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—Original—

Effects of Ovary Storage Time and Temperature on DNA Fragmentation and Development of Porcine Oocytes

Pimprapar WONGSRIKEAO[1)], Takeshige OTOI[1)], Ni Wayan Kurniani KARJA[1)], Budiyanto AGUNG[1)], Masahiro NII[2)] and Takashi NAGAI[3)]

[1)]Laboratory of Animal Reproduction, The United Graduate School of Veterinary Science, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, [2)]Tokushima Agricultural Institute for Agriculture, Forestry and Fisheries, Anan, Tokushima 774–0047, [3)]Department of Research Planning and Coordination, National Institute of Livestock and Grassland Science, Tsukuba, Ibaraki 305–0901, Japan

Abstract. The present study was conducted to investigate the effects of storage time and temperature of porcine ovaries on the quality and nuclear maturation *in vitro* of oocytes obtained from stored ovaries and their subsequent development after *in vitro* fertilization. The ovaries were stored in physiological saline for 0, 3, 6, 9 and 12 h at various temperatures (4, 15, 25 and 35 C). The pH of follicular fluid obtained from the ovaries, DNA fragmentation of the oocyte nucleus and meiotic competence of oocytes were examined. Some oocytes from ovaries stored at 15, 25 and 35 C for 6 h were fertilized *in vitro*, and then cultured for 7 days to examine the ability of embryos to develop to the blastocyst stage. When the ovaries were stored at 35 C, the pH of follicular fluid decreased and the proportions of oocytes with DNA fragmented nuclei increased as the storage time was prolonged, and the storage of ovaries for 6, 9 and 12 h resulted in lower maturation rates of oocytes. When the ovaries were stored at 4, 15, 25 and 35 C for 6 h, the storage at higher temperatures (≥15 C) decreased the pH of follicular fluid and induced nucleic DNA fragmentation in higher proportions of oocytes. None of the oocytes from ovaries stored at 4 C reached metaphase II. The storage of ovaries at 15 C reduced the rates of *in vitro* fertilized oocytes and subsequent embryo development, but there were no significant differences in the rates of fertilization and blastocyst formation between oocytes from ovaries stored at 25 C and 35 C. Our findings indicate that the storage of ovaries at 25–35 C for 6 h is effective for maintaining the developmental competence of porcine oocytes even though the development rates were lower than those of ovaries stored at 35 C for 3 h.

Key words: Ovary storage, *In vitro* culture, Porcine oocyte

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In studies using oocytes from slaughtered animals, a long transport time from the slaughterhouse to the laboratory before culture may adversely affect oocyte quality in terms of nuclear maturation and developmental competence after *in vitro* maturation (IVM) and fertilization (IVF) [1–7]. In cattle, ovary storage at 37 C for 8 h significantly decreased both rates of cleavage and blastocyst formation after *in vitro* culture (IVC) of IVM/IVF oocytes [2], and a similar phenomenon was seen when the ovaries were stored at 4 C for 12 or 24 h [3]. However, it was demonstrated that the storage of bovine ovaries at 15–21 C for 24 h did not affect the rates of matured oocytes, and subsequent cleavage and blastocyst formation after IVF in cattle [4]. In horses, ovaries can be stored for 6–8 h at a

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Correspondence: T. Otoi (e-mail: otoi@yamaguchi-u.ac.jp)

temperature of 27 C to 37 C during transportation without remarkable effects on the nuclear maturation and cytoplasmic membrane integrity of oocytes [6]. In sheep, when ovaries were stored at temperatures of 4, 22 or 37 C, the maturation rates of oocytes decreased with increasing storage time after slaughter, but the oocytes from ovaries stored at 22 C showed a higher maturation rate than oocytes stored at the other temperatures [5]. In pigs, however, no information is available on the effects of long-term storage of ovaries with respect to the quality of oocytes and their subsequent *in vitro* development after IVM/IVF.

During transportation of ovaries to the laboratory, the occlusion of blood flow reduces the supply of oxygen and energy to the ovaries and places them under ischemic and re-oxygenation conditions. The oxygen deprivation of tissue results in a switch from aerobic to anaerobic metabolism, and the main product, lactic acid by anaerobic metabolism, is accumulated within the cell. The ATP is broken down without being re-synthesized, and inorganic phosphate, which is a byproduct of the breakdown of ATP, interacts with water to become phosphoric acid. Then, accumulation of lactic and phosphoric acids causes a decrease in pH [8]. Moreover, reoxygenation is found to generate toxic free oxygen radicals [9] such as superoxide anion (O^-), toxic hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2), which react with proteins, lipids and DNA, resulting in inactivation of enzyme, membrane lipid peroxidation and DNA alteration [10, 11]. Webster *et al.* [12] reported that the contributions of acidosis, reoxygenation, and reperfusion under ischemic conditions strongly stimulate cells to undergo programmed cell death.

