Mammalian Oocyte Cryopreservation - Review

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Abstract
Although oocyte cryopreservation represents one of the main objectives of the reproductive medicine and cryobiology in the last years, until now, regardless of the studied specie, there is no freezing protocol that will assure satisfactory survival rates after thawing. Oocyte cryopreservation is now one of the most problematic issues in cryobiology aria, especially because they are extremely sensitive to low temperatures, and the maintaining of a normal development potential after thawing is very difficult. For animals cryopreservation is offering the possibility of preserving the genetically valuable genotypes or endangered species, with reducing the costs associated with conventional breeding (shelter, food, bed, drugs, human resources, etc.). The main factors affecting the viability of the oocyte are cryoprotectors, freezing method, disruption of meiotic spindle, zona pellucida hardening and oocyte maturation stage. The present paper is aiming to review the historic of oocyte cryopreservation, the main factor involved in oocyte viability and current trends in gamete preservation.

Keywords: cryoprotectors, oocyte cryopreservation, oocyte viability.

1. Introduction

Although oocyte cryopreservation represents one of the main objectives of the reproductive medicine and cryobiology in the last years, until now, regardless of the studied specie, there is no freezing protocol that will assure satisfactory survival rates after thawing. Oocyte cryopreservation is now one of the most problematic issues in cryobiology aria, especially because they are extremely sensitive to low temperatures, and the maintaining of a normal development potential after thawing is very difficult.

Mammalian oocytes are more sensitive to low temperatures compared with the embryos [1]. One of the reasons for which the survival rates are lower after thawing the oocytes is that they contain a higher water quantity, also inside the oocyte cytoplasm there are highly specialized structures, like the meiotic spindle with microtubules and microfibers, sensitive to the artificial environment conditions (temperature changes, osmolality). In order to successfully apply a freezing procedure to the oocytes, the meiotic spindle integrity must be maintained. If this is affected during the freezing procedure, than the chromosomes chromatids cannot be separated properly during the second division of the gametes. This leads to a disruption of the normal fecundation process, the abnormal dispersion of the chromosomes, a high incidence of the aneuploidy or polyploidy and the perturbation of the normal development process [2].

Recently, the fast freezing methods (vitrification) is assuring higher survival rates of the oocytes after thawing, compared with controlled freezing [3]. Vitrification is a rapid freezing method that is becoming more popular. The main principle is based on the fact that some solutions, register an excessive increase in viscosity when they are cooled, and they solidify without crystallization.

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The nonechilibrated freezing of the cells require the use of high concentrations of cryoprotective agents (4 to 8 M), a short exposure time (30 sec. – 1 min) and high cooling rates from 500°C/min to 20.000°C/min. The standard cooling rates are between 500°C – 3.000°C/min, and can be achieved if standard, 0.25 mL straws are used. The main drawback of the vitrification is the high concentration of cryoprotector necessary, which is toxic for the cells [4, 5, 6, 7, 8].

Oocyte cryopreservation is much more difficult, compared with embryo, sperm of cell cryopreservation, mainly because of the fact that the oocytes are one of the bigger cells in the organism and in cryobiology the size is negatively correlated with survival rate after thawing [9]. Cell division after the first week following fecundation, leads to a reducing of the cell volume and a reducing of the water content, also of the cryoprotective agent necessary. An embryo in blastocyst sage can have over 100 cells, meaning a lower individual cell volume [9, 10, 11]. More over the fact that embryos have a higher number of cells, is assuring an advantage for freezing, an embryo can survive even if 50% of the cells are damaged [11].

Besides the shape of the oocyte, there are other factors involved in oocyte survival after cryopreservation: depolimerisation of the microtubules of the meiotic spindle, damage to the spindle and the defective alignment of the chromosome, lesions to the cell membrane, the osmotic shock, the early expel of the cortical granules and the hardening of the pelucida zone etc., [1, 2, 9, 12].

From the factors involved in viability of the oocytes after thawing the main one are: ice crystal forming, osmotic dehydration of the cell and the toxicity of the cryoprotectors [6, 13, 14].

The present paper is aiming to review the historic of oocyte cryopreservation, the main factors involved in oocyte viability and current trends in gamete preservation.

2. The influence of the cryoprotective agents of the in vitro fertilization rates of the oocytes:

The shape and the dimension of the oocytes require higher concentrations of penetrating cryoprotectors. In order to facilitate the penetration of the cryoprotectors in the cells and to reduce the concentration, the freezing media use mixtures of penetrating a non penetrating cryoprotectors. The most used nonpenetrating cryoprotector is sucrose, although trehalose seems to assure the higher survival rates [9,15].

