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Effect of different transport temperatures on in vitro maturation of oocytes collected from frozen-thawed sheep ovaries

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Abstract: The aim of this study was to determine the effects of 2 different transport temperatures on the in vitro maturation of oocytes collected from frozen-thawed sheep ovaries. Sheep ovaries were transferred into saline at temperatures of 4 °C and 32 °C. After the 2 experimental groups (A: fresh cortex, B: frozen-thawed cortex) were formed, each group was divided into 2 subgroups (group A1: 4 °C, group A2: 32 °C [control]; group B1: 4 °C, group B2: 32 °C). The cortexes were dissected into slices 1-3 mm thick and pieces of 0.5 cm^2. For groups B1 and B2, 1-2 cortex pieces were placed in cryogenic vials containing 1 mL of freezing medium modified with Earle's salts (TCM-199) and supplemented with 10% fetal calf serum (FCS) (FCS + 2.5 M ethylene glycol + 0.1 M sucrose). The vials were then cooled to −7 °C at 2 °C/min and held at −7 °C for 10 min for manual seeding. The temperature was then lowered by −0.3 °C/min to −35 °C and thereafter by −10 °C/min to −75 °C. Vials were plunged into −196 °C liquid nitrogen and stored. Cortexes were thawed at 37 °C. Collected oocytes were matured in their own groups in 700 µL of TCM-199 (supplemented with luteinizing hormone, follicle-stimulating hormone, pyruvate, and FCS) for 23 h in a gas mixture of 5% CO_2, 5% O_2, and 90% N_2 at 38.8 °C. After maturation, oocytes were fixed in acetic acid and ethyl alcohol (1:3) for 48 h. Oocytes were stained with aceto-orcein and then examined. At the end of the study, maturation rates for reaching metaphase I (MI) were similar in all groups (group A1: 30.76%, group A2: 38.09%, group B1: 30.65%, and group B2: 33.33%). The rates at which metaphase II (MII) was reached were 18.58%, 34.69%, 7.25%, and 6.48%, respectively. The best development was seen in group A2 ($P < 0.001$). Sheep oocytes obtained from fresh and frozen-thawed cortexes reached the MII stage if transported at 4 °C.

Key words: Sheep, oocyte, maturation, ovary cortex, transport temperature

1. Introduction

In the past century, hundreds of animal species have vanished, and three-quarters of all mammals have become endangered species. Cryobiology and preservation studies are being carried out in many countries in order to preserve gene sources of endangered species (1,2). Present-day animal experiments have proven that the ovary cortex containing the primordial follicle can be frozen (3-7). In human studies, ovulation has been achieved by transplantation of the frozen ovary cortex (8-10). As a result of frozen ovary cortex transplantation, pregnancy has also been reported in mice, rats, and sheep (7,8,11,12). Ovaries contain a limited number of follicles, and the oocytes within these follicles are in the germinal vesicle breakdown stage (13-15). By freezing the ovary cortex, it is possible to store immature female gamete cells and create gene banks for endangered species (2,4,9,16). In this manner, the reproductive capacity of both humans and animals can be preserved. In human medicine, the risk of losing gamete cells due to chemotherapy or radiotherapy in connection with an illness will be eliminated (8,9,17).

Although ovary tissue has been successfully frozen, problems are still being encountered in the freezing stage for ovary tissue and in embryos of large animals such as cattle and sheep, as well as in humans (9,11,16,18,19). In addition, ovary transport is still problematic. There is a need for existing cryopreservation techniques to be developed further. Storing the ovaries for 5-8 h at average temperatures of 37-38 °C decreases the maturation of the oocytes and adversely affects their development to the blastocyst stage (20,21). In studies carried out in cattle, it has been reported that long-term storage of ovaries at

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20-25 °C and 24-h storage at 4 °C has no effect on the in vitro maturation of oocytes and their development to the blastocyst stage after in vitro fertilization (20-22). The metabolic activities of cells, both human and animal, are slowed down or completely arrested at low temperatures (15,23). Cryopreservation of ovarian tissue could present a means for enlarging the gene pool. Cryopreserved ovarian tissue could be used in auto- or xenografts or for in vitro maturation of primordial follicles (24).

