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Effects of Different Transport Temperatures on *in vitro* Development of Queen Oocytes

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Abstract: Today, many species, especially feline are endangered. For this, domestic cats used as a model for *in vitro* culture studies. So, many investigators have problems transporting ovaries to their laboratories. In this study effects of different transport temperatures on *in vitro* maturation of feline oocytes were investigated. Ovaries were collected from 12 ovariectomised queens of 2-3 years old, four of which were at oestrus and eight at anoestrus. One ovary of each pair was brought to the laboratory in PBS at 4°C and other one at 37°C. Two main groups as oestrus and anoestrus were established and each were divided into further 2 subgroups as 4 and 37°C. Oocytes were collected in TCM-medium and matured for 24 h under 5% CO_2 at 38.5°C. Matured oocytes were fertilized with fresh semen at a final concentration 1×10^6 mL^{-1} in Sperm-Talp medium under 5% CO_2 for 18-24 h. After removing cumulus oophorus cells and zygotes were divided into groups and *in vitro* cultured for 72 h in 100 μL SOF medium under 5% CO_2, 5% O_2 and 90% N_2 gas mixture. At the 48th h of incubation, the best cleavage was 44.4% (8/18) at 37°C oestrus group and the lowest was 14% (7/50) in the oestrus group at 4°C. These rates were 22.72% (15/66) and 28.57% (8/28), respectively for the anoestrus group. At the 72 h of culture in 37°C oestrus group 7 embryos stayed at 4-8 blastomere stage and 1 embryo reached 16-32 blastomere stage. This result was significant when compared to the other groups ($p<0.001$). In 4°C anoestrus group only 5 embryos have reached 4-8 blastomere and no significant difference among the results was observed. It is concluded that cat ovaries oestrus are better transported at 37°C while anoestrus ovaries could be carried at 4°C.

Key words: Queen, transport, temperatures, oocyte, *in vitro* fertilization, Turkey

INTRODUCTION

In the last 400 years, 2/3 of hundreds of animal species have been destroyed in the world and 3/4 of the remaining mammals face with the danger of extinction. Especially, the number of the wild cat species decreased in large amounts in order to the men's hunting in the forbidden regions and the restriction of these animal's habitats (Cocchia *et al*., 2010). Reproductive studies conducted on wild cats all over the world are quite limited. Now a days, domestic cats are extensively used in the studies to understand the function of the gamete cells and utilization of the reproductive techniques in endangered wild cat populations (Freistedt *et al*., 2000; Gomez *et al*., 2003; Nagano *et al*., 2008). In recent years, *In Vitro* Maturation (IVM), *In Vitro* Fertilization (IVF) and *In Vitro* Culture (IVC) studies are increased in the obtained oocytes from the domestic cats (Freistedt *et al*., 2000). Although, these kinds of studies are still continuing, embryo production, freezing and obtaining an offspring from the domestic cat could not reach at the desired level of success yet (Gomez *et al*., 2003). In order to protect the genetic resources of the wild and domestic cats, researchers are performing oocyte freezing and fertilization studies from the reproductive techniques (Cocchia *et al*., 2010).

Many researchers reported that the obtained oocytes can maturate and achieve an embryo due to the storage or transfer of the ovaries of the farm animals in different temperatures (4-32°C) (Yang *et al*., 1990; Solano *et al*., 1994; Abe and Shioya, 1996; Ozda *et al*., 2006). The researchers reported that when they store the cat ovaries at different temperatures [4°C (Wolfe and Wildt, 1996; Freistedt *et al*., 1999), 10°C (Ksiazkiewicz *et al*., 2003), 23-25 and 37°C (Ozda *et al*., 2006)], the oocytes that were obtained from these ovaries could also reach to

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metaphase 2 stage either at low temperatures (Naoi *et al*., 2007). The storage of the ovaries at low temperatures temporarily provides the opportunity to rescue the wild feline oocytes that have been ovario-hysterectomized for medical reasons or died suddenly in their life. Submitting both domestic and wild animal's ovaries which were stored at low temperatures to the advanced centers could provide the possibility of the preservation of genetic resources in undeveloped areas in the absence of *in vitro* culture laboratory. But there isn't enough information about the storage period and temperature for the potential recovery of the oocytes from the ovaries (Naoi *et al*., 2007). The aim of the present study is to determine the effect of different transport temperatures to the *in vitro* embryo development from the different stages of the reproductive cycle of the street cat's ovaries. According to the current knowledge, there is no study in the world about the effects of different transport temperatures (4 and 37°C) of the oocytes *in vitro* fertilization that were collected from different reproductive stages of the cat's ovaries.

