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Piglet production by non-surgical transfer of vitrified embryos, transported to commercial swine farms and warmed on site

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Abstract

We investigated the feasibility of piglet production by non-surgical embryo transfer (Ns-ET) of vitrified porcine blastocysts and expanded blastocysts transported to commercial farms and warmed on site (V/T/W embryos). Ns-ET was performed by depositing 11–20 vitrified and warmed embryos at a proximal site within the uterus via a catheter. In Experiment 1, the effect of donor–recipient estrous cycle asynchrony on the efficiency of Ns-ET of vitrified and ordinary warmed embryos was investigated at the experimental facility. With a 1-day delay recipients relative to that of donor, the farrowing rate was 50.0% and the survival rate to term was 21.1%. In Experiment 2, Ns-ET using recipients with a 1-day delay and vitrified embryos after one-step warming and dilution was evaluated at the experimental facility. Although the resulting farrowing rate was 42.9%, the survival rate was 6.4%. In Experiment 3, Ns-ET was conducted using V/T/W embryos at four commercial farms, where piglets derived from them were produced. When artificial insemination was conducted prior to Ns-ET, the farrowing and survival rates obtained using V/T/W embryos were 75.0%, and 21.3%, respectively. These results show that Ns-ET of V/T/W embryos using this protocol would be feasible for piglet production at farms.

KEYWORDS

artificial insemination, commercial farm, non-surgical porcine embryo transfer, one-step warming and dilution, vitrified and warmed embryos

1 | INTRODUCTION

Long-term storage of embryos by cryopreservation is indispensable for the practical application of ET. In recent years, surgical embryo transfer (S-ET) of vitrified and warmed (V/W) embryos has yielded acceptable results in terms of reproductive performances such as pregnancy and farrowing rates, and the ability of transferred embryos to survive to term (Cuello et al., 2016; Fujino et al., 2008; Martinez et al., 2015; Misumi et al., 2013). However, it is difficult to apply S-ET at commercial farms because most of them lack suitable facilities and equipment for S-ET. In this context, non-surgical embryo transfer (Ns-ET) of V/W embryos, without such facilities and equipment for S-ET, offers considerable promise for improving the efficiency of ET in the pork industry.

Recently, it has also become possible to deposit embryos into deep intrauterine sites using non-surgical procedures, and several

studies have demonstrated that piglets can be produced by Ns-ET of V/W embryos using various forms of catheter (Cuello et al., 2005; Gomis et al., 2012; Martinez et al., 2015; Tajima et al., 2020b). On the other hand, Misumi et al. (2020) have confirmed that Ns-ET of V/W embryos into the uterine body is able to produce piglets, and the recipient's reproductive performance is equivalent to that achieved using S-ET into the caudal quarter of the uterine horn. More recently, a disposable catheter for Ns-ET of porcine embryos into the "proximal" uterus (uterine body or uterine bifurcation area) has been developed (Kurenai-3 Prototype, Misawa Medical Industry, Ibaraki, Japan) (Hirayama et al., 2020). Ns-ET into a proximal uterine site allows embryos to be deposited using a markedly shorter catheter insertion length. Therefore, it is inferred that Ns-ET with this type of catheter may be advantageous for the ease of handling with only a low risk of accidental injury to the uterine endometrium.

A procedure for warming and dilution of vitrified porcine embryos—the so-called "one-step" method—has been developed (Cuello et al., 2004, 2005). Ns-ET after the one-step method in the field requires no special manipulations such as stepwise dilution and embryo manipulation under stereomicroscopic guidance. Takishita et al. (2020) have also developed a one-step method for S-ET of V/W embryos based on the micro volume air cooling (MVAC) method (Misumi et al., 2013) and achieved acceptable reproductive performance. However, it has not yet been clarified whether an acceptable reproductive performance can be obtained with Ns-ET of V/W embryos using one-step method based on the MVAC method.

