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Effects of Preservation Media on *In Vitro* Maturation of Porcine Oocytes

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Abstract

The effects of preservation media for ovaries on *in vitro* maturation of porcine oocytes was studied. The cumulus-oocyte complexes (COCs) obtained from ovaries that had been preserved in three different media at various temperatures for different time intervals were cultured in the M199 maturation medium. The preservation media used were 0.9% saline solution, BCS (Braun-Collins solution) and Dulbecco's phosphate buffered saline solution (PBS). Mature oocytes obtained from the ovaries preserved in three preservation media for 8 h were electrically activated. The activated oocytes were then cultured in the NCSU23 embryo culture medium for 16 h to observe activation, or for 144 h to observe embryo development. It was found that the preservation temperature significantly affected maturation of the porcine oocytes. A preservation temperature of about 25°C showed an optimal maturation rate for a preservation time of 8 h for the three preservation media. Although the preservation temperature was a major factor influencing the maturation rate, different preservation media at 25°C for 8 h also significantly affected the maturation rate, activation rate and embryo development. Among these three preservation media, PBS exhibited the highest cleavage rate indicating that PBS should be a better preservation medium for porcine ovaries at 25°C for 8 h or longer periods.

Key Words: porcine oocyte, *in vitro* maturation, preservation medium

Introduction

Mammalian ovaries obtained from slaughterhouses have been used in assisted reproduction and experimental approaches of nuclear transfer (14). One practical problem often encountered is the effect of transportation and preservation (23). Improper transporting and preserving conditions might cause failure in oocyte maturation. Various treatments including preserving temperature (8, 14, 19, 23), preserving methods (11) and preserving media (3-5) have been described in the literature. The effects of preservation conditions on maturation of oocytes were found to depend on the animal species.

Porcine oocytes are more sensitive to temperature than those of other live-stocks such as cattle and goat. Thus, studies on preservation of porcine ovaries may help assisted reproduction of pigs. Some studies have indicated that successful *in vitro* fertilization and development of *in vitro* matured porcine oocytes from ovaries that have been preserved for 1 h could be obtained (23). Pigs are often slaughtered at midnight in conventional slaughterhouses in Taiwan. If porcine ovaries can be preserved for more than 8 h, it will be more convenient for the operators to do the routine treatments in normal working hours. In this study, porcine ovaries obtained from a local slaughterhouse were preserved for various time intervals at

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various temperatures in three different preservation media. The effects of the preservation media on the maturation of porcine oocytes cultured in a maturation medium were studied, and effects on activation and embryo development of the matured oocytes were investigated.

Materials and Methods

Preparation of Supplemented Maturation Culture Medium

The M199 maturation culture medium (Gibco Cat. No. 21200-076, Paisley, UK) was prepared as described by Wang *et al*. (21). In the culturing experiments, the M199 medium was supplemented with 0.5 μg/ml estradiol (E2; Sigma Cat. No. E-2257, St. Louis, MO, USA), 2.5 μg/ml follicle-stimulating hormone (FSH; Sigma Cat. No. F-2293), 5 IU/ml luteinizing hormone (LH; Sigma Cat. No. L-9773), 20 ng/ml luteotropic hormone (LTH; Sigma Cat. No. L-6520) and 20% porcine follicular fluid (pFF). The medium was filtered with a 0.2 μm filtration membrane (Minisart®, Sartorius, St. Louis, MO, USA) and was stored in the refrigerator at 4°C.

Collection of Cumulus-Oocyte Complexes (COCs) and In Vitro *Maturation Culture*

Porcine ovaries from gilts (about 5 months and around 100 kg) were collected from a local slaughterhouse and were transported to the laboratory within 30 min in different preservation media at 35°C-37°C. The preservation media used were 0.9% saline solution, Dulbecco's phosphate buffered saline solution (PBS, Gibco Cat. No. 450-1300) (20) and BCS (Braun-Collins solution) (3). Some ovaries were preserved in different preservation media at various temperatures (4, 18, 25 and 37°C) for various time intervals. Some ovaries were directly used for culture as the control. The ovaries were placed in a dish (6 cm diameter) (Cat. No. 353001, Bacton Dickinson Labware Franklin Lakes, NJ, USA) supplemented with PBS. COCs were obtained by cutting off the antral follicles (3-5 mm in diameter) with a dissection blade at room temperature. COCs surrounded with more than three layers of intact cumulus cells were selected and collected, and washed three times with PBS, then washed once with the supplemented maturation medium. Three drops of COCs (about 10 COCs in each 50 μl microdrop) were cultured in the complemented maturation culture medium which was covered with 2 ml mineral oil (Sigma Cat. No. M-8410) and incubated in a 95-100% humidified atmosphere of 5% CO_2 for 48 h.