The objective of this study was to investigate the effects of storage of porcine ovaries for different periods at different temperatures on the quality and nuclear maturation of oocytes obtained from stored ovaries and their development after IVM and IVF.

Materials and Methods

Recovery and maturation culture of oocytes

Ovaries were retrieved from prepubertal crossbred gilts at a local slaughterhouse and transported to the laboratory and stored in physiological saline (0.85% [w/v] NaCl). Cumulus oocyte complexes (COCs) from follicles (3 to 6 mm in diameter) were aspirated using an 18-guage needle attached to a 5-ml syringe. After being washed twice with modified phosphate-buffered saline (Embryoteck; Nihonzenyaku, Fukushima, Japan), only oocytes with uniform ooplasm and compact cumulus cells were used. The selected COCs were transferred into maturation medium, a modified North Carolina State University (NCSU)-37 solution [13] supplemented with 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma, St. Louis, MO, USA), 10 IU/ml equine chorionic gonadotropin (eCG; Teikokuzoki, Tokyo, Japan), 10 IU/ml human chorionic gonadrotropin (hCG; Teikokuzoki, Tokyo, Japan), 50 μg/ml gentamicin (Sigma) and 10% (v/v) porcine follicular fluid (pFF). About 50 COCs were cultured, each in 500 μl of the maturation medium under a layer of mineral oil (Sigma) for 22 h. They were subsequently cultured in the maturation medium without hormones and dbcAMP for an additional 22 h. All cultures for IVM, IVF of oocytes and IVC of embryos were performed in a 38.5 C humidified incubator containing 5% CO_2 in air.

Preparation of porcine spermatozoa for IVF

The sperm-rich fractions of ejaculates were obtained from a Large White boar and frozen according to the method described by Kikuchi *et al.* [14] with minor modifications. Briefly, the suspension of spermatozoa in a 50-ml glass tube was placed in a refrigerator at 15 C for 3 h and was then centrifuged at 800 × g for 10 min. Precipitated spermatozoa were diluted with the first extender, designated NSF-1 extender, which consisted of 8.8% (w/v) lactose (Wako Pure Chemical, Osaka, Japan), 200 μg/ml ampicillin (Mitaka, Tokyo, Japan) and 20% (v/v) egg yolk in distilled water. The diluted spermatozoa were equilibrated in a water bath at 4 C for 2 h. After equilibration for 2 h, the second extender [NSF-II; NSF-I extender supplemented with 6% (v/v) glycerol and 1.48% (v/v) orvus ES paste (Miyazaki Kagaku, Tokyo, Japan)] was added. The spermatozoa were then equilibrated at 4 C for an additional 5 min. At the end of the equilibration period, the same volume of NSF-II extender was added at 4 C. The sperm concentration was adjusted to 2×10^8 cells/ml. The spermatozoa were immediately loaded into 0.25-ml

French straws (no. AAA201; I.M.V., l'Aigle, France), and frozen by placing the straw (4 cm in height from the surface of liquid nitrogen) on a styrofoam plate in liquid nitrogen vapor for 20 min, and subsequent storage in liquid nitrogen. On the day of insemination, the straw was placed in air for 5 sec, and submerged into a 30 C water bath for 30 sec for thawing.

In vitro fertilization and culture

In vitro fertilization was carried out according to the method described by Kikuchi *et al.* [15]. Frozen-thawed boar spermatozoa were diluted in tissue culture medium (TCM) 199 with Earle's salts (Gibco, Grand Island, NY, USA) adjusted to pH 7.8 and then centrifuged at 200 × g for 2 min. After centrifugation, the supernatant was removed and the sperm pellet was preincubated for 15 min at 38.5 C before fertilization. A portion (10 μl) of preincubated spermatozoa was introduced into a 90-μl drop of fertilization medium containing 10–20 matured COCs to give a concentration of 1×10^6/ml. The fertilization medium consisted of 90 mM NaCl, 12 mM KCL, 25 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate, 3 mg/ml bovine serum albumin (BSA; fatty acid free, Sigma), 5 mM caffeine (Sigma) and 50 μg/ml gentamicin. After co-incubation of oocytes with spermatozoa for 5 h, the inseminated oocytes were denuded from cumulus cells and attached spermatozoa by mechanical pipetting and then transferred to culture medium. Presumptive zygotes were cultured in NCSU-37 supplemented with 4 mg/ml BSA, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate and 50 μg/ml gentamicin. At 72 h after insemination, all cleaved embryos were transferred into fresh culture medium: NCSU-37 supplemented with 4 mg/ml BSA, 5.55 mM D-glucose, and 50 μg/ml gentamicin. The cleaved embryos were cultured for an additional 4 days to evaluate their ability to develop to the morula and blastocyst stages.

Analysis of pH of porcine follicular fluid

Porcine follicular fluid (pFF) was aspirated immediately after storage of ovaries at each time and temperature. About 2 ml of follicular fluid was placed in an Eppendorf tube and the pH was evaluated twice within 2 min after the collection, using a pH meter (Hanna instrument, Ann Arbor, MI, USA). The pH record per sample was the mean of the two measurements.

