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Effect of different transport temperatures of cattle and sheep ovaries on in vitro maturation of oocytes

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Summary

The aim of study is to determine the effects of different transport temperatures (4°C, 32°C) of sheep and cattle ovaries on the in vitro maturation of oocytes. Two experimental groups were formed. Sheep and cattle ovaries were put into saline solution at 32°C. The ovaries were transported to the laboratory, at the same temperature (Group I) or at 4°C following 10 minutes of incubation at room temperature (Group II) (n=6). Oocytes were collected from ovaries using the dissection method. Oocytes matured in their own group in 700 ml TCM-199 (supplemented with pyruvate, LH, FCS) for 23 h at a gas atmosphere of 5% CO2, 5% O2, 90% N2 and at 38.8°C. After maturation, oocytes were fixed in acetic acid-ethyl alcohol (1:3) for 48 hours. The stages of development up to MII, of the oocytes stained with aceto-orsein were then examined. The Chi-Square test was used for statistical analysis. While in the 4°C group, sheep oocytes reached 30.6% (MI), 15.3% (MII) and cattle oocytes reached 17.3% (MI), 46.8% (MII), in the 32°C group these percentages were respectively 38.3%, 33.1% in sheep and 19.3%, 55.4% in cattle. While oocytes obtained from sheep ovaries transported at 32°C reached the MII stage at a higher rate compared to those at 4°C (P<0.001), no statistically significant difference was observed between maturation to the MII stage of oocytes obtained from cattle ovaries transported at 4°C and 32°C. As a result of this study, it was established that cattle ovaries could be transported both at +4 C, +32°C and that there was no difference in oocyte maturation.

Keywords: Ovary transport, in vitro maturation, bovine, sheep

Blastocyst rates in embryos obtained from the *in vitro* maturation of oocytes and their fertilization and culture, is still not at the desired level in comparison to embryos produced in vivo (4, 12, 17). One of the most important problems encountered in *in vitro* culture studies, is seen in the maturation period of oocytes until they reach a fertilizable level (10, 11). The use of ovaries obtained from animals slaughtered in abattoirs is a practical method employed for the *in vitro* production of embryos, and also for experimental studies in human medicine (9, 12, 20). Transport time, storage of the ovaries and, in particular, temperature of the medium used during transport are among the factors affecting complete maturation (15, 22). Storage of the ovaries for 5-8 hours at a temperature of 37-39°C decreases maturation of the oocytes and adversely affects their development to the blastocyst stage after *in vitro* fertilization (1, 15, 16, 22). In contrast, storage of ovaries for 8 hours at a temperature of 20-25°C has been reported to have no effect either on oocyte maturation rates or development to blastocyst following *in vitro* fertilization (1, 22). Solano et al reported that, storage of ovaries for 24 hours at a temperature of 4°C had no effect on oocyte maturation or early cleavage rates, however, that the potential to develop to blastocyst was unknown for these oocytes (19). Oocytes used in *in vitro* culture studies are obtained from follicles with a radius of 2-10 mm, and these oocytes are present in an immature state in the germinal vesicle (GV) stage (2, 5, 9). Oocytes resume meiosis in suitable *in vitro* medium in which gonadotrophic hormones are also present and reach the MII stage by continuing nuclear maturation (2, 5, 8, 18). Meiotic development occurs as a result of the activation of the M-phase promoting factor (MPF), P34 (p34cdc2), cerine-thereonine kinase and cyclin B (9). Nuclear maturation also begins due to metabolic changes around the oocyte cytoplasma and the use of lipid drops and a connection is present between them MPF shows differences (7, 12, 13). In addition to this, while there is a correlation between the oocyte diameter and follicle diameter in the meiotic development capacity of the oocyte, oocytes are able to continue their normal development in suitable temperature and medium (11, 22). Again, in similar medium, expansion of the *cumulus* cells plays a significant role in the cytoplasmic maturation of the oocyte (9, 11, 20). In

cryobiology and transplantation studies, it is stated that metabolic activities of cells, whether human or animal, are slowed down or completely arrested in low temperatures (9, 20). According to our current information, there are no comparative studies among species, on *in vitro* maturation development of oocytes obtained from ovaries transported at 4°C. In the light of the hypothesis that cell metabolism slows down or is completely arrested in low temperatures, the aim of this study is to determine the effects of different temperatures (4°C, 32°C) during transport of sheep and cattle ovaries, on the *in vitro* maturation of oocytes.