The toxic effect of the cryoprotectors, on the viability of the cells is stated in specialty literature but the mechanism thru which it accts is not fully known. Huang et. al. (2006) has performed studies that are suggesting that the toxicity of the cryoprotectors is due to specific and nonspecific effects like dehydration and chemical cytotoxicity [2]. Mouse oocytes exposed for 20 minutes at a 1 M sucrose solution have normal morphologic appearance but could not be fertilized [3]. The low fertility rates of the oocytes can be caused by the perturbation of the division spindle of the second meiosis division. It is assumed that the disruption of the division spindle is caused by the osmotic stress. In cryoprotective solutions, because of the higher osmotic pressure, a dehydration of the cells takes place, which leads to disturbing the cell cytoskelet. Moreover, some studies showed that treating the oocytes with a hypotonic solution can induce the release of the cortical granules and a precocious hardening of the pellucid zone. The studies performed by Huang et. al. (2006) showed that the maxim osmotic tolerance of the oocytes exposed to a 2 M solution of sucrose is 3 minutes, despite the fact that oocytes appear normal even after 20 minutes of exposure [2].

With the advances of the studies in cryobiology, more and more cryoprotectors are used. In figure 1, we represented a toxicity scale of the cryoprotectors from the least toxic to the more toxic [14].

Figure 1. The toxicity of the cryoprotectors (after Moore K. et.al. 2006)

The toxicity scale is relative because it varies with the specie, the developmental stage and the exposure time. For example: ethylene glycol is presented as the least toxic, also it has a high rate of penetration into the cells which is desirable, because it implies a shorter exposure time prior to

freezing and it has a high rate of leaving the cell [16]. Interestingly is that ethylene glycol has a low vitrification capacity and, usually, is used in combination with other cryoprotective agents, more fervently with DMSO [17]. The studies performed on the embryos are showing that ethylene glycol is the least toxic of the cryoprotectors. For oocytes cryopreservation it has been demonstrated that the toxicity of the ethylene glycol is directly related with concentration, temperature and exposure time. The exposure of the oocytes at a solution of 30% ethylene glycol, at room temperature, for 10 minutes, had no effect on the morphology of the oocytes, 100% from the oocytes had normal morphologic aspect, but when the temperature of exposure was 30°C, only 24% of the oocytes were normal [2].

From the penetrating cryoprotectors glycerol is the most used for embryo cryopreservation. For oocytes cryopreservation, glycerol is no recommended because it has low permeably through membranes. Exposing the oocytes to a solution of 1 M glycerol has no effect on the fertility rates but when the concentration is raised to 2 M, the fecundity rated decreases significantly, demonstrating that glycerol is not recommended [2].

For cell cryopreservation, the most used crioprotector is DMSO (dimethyl sulfoxide), especially because it's high permeability. Studies performed by Huang et. al. (2006), showed that the oocytes exposed to DMSO 1M, have no decreases in fertility rates. When the concentration of DMSO is higher, 2M the fecundity rates decrease, but the studies of Huang et. al. (2006), showed that DMSO has a stabilization role for the microtubules in oocytes cytoplasm [2].

1,2 propandiol is a cryoprotective agent that was rarely used in embryo or cell cryopreservation but is more used for oocyte cryopreservation. This is used mainly for its high permeability through cell membranes. The studies in literature state that 1.2 pronandiol (PROH) is inducing the partenogenetic activation of the oocytes [18]. Huang et. al. (2006), showed that the fecundity rates and the blastocyst formation rates are lower in the case of oocytes that are exposed to a concentration of 1.5 M PROH [2].

3. The influence of the cryopreservation method on the fecundity rates of the oocytes

The cryopreservation of the oocytes has become part of the Assisted Reproductive Technologies (ART) together with the embryo cryopreservation. Generally the cryopreservation methods are divided into two categories: controlled freezing (classic or equilibrated) and rapid (vitrification or nonequlibrated) [19].

Controlled freezing is equilibrated freezing of the cells, suspended into a solution that contains small quantities of cryoprotectors (usually 1.5 M), with a low temperature decreasing rate, between 0.3 and 2 °C/min. until approximately -30° or -40°C and immersing it directly into liquid nitrogen (-196°C). For thawing the temperature increasing rate is approximately the same as the one in freezing, than the cryoprotective agent is removed from the cells [4, 6, 20, 21]. Controlled freezing is rarely used for oocytes. In the specialty literature it is stated as being used for freezing human oocytes, so in 2003, Fosas N. et. al., reported the birth of 4 children from oocytes recovered from donator females. After recovery the oocytes were controlled freeze with 1,2 propandiol (1.5 mol./L) and sucrose (0.3mol./L) [22]. Dessolle L. et. al. (2009) obtained survival rates of 70% for controlled frizzed human oocytes and 80% fecundity rate [23].