Staining of Cumulus Cells after Preserving for 8 h

Cumulus cells obtained from the ovaries preserved in the three different media at 25°C for 8 h were stained with 0.4% Trypan blue (Gibco Cat. No. 15250-061) (24). The cumulus cells were then observed on a phase-contrast microscope (Olympus BX50, Tokyo, Japan). Live cumulus cells which were free from staining remained clear, and dead cumulus cells were stained deep blue to dark blue. The fraction of the cumulus cells that remained alive was assessed.

Activation of Matured Oocytes

An activation procedure described by Prather *et al*. (15) was used. Cumulus cells were removed by vortexing the matured COCs (obtained from the ovaries preserved at different temperatures for 8 h) in PBS containing 0.1% (w/v) hyaluronidase (Sigma Cat. No. H-4272). The matured oocytes were placed in an electric activation medium (0.3 M mannitol, Sigma Cat. No. M-9546; 0.1 mM $CaCl_2$, Sigma Cat. No. C-7902; and 0.1 mM $MgSO_4$, Sigma Cat. No. M-7774) and stood for 5 min before they were transferred to an electric activation chamber with two electrodes separated by 0.5 mm (No. 450; BTX Inc., San Diego, CA, USA). Activation was carried out at 5 V AC for 10 s and DC pulses of 1.2 kV/cm for 30 μs (7).

In Vitro *Culture of Oocytes*

Oocytes after activation were washed three times with the NCSU23 embryo culture medium (13). Then, 30 oocytes in 150 μl of the NCSU23 embryo culture medium covered with 2 ml mineral oil were cultured in a 95-100% humidified atmosphere of 5% CO_2 at 39°C for 16 h to observe activation, or 144 h to observe embryo development.

Characterizations
Fixing and Staining

Oocytes or embryos were mounted on glass slides and covered with a coverslip supported by droplets of a paraffin-petroleum jelly mixture (1:1, v/v). Then, the oocytes or embryos were fixed with a fixative of anhydrous alcohol/glacial acetic acid (1:3, v/v) for 48 h and stained with 1% lacmoid (Sigma Cat. No. L-7512) according to the method described by Abeydeera *et al*. (1). Maturation, activation and embryo developmental competence of the oocytes or embryos were examined on a phase-contrast microscope (Olympus BX50, Tokyo, Japan).

Evaluation of Oocyte Maturation

Oocytes in metaphase of meiosis II were con-

Table 1. Effects of preserving media at various temperatures for 8 h on *in vitro* maturation of porcine oocytes

Preservation media	Preservation temperature (°C)	No. of oocytes examined (Replicates)	GV (%)	Met I (%)	Met II (%) (Maturation rate)
Saline solution	Control	90 (3)	4.4 ± 1.8	8.9 ± 2.1	86.7 ± 0.5
	37°C	89 (3)	96.6 ± 0.2[a]	2.3 ± 2.0[c]	1.1 ± 1.9[c]
	25°C	90 (3)	16.6 ± 3.1[b]	17.8 ± 2.0[b]	65.6 ± 1.1[a]
	18°C	95 (3)	23.2 ± 1.9[b]	30.5 ± 0.7[a]	46.3 ± 2.4[b]
	4°C	89 (3)	97.7 ± 2.0[a]	1.2 ± 2.1[c]	1.1 ± 2.0[c]
BCS	Control	88 (3)	3.4 ± 0.2	9.0 ± 1.7	87.6 ± 1.5
	37°C	89 (3)	95.5 ± 1.9[a]	3.3 ± 0.1[c]	1.1 ± 1.9[c]
	25°C	84 (3)	11.9 ± 1.1[c]	16.7 ± 1.1[b]	71.5 ± 0.5[a]
	18°C	88 (3)	22.8 ± 2.5[b]	26.0 ± 4.3[a]	51.2 ± 2.1[b]
	4°C	92 (3)	96.7 ± 3.3[a]	1.1 ± 1.8[c]	2.2 ± 1.9[c]
PBS	Control	91 (3)	3.3 ± 0.3	8.8 ± 2.0	87.9 ± 2.2
	37°C	92 (3)	93.5 ± 0.3[a]	3.2 ± 0.1[c]	3.2 ± 0.1[c]
	25°C	91 (3)	10.9 ± 1.4[c]	14.3 ± 2.5[b]	72.6 ± 4.0[a]
	18°C	91 (3)	22.0 ± 1.7[b]	26.4 ± 3.4[a]	51.6 ± 1.7[b]
	4°C	95 (3)	94.8 ± 1.7[a]	2.0 ± 1.8[c]	3.1 ± 0.2[c]