Assessment of nuclear status, fertilization and embryo development

At the end of IVM culture, oocytes were mechanically denuded from cumulus cells in Dulbecco's phosphate-buffered saline (PBS; Gibco) supplemented with 1 mg/ml hyaluronidase (Sigma). They were then fixed and permeabilized for 15 min at room temperature in PBS supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma), and then placed in PBS containing 0.3% (w/v) polyvinylpyrrolidone for 15 min at room temperature. The oocytes were transferred into a small drop comprising PBS supplemented with 90% glycerol (v/v) (Wako Pure Chemical) and 1 μg/ml bis-benzimide (Hoechst 33342; Sigma) on a slide. Subsequently, the oocytes were overlaid with a cover slip supported by 4 small droplets of vaseline/paraffin and incubated overnight at 4 C. The oocytes were examined using a fluorescence microscope with a 320 nm wavelength excitation filter and classified as being at the germinal vesicle (GV), condensed chromatin (CC), metaphase I (MI), anaphase I/telophase I (AT) or metaphase II (MII) stages. Oocytes showing an abnormal chromatin configuration or no chromatin at all after staining were considered as being degenerated.

At 16 h after IVF, the presumptive zygotes were mounted on a slide, and fixed with acetic acid: ethanol (1: 3 v/v) for 48–72 h. The fixed zygotes were stained with acetic-orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope. Oocytes containing both female and male pronuclei were considered as fertilized and categorized as normal or polyspermic fertilization, according to the number of swollen sperm heads and pronuclei in the cytoplasm. For examination of the embryo development, on Day 7 (Day 0= insemination) all embryos were fixed and stained with Hoechst 33342 as described above. An embryo with a clear blastocoele and more than 20 cells was defined as a blastocyst. The numbers of cleaved embryos, blastocysts and their cell numbers were recorded.

Analysis of DNA damage of oocytes

At the onset of IVM culture the oocytes were denuded of cumulus cells and analysed for DNA damage using a combined technique for

simultaneous nuclear staining and terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL) by a modification of the procedures previously described by Otoi *et al.* [16]. Briefly, the denuded oocytes were washed four times in PBS containing 3 mg/ml polyvinylalcohol (PBS-PVA), and fixed overnight at 4 C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, the oocytes were washed four times in PBS-PVA, permeabilized in PBS containing 0.1% (v/v) Triton-X100 for 1 h, and incubated in PBS containing 10 mg/ml BSA (blocking solution) overnight at 4 C. They were then washed four times in PBS-PVA and incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; Roche Diagnostics, Tokyo, Japan) for 1 h at 38.5 C under 5% CO_2 in air. Positive controls (one or two oocytes per TUNEL analysis) were incubated in 1000 IU/ml deoxyribonuclease I (DNase; Sigma) for 30 min at 38.5 C under 5% CO_2 in air, and washed twice in PBS-PVA before TUNEL staining. Negative controls (one or two oocytes per TUNEL analysis) were incubated in fluorescein-dUTP in the absence of TdT. After TUNEL staining, the oocytes were washed three times in PBS-PVA and counterstained with 25 μg/ml bis-benzimide (Hoechst 33342; Sigma) for 30 min. They were then washed in blocking solution, treated with an anti-bleaching solution (Slow-Fade; Molecular Probes, Eugene, OR, USA), mounted on a glass slide, and sealed with clear nail polish. Labeled oocytes were examined using a Nikon Diaphot microscope fitted with epifluorescent illumination. Two standard filter sets were used, a filter with an excitation wave length of 450–490 nm and barrier filter of 520 nm was used to detect fluorescein isothiocyanate (FITC) alone. A filter with an excitation wavelength of 330–380 nm and barrier filter of 420 nm was used to detect the nuclear status of oocytes stained by Hoechst 33342. To assess DNA damage of oocytes from ovaries stored at each time and temperature, the number of nuclei labeled by TUNEL were counted.

Experiment 1:

We evaluated the effects of ovary storage time on the pH of pFF, oocyte nuclear damage, and meiotic competence of oocytes from ovaries stored at 35 C. Ovaries were randomly assigned to 5 groups: ovaries stored for 0 h, 3 h, 6 h, 9 h and 12 h. In the 0 h group (control group), pFF containing COCs was aspirated immediately after ovary collection. The pH of pFF was evaluated immediately after aspiration of follicular fluid. The COCs were selected and cultured in maturation medium at 38.5 C in a portable incubator (Fujihira Tokyo, Japan) during transportation from the slaughterhouse to the laboratory. After transportation, the COCs were transferred into fresh maturation medium and cultured as described above. In other treatment groups, the pFF and COCs were collected from ovaries stored for 3 h, 6 h, 9 h, and 12 h, and then COCs were matured *in vitro* for 44 h as described above. Oocytes in each group were assessed for the nuclear status and DNA fragmentation.

