantation embryos includes three technological steps: the *in vitro* maturation (IVM) of immature oocytes, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of zygotes. Successful large-scale IVP of embryos will enable us to reduce the cost and time required and will be valuable for research in reproductive physiology, agriculture and biotechnology.

In this paper, (1) development of chemically defined media for IVP of porcine embryos, (2) *in vivo* viability of IVP embryos after surgical transfer to recipients and (3) improvement of a procedure for non-surgical transfer of *in vivo* derived or IVP embryos deep into one uterine horn of non-sedated recipients were described.

IN VITRO PRODUCTION OF PORCINE EMBRYOS

The first successful production of piglets from *in vitro*-matured and *in vitro*-fertilized oocytes was reported by Mattioli *et al*. (1989) in which 2- to 4-cell embryos at 44 h after IVF were transferred into recipients. Since then, some laboratories have succeeded in producing piglets from cleaved embryos at the 2- to 4-cell stage cultured for 24–36 h (Yoshida *et al.* 1993, Funahashi *et al.* 1996, Funahashi *et al.* 1997) and from 8-cell to morula-stage embryos cultured for 96 h (Abeydeera *et al.* 1998) following IVM and IVF. Moreover, in the present decade it has been demonstrated that porcine IVP blastocysts cultured for 5 or 6 days after IVF could develop to full term (Marchal *et al.* 2001, Kikuchi *et al.* 2002). However, despite recent improvements in porcine IVP techniques, the developmental rate of embryos matured and fertilized *in vitro* to the blastocyst stage and their quality are still low compared with *in vivo* derived embryos (Nagai *et al.* 2006). The low developmental competence of porcine IVP embryos might be caused by several factors, including a reduced incidence of male pronuclear formation, a high incidence of polyspermy and suboptimal conditions for embryo culture (Funahashi and Day 1997, Abeydeera 2002*)*.

Numerous media, such as modified Whitten's medium (Beckmann and Day 1993), North Carolina State University (NCSU) 23 medium (Petters and Wells 1993), Beltsville embryo culture medium (BECM)-3 (Dobrinsky *et al.* 1996), modified synthetic oviduct fluid (mSOF) (Marchal *et al.* 2001) and sequential media of NSCU37-PyrLac and NSCU37-Glu (Kikuchi *et al.* 2002) are available for the successful culture of embryos to the blastocyst stage. We previously developed a chemically defined medium, porcine zygote medium (PZM), for IVC of porcine zygotes (Yoshioka *et al.* 2002). The inorganic and energy substrate composition of PZM was calculated according to the reported concentrations of pig oviductal fluid (Iritani *et al.* 1974, Nichol *et al.* 1992). This medium also contains glutamine, hypotaurine and premixed solutions of basal medium Eagle's (BME) essential amino acids and minimum essential medium (MEM) non-essential amino acids. *In vivo* derived zygotes cultured for 4 days in PZM showed full-term developmental potential without any severe reduction in embryo viability after surgical transfer (Yoshioka *et al.* 2002). Moreover, we have also developed a suitable media for IVM of oocytes (porcine oocyte medium; POM) (Yoshioka *et al.* 2008), IVF (porcine fertilization medium; PFM) (Yoshioka *et al.* 2003) and IVC of embryos beyond the blastocyst stage (porcine blastocyst medium; PBM) (Mito *et al.* 2012) by modification of the composition of PZM. These media are chemically defied and 15–35% of presumptive zygotes after IVF could develop to blastocysts in our IVP systems for porcine embryos (Yoshioka *et al.* 2003, Suzuki and Yoshioka 2006, Yoshioka *et al.* 2008, Mito *et al.* 2013), while different batches of semen had a wide range in potential for fertilization and subsequent embryo development (Suzuki *et al.* 2005, Noguchi *et al.* 2013). The chemically defined medium is useful for a precise analysis of the physical action of substances, such as inorganic compounds, energy substrates, hormones, cytokines and vitamins, on oocyte maturation, fertilization or early embryo development, since it eliminates the presence of undefined factors in biological materials such as serum or serum albumin. Use of a chemically defined medium also improves the reliability of media formulations, yields a higher reproducibility of results and ensures biosafety of culture media by elimination of protein preparations, which risk contamination by pathogens (Bavister 1995). Such a medium could also serve as a powerful tool for optimizing the IVP system and maximizing the number of embryos that survive after transfer and therefore has certain advantages for research and commercial purposes. All of these media can be purchased from Research Institute for the Functional Peptides Co., Ltd. (Higashine, Japan).