Vitrification is nonequilibrate freezing through which oocytes exposed at high concentrations of cryoprotectors (4 and 8 M), for a short period of time (30 sec.-1 min.), with temperature decreasing rates from 500°C/min. to 20.000°C/min.

Vitrification is the process through which the biologic material is transformed into solid state (at -196°C) without ice crystal formation, in less than one second. The rapid chance of aggregation state, makes that the biologic processed to be instantaneous stopped, which assures an optimization of the preservation process. In other order of idea, vitrification is arresting the biological processes, without disturbing the natural order of living cells [24].

Hettig A. et. al. (2010) performed immature swine oocyte vitrification by OPS (Open Pulled Straw) method, a technique using thinner wall straw that allows for higher temperature decrease rate, and obtained 46,30% viable cells, for a concentration of 45% Ethylene Glycol in the vitrification media [25].

Saeed Zavareh et. al. (2009) performed studies to establish the influence of the vitrification method and the time of exposure on to the fecundation rate of the oocytes and embryo development. They used OCC (oocyte cumulus complex), and tested two vitrification methods. After the experiments, for the denuded oocytes the best survival rate was obtained after cryotop vitrification (63.9%) compared with straw vitrification (56.8%). The survival rates were expressed depends the percent of oocytes that reached MII stage, after thawing. For OCC, 64.5% survived after cyotop vitrification and after 0.25 mL straw vitrification, with 5 minutes exposure time, 56.2% from the oocytes were appreciated as viable [26].

4. The influence of the maturation stage of the oocyte on to the in vitro fecundity rate

Oocytes can be frozen in different developmental stage: mature oocytes in metaphase II (MII) and immature oocytes, in germinal vesicle stage (GV). The freezing protocol varies depending of the maturation stage of the oocyte.

Mature oocytes, in metaphase II of the cell division have passed the nuclear and cytoplasmatic maturation, the first polar body was eliminated, and the chromosomes were condensed and attached at the division spindle filaments. Mature oocyte freezing is accomplished with difficulty because of the fact that the mature oocytes have a short fertile life and are sensitive to the lesions that are caused by the low temperatures. From the methods of freezing the mature oocytes, vitrification has proven to be the best method [27]. During freezing the mature oocytes are more sensitive, because of the division spindle that can be disturbed and aneuploidy can set in. The cytoskelet of the oocyte can be disturbed by the ice crystals or some cryoprotectors like DMSO [28]. In literature there are dates that suggest that cryoprotectors are involved in the passive influx of the Ca++ ions, which leads to phospholipaze, protease, ATP-ase and endonuclease activation, altering the integrity of the membranes and to denaturation of the cytosolic proteins and starting of the apoptosis [27, 29]. The presence or absence of the cumulus cells is one of the factors that influence the survival of oocytes. The partenogenenetic activation of the oocyte is also one of the effects of the cryoprotector on to mature oocyte, a solution of 2 M cryoprotectors can rise the percent of pertenogenetic activated oocyte significantly compared with controlled groups [2].

Immature oocytes (germinal vesicles, GV) are oocytes in diploten state on the prophases of the first meiotic division. After recovery, immature oocytes need to undergo a maturation stage for fertilization. In literature there are noted higher survival rates compared to the mature oocytes. The resistance of the mature oocytes to low temperature can be due to the fact that the cytoplasm is not that highly specialized. Garg N. and G.N. Purohit (2007), performed experiments in order to fast freeze the immature oocytes, after thawing and in vitro maturation, 31.49% of the oocytes matured in vitro, after vitrification and thawing [30].

The fact that the immature ooctytes must pass through a maturation stage, after thawing, leads to obtaining some fecundation percents comparable to the one obtained after freezing the mature oocytes. The studies performed by Garg N. and G.N. Purohit (2007), are underlying some survival rates of 94.0%, for the immature oocytes. The evaluation of the survival rate was performed by morphologic evaluation. After thawing oocytes were matured in vitro, the percent of oocytes mature obtained was 41.89% [30]. The immature oocytes are less sensitive to cryoinjueries because of their smaller dimension. The fact that they need a longer cultivation period until fecundation it might provide the time necessary for reparation of the cytoskelet [27].

