Seasonal influence on the development of parthenotes and cloned embryos in pigs*

Zhang Kun[1], Zhang Yunhai[3,4], Pan Dengke[5], Ma Yufang[1], Wei Hengxi[1], Zhang Jian[6], Li Xuyang[7], Wang Shaohua[1], Li Yan[2], Dai Yunping[1,2] and Li Ning[1]**

(1. State Key Laboratory for Agrobiotechnology, College of Biological Science, China Agricultural University, Beijing 100094, China; 2. JiPulin Biotech Ltd., Beijing 100094, China; 3. Danish Institute of Agricultural Sciences, 8830 Tjele, Denmark; 4. College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, China; 5. Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100094, China; 6. Animal Clinical Hospital, China Agricultural University, Beijing 100094, China; 7. Hebei Minghui Group of Pigs Breeding, Sanhe 065200, China)

Accepted on March 5, 2007

Abstract Seasonal influence on the developmental ability of porcine embryos was studied by comparisons of the number of cumulus-oocytes-complexes (COCs) per ovary pair and meiotic maturation ability of oocytes in different seasons; and by the observations on developmental competence of the parthenogenetic oocytes and cloned embryos collected from different seasons. We found that the number of COCs per ovary pairs was significantly higher in spring compared to the other seasons ($P < 0.05$). However, no significant difference was found in the rate of oocytes progressing to M Ⅱ in four seasons ($P < 0.05$). In addition, the rate of blastocyst formation of parthenogenetic embryos in summer was obviously declined compared to the other seasons ($P < 0.05$), and an increased blastocyst rate of cloned porcine embryos was found in spring compared to autumn and winter ($P < 0.05$). The results suggest that there should be a seasonal influence on the developmental competence of porcine embryos.

Keywords: pig, cloned, parthenogenetic, season.

Somatic cell nuclear transfer (SCNT) in pigs provides many potential applications for biomedicine, agricultural and biological basic research[1,2]. However, its efficiency is still very low in porcine SCNT (often less than 1% according to percentage of pregnancy to terms from oocytes used) like other species[3]. It is generally acknowledged that many factors contribute to the inefficiency such as laboratory variation, oocyte source, cell type of nuclei donor, treatment of donor cells prior to nuclear transfer, conditions of embryo culture, and etc.[1,3].

In pigs, it is well known that early embryo mortality accounts for most of overall embryo death. The major proportion of the death occurs at preimplantation stage. Among many factors that have been acclaimed to cause embryo death, oocyte source and quality variation in pigs could be the major factors. In addition, it is generally accepted that environmental influences, such as high ambient temperature and humidity[4], are correlated with marked seasonal declines in the reproductive performance of pigs in hot climate[2,5,6]. The current study was designed to investigate seasonal effects on *in vitro* maturation (IVM) of porcine oocytes derived from slaughterhouses, *in vitro* developmental competence of parthenogenetic and SCNT embryos and on *in vivo* developmental ability of SCNT embryos.

1 Materials and methods

Unless otherwise noted, all chemicals were bought from Sigma-Aldrich Corp (St. Louis, USA).

1.1 IVM of porcine oocytes

Gilt ovaries were obtained from a local slaughterhouse and transported to the laboratory in physiological saline added with antibiotics at 25—35°C within 2—4 h. Cumulus-oocytes complexes (COCs) were aspirated from follicles with 3—6 mm in diameter using a 18-gauge needle connected to a 10 mL disposable syringe. Grade A/B COCs (Oocytes surrounded by at least 3 layers of compacted cumulus cells and with uniform cytoplasm were defined as Grade A, and oocytes surrounded by 2—3 layers of cumulus cells and with uniform cytoplasm were defined as

* Supported by Natural Science Foundation of Beijing (Grant No. 5030001)
** To whom correspondence should be addressed. E-mail: ninglbau@public3.bta.net.cn

Grade B) were selected after being washed in the maturation media (NCSU-23) three times. Of the 50—80 COCs were moved to 500 μL maturation medium covered with mineral oil in a four-well multidish (Nunc). BSA-free NCSU-23 was supplemented with 10 IU/mL pregnant mare serum gonadotrophin (PMSG), 10 IU/mL human chorionic gonadotrophin (hCG), 10% porcine follicular fluid (PFF), 0.57 mmol cysteine and 10 ng/mL epidermal growth factor (EGF). PFF was sucked from follicles with 5—8 mm in diameter, centrifuged at 1600 *g* for 30 min at 4℃, then supernatant was harvested and passed through 0.45 μm filters. Aliquots were stored at 20℃ until used. Maturation media were balanced in the incubator at least 3 h before IVM. COCs were maturated for 42—44 h at 39℃ and 100% humidity in 5% CO_2 air.