DEVELOPMENTAL COMPETENCE OF IN VITRO-PRODUCED EMBRYOS AFTER SURGICAL TRANSFER

Production of offspring by embryo transfer is the only method for validating the normality of IVP embryos unequivocally. Without this validation, the physiological relevance of data from oocyte maturation to early embryo development would be uncertain. Marchal *et al*. (2001) reported that the transfer of 80 IVP blastocysts to four recipients (14–26 blastocysts per recipient) resulted in one pregnancy (pregnancy rate of 25%) and the birth of only two piglets (efficiency of piglet production of 2.5%). Kikuchi *et al*. (2002) obtained an efficiency of piglet production of 13% by transferring 150 IVP blastocysts into three recipients (50 blastocysts per recipient): all recipients became pregnant and farrowed. Pregnancy results after surgical transfer of porcine blastocysts produced *in vitro* in PZM are summarized in Table 1. Overall, transfer of 18–25 blastocysts per recipient into 11 recipients resulted in pregnancy rates of 100% and average litter size of 5.5 ± 2.5 (mean ± SD) (Yoshioka *et al.* 2003, Akaki *et al.* 2009, Mito *et al.* 2009). On the other hand, farrowing rate (72.5%) and average litter size (4.4 ± 1.5) appeared to decrease when 15 blastocysts per recipient were transferred.

Table 1. Pregnancy results after surgical transfer of porcine blastocysts produced *in vitro* in PZM

	No. of blastocysts per recipient	
	18–25*	15
Blastocysts transferred	239	270
Recipients	11	18
Farrowing (%)	11 (100)	13 (72.2)
Piglets born (average litter size)	61 (5.5 ± 2.5)	57 (4.4 ± 1.8)
Body weight of piglets at birth (kg)	1.23 ± 0.30	1.24 ± 0.32
Piglet production efficiency[§]	25.5%	21.1%

*The data of this column are pooled from Yoshioka et al. (2003), Akaki et al. (2009) and Mito et al. (2009).

Average data are expressed as mean ± SD.

[§]Percentage of piglets born / embryos transferred.

As a polytocous species, maintenance of pregnancy is dependent on a minimum fetal occupancy within the uterus. In pigs, this is most critical between approximately day 11 and day 12 of gestation, when conceptus-derived estrogen is responsible for maternal recognition of pregnancy (Geisert *et al.* 1990). During this time, a minimum of four to five viable embryos (Polge *et al.* 1966) or at least 50% uterine occupancy (Geisert *et al.* 1990) is required for pregnancy maintenance. In the surgical transfer of IVP blastocysts, a certain number of embryos may be needed to produce piglets after ET. We also have succeeded in producing piglets after surgical transfer of cryopreserved IVP blastocysts, which were vitrified (Table 2) using the Cryotop method described by Misumi *et al.* (2013). Now, IVP of pig embryos can provide viable embryos more efficiently in terms of cost and time when compared with the collection of *in vivo* derived embryos.

Table 2. Pregnancy results after surgical transfer of porcine IVP blastocysts vitrified using the Cryotop method

Recipient	No. of blastocysts transferred	Gestation period (days)	Piglets born (%)*	Body weight of piglets at birth (kg)
G10	30	115	6 (20.0)	1.48 ± 0.20
G11	30	119	1 (3.3)	1.40
G12	30	118	7 (23.3)	1.30 ± 0.35
Overall	90	117.3 ± 2.1	14 (15.6 ± 10.7)	1.39 ± 0.28

Average data are expressed as mean ± SD.