Each value represents the means ± SEM.
Different superscripts (a, b, c) in the same column indicate significant differences ($P < 0.05$).

sidered to be matured. Oocytes exhibiting a germinal vesicle (GV) and metaphase I (Met I) of meiosis were termed as immature (9).

Evaluation of Activation of Oocytes

Activation of oocytes was examined according to the method described by Wang *et al*. (22). An oocyte was judged to be activated when pronuclear formation was observed.

Evaluation of Embryos

Determination of the development stage of embryos was examined according to the method described by Lee *et al*. (10). The embryos were placed on an inverted phase-contrast microscope to observe the number of blastomeres, and fixed and stained to confirm the nucleus number of the blastomere.

Statistical Analysis

There were at least three replicates for each treatment. Data of maturation, activation and embryo development of reconstructed oocytes were analyzed by ANOVA in the SAS* software. The data were expressed as means ± SEM. Differences of $P < 0.05$ were considered to be statistically significant.

Results

Porcine oocytes were cultured in M199 maturation medium for 48 h to evaluate maturation. The oocytes might remain in the germinal vesicle stage (GV), or progressed to Met I or metaphase II (Met II). Only those progressed to metaphase II stage were considered to be completely matured. Table 1 summarizes the effects of preservation temperature in three different preservation media for 8 h on the maturation of porcine oocytes. The preservation temperature affected the maturation rate significantly. A preservation temperature of 25°C gave the best maturation rate in all three media. Preservation media affected the maturation rate slightly but significantly.

The plots of the maturation rate of the porcine oocytes and the preservation time at various preservation temperatures in the three different preservation media are shown in Figs. 1-3, respectively. These kinetic plots are more informative about the preservation capability of the preservation media. A preservation temperature of 25°C seemed to be the optimal condition for the preservation for around 8 h.

The effects of the preservation media on the percentage of cumulus cells remained alive are shown in Table 2. The proportion of cumulus cells that remained alive seemed to be related directly to the maturation rate.

*SAS. User's Guide: Statistics. Release 8.02 Edition. SAS Institute Inc., Cary, NC. 1999.

Table 2. Effects of preservation media at 25°C for 8 h on the percentage of cumulus cells remaining alive

Group	No. of cumulus cells examined (Replicates)	Percentage of cumulus cells remained alive (%)
Saline solution	300 (3)	63.5 ± 2.1^{b}
BCS	300 (3)	72.7 ± 1.2^{a}
PBS	300 (3)	74.3 ± 2.1^{a}

Each value represents the means ± SEM.
Different superscripts (a, b) in the same column indicate significant differences ($P < 0.05$).

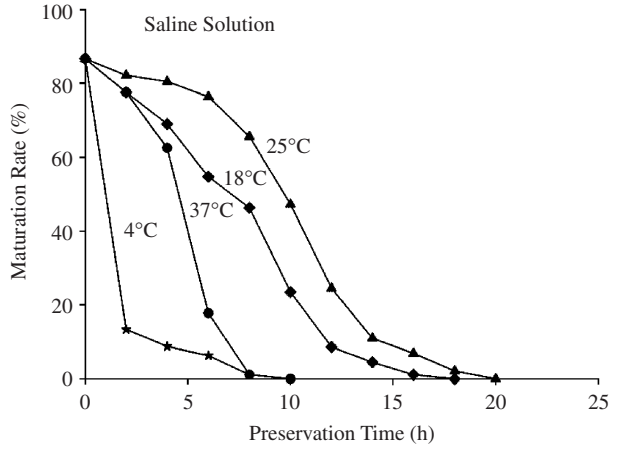

Fig. 1. Effects of preservation time in saline solution on the maturation rate of porcine oocytes at various preservation temperatures.