Immediate transport of ovaries to the laboratory, as well as intercity and international transport, is still a problem for many research centers. Currently there is no study on the in vitro development of oocytes obtained from the fresh and frozen-thawed cortexes of sheep ovaries transported at different temperatures. The aim of this study was to investigate the effects of different transport temperatures (4 °C and 32 °C) on in vitro maturation of fresh and frozen-thawed oocytes obtained from the ovarian cortexes of sheep.

2. Materials and methods

Ovaries belonging to sheep that had reached puberty and were then slaughtered at an abattoir out of season (between March and June) were used in this study. The study was repeated 6 times (n = 6). Ovaries were transferred to thermoses containing 0.9% NaCl and transported to the laboratory within 2-4 h. Two main groups were formed: group A and group B. From each sheep, 1 ovary was put into a thermos containing 0.9% NaCl at 32 °C; after being held at room temperature for 10 min, the other ovary from the same animal was transferred to a thermos containing 0.9 % NaCl at 4 °C.

Two experimental groups, including a control group, were formed, and each group was divided into 2 subgroups.

Experiment A: Control groups

In the first experiment, oocytes were collected via dissection from ovaries brought to the laboratory after slaughter. Experimental group A was divided into 2 subgroups; group A1 at 4 °C (control) and group A2 at 32 °C (control). Dissection of the oocytes for group A1 was performed inside a cold cabinet at 4 °C. Dissection of the oocytes for group A2 was performed at 32 °C.

Oocytes in both groups were obtained from follicles with a diameter of 2-8 mm. The oocytes selected for maturation had 3-4 rows of cumulus oophorus cells surrounding them, their vitellus was homogenous, and zona were smooth. Selected oocytes were cleansed 3 times in washing medium (TCM-199 supplemented with 50 µg/mL gentamicin sulfate and 1 mM L-glutamine) and once in the in vitro maturation medium (IVM) (TCM-199 supplemented with 1 mM L-glutamine, 0.2 mM Na-pyruvate, 50 µg/mL gentamicin sulfate, and 24 IU/mL 10% fetal calf serum (FCS)). They were then matured for 23 h in 4-well dishes at 38.8 °C in a medium of 5% CO_2, 5% O_2, and 90% N_2. Following maturation, oocytes were cleansed of their cumulus oophorus cells using a mechanical and chemical method. Hyaluronidase was used (1.0 µg/mL). Oocytes were then fixed for 48 h in acetic acid and ethyl alcohol (1:3). After fixation, oocytes were stained with 2% aceto-orcein. Developmental stages of the oocytes up until metaphase II (MII) were assessed using a phase-contrast microscope.

Experiment B: Cortex dissection and freezing

For experiment B, 2 groups were formed: group B1 at 4 °C (frozen-thawed cortex) and group B2 at 32 °C (frozen-thawed cortex). Dissections of the ovaries were carried out inside a cold cabinet at 4 °C for group B1 and at 32 °C for group B2.

The ovaries were dissected after being washed in sterile saline. The ovary cortex was dissected to a thickness of 1-3 mm. The dissected sections were separated into pieces of 0.5 cm^2. It was ensured that follicles of 2-3 mm in diameter were present in the cortex pieces. Cortex pieces were then placed in cryotubes containing 1.0 mL of freezing medium (TCM-199 + 10% FCS + 2.5 M ethylene glycol + 0.1 M sucrose). A total of 1-2 cortex pieces were placed inside each tube. In group B1, the cryotubes containing freezing medium were cooled to 4 °C before the cortex pieces were placed inside. The tubes were then chilled to -7 °C at 2 °C/min. The cryotubes were left at -7 °C for 10 min for manual seeding. The temperature was then lowered from -7 °C to -35 °C at 2 °C/min, after which it was lowered at 10 °C/min to -75 °C. The cryotubes that reached -75 °C were dipped into -196 °C liquid nitrogen and stored.

