—Brief Note—

Transportation of preimplantation porcine embryos without cryopreservation using a novel embryo carrier

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Abstract: The objective of this study was to develop a transportation system for fresh porcine embryos in a chemically defined medium without reducing their viability using a novel embryo carrier. When embryos were introduced in 0.25-ml straws with porcine blastocyst medium (PBM) and given a vibration load on a shaker for 20 h to mimic transportation conditions at 38 °C, there was no significant difference in survival rates between the vibration and no-vibration groups. Next, an embryo carrier was developed that can maintain interior conditions of 5% CO_2 and 38 °C for the transportation of embryos. The embryos on Day 5 after insemination were divided into three groups. In the first group, the embryos were introduced to straws with PBM and the straws were sealed (sealed group). In the second group, the embryos were transported in a tube with a CO_2 pouch (CO_2 gas group). They were transported by a door-to-door delivery service. In the last group, the embryos were cultured in an incubator (not-transported group). Although the survival rate of embryos in the sealed group was significantly lower than that of not-transported group, the survival rates and the hatching rate of the embryos in the CO_2 gas group were similar to those of not-transported group of embryos (100% vs 97.4% and 57.9% vs 47.5%, respectively). Our results demonstrate that *in vivo*-derived porcine embryos can be transported without harm in PBM using our carrier.

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Introduction

The pig is a multiparous animal, having long uterine horns. In general, porcine embryos are collected surgically by uterine flushing. It is difficult to perform embryo collection at farms. Therefore, embryos are collected at facilities equipped with surgical and examination equipment, and then – either fresh or cryopreserved – transported to a farm for embryo transfer. Although piglets can be produced from cryopreserved embryos after embryo transfer [1, 2], the piglet production rate (percentage of piglets born per embryos transferred) is low compared to fresh embryos, due to the extremely high sensitivity of porcine embryos to low temperature. The development of a method for the transportation of porcine embryos without serious damage to their viability may expand the use of embryo transfer technologies in the swine industry. While post-transportation survival of fresh embryos and successful conception of transported embryos have been reported with bovine [3, 4] and human embryos [5], there are few reports of successful deliveries in pigs after non-surgical transfer of fresh embryos transported long distances. Nakane *et al.* [6] reported that for porcine *in vitro*-produced embryos, transportation of embryos in a serum-supplemented transportation medium led to a lower pregnancy rate among recipients after non-surgical embryo transfer compared with recipients receiving

non-transported embryos.

On the other hand, Ozawa *et al.* [7] reported that when *in vitro*-derived porcine embryos were cultured in a glass tube in a gas-equilibrated medium under atmospheric conditions, the pH and osmolality of the medium decreased, resulting in a reduced blastocyst formation rate and a low number of cells in the blastocysts. Thus, the transportation medium and the conditions of transportation appear to influence the development of the embryos.

Mito *et al.* [8] have developed a chemically defined medium for porcine *in vitro*-produced blastocysts called porcine blastocyst medium (PBM). They reported that culture of *in vitro*-produced blastocysts in PBM increased their survival and hatching rates, and the number of cells and ATP content in blastocysts compared with culture in PZM-5 [9]. The use of a chemically defined medium is desirable for the transportation of embryos in order to prevent the spread of diseases. Therefore PBM is a good candidate for the transportation of embryos without damaging their viability.

There are a few reports describing the effect of transportation on embryo viability. According to Lewis *et al.* [10], the cytoskeleton of cells subjected to low frequency vibration may be disrupted, and Vandenberg *et al.* [11] suggested that vibrations negatively affect embryo morphogenesis. On the other hand, Isachenko *et al.* [12] reported that in *in vitro* culture of human embryos, mechanical agitation of 6 Hz dramatically increased pregnancy rate. Nevertheless, it is unclear how vibration is generated during transportation by trucks and whether such vibration affects the viability or developmental competence of the transported embryos.

In this study, we developed and tested a transportation system for fresh porcine embryos in a chemically defined medium which did not reduce their viability, using a novel embryo carrier which can maintain interior conditions of 5% CO_2 and 38 °C.

Materials and Methods

The animal experiments in this study were approved by the Institutional Animal Experiment Committee of Kanagawa Prefectural Livestock Industry Technology Center.