Tab. 1. Stages of development in sheep and cattle oocytes after 23 hours of culture

Species	Transport Temperature	Used Oocytes	GV	GVBD	MI	AI-TI	MII	UDNM
Sheep	+4°C	144/175	32[b] 22.2%	14[b] 9.7%	44[a] 30.6%	3a 2.1%	22[b] 15.3%	29[a] 20.1%
	+32°C	133/158	11[a] 8.3%	3[a] 2.3%	51[a] 38.3%	5[a] 3.8%	44[a] 33.1%	19[a] 14.3%
Cattle*	+4°C	104/129	7 6.7%	6 5.8%	18 17.3%	5 4.8%	48 46.8%	20 19.2%
	+32°C	101/126	4 4.0%	4 4.0%	20 19.8%	5 5.0%	56 55.4%	12 11.9%

Explanation: a, b – rates with different letters in the same column are statistically significant in sheep ($P < 0.001$). GV – germinal vesicule; GVBD – Germinal vesicule break down; MI – metaphase I; AI-TI – anaphase-telephase I; MII – Metaphase II; UDNM – undifened material

Material and methods

In this study, cattle and sheep ovaries from the slaughterhouse were used. The study was repeated six (n = 6) times. The ovaries were transferred into thermos flasks containing sterile saline solution and brought to the laboratory in 2-4 hours. Two experimental groups were formed (where ovaries belonging to each species were in their own group). While one of each of the cattle and sheep ovaries was transferred into the thermos flask containing 0.9% NaCl at a temperature of 32°C, the other ovary belonging to the same animal was first put into 0.9% NaCl at 32°C for 10 minutes at room temperature, after which it was transferred to a thermos flask with 0.9% NaCl at 4°C and brought to the laboratory without delay. Ovaries belonging to each group were washed three times using sterile saline solution.

Obtaining oocytes and maturation. Cumulus oocyte complexes (COCs) were obtained by dissection from 2-10 mm follicles from slaughtered cattle and sheep ovaries. Oocytes were selected depending on the structure of the *vitellus*, shape of the *zona* and presence of 3-4 layers of *cumulus oophorus* cells surrounding them. Using the dissection method, 333 oocytes were obtained from sheep and 277 (83.1%) of these were used. In the cattle, of the 255 oocytes collected, 205 (80.39%) were selected for maturation. The selected oocytes were passaged three times in the washing medium (TCM-199 supplemented with 50 microgram/ml gentamycine sulphate and 1 mM L-glutamin) and once in the maturation medium (Hepes buffered TCM-199 supplemented with 1 mM-L-glutamin, 0.2 mM Na-pyruvate, 50 µg/ml gentamycine sulphate, 24 IU/ml LH, 10% FCS). Groups of approximately 20-30 COCs were then placed in 700 µl *in vitro* maturation medium in 4 well dishes for 23 h culture under an atmosphere of 5% CO_2, 5% O_2, 90% N_2 gas atmosphere at 38.8°C. At the end of maturation, the oocytes were cleansed from their cumulus cells with the chemical-mechanical method using hyaluronidase. Oocytes were then put into 0.7% KCl for 5 minutes to prepare the slides. Slides were fixed in acetic acid-ethyl alcohol (1 : 3) for 48 hours. The developmental staged up to MII, of the oocytes stained with aceto-orcein were then examined under the phase contrast microscope. The Chi-Square test was used for statistical analysis.

Results and discussion

Results were obtained after 23 hours of culture of cattle and sheep oocytes. While a large proportion (22.2%) of the oocytes obtained from sheep ovaries transported at 4°C remained at the germinal vesicle stage, this percentage was 8.3% in the 32°C group. On the other hand, oocytes in the 4°C cattle group exhibited better development compared to sheep, where only 6.7% of the oocytes remained in the GV stage. This rate was 4.0% in the 32°C cattle group. While in the 4°C group, sheep oocytes reached the MI stage at a rate of 30.6%, the Anaphase-Telephase I (AI-TI) at 2.1% and the Metaphase II at 15.3% a statistically significant difference was found in the oocyte percentage reaching MII as a result of *in vitro* maturation of the oocytes obtained from sheep ovaries transported at 32°C and 4°C. The difference was $p < 0.001$ (tab. 1).