Experiment 2:

In Experiment 1, we found that the meiotic competence and DNA of oocyte nuclei were impaired by the storage of ovaries for 6–12 h. To improve this situation we investigated the effects of ovary storage temperature, another important factor determining the viability of oocytes, on the pH of pFF, oocyte nuclear damage, and meiotic competence of oocytes. Ovaries were randomly stored at 4, 15, 25 and 35 C. After storage of 6 h at each temperature, the pFF containing COCs were collected from ovarian follicles. The pH of pFF was evaluated. Oocytes in each group were assessed for nuclear status and DNA fragmentation. Some COCs from ovaries stored at 35 C for 3 h were used as a control group.

Experiment 3:

In Experiment 2, we found that none of the oocytes reached MII after maturation culture when the ovaries were stored at 4 and 15 C for 6 h. COCs from ovaries stored at 15, 25 and 35 C for 6 h were used in Experiment 3. We evaluated the effects of ovary storage temperature on the IVF and developmental competence of oocytes obtained from ovaries stored for 6 h.

The COCs obtained from ovaries stored at 15, 25 and 35 C for 6 h were matured for 44 h and then subjected to IVF and *in vitro* culture (IVC), as described above. Some COCs obtained from ovaries stored at 35 C for 3 h were used as a control group.

Statistical analysis

Data are expressed as means ± SEMs. The percentages of oocytes reaching each stage of

meiosis, oocytes fertilized, embryos developed after IVF, and oocytes with DNA fragmented nucleus were subjected to arc-sin transformation before analysis of variance (ANOVA). The transformed data, mean of pH and total cell number of blastocysts were tested by ANOVA followed by a post hoc, Fisher's protected least significant difference test (PLSD test) using the Statview program (Abacus Concepts, Inc, Berkeley, CA). Differences with a probability value (P) of 0.05 or less were considered to be significant.

Result

Experiment 1

As shown in Fig. 1A, the pH of pFF from non-stored ovaries (0 h group) was 7.53 and significantly decreased even when ovaries were stored for 3 h (pH 7.28, P<0.05). Moreover, the pH of pFF from ovaries stored for more than 6 h significantly decreased, as compared with that from ovaries stored for less than 3 h. However, there were no significant differences in the pH of pFF among the treatments with storage of ovaries for more than 6 h: pH 7.03, 6.93 and 6.95 in pFF from ovaries stored for 6 h, 9 h and 12 h, respectively.

A total of 405 oocytes were examined for DNA fragmentation of the nucleus after various storage times of ovaries (Fig. 1B). There was no significant difference in the percentages of oocytes with DNA fragmented nuclei between storage of ovaries for 0 h and 3 h. However, when the ovaries were stored for more than 6 h, the percentages of DNA fragmentation significantly increased with increasing storage time (P<0.05).

After maturation culture there were no significant differences in the percentages of oocytes undergoing germinal vesicle breakdown (GVBD) and maturing to MII between oocytes obtained from ovaries stored for 0 h and 3 h (Table 1). However, the percentages of GVBD and MII in oocytes from ovaries stored for 6, 9 and 12 h significantly decreased, as compared with those from ovaries stored for 3 h or no storage. The percentages of degenerated oocytes increased with increasing storage time.