Embryo collection from gilts

A total of nine prepubertal gilts (Large White, Yorkshire, Landrace and Duroc, 6.6–8.3 months old) were used for the collection of embryos, as previously described with some modifications [13, 14]. Briefly, superovulation was induced by an administration of equine chorionic gonadotropin (eCG, Peamex, 1,500 IU intramuscular (im), Sankyo, Tokyo, Japan), followed 72 h later by an administration of human chorionic gonadotropin (hCG, Puberogen, 500 IU im, Sankyo). The gilts were artificially inseminated twice, in the afternoon one day after hCG treatment and in the morning two days after hCG treatment. In the morning of Day 5 (Day 0=the day of the first AI), the embryos were recovered from the uterine horns by laparotomy under general anesthesia (4 to 5% [v/v] isoflurane) by flushing with a porcine oocyte/embryo collection medium (POE-CM) [15] (Research Institute for the Functional Peptides [IFP], Yamagata, Japan).

Morphological evaluation

The embryos were observed under an inverted microscope (×200) and were classified as normal, degenerated or unfertilized eggs. Normal embryos were morphologically assessed in terms of developmental stage, proportion of degenerated cells, etc., and only embryos of quality Code 1 according to the IETS manual [16] were used in the experiment.

Measurement of the oxygen consumption rate

To objectively evaluate the quality of the embryos, the oxygen consumption rate of the embryos was measured using an embryo respirometer (HV-405; IFP) according to the method of Abe *et al.* [17]. Briefly, an embryo was placed at the bottom of a cone-shaped microwell on a respiration assay plate (RAP-1; IFP) which was filled with an embryo respiration assay medium (ERAM-2; IFP). A platinum microelectrode was set near the embryo, and a voltage of -0.6 V *versus* Ag/AgCl was applied to the microelectrode to reduce oxygen. The oxygen consumption rate gradients in the solution surrounding the embryo were measured by scanning the z-axis (i.e., vertical direction) at a speed of 30.0 μm/sec over a scanning distance of 160 μm. The oxygen consumption rate (respiration rate) of the embryos was calculated using analytical software based on spherical diffusion [18].

Specially developed embryo carrier

The temperature inside the carrier that was specially developed for transportation of embryos was maintained at 38 °C using a heater powered by lithium ion batteries (12 V, 60 A) (Fig. 1A). Assuming that straws or sampling tubes will be used to carry embryos, the straw container was designed to hold 10 × 0.25-ml straws (Fig. 1B) and the tube container was designed to hold 8 × 1.5-ml sampling tubes (Fig. 1C). The containers can be fixed in an aluminum sealed trunk (0.5 l) and the 5% CO_2 and humidified environment can be maintained using Culture

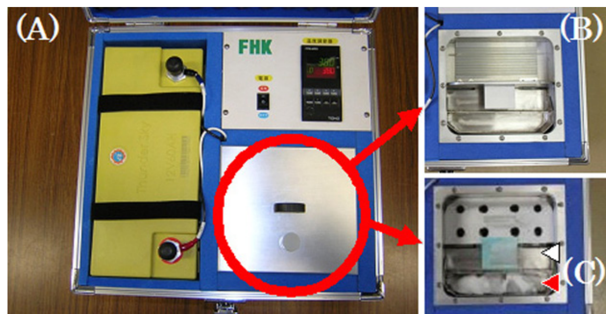

Fig. 1. Specially developed embryo carrier. (A) Temperature can be maintained at 38 °C for over 120 hours using a heater powered by lithium ion batteries. (B) Straw container carrying up to 10 × 0.25-ml straws. (C) Tube container carrying up to 8 × 1.5-ml sampling tubes. A CO_2 pouch is placed at ◁ and Milli-Q soaked absorbent cotton are placed at ◀.

Pal® CO_2 (Mitsubishi Gas Chemical, Tokyo, Japan) and Milli-Q water soaked absorbent cottons.

Evaluation of cell numbers in embryos

Some of the embryos were subjected to double staining of viable and dead cells as previously described [13]. Briefly, after washing with TCM199 containing 20% inactivated fetal bovine serum (FBS; Bio Fluids, Rockville, MD, USA) warmed to 38.5 °C, the embryos were cultured in a staining solution for 30 mins for double staining of viable and dead cells. TCM199 supplemented with 10 μg/ml bisbenzimide (Hoechst 33342, Calbiochem, San Diego, CA, USA) and 10 μg/ml propidium iodide (Sigma-Aldorich, St. Louis, MO, USA) was used as the staining solution. The stained embryos were washed with TCM 199 containing 20% FBS, placed on a slide with a small amount of medium and covered with a cover glass. The embryos were observed under an inverted microscope equipped with a fluorescent light source (TE-300, Nikon, Tokyo, Japan).

