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—Original Article—

## Effects of Seasonal Changes on *In Vitro* Developmental Competence of Porcine Oocytes

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**Abstract.** The aim of this study was to investigate whether seasonal changes affected *in vitro* developmental competence of porcine oocytes. The relationship between atmospheric temperature and embryonic development of *in vitro* matured porcine oocytes following intracytoplasmic sperm injection was examined throughout the year. The blastocyst rate (31.1%) in winter (mean atmospheric temperature during December to February:  $-3.8$  C) was significantly higher ( $P < 0.05$ ) than those of other seasons in 2008/2009 (19.7–23.5%; 6.3–17.5 C). The monthly mean blastocyst rates were negatively correlated with the temperatures ( $r = -0.5944$ ,  $P < 0.05$ ). The results of the present study suggest that porcine embryos could be produced throughout the year, but the *in vitro* production efficiency was significantly affected by season, i.e., atmospheric temperatures. Furthermore, the results showed that winter is a favorable season for blastocyst production in the region of Obihiro, Hokkaido, Japan.

**Key words:** Embryonic development, Pig, Seasonal changes, Temperature

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*In vitro* production (IVP) of porcine embryos including *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) has been utilized for research on embryology and commercial purposes. Porcine IVP can be performed throughout the year, because porcine ovaries are available from slaughterhouses at any time of the year. In our laboratory, porcine blastocysts have been produced *in vitro* using defined culture media after intracytoplasmic sperm injection (ICSI) [1–3]. A significant problem for porcine IVF is a high polyspermy rate (50–60%) that results in embryo polyploidization [4, 5]. At present, it seems that ICSI is the only method that can be used with porcine IVP to completely eliminate the risk of polyspermy and produce normal diploid embryos.

Summer heat stress induces low developmental competence in bovine embryos and low conception rates [6–8]. Thus, seasonal infertility may be strongly related to high atmospheric temperatures. Some reports show the detrimental effects on oocyte cytoskeletal organization [9] and meiotic [10] and developmental competence [11] caused by subjecting porcine ovaries or oocytes to heat stress. On the other hand, porcine oocytes and embryos have high sensitivity to low temperatures compared with those of other mammalian species [12]. Since porcine oocytes are very sensitive to extreme temperatures, the IVP efficiency may be influenced by seasonal changes. To our knowledge, however, the effects of seasonal changes on the efficacy of porcine IVP have not been investigated. Furthermore, it is not known whether there is seasonal infertility in pigs in Hokkaido, a region with a comparatively cooler climate and shorter summer period than the mainland of Japan.

In the present study, we investigated the effects of seasonal

changes on *in vitro* embryonic development of *in vitro* matured porcine oocytes following ICSI throughout the year.

### Materials and Methods

The present study was approved by the Animal Experimental Committee of Obihiro University of Agriculture and Veterinary Medicine in accordance with the Guiding Principles for the Care and Use of Research Animals.

#### Experimental period and temperatures in Obihiro

The present study was performed from September 2008 to August 2009. The monthly temperature data in Obihiro (42°55'N, 143°12'E), Hokkaido, Japan, were obtained from the Japan Meteorological Agency (<http://www.jma.go.jp/jma/indexe.html>).

#### Oocyte collection

Ovaries were obtained from prepubertal gilts at a local slaughterhouse, and transported to the laboratory in a sterilized saline (0.9% NaCl) solution at approximately 37 C within 3 h and washed twice in sterilized saline solution. Cumulus-oocyte complexes (COCs) were aspirated from 3 to 8 mm follicles using an 18-G needle attached to a 5-ml disposable syringe. Subsequently, the COCs were washed three times in a HEPES-buffered Tyrode's medium containing 0.05% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA; TLH-PVA). COCs with two or three layers of cumulus cells and uniform cytoplasm were selected for IVM culture.

#### *In vitro* maturation (IVM)

The medium for IVM was Medium 199 (with Earle's salts, L-glutamine, and 2,200 mg/l sodium bicarbonate; Sigma) supplemented with 0.05% PVA, 3.05 mM glucose (Wako Pure Chemical Industries, Osaka, Japan), 0.91 mM Na-pyruvate (Wako), 100  $\mu$ M

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cysteamine (Sigma), 10 ng/ml epidermal growth factor (Sigma), and 75 mg/l kanamycin (Sigma). Selected COCs were washed three times in IVM medium, and then 10–15 COCs were cultured in a 100  $\mu$ l droplet of the IVM medium covered with mineral oil (Sigma) for 44 h at 39 C in a humidified atmosphere of 5% CO<sub>2</sub> in air. In the first 22 h of culture, COCs were cultured with 10 IU/ml PMSG (ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml hCG (ASKA), and in the last 22 h, they were cultured without these hormones.