MATERIALS AND METHODS

Twelve ovaries that were collected during the ovario-hysterectomie operation were used in this study from the cats in different reproductive stages in February at the Faculty of Veterinary Medicine of Istanbul University. Eight of them were at anoestrus period. While one of each of cat ovaries were transferred to the laboratory at 4°C in thermos containing PBS with 5% Fetal Calf Serum (FCS), the other ovary was transferred in the same buffer but at 37°C. Ovaries were incubated in PBS containing 5% FCS for 3 h at 4 and 37°C separately in the laboratory. After this stage, ovaries were washed with PBS for three times and then they were transferred in TCM-199 solution for dissection. The ovaries were allocated to two main experimental groups, oestrus and anoestrus group (group A and B), respectively. These experimental groups were divided into two sub-groups as 37 and 4°C, respectively (group A1: 37°C, A2: 4°C, B1: 37°C and B2: 4°C).

Oocyte collection and maturation: Ovaries were sliced with a scalpel to maintain the purifying of the Cumulus Oocyte Complexes (COCs) and transferred to the 90 mm culture dish containing TCM-199 wash medium at 37°C. The oocytes selected for maturation were ensured to have 3-4 layers of cumulus oophorus cells surrounding them that their vitellus was homogenous and zona were smooth. Selected oocytes were cleansed 3 times in the washing medium (TCM-199 supplemented with 50 μg mL^{-1} gentamycine sulphate and 1 mM L-glutamin) and once in the *In Vitro* Maturation medium (IVM) (TCM-199 supplemented with 1.0 mM L-glutamin, 0.2 mM Na-pyruvate, 50 μg mL^{-1} gentamycine sulphate, 0.1 IU mL^{-1} FSH-LH, 25 ng mL^{-1} EGF, 10% FCS after which they were matured for 24 h (Nafano *et al*., 2008) in 4 well dishes at 38.5°C and a under an atmospheric mixture of 5% CO_2, 5% O_2 and 90% N_2.

Oocyte *in vitro* fertilization and embryo culture: At the end of culture, oocytes of each groups showing cumulus expansion were washed 3 times in the semen-TALP medium for fertilization. The matured oocytes were then transferred to 4-chambered fertilization flasks including 400 μL of IVF-TALP medium (group A1, A2, B1, B2). Fertilization was performed by using fresh semen of tom cat. The concentration of the semen prepared in accordance with the swim-up method with Sperm-TALP medium was adjusted to 1×10^6 mL^{-1} (Zambelli *et al*., 2008) concentration and transferred to the fertilization flasks, each cell including 20-25 matured oocytes. The oocytes and spermatozoa were incubated for fertilization at 38.8°C for 18-24 h under an atmospheric mixture of 5% CO_2, 5% O_2 and 90% N_2. At the end of the fertilization, embryos considered to be fertilized were transferred into tubes containing 1.0 mL of TCM-199 washing medium in order to eliminate the surrounding cumulus cells and spermatozoa. The tubes were mixed by vortex for 99 sec and the embryos were washed 3 times in washing medium and one time in SOF culture media. After removing cumulus oophorus cells, zygotes were divided into groups and *in vitro* cultured for 72 h in 100 μL SOF medium under 5% CO_2, 5% O_2 and 90% N_2 gas mixture.