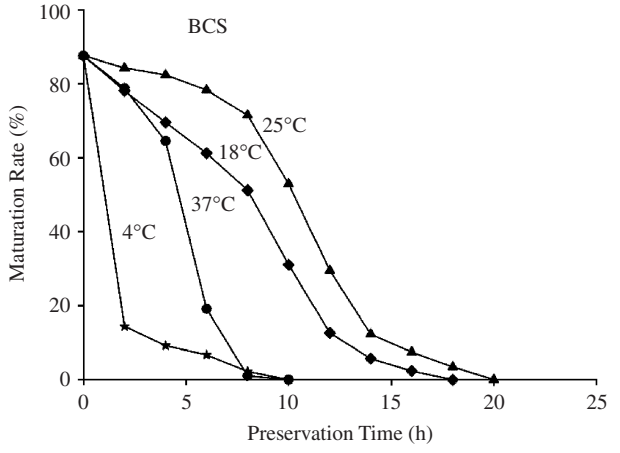

Fig. 2. Effects of preservation time in BCS on the maturation rate of porcine oocytes at various preservation temperatures.

The maturated oocytes of those preserved at various temperatures for 8 h were activated by 1.2 kV/cm electric pulses. After electrical activation, the oocytes were cultured in the NCSU23 embryo medium for 16 h to check for activation and pronuclear formation, or for 144 h to evaluate embryo development. The effects of different preservation media on the

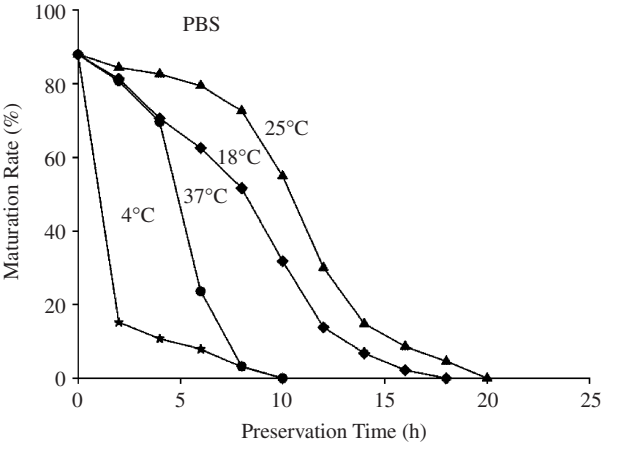

Fig. 3. Effects of preservation time in PBS on the maturation rate of porcine oocytes at various preservation temperatures.

activation and embryo development of the oocytes are summarized in Tables 3 and 4, respectively. The activation rates of the PBS and BCS groups are significantly higher than that of the saline group. The cleavage rate of the PBS group (53.6%) are significantly higher than that of the BCS group (48.6%) which was, in turn, significantly higher than that of the saline group. The trend for advanced development also followed the same order: PBS group > BCS group > saline group.

Discussion

Saline solution has been used as a preservation medium for ovary transport (2, 12, 16-18). Thus, 0.9% saline solution was first chosen in this study to serve as the medium to preserve porcine ovaries at 37°C for 8 h. However, the maturation rate of the porcine oocytes was very low (1.1%) after this method of preservation. Braun-Collins solution (BCS) (4) and Dulbecco's phosphate buffered saline (PBS) (8) were two other choices used in this study. The maturation rate of the porcine oocytes after preservation in BCS at 37°C for 8 h was also very low (1.1%), and that in PBS (3.2%) was slightly higher, but still very low.

The effects of preservation time at different

Table 3. Effects of preservation media at 25°C for 8 h on activation of oocytes

Preservation media	No. of oocytes examined (Replicates)	Activation rate (%)	1N (%)	1N2P (%)	≥ 2N (%)
Saline solution	126 (4)	56.9 ± 4.8^{b}	4.7 ± 3.5^{b}	28.3 ± 3.2^{b}	23.8 ± 2.9^{b}
BCS	116 (4)	71.0 ± 2.3^{a}	9.8 ± 1.2^{a}	31.3 ± 0.9^{b}	30.0 ± 2.6^{a}
PBS	102 (4)	73.0 ± 0.7^{a}	9.7 ± 3.4^{a}	41.8 ± 3.8^{a}	21.5 ± 1.2^{b}

Each value represents the means ± SEM.
Different superscripts (a, b) in the same column indicate significant differences ($P < 0.05$).