Experimental design

Experiment 1: Effects of vibration on the viability and oxygen consumption rate of in vivo-derived porcine embryos: After measurement of the oxygen consumption rate, the early blastocysts on Day 5 after artificial insemination (n=60) were divided into two groups, and the embryos were introduced into 0.25-ml crystal straws (005592, IMV Technologies, L'Aigle, France) (4–6 embryos per straw) with PBM [8] at the volume of 5 μl/embryo. The straws were sealed and stored in the carrier at 38 °C (Fig. 1 B).

Vibration data during transportation by car were collected using Data Station (Ono Sokki, Yokohama, Japan) and a three-axis accelerometer (PCB, Depew, NY, USA), which measured the center of the bed of a truck three times while the truck was running on the Tomei Expressway at a speed of 80 km/h. The data were converted to acceleration power spectral density for fast Fourier transform vibration analysis. Based on the analysis, the vibration was reproduced using a vibration tester (VS-2000-140T, IMV, Osaka, Japan). The vibration group carrier was placed on the vibration tester and given a vibration load for 20 h. The no-vibration group carrier was placed in a stationary state for 20 h. Then, the survival and oxygen consumption rates of the embryos were measured, and cell counts were made for both groups.

Experiment 2: Developmental ability of the in vivo-derived porcine embryos transported in a straw or sampling tube with PBM: We examined the viability of embryos transported in a straw or sampling tube. To transport the embryos in straws, four to six compacted morulae collected on Day 5 after artificial insemination were introduced into each 0.25-ml straw with PBM at 5 μl /embryo. The straws were sealed and placed in the carrier maintained at 38 °C (Fig. 1 B). To transport the embryos in a sampling tube, the embryos were loaded in 1.5-ml polypropylene sampling tubes (72.690.001SX, Assist, Tokyo, Japan) with 150 μl of PBM at five or six embryos per tube and topped with liquid paraffin (26137–85, Nacalai Tesque, Kyoto, Japan). The tubes were sealed with a semitransparent membrane (Breathe-easier, BERM-2000, Diversified Biotech, MA, USA) instead of their lids and were transported in a CO_2 pouch (Fig. 1 C). The embryos were transported from Ebina City in Kanagawa Prefecture to Yamagata City in Yamagata Prefecture, Japan, using a door-to-door delivery service. Vibration during the transportation was measured using a tri-axial accelerometer (G-MEN DRα, SRIC, Matsumoto, Japan) which was fixed to the bottom of the carrier. The temperature immediately below the embryos in the carrier was measured using a temperature data logger with a resolution of 0.1 °C (Thermochron, KN Laboratories, Ibaraki, Japan). The oxygen consumption rate of the embryos was measured before and after transportation, and the transported embryos were cultured in 50 μl of PBM in a Reproplate (IFP) individually at 38.5 °C under 5% CO_2 and 5% O_2 (low oxygen condition) for 48 h until immediately after transportation to determine survival and hatching rates. Not-transported embryos were individually cultured in 50 μl of PBM in a Reproplate under a low oxygen condition for 48 h and their survival and hatching rates were also measured.

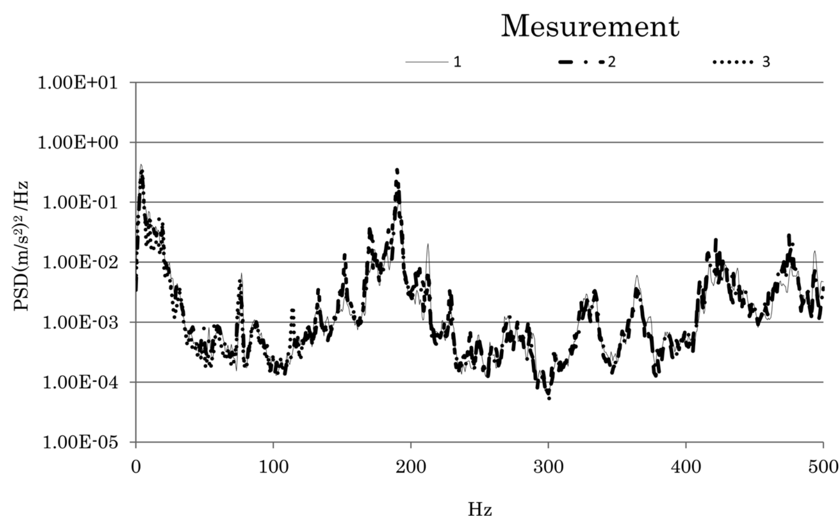

Fig. 2. The acceleration power spectrum densities measured by an accelerometer mounted at the center of a truck bed while the truck was running on the Tomei Expressway at 80 km/h (vertical direction: Z-axis).