It has been reported that the reproductive performance achieved with Ns-ET is inferior to that of S-ET (Hazeleger et al., 2000; Martinez et al., 2004, 2015; Yoshioka et al., 2012). However, in our previous study (Tajima et al., 2020b), we demonstrated that artificial insemination (AI) prior to Ns-ET of V/W embryos into a deep uterine site was able to overcome the disadvantages of Ns-ET compared to S-ET. It has also been reported that Ns-ET of V/W porcine embryos into a proximal uterine site is able to enhance the efficacy of Ns-ET while reducing labor and invasiveness for recipients (Hirayama et al., 2020). So, it is expected that AI prior to Ns-ET of V/W embryos into a proximal uterine site would improve yield and efficiency of ET.

In the present study, we investigated the feasibility of Ns-ET of V/W embryos prepared using the MVAC method into a proximal intrauterine site, and attempted to optimize the Ns-ET procedure using a one-step method with the aim of practical application to commercial swine farms. Finally, we conducted Ns-ET of vitrified, transported and warmed (V/T/W) embryos at four farms and assessed the practical utility of our Ns-ET procedure. In this trial, we investigated the effect of AI prior to Ns-ET and verified its applicability for stable piglet production.

2 | MATERIALS AND METHODS

2.1 | Animals

All animals used in the present study and experimental procedures were approved by the Animal Care and Use Committees of both Aichi Agricultural Research Center and the Saga Prefectural Livestock Experiment Station.

2.2 | Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MA, USA) unless otherwise indicated.

2.3 | Embryo collection from donors

Embryos were produced from 44 estrous-cycling gilts aged between 8 and 22 months (Duroc (D), Landrace (L), Large White (W) and L × W crossbred (LW))and 12 sows of the 1st–8th-parity (D, L, W, and LW). Luteolysis of donors was induced using one of the following methods reported previously (Misumi et al., 2013; Tajima et al., 2020b): (a) administration of 0.562 mg sodium cloprostenol (Planate, MSD animal health K.K., Tokyo, Japan) intramuscularly twice with a 24-hr interval during 20–40 days of gestation or (b) administration of 0.276 mg sodium cloprostenol intramuscularly twice with a 12-hr interval during Days 13–16 of the estrous cycle (Day 0: onset of estrus). These donors were then given 1,500 IU of equine chorionic gonadotropin (eCG, Peamex, Zenyaku Kogyo Co., Ltd., Fukushima, Japan) intramuscularly during or 24 hr after the second injection of sodium cloprostenol. Then an intramuscular injection of 500 IU of human chorionic gonadotropin (hCG, Puberogen 500 U; Zenyaku Kogyo, Tokyo, Japan) was followed at 72 hr after the eCG treatment to induce ovulation. The semen used for artificial insemination (AI) was collected from L, W, and D boars in advance on the day of AI or one day before. The semen samples were examined microscopically to determine sperm characteristics, extended with Modena solution (Weitze, 1991) to 1×10^8 sperms/mL, and stored at 15°C until use. The hormone-treated gilts and sows were artificially inseminated conventionally with semen from the same breed of boars two times at 24 hr and 40–42 hr after hCG injection (Tajima et al., 2020b).

Around 160 hr after hCG injection, embryos were recovered surgically from donors under general anesthesia by flushing of the uterine horns with embryo collection medium, POE-CM (Mito et al., 2015). The recovered embryos were transferred to a 35-mm plastic dish (Falcon 353,001, Corning, NY, USA) containing 20 mM Hepes- buffered PBM (Hepes-PBM) (Mito et al., 2015) and washed twice under a stereomicroscope. After the quality of the embryos had been assessed, zona-intact embryos at the blastocyst (BL) to the expanded blastocyst (ExB) stages were selected and cultured temporarily (within 60 min) in 100 μL PBM (without Hepes, Mito et al., 2015) using a multidish (Reproplate, IFP9670; Research Institute for the Functional Peptides, Yamagata, Japan) under a humidified atmosphere of 5% O_2 and 5% CO_2 in air at 38.5°C.