#### *Intracytoplasmic sperm injection (ICSI)*

After IVM culture, the oocytes were denuded from their cumulus cells by gentle pipetting in the IVM medium. Denuded oocytes were washed three times and kept in fresh IVM medium until ICSI. As in our previous studies [1–3], the same batch of pelleted frozen semen derived from ejaculates of a Duroc boar was used throughout the study. The frozen semen was thawed in prewarmed (39 C) Dulbecco's phosphate buffered saline (PBS; Gibco-BRL, Grand Island, NY, USA) containing 0.1% PVA (PBS-PVA) and was washed by centrifugation at 300  $\times$  g for 3 min in the same medium.

The procedure for ICSI was performed as previously reported [13] with slight modifications. Briefly, manipulation was conducted with the aid of a pair of micromanipulators (Leitz, Wetzlar, Germany) under an inverted microscope. Several drops of PBS-PVA (5  $\mu$ l each) containing spermatozoa and TLH-PVA containing denuded oocytes were placed on the lid of a 50  $\times$  9 mm petri dish (Falcon 1006, Franklin Lakes, NJ, USA) and covered with mineral oil. A 1- $\mu$ l supernatant portion of washed semen was added into PBS-PVA on a manipulation dish. An injection pipette was prepared by breaking the tip of a pulled capillary tube (G-100; Narishige, Tokyo, Japan) with an inner diameter 3–4  $\mu$ m. From the edge of the sperm-containing droplet, a motile spermatozoon was aspirated into the injection pipette tail-first without an immobilizing treatment, such as tail-scoring/cutting, and transferred to the drop containing oocytes. An oocyte was held with its polar body at either the 6 or 12 o'clock position using the holding pipette. A spermatozoon was injected into the oocyte cytoplasm and mixed with the cytoplasmic components by gently sucking and blowing back a small part of cytoplasm using a mouth regulated open tube.

#### *In vitro culture (IVC)*

After ICSI, oocytes were washed three times in IVC medium comprised of Porcine Zygote Medium-4 [14] supplemented with 2.77 mM *myo*-inositol (Sigma), 0.34 mM tri-sodium citrate (Merck, Darmstadt, Germany) and 50  $\mu$ M  $\beta$ -mercaptoethanol (Sigma). Immediately after washing, groups of 10–15 oocytes were cultured under 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> at 39 C in a 30  $\mu$ l drop of IVC medium covered with mineral oil.

#### *Observations of maturation, fertilization, cleavage and blastocysts*

In this study, an oocyte with an extruded first polar body was considered to be mature. After 12 h of ICSI, some oocytes were fixed in 25% (v/v) acetic acid (Wako) in ethanol (Wako) for 24 h and stained with 1% (w/v) orcein (Sigma) in 45% acetic acid solution for the determination of fertilization rates. Oocytes with male and female pronuclei, two polar bodies and a sperm tail were con-

sidered to be normally fertilized. The rates of cleavage and blastocyst formation were determined at 48 and 144 h from the start of IVC, respectively. The number of cells per blastocyst was determined by staining with 5  $\mu$ g/ml Hoechst 33342 (Sigma) for 15 min and using a fluorescence microscope.

#### *Experimental design*

In this study, when the monthly mean temperatures were less than 0 C, it was considered to be winter (December, January, and February), and the remaining seasons were classified into the following three months intervals: March to May (spring), June to August (summer), and September to November (autumn). The rates of maturation, fertilization, cleavage and blastocyst formation were calculated for each month of the year and grouped into four seasons to determine the effect of seasonal changes on *in vitro* developmental competence of porcine oocytes. Thereafter, the correlation between monthly mean temperatures and developmental competence of porcine oocytes was investigated.

#### *Statistical analysis*

The experiment was repeated at least four times per month. Statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). The rates of maturation, cleavage and blastocyst formation were analyzed by logistic regression following a binomial distribution using the following model:  $\ln(\alpha/1-\alpha) = \beta + \text{main factor (seasons)}$ , where  $\alpha$  = the frequency of positive outcome and  $\beta$  = the intercept. The comparison of blastocyst cell numbers was conducted by one-way ANOVA. Post-hoc analysis was performed by Tukey-Kramer multiple comparison tests to determine significant differences. The correlation between the atmospheric temperature and embryonic development was analyzed by Pearson's product-moment coefficient of correlation. Differences were considered significant when the P value was less than 0.05.