Table 1. Survival rate, oxygen consumption rate, and number of cells of *in vivo*-derived porcine embryos which were subjected to vibration for 20 hours

Experimental group	No. of embryos (No. of replicates)	No. of embryos survived (Survival rate: %)	Oxygen consumption rate [1)] ($F \times 10^{14}$ /mol s^{-1})		Number of cells After treatment[1)]	
			Immediately after collection	20 hours later	Viable	Dead
Vibration	35 (5)	31 (88.6)	0.84 ± 0.05	0.90 ± 0.08	63.3 ± 4.4	2.3 ± 1.3
No-vibration	25 (5)	23 (92.0)	0.84 ± 0.05	1.05 ± 0.09	68.8 ± 4.7	2.2 ± 1.8

[1)]: Data are expressed as the mean ± S.E.M. There were no significant differences between the groups.

Statistical analysis

Statistical analysis was conducted using SPSS (SPSS 16.0 J. User's Guide, SPSS Inc., Tokyo, Japan). To compare the oxygen consumption rate among the embryos, one-way analysis of variance (ANOVA) was performed. The numbers of cells in the embryos were subjected to logarithmic transformation. The chi-square test was used to compare the survival rates and hatching rates of embryos. A P value of less than 0.05 was considered statistically significant.

Results and Discussion

Experiment 1: The acceleration power spectrum densities (PSD) measured at the center of the truck bed are shown in Fig. 2. The three measurements were almost the same. The vibration load, determined based on the PSD data, was applied for 20 h; there was no significant difference in the survival rates of the embryos, the oxygen consumption rates, and the number of viable cells between the vibration and the no-vibration groups (Table 1).

Although we hypothesized that vibration would have negative effects on the development of embryos, the results indicate there were no significant differences in the survival rate, oxygen consumption rate and number of viable cells between the vibration and no-vibration groups. This finding is in agreement with a previous report by Mizobe *et al.* [19] who reported there was no significant difference in blastocyst formation rates between mechanically agitated (five seconds at 60-min intervals) and not-vibrated groups during *in vitro* culture of miniature pig embryos. In the present study, the vibration load test was determined based on the oscillatory waveform data collected on a truck. Thus, the vibration during transportation by truck may have no detrimental effect on the viability of embryos transported in PBM.