RESULTS

The highest cleavage rate was found as 44.44% in the A1 group that was in the oestrus period and the ovaries transported at 37°C. However, the division rate of the ovaries taken from the cats at the oestrus period decreased to the 14.00% when transported in cold (Table 1) and this difference is statistically significant ($p<0.01$). While the fertilization rate of the oocytes that were obtained from the cat's ovaries from the anoestrus period transported at 4°C was found as 28.57% in the

Table 1: Fertilization rate of oocytes obtained from the oestrus period of the cats

Transport temparature	Used oocytes	Degenerated oocytes	Cleavage rate	4-8 blastomere	8-16 blastomere
Group A1 (37°C)	18/34	3.00 (16.66%)	8.00[a] (44.44%)	7.0[a] (38.88%)	1.00[a] (5.55%)
Group A2 (4°C)	50/73	12.00[a] (24.00%)	7.00[b] (14.00%)	2.00[b] (4.00%)	0.00[b] (0.00%)

[a,b]Rates with different letters in the same column are statistically significant in cats ($p<0.01$)

Table 2: Fertilization rate of oocytes obtained from the anoestrus period of the cats

Transport temparature	Used oocytes	Degenerated oocytes	Cleavage rate	4-8 blastomere	8-16 blastomere
Group B1 (37°C)	66/80	29.00 (43.93%)	15.00 (22.72%)	7.00 (10.60%)	3.00 (4.54%)
Group B2 (4°C)	28/48	8.00 (28.57%)	8.00 (28.57%)	5.00 (17.85%)	0.00 (0.00%)

Table 3: Fertilization rate of oocytes obtained from oestrus and anoestrus period of the cats

Transport temparature	Cleavage rate	4-8 blastomere	8-16 blastomere
Group A1 (37°C)	8/18[a] (44.44%)	7[a] (38.88%)	1 (5.55%)
Group A2 (4°C)	7/50 (14.00%)	2 (4.00%)	0 (0.00%)
Group B1 (37°C)	15/66[b] (22.72%)	7[b] (10.60%)	3 (4.54%)
Group B2 (4°C)	8/28 (28.57%)	5 (17.85%)	0 (0.00%)

[a,b]Rates with different letters in the same column are statistically significant in cats ($p<0.01$)

group 37°C was found as 22.72% and there was statistically no difference between them (Table 2). Reaching rate to the 4-8 blastomere stage of the cleavaged oocytes was found as 38.88% (A1), 4.00% (A2), 10.60% (B1), 17.85% (B2), respectively (Table 3). While statistically difference was found between group A1 and A2, statistically difference was not found between group B1 and B2. Chi-square statistical analysis was used for the study.

DISCUSSION

As a result of this study, it is found that the oocytes can be fertilized when the cat's ovaries transported at 4°C and kept for 3 h at the same temperature. Although, some researchers reported in their studies that it is possible to obtain embryos from different farm animals' ovaries which were transported and stored at different temperatures there are no such reported researches in felines (Yang *et al*., 1990; Solano *et al*., 1994; Abe and Shioya, 1996; Ozda *et al*., 2006). Although, there are studies related to the different temperatures storages of the cat's ovaries at different reproductive period, this study indicates the effects of 4 and 37°C transport temperatures to the cats' fertilization. While the cleavage rate of the cat's ovaries from the oestrus period (group A1) was found as 44.44% when transported at 37°C this rate was found as 22.72% of the cat's ovaries from anoestrus period (group B1) and there are found statistically significance between them in favor of the A1 group ($p<0.01$). About 50-65% of the oocytes obtained from the ovaries can reach to MII stage in domestic cats and 20-30% of them can reach to blastocyst stage (Naoi *et al*., 2007).

Pope *et al*. (2006) reported that as a result of fertilization of cat's oocytes demonstrate a 45-55% cleavage rate. According to these information the cleavage results achieved from the oestrus period of the cats are successful.

However, at the anoestrus cats this rate decreased almost by half. *In vitro* maturation and fertilization of the cat's oocytes depends on so many factors such as oocyte quality, culture environment (conditions), hormonal effects and especially seasonal effect and oestrus cycle. Cleavage rate can decrease out of the season (Spindler and Wildt, 1999; Ksiazkiewicz *et al*., 2003; Pope *et al*., 2006). The finding at the anoestrus group (22.72%) cats supports the thesis about the out of the season cats have oocytes with bad quality and uncapable of development (Pope *et al*., 2006). When researchers examine the oestrus and anoestrus 4°C transport temperature groups, the cleavage rates were 14.00 and 28.57%, respectively. When researchers examine the 4°C transport temperature in both oestrus and anoestrus groups, the cleavage rates were 14.00 and 28.57%, respectively. The ovaries at the anoestrus group transported at 4°C when compared to oestrus group showed higher cleavage (Table 3). The result is parallel to Karja *et al*. (2003) that the cleavage rates of oocytes obtained from follicular ovaries are lower than luteal phase and those obtained from inactive ovaries.