Experiment 2

The pH of pFF from ovaries stored at 4 C was

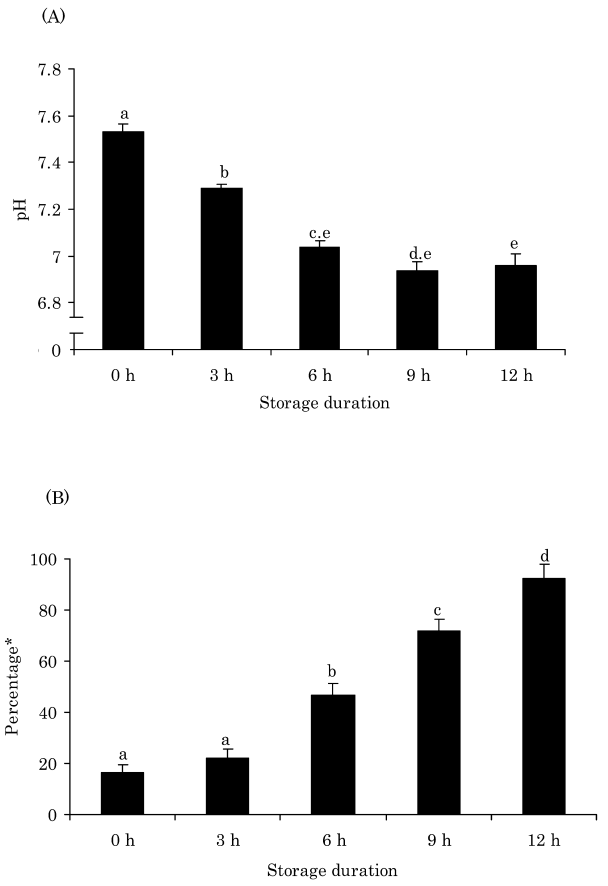

Fig. 1. Effects of ovary storage time on the pH of follicular fluids (A) and percentage of oocytes having a nucleus with DNA fragmentation (percentage within the number of the oocytes examined) (B) from ovaries stored at 35 C. A) The evaluation of pH of follicular fluids was replicated eight times. B) The experiment for DNA fragmentation was conducted at least four times using oocytes from ovaries stored for 0 h (n=74), 3 h (n=99), 6 h (n=77), 9 h (n=75) and 12 h (n=80). The results are expressed as mean ± SEM. Values with different superscripts are significantly different (*P*<0.05). * Percentages were calculated by dividing the number of oocytes with DNA fragmented nuclei by the total number of oocytes examined.

significantly higher (P<0.05) than that of the other groups (P<0.05), but similar to that in the control group (Fig. 2A). There were no significant differences in pH among the 15 C, 25 C and 35 C storage groups.

A total of 430 oocytes were examined for DNA fragmented nuclei after storage at various temperatures (Fig. 2B). There was no significant difference in the percentages of oocytes with DNA

Table 1. Nuclear status of *in vitro* matured porcine oocytes from ovaries stored for various time at 35 C

Storage time (h)	No. of trials	No. of oocytes examined	No. (mean ± SEM) of oocytes		No. (mean ± SEM) of degenerated oocytes
			GVBD	MII	
0	5	101	96 (95.1 ± 2.4)[a]	74 (72.2 ± 1.1)[a]	1 (0.9 ± 0.9)[a]
3	5	102	95 (94.4 ± 2.8)[a]	74 (72.4 ± 3.1)[a]	1 (0.6 ± 0.6)[a]
6	5	107	90 (84.8 ± 3.5)[a,b]	48 (44.6 ± 1.3)[b]	6 (5.4 ± 2.6)[a,b]
9	5	100	75 (75.0 ± 2.5)[b]	35 (35.3 ± 1.5)[c]	10 (9.6 ± 3.4)[b]
12	5	106	62 (57.2 ± 5.5)[c]	20 (19.1 ± 3.0)[d]	25 (24.4 ± 2.8)[c]

GVBD, germinal vesicle breakdown; MII, metaphase II.
[a-d] Values with different superscripts in the same column are significantly different ($P<0.05$).

fragmentation between the 4 C storage group and the control group. The percentage of oocytes with DNA fragmentation gradually increased with increasing storage temperatures.

The percentages of oocytes undergoing GVBD and maturing to MII in oocytes obtained from ovaries stored for 6 h at 4 and 15 C were significantly lower (P<0.05) than those of other groups (Table 2). None of oocytes from ovaries stored at 4 C and 15 C reached MII after maturation culture. The percentage of oocytes maturing to MII in oocytes obtained from ovaries stored for 6 h was significantly lower (P<0.05) than that of the control group, irrespective of storage temperature.

Experiment 3

The percentages of total, monospermic and polyspermic fertilization were significantly lower (P<0.05) in the 15 C storage group than in the other storage groups (Table 3). More oocytes in the 15 C storage group degenerated after IVF, compared with the other groups. The fertilization rates were significantly lower (P<0.05) for oocytes obtained from ovaries stored for 6 h than for oocytes in the control group, irrespective of storage temperatures.

Similar results were observed in the percentages of cleaved embryos, blastocyst formation and mean cell number of blastocysts (Table 4). There were no