2.4 | Embryo vitrification

Vitrification and warming of embryos were carried out by the MVAC method (Misumi et al., 2013). By this method, embryos were

vitrified using chemically defined media and cooled into straw out of contact with liquid nitrogen (LN_2). Media for vitrification and warming were from a commercial kit (PEV-SK, IFP16PVSK; Research Institute for the Functional Peptides). A group of 5–18 embryos were initially washed twice in Hepes-PBM and equilibrated with equilibration solution-1 (PES-1; Research Institute for the Functional Peptides) followed by equilibration with solution-2 (PES-2; Research Institute for the Functional Peptides) for 5 min each. The equilibrated embryos were washed briefly in vitrification solution (PVS; Research Institute for the Functional Peptides), transferred sequentially into 100-μl droplets of PVS, and finally loaded on the tip of the device designed for MVAC (Embryo-stick; Misawa Medical Industry, Ibaraki, Japan) in approximately 1 μl of PVS. The devices with the installed embryos were inserted into a straw placed vertically in LN_2 and cooled completely within 1 min of exposure to PVS. All procedures were performed on a warm plate at 38°C. The embryos were stored in LN_2 for at least 1 month.

2.5 | Warming and dilution of cryoprotectants

Warming and dilution were performed in conventional method (Misumi et al., 2013) or a one-step method as described previously (Takishita et al., 2020). In conventional method, warming and dilution were performed by submerging the tip of the device directly in 3 ml of warming and dilution solution (PWDS; Research Institute for the Functional Peptides) which had been pre-warmed for 3 min at 38°C in a 35-mm plastic dish. The V/W embryos were then placed in Hepes-PBM until used for the experiment. In a one-step method, the tip of the device containing the embryos was immersed in a 5-mL disposable syringe containing 3 ml of PWDS kept at 40°C. For each trial, one or two such devices were warmed in the same syringe. The V/W embryos were then immediately and directly transferred by connecting the syringe to the injector.

2.6 | Ns-ET of V/W embryos to recipients

Intrauterine catheters (Kurenai-3 Prototype) were used for Ns-ET of V/W embryos. These catheters were designed to deposit embryos in a proximal intrauterine site.

In an experimental facility, estrus synchronization in the recipients was induced in the same manner as that for the embryo donors. In commercial swine farms, sows had been synchronized by weaning, and ovulation was induced using one of the following methods: (a) administration of 1,000 IU of equine chorionic gonadotropin on the day of weaning was followed 72 hr later by an intramuscular injection of 500 IU of human chorionic gonadotropin or (b) administration of 1,000 IU of human chorionic gonadotropin 72 hr after weaning. Detection of estrus in the recipient gilts and sows was performed twice daily (every 12 hr) by exposure to a mature boar.

Before the Ns-ET experiment, we have checked the status of catheter insertion by laparotomy. Thirteen estrous-cycling gilts 9–15 months old (D and L) and 7 sows of the 1st to 5th-parity (D and L) were used to investigate the location of the catheter tip and transplantation site while maintaining the catheter insertion length within a range of 40–50 cm anterior to the vulva (Table S1). The obtained data confirmed that the catheters were functional, as reported by Hirayama et al. (2020).

