Assessment of the Viability of Cat Oocytes Subjected to Storage at Different Time Intervals

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Abstract

The aim of this work was to investigate prolonged storage at 4˚ C on the survival of cat cumulus oocyte complexes. The ovaries were obtained from 20 domestic cats. The ovaries were evaluated at different time intervals. The viability test for cumulus oocyte complexes was performed at 2 hours, 24 hours and 72 hours after being sampled. Tests for the viability of cumulus oocyte complexes were performed with Neutral red and Trypan blue. Storage conditions for cumulus oocyte complexes are a critical step in establishing fertility conservation protocols in animals, as well as for assisted reproduction.

Keywords: oocyte viability, neutral red, trypan blue

1. Introduction

The domestic cat is an important model for assisted breeding technology. Based on cumulus oocyte complexes, in vitro maturation and fertilization methods can be developed in endangered cats.

Domestic cats (*Felis catus*) are frequently used for research as a model for feline species that are threatened with extinction. In fact, many assisted breeding methods are being tested using the domestic cat model due to the widespread availability of this research material [1]

Mammalian oocytes originate from primordial cells that multiply, reaching several million cells during intrauterine life and migrate to the immature gonad, remaining at rest until recruitment, maturation and ovulation. These cells are a source of gametes for assisted reproduction [2]. The progress in the development of biotechnological techniques of assisted reproduction is based on the ability to obtain gametes (oocytes, sperm) that correspond quantitatively but also qualitatively.

The progress in the development of biotechnological techniques of assisted reproduction is based on the ability to obtain gametes (oocytes, sperm) that correspond quantitatively but also qualitatively. The collection of oocytes from live cats is done by laparoscopic aspiration, the harvested oocytes can be used for in vitro fertilization or for cloning. Harvesting oocytes from the ovaries can also be done post mortem, and if the recovered oocytes are immature, then they must be matured by in vitro methods. The number of cumulus oocyte complexes varies depending on the age and health of the female. It is also known that the number of oocytes, their quality and capacity for development decreases with increasing age [3].

The development capacity of oocytes from prepubertal animals has been studied and it has been successfully identified in vitro to obtain embryos from oocytes taken from such animals [4]. The in vitro rate of obtaining embryos from oocytes from prepubertal animals has been described as lower than that of using oocytes from mature animals [5], while other studies show similar results to those using adult oocytes. [6].

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Despite the fact that some researchers reported a birth of live kittens obtained from vitrified cat oocytes, cryopreservation of immature or mature cat oocytes has not been shown to be until now a reliable and repeatable tool [7,8]. However, only a small number of studies have shown a cleavage rate of cumulus oocyte complexes between 14% and 25% [9].

The neutral red cell viability test provides a quantitative estimate of the number of viable cells in a cell culture. It is one of the most widely used cytotoxicity tests, with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the neutral red surviving dye to the lysosomes. Most primary cells and cell lines of diverse origin can be used successfully in such studies [10].

The cell viability test with trypan blue determines the identification of dead cells, they appear strongly colored in blue. Oocytes at the beginning of their development are most sensitive to resorption trends. The uptake of trypan blue to be absorbed into the oocytes is normally proportional to the surface of the oocytes.

Staining of cumulus oocyte complexes with trypan blue was used to differentiate living and dead cat oocytes. It is very important to know the viability of oocytes before in vitro fertilization. Cumulus oocyte complexes remain stained for a maximum of 10 minutes, during which time oocytes must be differentiated from live and dead ones.

Trypan blue is a technique used to quantify living cells by exclusively labeling dead cells. Because living cells have an intact cell membrane, trypan blue cannot penetrate the cell membrane of living cells and cannot enter the cytoplasm. In a dead cell, blue trypan passes through the porous cell membrane and enters the cytoplasm. In light microscopy analysis, only dead cells appear colored blue.