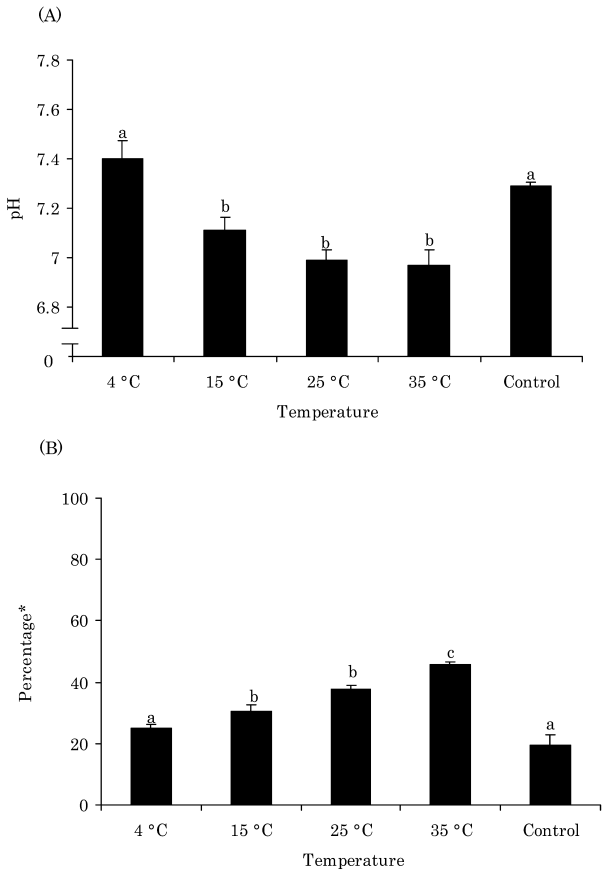

Fig. 2. Effects of ovary storage temperature on the pH of follicular fluids (A) and percentages of oocytes having a nucleus with DNA fragmentation (percentage within the number of the oocytes examined) (B) from ovaries stored for 6 h. A) The evaluation of pH of follicular fluids was replicated eight times. B) The experiment for DNA fragmentation was conducted five times using oocytes from ovaries stored at 4 C (n=84), 15 C (n= 82), 25 C (n=82), 35 C (n=79) and control (n=103) in which control oocytes were collected from ovaries stored at 35 C for 3 h. The results are expressed as mean ± SEM. Values with different superscripts are significantly different ($P<0.05$). *Percentages were calculated by dividing the number of oocytes with DNA fragmented nuclei by the total number of oocytes examined.

Table 2. Nuclear status of *in vitro* matured porcine oocytes from ovaries stored at various temperatures for 6 h

Storage temperature (C)	No. of trials	No. of oocytes examined	No. (mean ± SEM) of degenerated oocytes		No. (mean ± SEM) of matured oocytes
			GVBD	MII	
4	5	110	64 (58.1 ± 2.1)[a]	0 (0.0)[a]	24 (21.9 ± 0.5)[a]
15	5	105	64 (59.6 ± 5.2)[a]	0 (0.0)[a]	17 (16.3 ± 1.3)[b]
25	5	105	83 (78.8 ± 2.1)[b]	54 (52.1 ± 1.9)[b]	16 (16.2 ± 2.3)[b]
35	5	110	92 (83.6 ± 2.3)[b,c]	44 (40.1 ± 1.2)[c]	11 (10.0 ± 0.6)[c]
Control	5	92	85 (93.0 ± 3.1)[c]	67 (73.0 ± 3.9)[d]	1 (0.8 ± 0.8)[d]

GVBD, germinal vesicle breakdown; MII, metaphase II.
Control oocytes were collected from ovaries stored at 35 C for 3 h.
[a-d] Values with different superscripts in the same column are significantly different ($P<0.05$).

Table 3. Fertilization rates of *in vitro* matured porcine oocytes from ovaries stored at various temperatures for 6 h

Storage temperature (C)	No. of trials	No. of oocytes examined	No. (mean ± SEM) of oocytes reached MII	No. (mean ± SEM) of oocytes fertilized			No. (mean ± SEM) of degenerated oocytes
				Total	Monospermic	Polyspermic	
15	6	104	9 (8.6 ± 3.7)[a]	14 (13.8 ± 1.1)[a]	13 (12.2 ± 0.9)[a]	1 (1.5 ± 1.5)[a]	40 (36.8 ± 3.8)[a]
25	6	102	44 (40.3 ± 5.6)[b]	53 (51.2 ± 3.8)[b]	46 (45.0 ± 3.4)[b]	7 (7.6 ± 1.9)[b]	15 (15.8 ± 3.1)[b]
35	6	106	50 (46.8 ± 3.0)[b]	56 (52.5 ± 3.7)[b]	49 (46.0 ± 3.7)[b]	7 (6.6 ± 0.6)[b]	9 (8.9 ± 3.5)[b,c]
Control	6	96	64 (66.1 ± 1.3)[c]	70 (72.6 ± 1.2)[c]	57 (59.4 ± 2.5)[c]	13 (13.2 ± 1.6)[c]	1 (1.3 ± 1.3)[c]

Control oocytes were collected from ovaries stored at 35 C for 3 h.
[a-c] Values with different superscripts in the same column are significantly different ($P<0.05$).

Table 4. Developmental competence of *in vitro* matured porcine oocytes from ovaries stored at various temperatures for 6 h

Storage temperature (C)	No. of trials	No. of zygotes cultured	No. (mean ± SEM) of embryos		mean ± SEM of total cells number of blastocyst
			Cleaved	Developed to blastocysts	
15	6	308	30 (10.6 ± 1.2)[a]	0 (0.0)[a]	0.0[a]
25	6	471	247 (53.2 ± 3.3)[b]	42 (8.5 ± 1.2)[b]	32.5 ± 1.8[b]
35	6	473	272 (57.9 ± 0.6)[b]	48 (10.4 ± 1.2)[b]	32.2 ± 1.5[b]
Control	6	370	271 (72.0 ± 2.0)[c]	67 (18.1 ± 1.1)[c]	38.2 ± 1.7[c]

Control oocytes were collected from ovaries stored at 35 C for 3 h.
[a-c] Values with different superscripts in the same column are significantly different ($P<0.05$).

significant differences in the proportions of embryo development and the mean cell number between the 25 C and 35 C storage groups, whereas the 15 C storage group showed a significantly lower percentage of cleaved embryos than the other storage groups and no development to the blastocyst stage.