Ns-ET was conducted at 118–144 hr after injection of hCG under sedation with 10 mg midazolam (Dormicum; Astellas Pharma Inc., Tokyo, Japan) (Nakamura et al., unpublished data). After thorough cleaning of the perineal area of each recipient, an outer catheter (guide, length: 61.5 cm) was inserted through the vagina using a vaginal speculum and propelled into the cervix. A slideable inner tube (injector) was advanced about 5 cm to the site of intended deposition. After complete insertion of the catheter, warming and dilution of the vitrified embryos were conducted as described above. Then, 14–19 embryos were loaded into a 0.25-ml straw (NFA121; Fujihira Industry Co., Tokyo, Japan) using a 2.5-ml disposable syringe. Prior to introduction of the embryos, the injector was loaded and filled with 1 ml Hepes-PBM at 38°C from a 2.5-ml disposable syringe through a sterilizing filter. The 0.25-mL plastic straw including the embryos was attached to the injector. The content of the straw was then introduced and flushed with Hepes-PBM into the uterus. Finally, 1 ml of air was pushed into the injector through a sterilizing filter using a 2.5-ml disposable syringe to flush any residual content completely into the uterus.

The recipients were checked for return of estrus, and pregnancy was detected using ultrasonography (HS-1500V; Honda Electronics Co., Toyohashi, Japan) on day 30 after estrus. Pregnant recipients were allowed to carry pregnancies to term.

2.7 | Experimental design

2.7.1 | Experiment 1: Ns-ET of V/W embryos to asynchronized recipients

This experiment was carried out in an experimental facility without transportation of the embryos.

Nine estrous-cycling D, L, and LW gilts 9–10 months old and 5 D, L, and LW sows of the 1st to 2nd-parity were used as recipients for Ns-ET of V/W embryos warmed and diluted in conventional method, and the effects of asynchrony between the donor and recipient estrous cycles on pregnancy, farrowing, and the survival rates of embryos to term were evaluated. In this experiment, 14–18 vitrified embryos from a few donors were warmed for one trial. A total of 225 embryos were used for this experiment. The estrous cycle of the recipients was controlled by hormone treatment as described above. Ns-ET to asynchronous recipients was conducted on day 5 ($n = 5$) or 6 ($n = 9$) after hCG administration (i.e. when the estrous cycles of the recipients were asynchronous with a 2- or 1-day delay, respectively, relative to those of the donor: designated as the 2-day and 1-day groups, respectively).

2.7.2 | Experiment 2: Ns-ET of V/W embryos warmed and diluted with cryoprotectant in a one-step method

This experiment was carried out in an experimental facility without transportation of the embryos.

The reproductive performances such as pregnancy and farrowing rates, and the ability of transferred embryos to survive to term were investigated by Ns-ET of VW embryos warmed and diluted with cryoprotectant in a one-step method. Five estrous-cycling D and L gilts aged 9–11 months and 2 LW sows of the 1st-parity were used as recipients for Ns-ET of V/W embryos, and 14–19 vitrified embryos from a few donors were transferred non-surgically per recipient at 6 days after hCG administration. A total of 110 embryos were used for this experiment. The time from catheter insertion to removal was recorded.

2.7.3 | Experiment 3: Ns-ET of embryos after vitrification, transportation to commercial swine farms, and warming by one-step method on site

This experiment was conducted to assess the production of piglets by Ns-ET under field conditions at commercial swine farms using vitrified, transported, and warmed (V/T/W) porcine embryos. The embryos derived from D, W, and L donors were vitrified at Aichi Agricultural Research Center or Saga Prefectural Livestock Experiment Station and transported to four commercial swine farms located 20–0 km apart from each experimental facility in Aichi or Saga Prefecture using a LN_2 container (SC4/2V; Chart biomedical Co., Ball Ground, GA, USA) on the day of Ns-ET. A total of 16 LW sows of the 1st to 6th-parity were used as recipients. Ns-ET was conducted at 6 days after injection of hCG in a small pen or farrowing crate kept under acceptable hygienic conditions. Warming and dilution of vitrified embryos were conducted in a one-step method, and a total of 11–20 vitrified embryos from each donor in one or two devices were warmed for one trial. The period of time from insertion to removal of the catheter was recorded. A total of 251 embryos were used for this experiment.