## Results

In this study, 5646 oocytes were used for IVM and 2752 oocytes were inseminated by ICSI. As a whole, 421 blastocysts were produced throughout the year. The effects of seasonal changes on meiotic and developmental competence in porcine oocytes are shown in Tables 1 and 2, respectively. The rates of maturation and fertilization were not significantly different among the four seasons. The mean cleavage rate in summer (75.7%) was significantly ( $P < 0.05$ ) higher than in the other seasons (60.7–67.3%; Table 2), and the developmental competence up to the blastocyst stage was significantly ( $P < 0.05$ ) higher in winter (31.1%) than in the other seasons (19.7–23.5%). Furthermore, the mean cell number per blastocyst produced in autumn (43.8) was significantly ( $P < 0.05$ ) higher than those (25.6–27.9) in the other seasons (Table 2). A significant ( $P < 0.05$ ) correlation was found between the monthly mean blastocyst rates and the temperatures throughout the year ( $r = -0.5944$ , Fig. 1); however, correlation was not detected between the mean temperatures and cleavage rates.

## Discussion

The *in vitro* developmental competence of porcine oocytes is

still lower compared with that of *in vivo* produced embryos [15, 16], although many efforts [17] have been made to improve porcine IVP technologies. The present investigation of porcine IVP efficiency clearly demonstrated that the *in vitro* developmental competence of porcine oocytes up to the blastocyst stage was different among seasons, with the highest efficiency occurring in winter. Additionally, a negative correlation between the atmospheric temperatures and the blastocyst rates was observed in the present study. These results suggest that one of the dominant factors determining the *in vitro* embryonic development of porcine IVP is the monthly atmospheric temperature.

In this study, the stock solution for sampling of ovaries was stabilized at approximately 37 C, and culture of oocytes was performed at the same temperature throughout the year. Therefore, the monthly differences of *in vitro* developmental competence of porcine oocytes throughout the year, as shown in this study, were most likely due to seasonal changes, including the atmospheric temperature. It has been reported that summer heat stress reduces the developmental competence of bovine oocytes [6–8]. Additionally, heat stress was found to change the numbers of follicles and their diameters [6] and also to modify the fatty acid [6] and protein [18] composition of the porcine oocyte plasma membrane. Furthermore, heat stress induces reactive oxygen species and results in apoptosis of granulosa cells in mice and rats [19, 20]. As reported in previous bovine studies [6–8], the present results clearly showed a negative relationship between developmental competence of porcine oocytes and atmospheric temperatures.

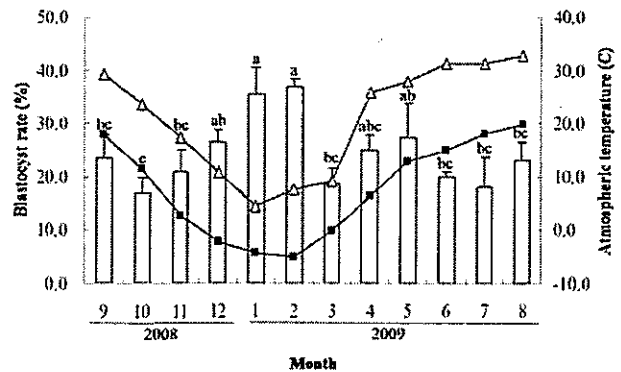
In contrast, Silva *et al.* [21] reported that the blastocyst rates of *in vitro* matured bovine oocytes were significantly higher in autumn than other seasons for a period of two years. Furthermore, Rivera *et al.* [22] indicated that season did not affect cleavage rates

or the subsequent development of bovine embryos. The reason for these contradictory findings is not clear, but at least, the present study has indicated that the seasonal changes affect porcine IVP performance. On the other hand, in the present study, the maturation rates were not significantly different among the four seasons, but the blastocyst formation was variable among seasons. It has been reported that subjecting porcine ovaries to elevated temperatures of 41–42 C does not affect the meiotic competence of oocytes but decreases the subsequent developmental competence [9]. Also, there are some reports that heat stress induces detrimental effects on porcine oocyte quality [7–9]. From these results, we conclude that seasonal changes do not directly affect nuclear maturation rates, whereas the oocyte quality including cytoplasmic maturation may be impaired for subsequent development. In other words, seasonal variations in the quality of oocytes affected the yields of blastocysts, as reported in bovine IVF [21]. In the present study, the cleavage rate in summer was significantly higher than in the other seasons. The reason for the high cleavage rate in summer is not clear; however, the rate of development of blastocysts to cleaved embryos was lowest in summer compared with the other seasons in this study. This result may have been caused by summer heat stress and suggests that porcine embryos produced during summer may

**Table 1.** Effect of seasons on *in vitro* maturation rate in porcine oocytes

Season (C)*	No. of oocytes	
	Cultured	Matured (% ± SEM)
Spring (6.3)	1147	841 (73.3 ± 2.1)
Summer (17.5)	837	588 (70.3 ± 3.1)
Autumn (10.5)	2124	1479 (69.6 ± 1.8)
Winter (-3.8)	1538	1096 (71.3 ± 2.6)

\*Seasons were classified by the monthly mean temperatures. Spring is March to May, summer is June to August, autumn is September to November and winter is December to February. C: mean temperatures of three months each.



