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Efficacy of mechanical micro-vibration in the development of bovine embryos during *in vitro* maturation and culture

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ABSTRACT. It is currently unclear how mechanical micro-vibration affects the *in vitro* culture of embryos in Japanese Black cow. In the experimental groups, immature oocytes and fertilized embryos were cultured using the micro-vibration culture system with the vibration set for 5 sec at intervals of 60 min and frequency of 20, 40 or 80 Hz, respectively, during *in vitro* maturation and *in vitro* development. Compared with the control group, the rate of blastocyst development significantly increased in the 40 Hz group. In addition, the number of blastocyst cells reduced significantly in the 80 Hz group. In conclusion, the development of blastocysts in cows is facilitated by providing moderate mechanical micro-vibration to immature oocytes and embryos during the *in vitro* maturation and *in vitro* development.

KEY WORDS: cow, embryo, *in vitro* development, *in vitro* maturation, mechanical micro-vibration

In cows, the *in vitro* production technology enables the commercial mass production of the embryos. Because ovary-derived oocytes from slaughterhouses are used for the mass and low cost production of beef cows, the efficient production of bovine embryos will contribute to a stable supply of beef. However, the efficiency of the bovine embryo production system based on the current *in vitro* technology is not fully developed when it is compared with *in vivo* embryo production based on controlled ovarian superovulatory response. To improve the *in vitro* development efficacy of bovine embryos, many studies have analyzed the compositions of culture media and added specified substances. During *in vitro* maturation and development, culture conditions such as temperature and the compositions of culture media are set to approximate the *in vivo* conditions, and oocytes and embryos are cultured in a petri dish in static conditions. The early development of embryos occur in the oviducts and uterus. Ovulated oocytes taken up into the oviducts by the movement of the fimbria are transported through the tube by the movement of ciliated epithelia and muscles. However, a recent study using porcine oocytes showed that the rate of blastocyst development was enhanced by mechanical micro-vibration provided during *in vitro* maturation and *in vitro* development [7]. Similarly, mechanical micro-vibration during *in vitro* culture improved the rate of blastocyst development in humans [5] and mice [4], and the pregnancy rate was also improved in humans [5].

However, the effect of mechanical micro-vibration on the *in vitro* development of bovine embryos is currently unknown. In this study, we therefore investigated how mechanical micro-vibration provided during *in vitro* maturation and *in vitro* development affect the development of bovine embryos.

Collection of oocytes: Bovine ovaries were collected from Japanese Black cows at a slaughterhouse and were transported at 20°C in physiological saline containing 0.5 mg/m*l* of injectable kanamycin sulfate Meiji 5 g (Meiji Seika Pharma Co., Ltd., Tokyo, Japan). Ovaries were washed with 70% ethanol for 1 min, and immature oocytes were aspirated along with the follicular fluid from 2–6 mm follicles using an 18 G (1·1/2" (38 mm) S.B.) syringe needle (Terumo Corp., Tokyo, Japan) and 5 m*l* syringe (SS-05SZ; Terumo Corp.). Immature oocytes thus collected were washed several times with a commercial flushing medium (Embryotech; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan). Based on the morphological classification criteria reported by Boni *et al* [1], the quality of the oocytes was evaluated under a stereomicroscope to select grade-A cumulus-oocyte complexes (A-COCs) which have a clear and compact cumulus completely surrounding the oocytes with at least three cell layers; the oocyte has a translucent and homogeneous ooplasm.

Designs of mechanical micro-vibration: In this study, the micro-vibration culture system (NSSB-300N; STREX Inc., Osaka,

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Table 1. Effect of mechanical micro-vibration at different frequencies during *in vitro* maturation and *in vitro* development of bovine oocytes and embryos

Group	Cleaved (%)	Blastocyst (%)
Control	47.0 ± 5.3	10.0 ± 0.8 a)
Vibration frequency		
20 Hz	54.0 ± 2.8	14.5 ± 2.1 a)
40 Hz	49.5 ± 2.5	14.0 ± 1.3 b)
80 Hz	49.0 ± 4.8	8.0 ± 1.7 a)

a, b) Values with different superscripts within a column are significantly different ($P<0.05$) compared with the control group. Values are means ± SE. In each group, experiments were repeated 8 times using 25 oocytes per drop in one experiment.