At one of the four farms, four recipients underwent AI with semen from a fixed single D boar kept at the farm, at a single time point 24 hr after hCG injection (designed the AI/Ns-ET group). At the other farms, a total of 12 recipients were assigned to Ns-ET without AI (the non-AI/Ns-ET group). In the AI/Ns-ET group, the LW recipients underwent AI with semen from D boars, and then V/T/W embryos from D or L donors were transferred. When D embryos were transferred, it was possible to distinguish the piglet origin according to coat color (i.e., piglets derived from AI were white or spotted, whereas those derived from V/T/W embryos from D donors were wholly brown). In the case of L embryo transfer, as the origin could not be distinguished by coat color, we requested genetic testing of tissue samples from both piglets and their parents and confirmed pedigrees by parentage test (Livestock Improvement Association of Japan, Maebashi, Japan).

2.8 | Statistical analysis

Data for survival rates to term of transferred embryos in Experiments 1 and 3 were analyzed by ANOVA using the General Linear Models procedures of the Statistical Analysis System (Ver. 9.2; SAS Institute Inc., Cary, NC, USA). Differences in percentages of pregnancy and farrowing from Experiments 1 and 3 were analyzed by Fisher's exact test. p-values of $< .05$ were considered to indicate significance.

3 | RESULTS

In the series of Experiments 1 – 3, none of the recipients showed symptoms of uterine infection such as vaginal discharge after Ns-ET.

3.1 | Experiment 1

The catheter insertion lengths in the 2-day and 1-day groups were 40.6 ± 4.1 cm and 43.6 ± 2.1 cm, respectively. As shown in Table 1, no recipient became pregnant in the 2-day group. Meanwhile, four recipients in the 1-day group became pregnant and farrowed a total of 27 live piglets. The farrowing rate was 50.0% (4/8), and the survival rate to term of the transferred V/W embryos in the 1-day group was 21.1% (27/128). There were no significant inter-group differences in the pregnancy, farrowing, and survival rates to term of transferred V/W embryos ($p = .110$). The mean ± SE birth weight of piglets in the 1-day group was 1.53 ± 0.06 kg (not shown in Table 1).

3.2 | Experiment 2

As shown in Table 2, three recipients became pregnant and farrowed seven piglets in total. The farrowing rate was 42.9%, and the survival rate to term of the transferred V/W embryos was 6.4% (7/110). The mean birth weight was 1.13 ± 0.13 kg (data not shown in Table 3). The length of catheter insertion was 41.3 ± 2.5 cm.

3.3 | Experiment 3

In all the four commercial farms, piglets derived from V/T/W embryos were successfully produced. As shown in Table 3, the pregnancy rate, farrowing rate, and survival rate to term of transferred V/T/W embryos in the non-AI/Ns-ET group were 58.3%, 33.3%, and 6.3%, respectively. In the AI/Ns-ET group, three of four recipients became pregnant and farrowed 30 piglets, of which 17 were found to be derived from embryos after AI fertilization, whereas 13 were generated from V/T/W and transferred embryos. The survival rate to term of V/T/W embryos was 21.3 ± 10.3%, and the farrowing rate was 75.0%. The survival rate to term of V/T/V embryos in the AI/Ns-ET group tended to be higher than that in the Non-AI/Ns-ET group ($p = .051$).

TABLE 1 Effect of asynchrony between donor and recipient estrous cycle on reproductive performance after non-surgical transfer (Ns-ET) of vitrified and warmed embryo by MVAC method into the proximal site of the uterine

Asynchrony between donors and recipients (days)[a]	Total no. of recipients	No. of embryos transferred (mean per recipient)	Inserted catheter length (cm)	No. of pregnant recipients (%)	No. of farrowed recipients (%)	No. [total] of piglets	% survival to term of transferred embryos[b]
2-day	4	64 (16.0 ± 1.1)	40.6 ± 4.1	0 (0.0)	0 (0.0)	*n*.d	0.0
1-day	8	128 (16.0 ± 0.3)	43.6 ± 2.1	4 (50.0)	4 (50.0)	5,6,8,8 [27]	21.1 ± 8.4

Note: Means ± *SEM*.