**Fig. 1.** The monthly mean blastocyst rates and atmospheric temperatures from September 2008 to August 2009 in Obihiro, Hokkaido, Japan. White bars show mean blastocyst rates in each month, and black square and white triangle lines indicate the monthly mean and maximum atmospheric temperatures, respectively. A significant ( $P < 0.05$ ) correlation was found between the blastocyst rates and temperatures ( $r = -0.5944$ ).

**Table 2.** Effect of seasons on *in vitro* fertilization and embryonic development following ICSI in porcine oocytes

Season (C)*	No. of oocytes		No. of oocytes		Blastocysts (% ± SEM)	Blastocysts/cleaved embryos: % ± SEM	Cells/blastocyst: mean ± SEM
	Examined	Fertilized (% ± SEM)	Cultured	Cleaved (% ± SEM)			
Spring (6.3)	213	125 (58.7 ± 3.7)	400	269 (67.3 ± 4.2) <sup>a</sup>	94 (23.5 ± 2.8) <sup>a</sup>	34.9 ± 4.8 <sup>a</sup>	26.5 ± 1.8 <sup>a</sup>
Summer (17.5)	138	72 (52.2 ± 5.4)	333	252 (75.7 ± 2.7) <sup>c</sup>	71 (21.3 ± 2.6) <sup>a</sup>	28.2 ± 3.3 <sup>a</sup>	27.9 ± 2.6 <sup>a</sup>
Autumn (10.5)	306	170 (55.6 ± 3.5)	593	360 (60.7 ± 2.6) <sup>b</sup>	117 (19.7 ± 2.4) <sup>a</sup>	32.5 ± 3.6 <sup>a</sup>	43.8 ± 6.2 <sup>b</sup>
Winter (-3.8)	322	177 (55.0 ± 4.7)	447	290 (64.9 ± 2.8) <sup>ab</sup>	139 (31.1 ± 2.3) <sup>b</sup>	47.9 ± 3.2 <sup>b</sup>	25.6 ± 1.2 <sup>a</sup>

<sup>a-c</sup>Values with different superscripts within the same column are significantly different ( $P < 0.05$ ). \*Seasons were classified by the monthly mean temperatures (Fig. 1). Spring is March to May, summer is June to August, autumn is September to November and winter is December to February. C: mean temperatures of three months each.

be more susceptible to fragmentation than those produced in other seasons. Furthermore, Wolfenson *et al.* [23, 24] and Roth *et al.* [25, 26] reported that follicular steroid production was reduced in autumn by a carry-over effect of summer heat stress and resulted in a reduced dominance of the selected follicles and affected bovine oocyte quality. In fact, the cleavage and blastocyst rates in autumn tended to be lower compared with those of the other seasons in the present study.

There was a significant seasonal difference in the cell numbers per blastocyst produced in our study. The mean cell numbers per blastocyst produced in autumn were higher than those in the other seasons. Although we could not clarify any certain reasons for the distinction of the cell numbers per blastocyst examined in the present study, it is possible that high-quality oocytes develop to blastocysts with a high cell number, whereas low-quality oocytes fail to develop to blastocysts under summer heat stress.

The present study was performed in Hokkaido in northern Japan, which has a comparatively colder climate than mainland Japan. It has been reported in pigs that seasonal infertility associated with atmospheric temperatures is observed in Britain, where the climate is as cold as in Hokkaido [27]. Furthermore, an environmental temperature range of 18 to 20 C has been found to support optimal reproductive performance of growing-finishing pigs and that signs of heat stress occur at above 22 C [28]. Additionally, the monthly maximum atmospheric temperatures during the summer season were over 30 C in Obihiro, Hokkaido, Japan (Fig. 1). However, not only the environmental temperatures but also the humidity, photoperiod and feeding systems are responsible for seasonal effects on reproduction [29]. Therefore, more information about seasonal factors influencing IVP efficiency is definitely needed. The present results clearly demonstrated that the atmospheric temperature is one of the dominant factors influencing on blastocyst production, which provides valuable information for the future study of porcine IVP.

In conclusion, the present results showed that porcine embryos could be produced throughout the year, but that the IVP efficiency was significantly affected by season, i.e., atmospheric temperatures. Furthermore, the results showed that winter is a favorable season for blastocyst production in the region of Obihiro, Hokkaido, Japan.

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