1.2 Establishment of porcine fetal fibroblast cell lines

A Landrace pig at 30 days of pregnancy was euthanized, and the uterus was transported to laboratory. Fetuses were taken out aseptically and were washed twice in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS; GIBCO), then cut to pieces after stripping of heads and internal organs using fine scissors and forceps. Afterwards, the pieces were seeded to a T-75 cell culture flask and cultured at 39℃, in 100% humidity and 5% CO_2 for 4—6 h until tissue explants attached. Then Dulbecco's modified essential media containing 20% fetal bovine serum and 1% nonessential amino acids and penicillin and streptomycin were added. At 70%—80% confluence as the cells grew, the medium was removed and the cells were washed three times with DPBS, then harvested by 0.25% trypsin and 0.02% EDTA treatment. The cells were subcultured two times and then frozen using DMEM with 10% DMSO. In order to be used as donor of nucleus in SCNT, cells were thawed and digested after they reached full confluence.

1.3 Reconstruction of porcine cloned embryos

Nuclear transfer[3, 7] was conducted under an inverted microscope TE2000U equipped with manipulation systems (Narishge, Japan). Micromanipulation drops comprised of Hepes buffered NCSU-23 with 7.5 μg/mL cytochalasin B (CB) covered with mineral oil. Groups of 20—30 oocytes and nuclear donor cells were transferred to the drop. After being positioned at 5—6 o' clock, the first polar body and 10%—15% of its surrounding cytoplasm probably containing metaphase plate were sucked with a beveled pipette (inner diameter of 15—20 μm). A round, smooth somatic donor cell was directly injected into the perivitelline space through the same opening. After SCNT, reconstructed embryos were transferred into drops of media covered with mineral oil for 1—2 h recovery until fusion and activation were conducted. The recovering medium was Hepes buffered NCSU-23 with 7.5 μg/mL CB.

1.4 Fusion/activation and parthenogenetic activation

The couplets were washed three times then balanced in the fusion/activation medium for 3 min. Groups of 4—5 couplets were put in the fusion chamber filled with the fusion/activation medium. They were manually aligned with a fine needle to make the contact plane parallel to electrodes. A single, 30 μs, direct current pulse of 1.8 kV/cm was applied with BTX ECM2001 instrument to induce fusion, and simultaneously activation. The fusion/activation medium was 0.25 mol/L mannitol, 0.1 mmol/L $MgCl_2$, 0.1 mmol/L $CaCl_2$, 0.5 mmol/L Hepes and 0.01% polyvinyl alcohol (PVA). After culturing couplets in drops of NCSU-23 plus 4 mg/mL BSA and 7.5 μg/mL CB for 30 min, fusion was observed under a stereomicroscope (SMZ1000, Nikon, Japan), and then they were cultured in embryo culture media to assess *in vitro* developmental competence[3, 8]. For parthenogenetic activation, oocytes were applied with the same parameters as were the NT embryos.

1.5 Embryos transfer

SCNT embryos cultured for 12—24 h were surgically transferred into oviducts of naturally cycling sows on the first day of standing estrus. Anesthesia was induced with 1 g Pentothal sodium. Pregnancy was maintained by injecting PMSG and hCG on day 10 and day 13, respectively (day 0 is the day embryo transfer was conducted). Gestation status was monitored monthly by ultrasound scanning.

1.6 Experimental design

COCs were collected from a local abattoir over two years, including all seasons from spring to winter. In Experiment 1, we compared the number of COCs (Grade A/B) per ovary pair obtained in differ-

ent seasons (COCs/ovary pair) and the rate of oocytes progressing to MⅡ stage. After the culture of the embryos, the developmental competence of the parthenogenetically activated oocytes (Experiment 2) was observed and the somatic cell nuclear transfer embryos (Experiment 3) produced from oocytes collected in four seasons were evaluated. In the latter two experiments, we compared the blastocyst rate and cleavage rate observed on day 2 and day 6, respectively. According to the preliminary result of Experiment 2 that showed the oocyte quality was poorer in summer than other seasons, in Experiment 3, we just tested the oocytes collected in spring, autumn and winter. In addition, *in vivo* developmental ability of SCNT embryos was assessed by embryos transfer in winter of 2004 and spring of 2005 and 2006 (Experiment 4).

1.7 Statistical analysis

All data obtained were subjected to one-way ANOVA with SPSS (version 13.0 for windows). Then the least significant difference (LSD) was applied for post hoc multiple comparison among groups. All data were expressed as mean±SEM. Differences among groups were considered significant at $P<0.05$.