In cattle, as a result of the *in vitro* maturation of oocytes obtained from ovaries transported at 4°C, the rate of reaching Metaphase I was 17.3%, that of Anaphase-Telephase was 4.8% and for Metaphase II it was 46.8%. These rates were 19.8%, 5% and 55.4% respectively in the 32°C group. According to these results, no statistically significant difference was found between the stages of development, in particular in the rates of reaching Metaphase II, following *in vitro* culture of oocytes collected from embryos transported at 4°C and 32°C in cattle (tab. 1).

Ovaries of animals slaughtered in abattoirs are routinely used experimentally in *in vitro* fertilization studies aimed at animal production. Oocytes are usually present in the GV stage within the primary follicle and continue their development to MII in suitable temperature and medium (11, 23). Some researchers have reported that, exposing the ovaries to temperatures of 37-39°C for 5-8 hours decreases the maturation rate of follicular oocytes and reduces their potential for developing to blastocyst following *in vitro* fertilization (1, 15, 22). A temperature related decrease in the potential for *in vitro* development will adversely affect the success rate in cases of laboratories with no ovary transport facilities, cell transportation, *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) studies in human medicine and where sperm cannot be obtained from the male due to psychological and mechanical reasons. Also, sheep ovaries are similar to human ovaries with respect to size and structure (3).

This study statistically shows that there is no decrease in the development to the MII stage of oocytes obtained from cattle ovaries transported at 4°C. However, in con-

trast, transporting sheep ovaries at 4°C adversely affects the *in vitro* development of oocytes. Parallel to the results of this study, Solano et al have stated that storage of cattle ovaries for 24 hours at 4°C had no effect on the development capacity of oocytes, however, that the development of these oocytes to blastocytes following fertilization was unknown (19). In addition, there was no decrease in the development to blastocyte following *in vitro* fertilization of oocytes obtained from ovaries stored at 18-25°C for 8 hours (1, 22). Both animal and human oocytes and embryos are very susceptible to cold and freezing (9, 20, 21). Exposure to the cold of GV stage oocytes or mature oocytes causes irreversible disruption in the spindle fiber in the nuclear structure of the oocyte (4, 9, 21). The intrafollicular environment surrounding the oocyte is protective of the oocyte against the adversary effects of low temperatures (14).

In this study, in the maturation of oocytes obtained from sheep ovaries transported at +4°C and +32°C, while most of the oocytes remained in the MI stage with 30.6% (44/144) and 38.3% (51/133) respectively, development to MII was found to be 15.3% (22/144) and 33.1% (44/133) respectively. In sheep, statistical significance was found to be at a level of $p < 0.001$ for reaching MII. In contrast, in the 4°C and 32°C group of cattle oocytes, most reached MII at rates of 46.8% (48/104) and 55.4% (56/101) respectively. These results suggest that, either sheep oocytes are more susceptible to the cold or that the follicular environment surrounding the oocyte is insufficient in protecting the oocytes against the cold. There is a close relationship between the mitochondrial endoplasmic reticulum and lipid in cattle oocytes and this continues as a result of the give-and-take of metabolytes in the cytoplasma microenvironment (7). According to these results, the low rates of sheep oocytes reaching the MII stage suggests it is related to the metabolic difference in the oocyte structure. Two other factors allowing oocyte maturation are the oocyte proteins, mitogen-activated proteinkinase and maturating promoting factor (MPF) cdc2-kinase (6, 9). The amounts of these species-specific proteins are different (9). Exposure to low temperatures of sheep oocytes may have caused degeneration in the structure of these protein and enzymes. Furthermore, exposure to low temperatures for a long period may cause degeneration of unknown re-programming factors (14).

Cell metabolism slows down in temperatures lower than body temperature. It is assumed that in cattle in the 4°C group the MPF of oocytes in the GV stage either were not adversely affected by the cold to a great extent, or slowed down or their metabolism stopped completely. When oocytes are placed in a suitable medium these supressed proteins begin maturation (9, 20). Despite there being differences between species, the fact that cattle oocytes can be transported at 4°C and there being no adverse effects on oocyte development potential, produces a model for other mammal species and humans. At the same time, before the oocytes are used, tests for zoonotic diseases such as Bovine Spongioform Encephalopathy (BSE) can be carried out and ovaries can be stored in low temperatures until negative results are established (14).

Finally, transportation of cattle ovaries at 4°C is not detrimental to the *in vitro* development capacities of oocytes. However, the development capacity of these oocytes to blastocytes following *in vitro* fertilization is unknown. The development capacity following IVF of these oocytes which form a model for both animals and humans must be investigated in future studies.

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