2. Materials and methods

The ovaries were taken from 20 domestic cats, using the ovariohysterectomy technique. From the collection room until they reached the laboratory for testing, they were stored in physiological serum. For the collection of oocyte cumulus complexes, the ovaries were sliced using a scalpel in a Petri plate with PBS medium.

The first group of oocytes were transferred to 6-well plates. The plates were subsequently incubated for 2 hours with a medium containing neutral red. The oocytes were subsequently washed by removing the dye from each well and the oocytes that were stained red were identified.

The second group of cumulus oocyte complexes were stained with trypan blue using a 0.16% trypan blue solution in TCM 199. Microscopic analyzes to identify living cells were performed under an Olympus microscope. Based on the number of living cells identified, the survival rate of oocyte-cumulus complexes was calculated. Statistical analysis was realized using Anova program.

3. Results and discussion

After ovariectomy, ovicite cumulus complexes were analyzed based on cell viability tests, which use 2 different color reagents: Neutral red and Trypan blue. Quantitative determinations were performed at different time intervals from sampling: 2 h, 24 h and 72 h. Figure 1 shows the values obtained after applying the cell viability tests with Neutral Red, respectively Figure 2 shows the data obtained when applying the test with Trypan blue. The obtained data were analyzed from a statistical point of view, finding or not differences between the two experimental groups, when applying the 2 cell viability tests.

Applying the cell viability tests to the cumulus oocyte complexes immediately after sampling, we find that there are no differences between the 2 groups in terms of the number of viable oocytes collected (p=1). Statistical analysis also indicates that there are no significant differences between the 2 groups, in terms of cell viability in cumulus oocyte complexes at 2 hours and 24 hours after sampling, the values recorded for p=0.65848 in both experimental variants. It is found, but there are some highly significant differences between the 2 groups, for the viability of the cumulus oocyte complexes when applying the 2 tests (p=0.00181).

In the experimental group 1, statistical analysis tests were applied and it was found that there were no significant differences in the viability of the cumulus oocyte complex in analyzes at 2 h compared to those at 24 h (p=0.12305). In the case of the statistical analysis of the data for the cellular viability of the cumulus oocyte complexes at 2 h shared with those of 72 h, very statistically significant differences are found (p=0.00047).

In the experimental group 2, statistical analysis tests were applied and it was found that there are significant differences in the viability of the cumulus oocyte complex in analyzes at 2 h compared to those at 24 h (p=0.03565). In the case of the statistical analysis of the data for the cellular viability of the cumulus oocyte complexes at 2 h shared with those of 72 h, highly statistically significant differences are found (p=0.0009).

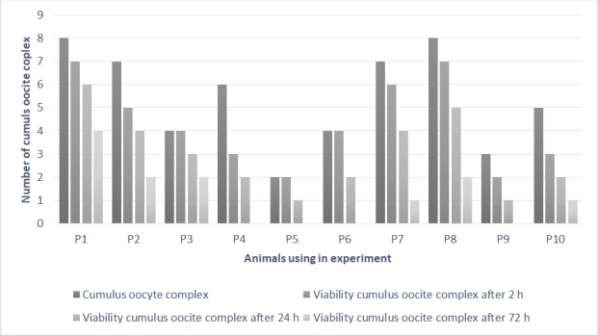

Figure 1. Analysis the viability of the cumulus oocyte complex in experimental animals based on the cell viability test with Neutral red

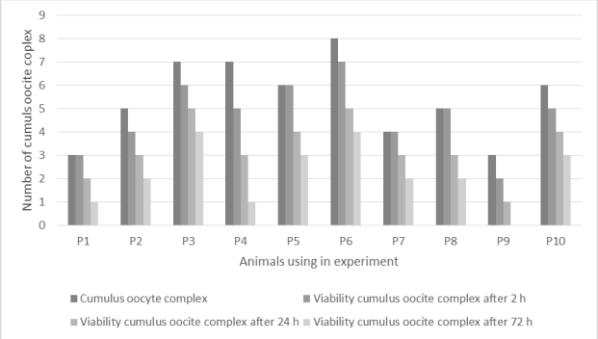

Figure 2. Analysis the viability of the cumulus oocyte complex in experimental animals based on the cell viability test with Trypan blue