2 Results

2.1 Seasonal effect on *in vitro* maturation of porcine oocytes

As seen in Table 1, maximal COCs/ovary pair was achieved in spring compared to summer, autumn and winter. However, no significant difference of the rate of oocytes developing to MⅡ stage was found for all seasons. In addition, from our general observation, the cytoplasm of matured oocytes from spring and autumn was more unique and sticky than that from summer and winter.

Table 1. Seasonal effect on *in vitro* maturation of porcine oocytes

Seasons	Ovary pair (*n*)	Replicates (*n*)	A/B COCs (*n*)	COCs/ovary pairs	MⅡ (%)
Spring (Mar to May)	1260	22	10803	7.6±3.5[a)]	75.3±3.3
Summer (June to Aug)	1082	18	7393	6.4±3.4	73.5±2.3
Autumn (Sept to Nov)	1341	26	11846	6.7±2.9	73.0±4.2
Winter (Dec to Feb)	1398	22	13386	6.7±3.5	76.5±6.7

a) There is significant difference between this group and other group ($P<0.05$)

2.2 Seasonal effect on *in vitro* developmental competence of parthenogenetic embryos derived from matured oocytes

As found in Table 2, no obvious difference in the cleavage rate of parthenogenetic embryos was observed between the seasons. However, the blastocyst rate in summer was remarkably lower than that of other seasons.

Table 2. Seasonal effect on *in vitro* developmental competence of parthenogenetic embryos derived from porcine matured oocytes

Seasons	Embryos cultured (*n*)	Replicates (*n*)	Cleavage embryos (%)[a)]	Blastocysts (%)[b)]
Spring (Mar to May)	874	14	440(69.5±3.0)	265(27.2±3.4)
Summer (June to Aug)	94	7	59(63.8±5.2)	9(10.1±2.6)[c)]
Autumn (Sept to Nov)	667	14	358(61.6±5.3)	154(22.4±3.5)
Winter (Dec to Feb)	991	20	514(66.1±3.9)	236(22.4±3.4)

a), b) Data are represented as mean±SEM.

c) There is significant difference between this group and other groups ($P<0.05$)

2.3 Seasonal effect on *in vitro* developmental competence of cloned embryos derived from matured oocytes

As seen in Table 3, no significant difference in cleavage rate of cloned embryos was found between seasons. However, the blastocyst formation of the cloned embryos in winter was obviously reduced when compared to spring and autumn.

Table 3. Seasonal effect on *in vitro* developmental competence of SCNT embryos derived from porcine matured oocytes

Seasons	Embryos cultured (n)	Replicates (n)	Cleavage embryos (%)[a)]	Blastocysts (%)[b)]
Spring (Mar to May)	1060	24	559(58.3±3.5)	137(12.2±1.3)
Autumn (Sept to Nov)	921	21	480(51.9±3.5)	90(10.2±1.2)
Winter (Dec to Feb)	1202	32	550(50.4±3.3)	108(8.1±1.4)[c)]

a), b) Data are represented as mean±SEM

c) There is significant difference between this group and other groups ($P<0.05$)

2.4 Seasonal effect on *in vivo* developmental competence of SCNT embryos derived from matured oocytes

From December 2004 to February 2005 a total of 824 cloned embryos at 1—2 cell stage were transferred to 9 receipts whereas from March to May in 2005 and 2006 a total of 1072 cloned embryos at 1—2 cell stage were transferred to 10 receipts. Both the rates of pregnancy and live birth were lower in former test than the latter (Table 4).

Table 4. Seasonal effect on *in vivo* developmental competence of SCNT embryos derived from porcine matured oocytes

Seasons	Surrogate mothers (n)	SCNT embryos transferred	Ratio of pregnancy	Ratio of viable offsprings
Winter (Dec to Feb)	9	824	1/9	0
Spring (Mar to May)	10	1072	3/10	1/10

3 Discussion

Previous studies indicate that environmental influences (such as hot climate in summer) are responsible for the functional declination of the porcine ovary, failure of fertilization, declination of the rate of viable embryo, and inclination of mortality[4]. The region where Beijing is located belongs to the climate of continental monsoon. It is dry and most freezing in winter and hottest in summer while every season has its unique characteristic. Therefore, the extreme temperature in summer and winter maybe accounts for the poor reproductive performance in pigs in these two seasons. In the present study, we found that the average number of COCs collected from the ovary pair was significantly higher in spring than that in summer, autumn and winter. This difference may be explained by the speculation that hot climate in summer elicited the heat stress in porcine ovary and inhibited the secretion of FSH, then caused declination of ovulation ability in pigs. However, no significant difference of the rate of oocytes progressing to M Ⅱ stage was observed between seasons. This phenomenon may be attributed to the fact that the quality of COCs we selected to mature *in vitro* was similar and that the condition of IVM was maintained at a stable level. Due to the lack of reliable markers, we could not compare the rate of cytoplasm maturation for seasonal effect[9]. However, in our manipulation of the cells, we found that the cytoplasm of porcine matured oocytes collected in spring and autumn was more unique and viscose than those collected in summer and winter, which indirectly indicated the cytoplasm maturation.