Table 2. Cell numbers of trophectoderm (TE) and inner cell mass (ICM) of expanded bovine blastocysts produced *in vitro* with mechanical micro-vibration at different frequencies during *in vitro* maturation and *in vitro* development

Group	n	Numbers of cells		
		TE	ICM	Total
Control	8	95.9 ± 7.9 a)	46.9 ± 4.7 a)	142.8 ± 11.6 a)
Vibration frequency				
20 Hz	8	103.1 ± 7.4 a)	47.3 ± 4.5 a)	150.4 ± 11.3 a)
40 Hz	8	101.3 ± 5.8 a)	43.5 ± 2.6 a)	144.8 ± 7.7 a)
80 Hz	8	70.0 ± 2.0 b)	31.4 ± 2.0 b)	101.4 ± 3.7 b)

a, b) Values with different superscripts within a column are significantly different ($P<0.05$) compared with the control group. Values are means ± SE.

Japan) was used to provide mechanical micro-vibration to experimental groups only during *in vitro* maturation and *in vitro* development, but not *in vitro* fertilization (IVF). According to the vibration conditions used in the study conducted by Mizobe *et al*. [7], vibration duration was set at 5 sec, vibration interval at 60 min, and vibration frequency at 20 Hz (vibration magnitude 2), 40 Hz (vibration magnitude 4), or 80 Hz (vibration magnitude 8). No mechanical micro-vibration was provided in the control group.

Maturation of immature oocytes: According to the culture method reported previously [13], a serum-free culture medium was used for *in vitro* maturation and *in vitro* development. For *in vitro* maturation of oocytes, 250 *µl* drops of the IVMD101 culture medium (Research Institute for the Functional Peptides Co., Ltd., Higashine, Japan) were placed in a 35 mm petri dish (MS-1135; Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and the medium was covered with Mineral Oil Heavy USP (Humco, Austin, TX, U.S.A.), and an O_2-CO_2 incubator (CPO_2-2301; Hirasawa Works Inc., Tokyo, Japan) was used for culture at 38.5°C in an atmosphere of 5% CO_2 in air. Selected A-COC immature oocytes were divided into groups of 25 COCs, washed with IVMD101, and subjected to *in vitro* maturation for 22 hr at 38.5°C. The total number of oocytes used in each group was 200 (25 oocytes × 8 cultures).

In vitro fertilization (IVF): Fifty *µl* drops of the IVF100 insemination fluid (Research Institute for the Functional Peptides Co., Ltd.) were placed in a 35 mm petri dish, and the medium was covered with mineral oil. Insemination was performed for 6 hr at 38.5°C in an atmosphere of 5% CO_2 in air using an O_2-CO_2 incubator. After 22 hr of *in vitro* maturation, 25 oocytes per drop were transferred to the insemination fluid. Frozen sperms from a Japanese Black cow was suspended with 50 *µl* of IVF100, and the diluted sperm was added to the insemination culture media containing oocytes with a final sperm concentration of 5×10^6/m*l*. The day of insemination was designated fertilization day 0.

In vitro embryo culture: Two hundred fifty *µl* drops of IVMD101 medium were prepared in a 35 mm petri dish coated with 0.01% (w/v) collagen type I solution (Research Institute for the Functional Peptides Co., Ltd.), to which 25 fertilized embryos per drop were transferred for 24 hr incubation at 38.5°C in atmosphere of 5% CO_2 in air. Then, the embryo was denuded by pipetting to remove the cumulus cells. Two hundred fifty *µl* drops of the IVD101 medium (Research Institute for the Functional Peptides Co., Ltd.) were placed in 35 mm petri dish, and the medium was covered with mineral oil, to which 25 denuded fertilized embryos were transferred for incubation at 38.5°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. At 48 hr after insemination, embryos at the 2 cell stage or later were defined as divided embryos, and the cleavage rate was calculated by comparing the number of embryos with the number of oocytes. On the 4th day of *in vitro* development, half of the medium was exchanged. The blastocyst development rate was calculated as the proportion of embryos that had grown to the blastocyst stage out of all the oocytes.

Measurement of the inner cell mass and trophectoderm cells in blastocysts: Fluorescent double staining method reported by Thouas *et al*. [9] was performed to calculate the number of cells in a blastocyst embryo. To count blastocyst cells, 8 expanded blastocyst embryos from individual experimental groups were examined according to the ultraviolet fluorescent microscopy. Images of blastocysts on a slide were created using a fluorescent microscope (U-RFC-T; OLYMPUS, Tokyo, Japan) with excitation filters for 330–385 and 530–550 nm to count the cell numbers of trophectoderm (TE) and inner cell mass (ICM).