This result supports the idea of the storage of the ovaries at 4°C for a long term (24 h) of the continuing oocytes development capacities and capabilities of fertilization (Wolfe and Wildt, 1996; Freistedt *et al*., 1999; Naoi *et al*., 2007). When we compare the groups in the oestrus period group A1 (37°C) and A2 (37°C) reaching to cleavage rate according to the A2 in favor of A1, it was found statistically significant ($p<0.001$). This result suggests that the oocyte obtained from the cats' follicles at the oestrus period is very sensitive to cold temperatures. Usually oocytes in the follicles exist in the immature Germinal Vesicule (GV) stage especially under the influence of hormones they continue to develop when they find a suitable environment (Gardner *et al*., 2004). These results suggest that during the oestrus period in cats with the effect of FSH and LH hormone in the oocyte M-phase Promoting Factor (MPF), P34, serine-thereonine kinase and cyclin-B activation and continued meiotic development, structural and lipid changes that occurred in the oocyte cytoplasm are all related to the cold sensibility (Gardner *et al*., 2004). Natural follicular wave causes the oocyte quality varieties (Wood *et al*., 1997). In study, the detection of so many zona ruptures in the oocytes obtained from the A2 group supports to the opinion. In the study the determination of many zona

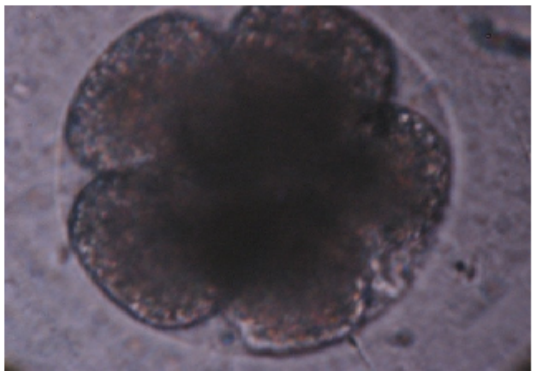

Fig. 1: 4 blastomere stage of cat oocyte

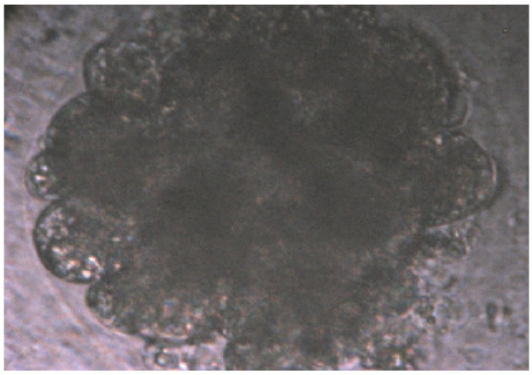

Fig. 2: 16 blastomere stage of cat oocyte

ruptures obtained from the A2 group oocytes supports this thinking. Cleavage rate is 22.72% in anoestrus group B1, it is 28.57% in B2 and there is no statistical difference between them. This result suggest there is no hormonal effect on oocyte maturation and suggest that these oocytes are cold-resistant. As a result of transporting the oocytes at low temperatures, the metabolism slows down and in order to the reduction of the enzymatic wastes, apoptosis slows down. It was determined that in B1 and B2 anoestrus group reaching to 4-8 blastomere stage (Fig. 1) is 10.60 and 17.85%, respectively and no statistically significant difference was found between them again.

The oocytes obtained from the ovaries that were transported at 37°C in oestrus and anoestrus group reached to 8-16 blastomere stage (Fig. 2) at a rate of 5.55% (A1) and 4.54% (B1), respectively and no statistically significant difference were found between them. This result supports the thesis of the good developmental capacity of the oocytes obtained from the inactivated ovaries in line with Karja *et al*. (2003).

CONCLUSION

The researchers can say the season is very important for the cat's *in vitro* culture experiments. Researchers can say the cleavage rate is higher in the anoestrus cat's ovaries transported at 4°C when compared with oestrus cat. Transporting the cat ovaries in cold at 4°C allows the oocytes meiotic development and fertilization even out of the season.

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