It is well acclaimed that amounts of lipid droplets exist in the cytoplasm of porcine oocytes and embryos[3,10], which caused the sensitiveness and fragility of the oocytes and embryos to exterior environment. This sensitiveness accounts for the difficulty of porcine embryos cryopreservation. Recently, Li et al. have successfully cryopreserved the porcine SCNT embryos after aspirating the lipid droplets. When they transferred these cryopreserved embryos into surrogate mothers, they finally got viable offsprings[10]. Our study indicated that blastocyst rate of parthenogenetic embryos in summer was strikingly lowered compared to other seasons, and the blastocyst yield of SCNT embryos in winter was remarkably declined in comparison with spring and autumn, these significant differences could be partly caused by the environmental temperature variation in a certain degree due to the difficulty in keeping the temperature constant in the experimental rooms for two years, but the seasonal effects on the developmental competence of porcine embryos did exist and this phenomenon was recently reported by others too[11—14].

We also found that both the rates of pregnancy and viable offspring were lower in winter than in spring when we conducted SCNT embryo transfer. This may be caused by the freezing climate in winter when the embryo viability, especially, the embryos at 1—2 cell stage, is low.

Taken together, we demonstrated that seasonal variation may influence the *in vitro* and *in vivo* development of porcine embryos derived from oocytes collected from slaughterhouse, but not the meiotic maturation of porcine oocytes.

Acknowledgements The authors would like to thank Ms. Fei Jing for technical assistance, to Mr. Wang Gang for help in ovary collection and transportation.

References

1 Lai L and Prather RS. Production of cloned pigs by using somatic cells as donors. Cloning Stem Cells, 2003, 5(4): 233—241
2 Peltoniemi OA and Virolainen JV. Seasonality of reproduction in gilts and sows. Reprod Suppl, 2006, 62: 205—218
3 Pan DK, Zhang YH, Sun XZ, et al. Cloned pigs derived from somatic cell nuclear transfer embryos cultured *in vitro* at low oxygen tension. Chinese Science Bulletin, 2006, 51(7): 839—844
4 Le Bellego L, van Milgen J and Noblet J. Effect of high temperature and low-protein diets on the performance of growing-finishing pigs. J Anim Sci, 2002, 80(3): 691—701
5 Boden MJ and Kennaway DJ. *Circadian rhythms* and reproduction. Reproduction, 2006, 132(3): 379—392
6 Dominguez JC, Pena FJ, Anel L, et al. Swine summer infertility syndrome in north west Spain. Vet Rec, 1996, 139(4): 93—94
7 Zhang YH, Pan DK, Sun XZ, et al. *In vitro* developmental competence of pig nuclear transferred embryos: effects of GFP transfection, refrigeration, cell cycle synchronization and shapes of donor cells. Zygote, 2006, 14(3): 239—247
8 Zhang Y, Pan D, Sun X, et al. Production of porcine cloned transgenic embryos expressing green fluorescent protein by somatic cell nuclear transfer. Science in China (C), 2006, 49(2): 164—171
9 Krisher RL and Bavister BD. Responses of oocytes and embryos to the culture environment. Theriogenology, 1998, 49(1): 103—114
10 Li R, Lai L, Wax D, et al. Cloned transgenic swine via *in vitro* production and cryopreservation. Biol Reprod, 2006, 75(2): 226—230
11 Peltoniemi OA, Love RJ, Heinonen M, et al. Seasonal and management effects on fertility of the sow: a descriptive study. Anim Reprod Sci, 1999, 55(1): 47—61
12 Love RJ, Evans G and Klupiec C. Seasonal effects on fertility in gilts and sows. J Reprod Fertil Suppl, 1993, 48: 191—206
13 Al-Katanani YM, Rivera RM and Hansen PJ. Seasonal variation in development of in vitro produced bovine embryos. Vet Rec, 2002, 150(15): 486—487
14 Silva CC, Rosa HJD and Knight PG. Seasonal variations in the developmental competence of bovine oocytes matured *in vitro*. Vet Rec, 2006, 158(14): 473