MVAC, micro volume air cooling.

[a]Estrus cycle in recipients were asynchronous to donors wiith delay.

[b]Calculated as follows: (number of piglets/number of transferred embryos) × 100.

TABLE 2 Effect of one-step warming and dilution procedure on reproductive performance after non-surgical transfer (Ns-ET) of vitrified and warmed embryo by MVAC method into proximal site

Number of recipients	No. of embryos transferred per recipient	Time required for Ns-ET (min's")	Inserted catheter length (cm)	No. of pregnant recipients (%)	No. of farrowed recipients (%)	No. [total] of piglets	% survival to term of transferred embryos[a]
7	110 (15.7 ± 0.6)	5'42" ± 0'37"	41.3 ± 2.5	3 (42.9)	3 (42.9)	2, 2, 3 [7]	6.4 ± 3.2

Note: Means ± *SEM*.

MVAC: micro volume air cooling

[a]Calculated as follows: (number of piglets/number of transferred embryos) × 100

4 | DISCUSSION

The present study clearly demonstrated that Ns-ET of V/W embryos using the MVAC method into a proximal site within the uterus achieved piglet production, even though warming and dilution of the vitrified embryos were performed by a one-step method. Moreover, at commercial swine farms, we succeeded in producing normal viable piglets derived from V/T/W embryos. It is anticipated that the procedure used in the present study for Ns-ET of porcine V/T/W embryos will be applicable at commercial swine farms that lack special facilities and equipment for manipulation.

Previously, catheters and procedures for Ns-ET have been designed to allow deposition of embryos into a more anterior (distal) site in the uterine horn (Martinez et al., 2004; Nakazawa et al., 2008; Yoshioka et al., 2012). On the other hand, Misumi et al. (2020) have recently confirmed that Ns-ET of V/W embryos into the uterine body can successfully yield piglets with a farrowing rate of 60% and survival rate to term of 17.9%. The present study employed a new catheter for Ns-ET designed to deliver V/W embryos to a proximal intrauterine site (Kurenai-3 Prototype) (Hirayama et al., 2020). It was found that Ns-ET with this type of catheter resulted in a reproductive performance (farrowing rate 66.7%, survival rate to term 15.4%) similar to those reported previously using recently developed deep intrauterine catheters (Cuello et al., 2005; Gomis et al., 2012; Martinez et al., 2015; Misumi et al., 2020). One of the advantages of Ns-ET into a proximal site is the shorter catheter insertion length required for embryo deposition, and thus the shorter insertion time. Therefore, it is expected that the use of Ns-ET for proximal deposition of embryos would be easier and safer without any risk of injury to the uterine endometrium. In the present Experiments 1 and 2, we attempted to optimize the procedure for Ns-ET deposition of embryos into a proximal site for practical application at commercial farms.

Moreover, we examined to clarify the secure rage of catheter insertion length for recipient gilts and sows. We confirmed that the tip of this catheter was mostly located in the uterine body or uterine horn (84.6% of gilts and 71.4% of sows) when inserted within a range of 40 – 50 cm anterior to the vulva (Table S1). In sows, there were no cases in which the tip of the catheter was located in one of the uterine horns. On the other hand, the catheter tip reached one of the uterine horns in 23.1% (3/13) of gilts. These results indicate that a range of 40 – 50 cm anterior to the vulva was secure for catheter insertion in recipient sows, but more delicate manipulation would be required in gilts, when inserting a catheter beyond 40 – 50 cm.