Classical methods of analyzing the viability of cumulus oocyte complexes are used, most of the invasive methods using microscopy techniques [11, 12]. As a result, the studied complexes are destroyed and prove useless for later cultural or transplant purposes. Neutral red (NR) is a non-toxic and water-soluble dye, it has been proposed as a non-invasive tool for performing cell viability tests to identify living / dead cells [10,13,14]. The principle of the method is based on the ability of living cells to incorporate it at the level of lysosomes [10, 13]. Although it has been shown to be very effective for testing cell viability, nothing is known about the effect of the neutral dye red NR on the viability of cumulus oocyte complexes that would be cryopreserved later.

Studies on a bovine model show that the method of testing cell viability with neutral red is a valuable tool, the limited toxic properties of the dye have been shown to be when using a concentration of 15 μg / ml and an exposure time of 30 min [16].

Oocytes collected from feline ovaries and stored at 4° C for 24 hours have a significantly higher rate of meiotic division, compared to those stored at room temperature or 38° C [17]. If the storage of cat ovaries after sampling takes place in saline at 4° C, oocytes are protected from morphological and structural changes: vacuolation and destruction of membrane integrity in granular cells and oocytes. If the ovaries after sampling are maintained at 4° C for 12 hours, there is an increase in the rate of apoptosis in the cumulus oocyte complexes, cellular enzymatic activity is affected, metabolic products accumulate and the apoptosis process is initiated [18].

Other studies report that prolonged storage at 4° C of the ovaries after ovariohysterectomy may reduce the quality of the ovaries and the ability to develop oocytes, increase the number of degenerated oocytes, the appearance of apoptotic and necrotic changes [19].

4. Conclusions

The viability of cumulus oocyte complexes registers statistically significant decreases with increasing time from sampling to use. The cellular viability test with neutral red was identified as the most suitable for highlighting the viability of cumulus oocyte complexes.

References

1. Donoghue, A. M., Johnston, L. A., Seal, U. S., Armstrong, D. L., Tilson, R. L., Wolf, P., Petrini, L. G., Simmons, T., Groos, T., Wildt, D. E., In vitro fertilization and embryo development in vitro and in vivo in the tiger (Panthera tigris).

2. Martins, J. L. A., Lopes, M. D., Souza, F. F., Possebon, F. S., Jewgenow, G. W. K., Cat preantral follicle survival after prolonged cooled storage followed by vitrification, Cryobiology, 2018, 81, 94-100.

3. Kochan, J., Nowak, A., Młodawska, W., Prochowska, S., Partyka, A., Skotnicki, J., Niz˙an´ski, W., Comparison of the Morphology and