Statistical analysis: Statistical analysis was performed using Statcel 2 (Add-on in Microsoft Excel; OMS Publishing, Japan). All data are expressed as means ± standard error (SE). All percentage data were subjected to an arcsine transformation in each replicate. The difference between the transformed values and numbers of cells in blastocysts was analyzed using the Student's *t*-test or Welch's *t*-test to determine statistical significance compared with the control group. For statistical analysis, the rates of cleavage and blastocyst development were measured in eight experiments (n=8) with 25 oocytes / experiment. A probability of $P<0.05$ was considered statistically significant. When $0.05 \le P < 0.1$, the difference was considered as a trend toward significance.

The effects of mechanical micro-vibration provided at different frequencies during *in vitro* maturation and *in vitro* development on the development of embryos are shown in Table 1. No significant difference in cleavage rates was observed among the groups. While the blastocyst development rate tended to increase in the 20 Hz group (P=0.08), the rate increased significantly in the 40 Hz group ($P<0.05$) compared with the control group.

Table 2 shows the effects of mechanical micro-vibration provided at different frequencies during *in vitro* maturation and *in vitro* development on the number of cells in expanded blastocyst embryos. In the 80 Hz group, trophectoderm cell count, inner cell

mass count, and total cell count were all significantly decreased when compared with the control group ($P<0.05$). No significant difference was observed in these numbers among the 20 Hz, 40 Hz, and control groups.

Mechanical micro-vibration provided at 40 Hz during *in vitro* maturation and *in vitro* development significantly increased the rate of blastocyst development, suggesting that the optimum vibration frequency for bovine oocytes and embryos is 40 Hz. This is higher than the frequency used in humans or pigs. The cilia in the oviducts reportedly moves around 5 Hz [8] to 20 Hz [10] in humans and similarly, a previous study using porcine oocytes was performed with vibration at 20 Hz [7]. Such treatment of micro-vibration is thought to be similar to the natural movement of oviduct fluid induced by the ciliated epithelium of the oviducts. In the study using porcine oocytes, the optimum duration and interval of vibration were determined to be 5 sec and 60 min, respectively [7], and the same conditions were used in this study, without investigating the optimum vibration duration and interval that are specific to cows. Therefore, the production efficiency for bovine embryos may be improved further by experimentally determining the optimum duration and interval of vibration. In addition, we failed to verify whether the improved rate of embryo development with mechanical micro-vibration was attributed to accelerated maturation of immature oocytes or accelerated embryonic development during *in vitro* culture. Furthermore, the examination of the mechanical micro-vibration effect will be necessary on the condition of high efficiency in the future, because the rate of cleavage and blastocyst development of bovine embryos were not high in this study generally.

When the vibration frequency was set up as high as 80 Hz, not only the blastocyst development rate, but also the number of blastocyst cells decreased. It was reported previously that the circulation of the culture medium for over 10 hr caused stress to cells in mouse embryos, resulting in the activation of MAPK8/9 that are involved in apoptosis, an increase in the number of TUNEL-positive cells, and a reduction in the cell number of blastocysts [12]. Furthermore, brief shearing stress due to pipetting upregulated phosphorylated stress-activated protein kinase / Jun kinase in mouse embryos [11]. In this study, blastocyst cell count was low in the 80 Hz group, suggesting that excessive vibration during culture is ineffective, presumably because apoptosis is induced through the activation of phosphorylated stress-activated protein kinases. This may have also adversely affected the quality of embryos, reducing the blastocyst development rate in the 80 Hz group compared with the 40 Hz group.

Two factors may be involved in the acceleration of embryo development by the vibration of oocytes and embryos. First, the acceleration of proliferation and maturation in embryos might have been due to activation of the intracellular response. For example, mechanical micro-vibration affects the expression of various genes in vascular endothelial cells [2]. It is therefore possible that mechanical micro-vibration might have affected genes involved in embryo development in this study. Secondly, the vibration of the culture media promoted the elimination of waste products from oocytes and embryos. In the still culture system, toxic substances such as ammonium [3] and oxygen-derived free radicals [6] generated during *in vitro* culture were shown to suppress the development of bovine embryos. Because the media used in this study contain amino acids, ammonium is produced due to amino acid degradation by cell metabolism in oocytes and embryos. However, mechanical micro-vibration provided in this study is thought to have removed ammonium and oxygen-derived free radicals in the vicinity of the COCs and embryos, thus reducing the suppression of embryo development by the toxic substances.

In conclusion, the provision of moderate mechanical micro-vibration to immature oocytes and embryos during *in vitro* maturation and *in vitro* development enhances the development of blastocysts in cows, whereas excessive vibration reduces the number of blastocyst cells, which may adversely affect the quality of embryos. In the future, it is necessary to elucidate the mechanisms of enhancement of embryo development by mechanical micro-vibration at which this occurs.

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