*Percentage of piglets born / embryos transferred.

NON-SURGICAL TRANSFER OF PORCINE EMBRYOS

IVP together with non-surgical ET technology could improve the dissemination of high value genetics to other farms at an affordable cost. We have succeeded in producing piglets derived from IVP blastocysts through non-surgical deep intrauterine ET using a flexible catheter with a stainless-steel coil between the inner and outer tubes (Suzuki *et al.* 2004). However, the pregnancy results (only one of six recipients farrowed [17% farrowing rate], producing seven piglets) were markedly inferior to those achieved by surgical transfer. To increase the utilization of IVP embryos for piglet production, improvements are required for the practical use of non-surgical

ET in farms in terms of techniques and the development of economically cheaper catheter materials with good performance. Thus, we developed a simple catheter made from polyethylene (Takumi® catheter; Fujihira Industry Co., Ltd., Tokyo, Japan) for non-surgical intrauterine transfer of pig embryos (Fig. 1) and examined the effects of insertion length for the catheter and asynchrony between the age of donor IVP blastocysts and the recipient estrous cycle (Yoshioka *et al.* 2012). Successful production of piglets derived from IVP embryos was achieved following non-surgical ET (Fig. 2), when the catheter was inserted at more than 30 cm anterior to the spiral guide spirette. Furthermore, the efficiency of piglet production was greater ($P<0.001$) in recipients whose estrous cycle was asynchronous to that of donors with a 1-day delay (8.3%) than in those with a 2- (1.5%) or 3-day (0.9%) delay, while pregnancy and farrowing rates (10–40%) did not differ among treatments (Table 3). Therefore, it was considered that the insertion length of the deep intrauterine catheter and the degree of asynchrony between donor embryos and recipient estrous cycle influenced on pregnancy and birth outcome following non-surgical transfer of IVP blastocysts. Movie manual for non-surgical embryo transfer in the pig is available on our website (NAROchannel 2013).

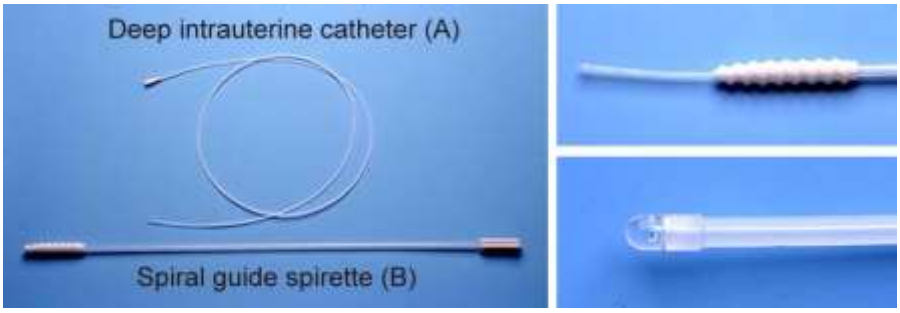

Fig. 1. Catheter used for non-surgical embryo transfer. The spiral spirette (length 60 cm; A) and the intrauterine catheter (length 1.2 m, outer diameter 3 mm and working canal 0.5 mm; B) were used as a guide to external uterine orifice and for deep intrauterine embryo transfer, respectively.

Fig. 2. Non-surgical embryo transfer (left panel) and piglets born after non-surgical transfer of IVP embryos (right panel).