Experiment 2: The average transportation time from

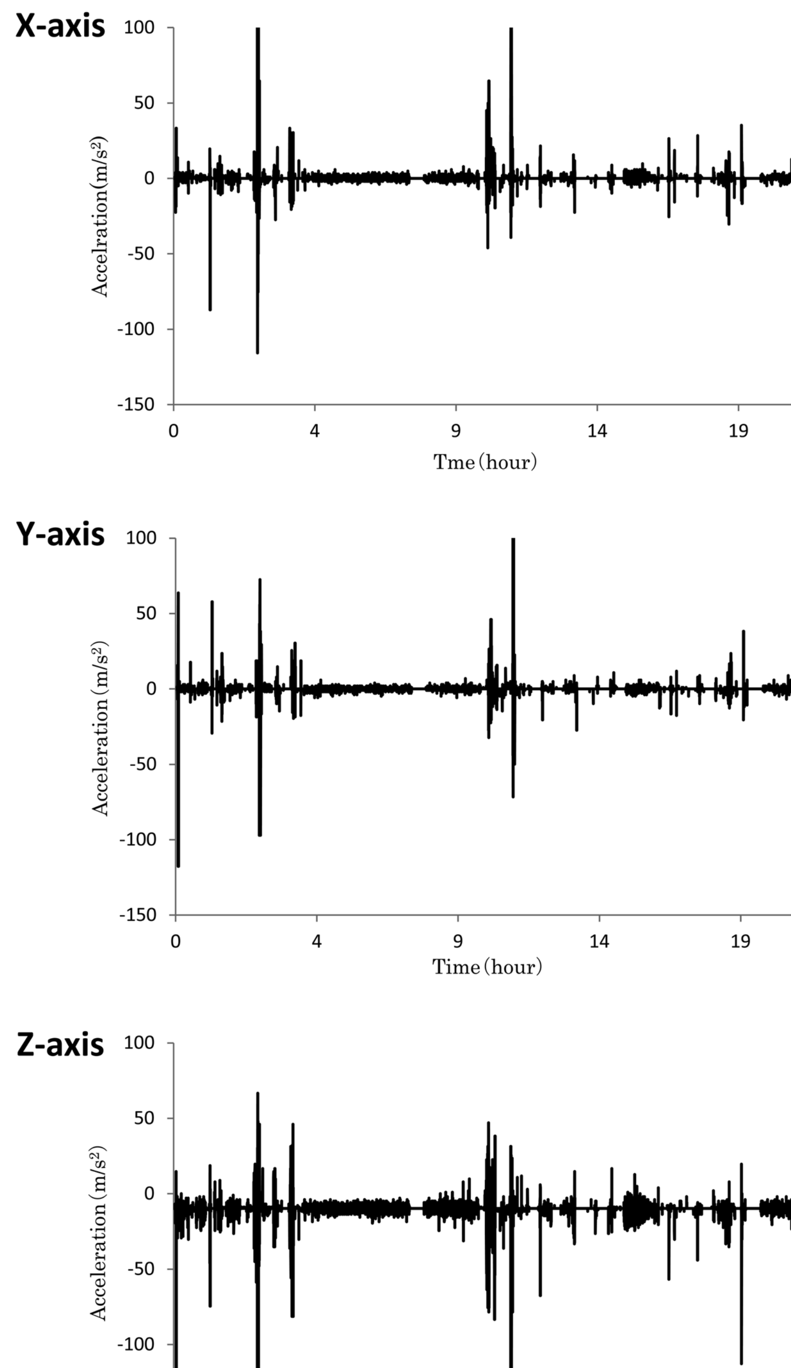

Fig. 3. Changes in acceleration, measured by a tri-axial accelerometer, along the X-, Y- and Z-axes during an approximately 22-hour transportation from Kanagawa prefecture to Yamagata prefecture.

Ebina City in Kanagawa Prefecture to Yamagata City in Yamagata Prefecture was 21.9 ± 0.3 h (means ± S.E.M.) in four or six replicates, and the transportation distance is about 357 km. The accelerations during the transportation are shown in Fig. 3, and the maximum accelerations (absolute values) were 116.6 m/s^2 along the X-axis, 117.6 m/s^2 along the Y-axis, and 119.6 m/s^2 along the Z-axis. The temperature below the embryos in the carrier did not change greatly (37.9 °C–39.0 °C). Although there was no significant difference in the oxygen consumption rates

Table 2. Oxygen consumption rate and developmental ability of *in vivo*-derived porcine embryos transported in straws, in sampling tubes, or not transported but cultured in a chemically defined medium

Experimental group	No. of embryos (No. of replicates)	Oxygen consumption rate[1)]: ($F \times 10^{-14}$ mol s^{-1})		No. of viable embryos immediately after transportation (Survival rate: %)	No. of hatched embryos 48 hours later (%)
		Before transportation	After transportation		
Sealed group (Transported in straws)	31 (6)	0.49 ± 0.02	0.56 ± 0.05	24 (77.4[a])	9 (29.0[a])
CO_2 gas group (Transported in Tubes)	19 (4)	0.47 ± 0.04	0.43 ± 0.05	19 (100[b])	11 (57.9[b])
Not transported group (Culture dishes)	40 (4)	–	–	39 (97.5[b])	19 (47.5[ab])

[1)]: Data are expressed as the mean ± S.E.M. [a-b]: Significantly different from the not-transported group (*: P <0.05).

between the sealed group and CO_2 gas groups, the survival rate of embryos in the sealed group was significantly lower than those of the other groups (Table 2). Also, the hatching rate of embryos after 48 h in the sealed group was significantly lower than that in the CO_2 gas group.

From the temporal data of accelerations it can be concluded that acceleration during transport on the road exists continuously without extreme changes. However, it became clear that during door-to-door delivery, and the in transit transfer of cargo that large accelerations occurred in the XYZ axes (the in transit times of cargo transfer were checked using the transporter's homepage). It is possible that the developmental ability of the sealed group might have been decreased by these large accelerations.

When embryos were kept under 5% CO_2 and 38 °C with PBM in sampling tubes, the survival rate and the hatching rate of the embryos after transportation were similar to those of not-transported embryos. Ozawa *et al.* [5] noted that reductions of pH and osmolality of the medium decreased the blastocyst formation rate and the number of cells in blastocysts of pigs. In the present study, the pH and osmolality of the medium might have decreased in the long-distance transport.

In conclusion, we succeeded in maintaining high viability of *in vivo*-derived porcine embryos that were transported long distance under conditions of 5% CO_2 and 38 °C in a chemically defined medium in our specially developed embryo carrier. Since the embryo carrier used in the present study can maintain these conditions for five days, it has an alternative use as an emergency incubator in the case of power supply interruption.

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