2.1. Oocyte collection from the frozen and thawed cortex

Cryotubes containing frozen cortexes were kept in a 37 °C water bath for 30 s. Cortexes belonging to groups B1 and B2 were then placed in the following medium for 5 min: TCM-199 + 10% FCS + 1.5 M ethylene glycol + 0.1 M sucrose. The cortexes were kept in the cleansing medium (TCM-199) for 10 min and passaged in the same medium 3 times. Oocytes were obtained from the cortexes using the slicing method. Collected oocytes were selected for maturation. Each of the selected oocytes was surrounded by 3-4 rows of cumulus oophorus cells, and their vitellus was homogenous and smooth. The selected oocytes were cleansed 3 times in the cleansing medium (TCM-199 supplemented with 50 µg/mL gentamicin sulfate and 1 mM L-glutamine) and once in the IVM (TCM-199 supplemented with 1 mM L-glutamine, 0.2 mM Na-pyruvate, 50 µg/mL gentamicin sulfate, and 24 IU/mL 10% FCS). They were then matured for 23 h at 38.8 °C in 4-well dishes containing 700 µL of IVM in a medium of 5% CO_2, 5% O_2, and 90% N_2. At the end of maturation, oocytes were cleansed from their cumulus oophorus cells using a mechanical-chemical method. Hyaluronidase (1 µg/mL) was used. After maturation, oocytes were fixed in acetic acid and ethyl alcohol (1:3) for 48 h. The developmental

stages of the oocytes stained with aceto-orcein up until MII were then examined. The chi-square test was used for statistical analysis.

3. Results

In experiment A (control), for which sample were transported at different temperatures (4 °C and 32 °C) but not exposed to the freezing process, the majority of oocytes matured in groups A1, and A2 remained at the metaphase I (MI) stage (Table 1). While 48 (30.76%) of the 156 oocytes left for maturation in group A1 reached MI, only 29 (18.58%) of them were able to develop to the MII stage. In group A2, 56 (38.09%) of the 199 oocytes left for maturation reached MI, and 51 (34.69%) reached MII.

Although there was a quantitative difference between oocytes reaching MI in groups A1 and A2, no statistical difference was found. However, a statistically significant difference was found between groups A1 and A2 (18.58% and 34.69%) with respect to oocytes reaching MII ($P < 0.001$).

In vitro maturation results for oocytes obtained from frozen-thawed ovary cortexes in the B groups showed a decrease in rates of oocytes reaching MII (Table 2). In group B1, 30.56% of in vitro-matured oocytes reached MI, and 7.25% reached MII. In group B2, on the other hand, these rates were 33.33% and 6.48%, respectively. No statistical difference was found between groups B1 and B2.

When groups with the same transport temperature were compared, there was no statistically significant difference between oocytes reaching MI in groups A1 and B1, but a significant difference ($P < 0.001$) was found in the rate of oocytes reaching MII (Table 3). A significant difference was also found between groups A2 and B2 in the rates of oocytes reaching MII ($P < 0.001$).

Table 1. Maturation rate of oocytes obtained from fresh sheep ovaries.

Transport temperature	Oocytes used	GV	GVBD	MI	MII	UDNM
Group A1 (control, 4 °C)	156/187	34[b] 21.79%	14[b] 8.97%	48[a] 30.76%	29[b] 18.58%	31[a] 19.87%
Group A2 (control, 32 °C)	147/199	15[a] 10.20%	5[a] 3.40%	56[a] 38.09%	51[a] 34.69%	20[a] 13.60%

[a,b]: Rates with different letters in the same column are statistically significant in sheep ($P < 0.05$). GV: Germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II; UDNM: undefined material.

Table 2. Maturation rate of oocytes obtained from frozen-thawed sheep ovaries.

Transport temperature	Oocytes used	GV	GVBD	MI	MII	UDNM
Group B1 (frozen-thawed cortex, 4 °C)	193/245	39 20.20%	51 26.42%	59 30.56%	14 7.25%	30 15.54%
Group B2 (frozen-thawed cortex, 32 °C)	216/277	54 25.00%	45 20.83%	72 33.33%	14 6.48%	31 14.35%

($P > 0.05$)

Table 3. Maturation rate of oocytes obtained from fresh and frozen-thawed sheep ovaries.