Table 4. Effects of preservation media at 25°C for 8 h on embryo development of the oocytes

Preservation media	No. of oocytes examined (Replicates)	Cleavage rate (%)	2 cells (%)	4 cells (%)	8 cells (%)	Morula	Blastocyst
Saline solution	108 (4)	38.9 ± 2.4^{c}	7.6 ± 5.0^{a}	25.0 ± 7.4^{a}	5.3 ± 3.5^{b}	0.9 ± 1.8^{c}	0.0 ± 0.0^{b}
BCS	107 (4)	48.6 ± 1.8^{b}	5.7 ± 2.5^{a}	22.4 ± 1.6^{a}	14.1 ± 2.1^{a}	4.6 ± 1.4^{b}	1.8 ± 2.1^{ab}
PBS	112 (4)	53.6 ± 1.6^{a}	4.4 ± 1.7^{a}	17.9 ± 3.9^{a}	14.4 ± 3.7^{a}	13.4 ± 1.4^{a}	3.5 ± 2.8^{a}

Each value represents the means ± SEM.
Different superscripts (a, b, c) in the same column indicate significant differences ($P < 0.05$).

temperatures in saline solution on the maturation rate are shown in Fig. 1. The maturation rate of the porcine oocytes decreased significantly as the preservation time was 4 h at 37°C, and dropped greatly as the preservation time was 6 h or longer. After leaving the body, porcine ovaries might suffer from autolysis (6). Thus, they could not sustain freshness during preservation around physiological temperature for a long period. In fact, some smell was found after preservation of 6 h or longer indicating the occurrence of degeneration. Thus, the maturation rate dropped for 6 h or a longer period of preservation at 37°C.

Degeneration of ovaries could be decreased by lowering the preservation temperature. However, porcine oocytes are very sensitive to temperature and frostbite is common. For example, it has been described that the *in vitro* maturation rate (Met II) of porcine oocytes dropped greatly to less than 31% as the ovaries were exposed to temperatures of 4-20°C for 1 h (23). As the preservation temperature in the saline solution in this work was 4°C, the maturation rate dropped to 13.4% for a preservation time of 2 h. In the case of a preservation temperature of 18°C, the maturation rate of the porcine oocytes was much better. The maturation rate dropped significantly as the preservation time was 10 h but the maturation rate after preserving in saline solution at 18°C for 8 h was low (46.3%). In the case of a preservation temperature of 25°C, the maturation rate was rather high at a period of up to 8 h as shown in Fig. 1. This preservation temperature was a compromise between suppressions of autolysis (6) and frostbite (11) due to low temperature and would be an optimal temperature for a preservation time of 8 h.

The effects of preservation time at different temperatures in BCS and PBS on the maturation rate are shown in Fig. 2 and Fig. 3, respectively. The trend is similar to that in the saline solution.

Although the preservation temperature was a major factor influencing the maturation rate, different preservation media (at 25°C for 8 h) also affected the maturation rate significantly as shown in Table 1. The maturation rates of PBS and BCS groups were significantly higher than that of the saline group. This might be due to the components other than salts in PBS and BCS as compared to the saline solution. The other factor might be the presence of bacteria. After culture, no significant bacterial colony was observed for the preservation system thus excluding bacterial contamination. Another factor might be the presence of some nutrition in PBS and BCS. The percentage of cumulus cells that remained alive as shown in Table 2 seemed to correlate well with the maturation rate (Table 1). It would be a reasonable deduction that the presence of the nutrients might have allowed more cumulus cells to survive after a long period of preservation.

Although the activation rates between the use of PBS and BCS (Table 3) showed no significant difference, the cleavage rate of the PBS group was significant higher than of the BCS group (Table 4). Moreover, the PBS group progressed to more advanced

development stage than the BCS group and saline group. This means that PBS should be as better preservation medium for porcine ovaries at 25°C for 8 h or longer.

In conclusions, the preservation temperature significantly affected the maturation of porcine oocytes. A preservation temperature of about 25°C showed optimal maturation rate for a preservation time of 8 h for the three preservation media tested. Although the preservation temperature was a major factor influencing maturation rate, different preservation media at 25°C for 8 h also evidently affected maturation rate, activation rate and embryo development. In particular, the cleavage rate and the trend for advanced development followed the order: PBS group > BCS group > saline group. This indicates that PBS should be a better preservation medium for porcine ovaries at 25°C for 8 h or longer.

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