Developmental Potential of Oocytes Obtained from Prepubertal and Adult Domestic and Wild Cats. Animals, 2021, 11, 20. https://dx.doi.org/10.3390/ani11010020.
4. Ptak, G., Loi PDattena, M., Tischner, M., Cappai, P., Offspring from one-month-old lambs: Studies on the developmental capability of prepubertal oocytes, Biol. Reprod., 1999, 61, 1568-1574.
5. Morton, K. M., Developmental capabilities of embryos produced in vitro from prepubertal lamb oocytes, Reprod. Domest. Anim., 2008, 43, 137-143.
6. Leoni, G. G., Palmerini, M. G., Satta, V., Succu, S., Pasciu, V., Zinellu, A., Carru, C., Macchiarelli, G., Nottola, S. A., Naitana, S., et al., Differences in the kinetic of the first meiotic division and in active mitochondrial distribution between prepubertal and adult oocytes mirror differences in their developmental competence in a sheep model, PLoS ONE, 2015, 20, e0124911.
7. Pope, C. E., Go´mez, M. C., Kagawa, N., Kuwayama, M., Leibo, S. P., Dresser, B. L., In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection and embryo transfer. Theriogenology, 2012, 77, 531-538.
8. Mikołajewska, N., Muller, K., Niżański, W., Jewgenow, K., Vitrification of Domestic Cat Oocytes – Effect on Viability and Integrity of Subcellular Structures, Reprod Dom Anim, 2012, 47 (Suppl.6), 295-299, doi: 10.1111/rda.12044, ISSN 0936–6768.
9. Tharasanit, T., Manee-In, S., Buarpung, S., Chatdarong, K., Lohachit, C., Techakumphu, M., Successful pregnancy following transfer of feline embryos derived from vitrified immature cat oocytes using stepwise' cryoprotectant exposure technique, Theriogenology, 2011, 76, 1442-1449.
10. Repetto, G., del Peso, A., Zurita, J. L., Neutral red uptake assay for the estimation of cell viability/cytotoxicity, Nat Protoc., 2008, 3(7), 1125-1131. doi:10.1038/nprot.2008.75.
11. Sanfilippo, S., Canis, M., Ouchchane, L., Botchorishvili, R., Artonne, C., Janny, L., et al., Viability assessment of fresh and frozen/thawed isolated human follicles: reliability of two methods (Trypan blue and Calcein AM/ethidium homodimer-1), J Assist Reprod Genet, 2011, 28(12), 1151-1156. doi:10.1007/s10815-011-9649-y.
12. Fransolet, M., Labied, S., Henry, L., Masereel, M. C., Rozet, E., Kirschvink, N., et al., Strategies for using the sheep ovarian cortex as a model in reproductive medicine, PloS One, 2014, 9(3):e91073. doi:10.1371/journal.pone.0091073.
13. Elliott, W. M., Auersperg, N.. Comparison of the neutral red and methylene blue assays to study cell growth in culture, Biotechnic Histochem: Off Publ Biol Stain Comm, 1993, 68(1), 29-35. doi:10.3109/10520299309105573.
14. Borenfreund, E., Puerner, J. A., Toxicity determined in vitro by morphological alterations and neutral red absorption, Toxicol Lett, 1985, 24(2-3), 119-124.
15. Chambers, E. L., Gosden, R. G., Yap, C., Picton, H. M., In situ identification of follicles in ovarian cortex as a tool for quantifying follicle density, viability and developmental potential in strategies to preserve female fertility, Hum Reprod (Oxford, England), 2010, 25(10), 2559-2568. doi:10.1093/humrep/deq192.
16. Langbeen, A., Jorssen, E. P. A., Granata, N., Fransen, E., Leroy, J. L. M. R., Bols, P. E. J., Effects of neutral red assisted viability assessment on the cryotolerance of isolated bovine preantral follicles, J Assist Reprod Genet, 2014, 31, 1727-1736. DOI 10.1007/s10815-014-0340-y.
17. Naoi, H., Otoi, T., Shimamura, T., Karja, N. W., Agung, B., Shimizu, R., Taniguchi, M., Nagai, T., Developmental competence of cat oocytes from ovaries stored at various temperature for 24 h, Journal of Reproduction and Development, 2007, 53, 271-277.
18. Wongsrikeao, P., Otoi, T., Karja, N. W., Agung, B., Nii, M., Nagai, T., Effects of ovary storage time and temperature on DNA fragmentation and development of porcine oocytes. Journal of Reproduction and Development, 2005, 51, 87-97.
19. Wlodarczyk, R., Bukowska, D., Jackowska, M. Mucha, S., Jaskowski, J. M., In vitro maturation and degeneration of domestic cat oocytes collected from ovaries stored at various temperatures, Veterinarni Medicina, 2009, 54(10), 491-497.