In Experiment 1, Ns-ET of V/W embryos prepared using the MVAC method into a proximal site was conducted. None of the recipients in the 2-day group became pregnant. On the other hand, recipients in the 1-day group showed a farrowing rate of 50.0% (4/8)

TABLE 3 Results of non-surgical embryo transfer (Ns-ET) of vitrified and warmed embryos by MVAC method into the proximal site at the commercial swine farm

Farm	Experimental group	Total no. of recipients	No. of embryos transferred (mean per recipient)	Inserted catheter length (cm)	Time required for Ns-ET (min's")	No. of pregnant recipients (%)	No. of farrowed recipients (%)	No.[total] of piglets (mean per recipient)	No. of recipients producing piglets derived from ET (%)	No. [total] of piglets derived from ET (mean per recipient)	% survival to term of transferred embryos[a]
1	Non-AI/Ns-ET[b]	6	98 (16.3 ± 0.8)	51.7 ± 1.5	7'12" ± 1'00"	3 (50.0)	2 (33.3)	3,3 [6] (1.0 ± 1.7)	2 (33.3)	3,3 [6] (1.0 ± 1.7)	6.1 ± 3.7
2		4	63 (15.8 ± 1.1)	48.3 ± 1.2	6'06" ± 0'17"	3 (75.0)	1 (25.0)	3 [3] (0.8 ± 0.8)	1 (25.0)	3 [3] (0.8 ± 0.8)	4.8 ± 5.0
3		2	29 (14.5 ± 3.5)	55.0 ± 0.0	6'40" ± 0'20"	1 (50.0)	1 (50.0)	3 [3] (1.5 ± 1.5)	1 (50.0)	3 [3] (1.5 ± 1.5)	10.8 ± 13.6
Subtotal		12	190 (15.8 ± 0.7)	55.0 ± 1.1	6'45" ± 0'29"	7 (58.3)	4 (33.3)	3,3,3,3 [12] (1.0 ± 0.4)	4 (33.3)	3,3,3,3 [12] (1.0 ± 0.4)	6.3 ± 2.6
4	AI/Ns-ET[c]	4	61 (15.3 ± 0.9)	54.0 ± 1.4	8'45" ± 0'35"	3 (75.0)	3 (75.0)	4, 12, 14 [30] (7.5 ± 3.3)	3 (75.0)	2,4,7 [13] (3.3 ± 1.5)	21.3 ± 10.3

Note: Mean ± *SEM*

MVAC, micro volume air cooling.

[a]Calculated as follows: (number of piglets/number of transferred embryos) × 100

[b]Non-AI/Ns-ET: Ns-ET into proximal site

[c]AI/Ns-ET: artificial insemination prior to Ns-ET into proximal site

and a survival rate to term of 21.1% (27/128) (Table 2). These results indicate that for V/W embryos prepared by the MVAC method, recipients whose estrous cycle is delayed by 1 day are adequate for Ns-ET into a proximal intrauterine site using this catheter. One of the major factors for successful ET is the degree of estrus synchrony between recipient and donor, and highest farrowing rate were found in recipients whose estrous cycle is delayed by 1 day with Ns-ET of fresh embryos. (Angel et al., 2014). It has been hypothesized that embryo manipulation and/or in vitro culture might cause a transitory delay of porcine embryo development (Almiñana et al., 2010; Blum-Reckow & Holtz, 1991; Macháty et al., 1998) and that porcine embryos might have tolerance for a "less advanced uterine environment" (Angel et al., 2014). In our previous report (Tajima et al., 2020b), we conducted Ns-ET of V/W-expanded blastocysts prepared by MVAC into a deep intrauterine site for recipients whose estrous cycle is delayed by 0, 1, or 2 days, and we demonstrated that recipients whose estrous cycle is delayed by 1 or 2 days are adequate. However, the present results contradict this. Under physiological conditions, blastocysts remain near the tip of the uterine horn until Day 6 or 7 of the estrous cycle and then progress toward the uterine body (Dziuk, 1985). Therefore, it is considered that transfer of blastocysts into the tip of the uterine horn might be advantageous in this respect (Angel et al., 2014; Martinez et al., 2015). In the environment of the uterine body, V/W blastocysts might retain tolerance for a shorter period than in the deep intrauterine environment.