Transport temperature	Oocytes used	MI	MII	Transport temperature	Oocytes used	MI	MII
Group B2 (frozen-thawed cortex, 32 °C)	216	72[a] 33.33%	14[b] 6.48%	Group B1 (frozen-thawed cortex, 4 °C)	193	59[a] 30.56%	14[b] 7.25%
Group A2 (control, 32 °C)	147	56[a] 38.09%	51[a] 34.69%	Group A1 (control, 4 °C)	156	48[a] 30.76%	29[a] 18.58%

[a,b]: Rates with different letters in the same column are statistically significant in sheep ($P < 0.05$).

4. Discussion

The ovaries of animals slaughtered in abattoirs are routinely used for in vitro culture and biotechnological studies carried out to preserve gene sources. Generally, oocytes in the germinal vesicle stage within the primary follicle continue their development to MII under suitable conditions (14,15,25). When ovaries are exposed to temperatures from 37 to 39 °C for 5-6 h, both the maturation rate of oocytes and their potential to develop into blastocysts decrease. The fact that the in vitro development potential of oocytes decreases with high temperatures is a problem for experimental studies in laboratories with transport difficulties (15,20,21). However, storage of cattle ovaries at 4 °C for 24 h has no effect on the in vitro maturation and development of ovaries (22).

It has been established in this study that fresh collected and frozen-thawed oocytes from sheep ovary cortexes transported at 4 °C are able to reach MII through in vitro culture. Oocytes of mammals are very susceptible to cold and freezing (4,9,15,18,23). However, the intrafollicular environment in which the oocytes are carried protects them from the damaging effects of the cold (2,15,26). While most of the oocytes collected from ovaries transported at 4 °C and 32 °C (groups A1 and A2) remained at the MI stage (30.76% [48/156] and 38.09% [56/147]), development to MII was 18.58% (29/156) and 34.69% (51/147), respectively. There is a statistically significant difference in favor of group A2 for the rate of oocytes reaching MII ($P < 0.001$). These result support the notion that oocytes are adversely affected by the cold; however, other studies have found that transporting ovaries from a different ruminant species, cattle, at 4 °C has no effect on oocyte development and fertilization (26,27). This suggests that there are significant differences in the structure of oocytes among species (15,27). Factors facilitating oocyte maturation are proteins in the structure (maturation-promoting factor, cell-division cycle kinase, and mitogen-activated protein kinase), mitochondria, endoplasmic reticulum, and fat (13-15,25).

In vitro development of oocytes collected from frozen-thawed sheep ovary cortexes transported at different temperatures was examined and the following was determined (Table 2). While most of the oocytes belonging to groups B1 and B2 remained at the MI stage (30.56% [59/193] and 33.33% [72/216]), few oocytes in either group reached MII after thawing (7.25% [14/193] and 6.48% [14/216]). This drop in the rate of oocytes reaching MII in groups B1 and B2 suggests that oocytes are adversely affected by the cold during freezing and that degeneration occurs in their enzyme structures, cytoplasm, and nuclei. Demirci et al. (24) reported the presence of abnormalities in frozen-thawed sheep cortexes and the oocytes collected from them. Furthermore, primary and primordial follicles found in the nonfrozen cortexes and two-thirds of the oocytes within them were under the influence of atresia and apoptosis. The fact that MII rates were low in the B groups suggests that the freezing and thawing of cortexes accelerates apoptosis. Despite this, oocytes in the germinal vesicle stage were mostly able to reach MI. Although oocytes are adversely affected by low temperatures, freezing, and cryoprotective substances, they do not completely lose their capacity to develop. Notably, the values determined between groups A1 and B1 and between groups A2 and B2 in experiments A and B were nearly identical with respect to reaching MI; no statistically significant difference was found between the groups. This reveals that in spite of negative factors, oocytes can protect their capacity to develop up to a certain stage.

In conclusion, no matter how low the development capacity is of oocytes collected before freezing the cortex and after freezing and thawing the cortex following transport of sheep ovaries at 4 °C, oocytes are able to reach MII. Although there may be differences between species, these results are a model for other animals and humans. In future studies, however, freezing techniques should be developed further, and fertilization of oocytes and their development to the blastocyst stage must be studied.

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