Discussion

Generally, ovaries collected at local slaughterhouses are the main source of oocytes for routine *in vitro* production of porcine embryos. Thus, ovaries must be transported from the slaughterhouses to laboratories. The storage of ovaries without blood supply may affect the oocyte quality by influencing the extracellular environment surrounding the oocytes. Our results show that when the ovaries were stored for 6–12 h,

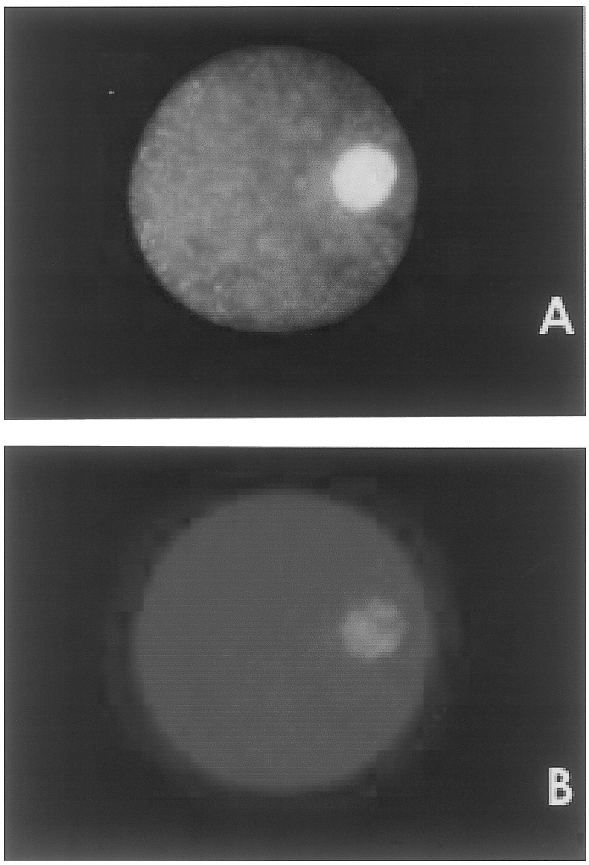

Fig. 3. Representative of GV stage of porcine oocytes with DNA fragmentation stained by A) terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL) and B) Hoechst 33342 (original magnification is × 100).

the pH of pFF from ovaries significantly decreased and the percentage of oocytes with DNA fragmented nuclei significantly increased. These results indicate that long-term storage may induce acidosis of follicular fluid by ischemia in the ovary, leading to DNA fragmentation of oocytes in follicles. The ATP breakdown and the shift from aerobic to anaerobic stage of the cell metabolism during the ischemic condition may induce the accumulation of acid byproducts such as lactic and phosphoric acids, which increase the accumulative number of H^+ [8]. The accumulated H^+ in the ovarian cell may be released into follicular fluid and induce acidosis in the environment surrounding oocytes. Dale *et al.* [17] suggested that the plasma membrane of the oocyte is highly permeable to H^+ ions and that no regulation of the concentration of H^+ occurs. Thus, when oocytes are placed in fluids with more acidic than the oocyte cytoplasm, the pH of oocytes might fall to the pH of the external medium leading to DNA fragmentation of oocytes. In experiments using slaughtered animals, the transport time between the slaughterhouse and laboratory and the handling time before culture can be long, and may adversely affect the oocytes quality in terms of nuclear maturation of oocytes, subsequent cleavage and blastocyst formation after IVF. Long-term storage (7–8 h) of ovaries under warm conditions (30–37 C) has been found to reduce the rate of blastocyst formation of oocytes after IVF in cattle [2, 18]. Moreover, it has been suggested that the loss of certain functions and degeneration of protein and DNA in post-mortem ovaries affected the viability of oocytes in horses [19] and in cats [20–21]. In the present study, the storage of ovaries for longer than 6 h at 35 C significantly decreased the meiotic competence of oocytes. These results indicate that alteration of follicular pH, occurrence of DNA fragmented nuclei and meiotic competence of the oocyte depends on storage time.