Table 3. Effect of asynchrony between donor and recipient estrous cycle on pregnancy after non-surgical transfer of IVP blastocysts

Asynchrony between embryos and recipients (days)	-3	-2	-1
Blastocysts transferred (per recipient)	264 (26.4 ± 2.4)	296 (29.6 ± 3.9)	276 (27.6 ± 3.2)
Recipients	10	10	10
Pregnancy (%)	4[††] (40.0)	1 (10.0)	4 (40.0)
Farrowing (%)	2 (20.0)	1 (10.0)	4 (40.0)
Piglets born (litter size)	2 (1.0 ± 0)	4 (4)	23 (5.8 ± 3.2)
Body weight of piglets at birth (kg)	1.6 ± 0.1	1.8 ± 0.2	1.5 ± 0.2
Efficiency of piglet production*	0.9%[a]	1.5%[a]	8.3%[b]

Modified from Yoshioka *et al*. (2012).

Average data are expressed as mean ± SD.

[††]Two recipients aborted.

*Percentage of piglets born / embryos transferred.

Different letters (a, b) in the same row indicate differences ($P < 0.001$).

Using the Takumi® catheter, a total of 1,622 *in vivo* derived fresh embryos that were collected at 5 or 6 days after artificial insemination were transferred into 97 recipients. Overall, 34 recipients were pregnant (35.1%) and 23 (23.7%) were farrowed a total of 150 piglets. As shown in Table 4, farrowing rate and efficiency of piglet production were significantly higher ($P<0.05$) in recipients into which Day 6 (Day 0 = the day of artificial insemination) embryos were transferred (34.0% and 13.8%, respectively) than in recipients of Day-5 embryos (14.0% and 4.9%, respectively).

Table 4. Pregnancy results after non-surgical transfer of *in vivo* derived fresh embryos

Age of embryos* (days)	5	6
Blastocysts transferred (per recipient)	822 (16.4 ± 2.4)	800 (17.0 ± 3.1)
Recipients	50	47
Pregnancy (%)	14 (28.0)	20 (42.6)
Farrowing (%)	7 (14.0)[a]	16 (34.0)[b]
Piglets born (litter size)	40 (5.7 ± 2.3)	110 (6.9 ± 2.3)
Body weight of piglets at birth (kg)	1.7 ± 0.3	1.7 ± 0.4
Efficiency of piglet production[§]	4.9%[a]	13.8%[b]

Average data are expressed as mean ± SD.

*The day after artificial insemination.

[§]Percentage of piglets born / embryos transferred.

Different letters (a, b) in the same row indicate differences ($P < 0.05$).

The combined use of cryopreservation and non-surgical ET could make ET a commercially viable technology in pigs. When *in vivo* derived blastocysts from individual donors were collected as a batch and vitrified using the micro volume air cooling method (Misumi *et al.* 2013), non-surgical transfer of a single batch of vitrified and warmed embryos resulted in a farrowing rate of 29.0% (Table 5). This protocol enables correct identification of the parents of piglets produced by ET.

Table 5. Pregnancy results after non-surgical transfer of in vivo derived blastocysts collected from individual donors and vitrified using the micro volume air cooling method

Farm	A	B	C
Blastocysts transferred	248	79	132
(per recipient)	(14–17)	(14–19)	(10–19)
Recipients	16	5	10
Pregnancy (%)	7 (43.8)	3 (60.0)	2 (20.0)
Farrowing (%)	4 (25.0)	3 (60.0)	2 (20.0)
Piglets born (litter size)	14 (3.5 ± 1.3)	13 (4.3 ± 3.5)	18 (9.0 ± 1.0)
Efficiency of piglet production*	5.6%	16.5%	13.6%

Average data are expressed as mean ± SD.

*Percentage of piglets born / embryos transferred.

CONCLUSION

In vitro production of porcine embryos can now provide viable embryos more efficiently with less cost and time compared with the collection of *in vivo*-derived embryos. Moreover, non-surgical ET and embryo storage technology could alter the dissemination structure of desirable embryos in the swine industry. However, the farrowing rate and the efficiency of piglet production after the non-surgical ET are still inferior to those achieved by surgical ET. Further modifications of non-surgical transfer techniques will be needed with respect to such factors as the appropriate number of embryos to transfer, the developmental age of embryos and the composition of the transfer medium and the procedure for non-surgical ET in pigs

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