A one-step method would be ideal for direct Ns-ET under field conditions on a farm without equipment for special manipulation techniques. Takishita et al. (2020) developed a one-step method for MVAC method and obtained a farrowing rate of 60% (3/5) and a survival rate to term of 27.5% (19/69) by S-ET of V/W embryos. In Experiment 2, we confirmed the reproductive performance of one-step Ns-ET for V/W embryos prepared using MVAC. The farrowing rate was 42.9% (3/7), which was similar that in Experiment 1 (50.0%; 4/8). However, the embryo survival rate was 6.4% (7/110), which was inferior to that in Experiment 1 (21.1%; 27/128). Gomis et al. (2012) assessed the effect of time required for completion of warming by a one-step method, and showed that in vitro and in vivo development of V/W embryos was significantly decreased when a longer time was needed for immersing the tip of the device containing the embryos into warming and diluting solution in a syringe, compared with immersing the device into the solution in a plastic dish. In the present study, although we completed warming and dilution within 1 min, we did not check the precise relationship between embryo development and the time required for our one-step method, and it is possible that this issue may cause a low survival rate. Further experiments will be required to clarify these effects and improve the technique for Ns-ET after one-step method.

In the present study, we demonstrated that piglet production was possible on commercial swine farms using Ns-ET of V/T/W embryos. In addition, piglets can be obtained from V/T/W embryos even if the locations and managers differ. To our knowledge, this is the first report to document successful production of piglets from V/T/W embryos using Ns-ET after the one-step method

on commercial farms without special manipulation or equipment. Moreover, we had no difficulty with smooth catheter insertion into a proximal site, and the time required for Ns-ET was less than 10 min. In addition, none of the recipients showed any sign of uterine infection such as vaginal discharge after Ns-ET. It is therefore considered that the present procedure is safe and well tolerated by recipient sows. At one of the four commercial farms, AI prior to Ns-ET was conducted for recipients. It is well known that at least four conceptuses are needed (i.e. that four embryos need to be implanted) for establishment and maintenance of pregnancy during early gestation in pigs (Polge et al., 1966). Even if less than four viable embryos implanted during early gestation, they could not complete fetal development and resulted in failure of pregnancy in many cases. We considered that major part of failure to pregnancy from Ns-ET of V/T/W embryos may be related to this reproductive characteristic of pigs. In our previous study (Tajima et al., 2020b), we performed AI prior to Ns-ET using a deep intrauterine catheter and achieved 25.2% of the survival rate to term of transferred V/W expanded blastocysts. We considered that creating adequate numbers of "supporting embryos" using AI prior to Ns-ET would be a promising strategy for ensuring pregnancy in the recipients, and that consequently, even if a limited number of V/W embryos were transferred, a few of them would develop to term (Tajima et al., 2020a). As shown in Table 3, when four recipient sows were inseminated prior to Ns-ET of V/T/W embryos, three of them farrowed and all three (75.0%) produced piglets derived from V/T/W embryos. The survival rate of V/T/W embryos to term was 21.3%, regardless of the semen origin. These results were equivalent to the farrowing rate and survival rate to term in Experiment 1 (50.5% and 21.1%, respectively). Consequently, it is considered that AI prior to Ns-ET could overcome any inherent disadvantage of the one-step method used in the present study. However, due to an outbreak of classical swine fever in Aichi in 2019, the total number of AI/Ns-ET trials was very limited, and therefore accumulation of further more solid data will be necessary for confirming the present findings.

In conclusion, the results of the present study demonstrate that Ns-ET deposition of V/T/W embryos prepared using the MVAC method into a proximal intrauterine site can achieve normal viable piglet production at commercial swine farms without the need for special manipulation or equipment. Although further improvements in the Ns-ET technique for V/W embryos will be needed, it appears promising for future application at commercial swine farms.

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CONFLICT OF INTEREST

All authors declare no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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