In the present study, 6-h storage of ovaries at 4 C did not alter the pH of follicular fluid, while the storage of ovaries in warmer temperatures showed decreases in pH. The percentage of DNA fragmentation of oocytes from ovaries stored at 4 C was similar to that of oocytes from ovaries stored at 35 C for 3 h, whereas significantly more oocytes with DNA fragmented nuclei were found in oocytes from ovaries stored at higher temperatures (≥15 C). Storage of ovaries at a low temperature may have delayed the accumulation of acid byproduct and the apoptotic processes, since the metabolism and enzymes present in warm-blooded animals work most efficiently at body temperature [8]. However, metabolism is not totally arrested in oocytes stored in an unfrozen state unlike storage at cryogenic temperatures. Thus, sustained exposure of ovaries to a low temperature would inevitably lead to ion concentration disequilibrium, resulting from metabolic arrest and temperature-dependent destabilization of membrane function [22]. It has been demonstrated that keeping horse ovaries at 30 C for more than 3 h or at room temperature for longer than 5 h increased the apoptosis of granulosa cells [19], while feline ovaries stored at 4 C did not show any increase in apoptosis of granulosa cells until 12 h of storage [21]. In the present study, a lower number of oocytes with

DNA fragmented nuclei were found when the ovaries were stored at a low temperature (4 C), coupled with a smaller decrease in pH of follicular fluid. Our results indicate that the storage of ovaries at a low temperature delayed the onset of DNA fragmentation, but storage at a higher temperature increased proportions of oocytes with DNA fragmented nuclei by alteration of follicular pH.

The effect of cooling oocytes varies in different species. Bovine oocytes from ovaries held at 25 C have more developmental competence than oocytes from ovaries held at 37 C [2]. Additionally, ovaries stored between 15–25 C or 24–25 C for more than 12 h did not decrease their capacity of fertilization and development to blastocysts in bovine oocytes [2, 4]. Cat oocytes recovered from ovaries stored at 4 C for 24 h are capable of maintaining the competence of IVM and development after IVF [20, 23]. The storage of horse ovaries at 25 C and 35 C for 5–8 h and 3–15 h, respectively, had no effects on the meiotic competence of oocytes [6, 24, 25], but a lower temperature (4 C) was detrimental as compared with room temperature [26]. Also, sheep oocytes were sensitive to cooling to 20 C at various stages of meiosis of oocytes, resulting in the induction of chromosomal abnormalities [27]. In the present study, the storage of ovaries for longer than 3 h impaired the oocyte quality in terms of maturation and subsequent development after IVF. Nevertheless, in the case of long-term storage, oocytes obtained from ovaries stored at 25 C and 35 C were able to maintain the ability to mature to MII and to develop to the blastocyst stage. Both of the rates, however, were lower than those of control oocytes from ovaries stored at 35 C for 3 h. It has been demonstrated that exposure of cumulus-intact GV oocytes to temperatures at or below 15 C adversely affects the viability of porcine oocyte [28, 29]. In the present study, similarly, we observed that few oocytes from the ovaries stored at lower than 15 C reached MII at the end of maturation culture. Guignot *et al.* [6] reported that the period and temperature of ovary storage affected the quality of oocytes, in which cytoplasmic membrane [30, 31], microtubule [32], cytoskeleton [33] and zona pellucida [34] may be sensitive to low temperatures. It has been known that porcine oocytes are highly sensitive to chilling, resulting in a loss of membrane integrity when cooled below 16 C [35]. In bovine oocytes, the membrane lipid of oocytes at the GV stage have a transition phase at temperatures between 13 C and 20 C and the plasma membrane of oocytes is very sensitive to cooling at or below 20 C [36]. At the ambient temperature, the membrane lipids undergo phase transition from a more liquid crystal phase to a more rigid gel phase, which results in a hardening of the zona pellucida [36]. The transition of membrane lipids is associated with the disruption of microtubules of the meiotic spindle and causes the loss of the ability of oocytes to be fertilized [37, 38]. In the present study, porcine oocytes from ovaries stored at 15 C had significantly decreased rates of fertilization, cleavage and development to blastocysts. In addition, more oocytes from ovaries stored at 15 C degenerated after IVF. Therefore, the low efficiency in the fertilization and subsequent development of oocytes from ovaries stored at 15 C might result, in part, from disruption of the meiotic spindle, loss of membrane integrity and oocyte zona hardening. The storage of ovaries at lower temperatures (≤ 15 C) irreversibly has a detrimental effect on the meiotic and developmental competence of oocytes. There was no significant difference in the rates of fertilization and embryo development after IVF and the mean cell number of blastocyst between oocytes from ovaries stored at 25 C and 35 C. These results indicate that the storage of porcine ovaries at from 25 C to 35 C can maintain the developmental competence of collected oocytes even when the ovaries are stored for 6 h.

In summary, the present study shows that (i) high storage temperature and long- term storage or transportation of porcine ovaries increases the number of oocytes with DNA fragmented nuclei by inducing acidosis in follicular fluids; (ii) a cool storage temperature can maintain the pH of follicular fluid and decrease the occurrence of DNA fragmentation in oocytes but has a detrimental effect on oocyte viability, maturation, fertilization and subsequent development after IVF; (iii) storage of ovaries for longer than 3 h significantly impairs the oocyte quality, but in the case of long-term storage needed for long distance transportation of ovaries, porcine ovaries can be stored between 25 C to 35 C for 6 h with oocytes maintaining competence of *in vitro* maturation and subsequent development after IVF, even though the rates are lower than those of ovaries stored for shorter than 3 h.

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