Immature oocytes cryopreservation can be performed by *ovarian tissue freezing,* but the technique is still in early stage. The main disadvantage of this technique is that the tissue comports differently at freezing compared to individual cells. Also during thawing there is the risk of recrystallization of the media and forming large ice crystals [27, 31].

Haidari et al. (2007), studied the ultrastructural characteristics of the mature oocytes, maured in vitro after vitrification. For the study, they recovered ovaries from the mouse females 14 days old. A part of the ovaries were cultured in vitro and they were vitrified. After tissue thawing, the immature oocytes were recovered from the ovaries by mechanic methods and they were matured in vitro. The results showed that immediately after thawing there were some ultrastructural modifications, which disappeared after 4 days of

in vitro culture. The results obtained by Haidari et. al. (2007), suggest that the in vitro cultivation allows sufficient time for repairing some problems encountered in freezing/thawing [32].

5. Current trends in oocyte preservation

Most of the freezing methods used for oocytes cryopreservation are protocols developed and tested on embryos. The oocyte cryopreservation is more difficult, compared with embryo because of the shape and the physiology of the oocytes [9, 10, 11, 33].

Leibo S.P. 2008, suggest that the developing of a method that is specially developed for oocytes is needed, because the cryoprotectors acts different with the membrane of the oocyte compared with the embryos. The shape of the oocyte, almost perfect sphere, makes the distribution of the cryoproetctant not to be equal. At freezing there is a larger quantity of cryoprotector in the exterior of the cell and at thawing the cryoprotector remains a longer period of time in the inside of the oocyte, being more toxic [9].

Although, in the last 30 years there were significant improvement made to the freezing technique of the oocytes, at the world level there is an interest in getting better results [6].

The reducing of the toxicity of the cryoprotecors and the evaluation of the effect of the cryoprotectors on to the physiology of the cells is also an insufficient researched problem in oocytes cryobiology.

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References

1. Stachecki, J. J, Cohen, J., An overview of oocyte cryopreservation. Reprod Biomed, 2004, 9, 152–163
2. Huang Jack, Y. J., Hai-Ying Chen, Seang Lin Tan si Ri-Cheng Chian, Effects of Osmotic Stress and Cryoprotectant Toxicity on Mouse Oocyte Fertilization and Subsequent Embryonic Development In Vitro, Cell Preservation Technology, 2006, 4, 3
3. Kuleshova, L. L., MacFarlane, D. R., Trounson, A.O., Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. Cryobiology; 1999, 38, 119–130
4. Rall, W. F., and Fahy Ice Free Cryopreservation of mouse embryo at – 196 C by Vitrification, Nature 1985, 313, 73-575
5. Leibo, S. P., si Mazur, P., Methods for the preservation of mammalian embyos by freezing. Methods in Mammalian Reproduction, Jr. Academic Press, New York 1978, 179-201
6. Leibo, S. P., Cryopreservation of oocytes and embryos: Optimization by theoretical versus empirical analysis, Theriogenology, 2008, 69 37–47
7. Andrabi, S. M. H., Maxwell, W. M. C., A review on reproductive biotechnologies for conservation of endangered mammalian species, Animal Reproduction Science, 2007, 99 223–243
8. Khosravi Farsani Somaye, Reza Mahmoudi, Mir Abbas Abdolvahhabi, Mehdi Abbasi, Fateme Malek, Aligholi Sobhani, Comparing the viability and in vitro maturation of cumulus germinal vesicle break down (GVBD) oocyte complexes using two vitrification techniques in mice, Iranian Journal of Reproductive Medicine, 2007, 5, 4. 165-170
9. Kuwayama, M., Oocyte Cryopreservation, J. Mamm. Ova.Res., 2007, 24, 2-7
10. Vanderzwalmen, P., Bertin, G., Debauche Ch. Standert, V., Van Roosendaal, E., Vandervorst, M., Bollen, N., Zech, H., Mukaida, T., Takahashi, K., si Schoysman, R., Births after morula and blastocist stages: efect of artificial reduction of the blastocelic cavity before vitrification, Hum. Reprod., 2002, 17, 744-751
11. Hiraoka, K., Kinutani, M., Kinutani, K., Blastocele collapse by micropipetting prior to vitrification gives ecellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocyst, Hum. Reprod., 2004, 19, 2884-2888
12. Fabbri, R., Porcu, E., Marsella, T, Rocchetta, G., Venturoli, S. si Flamigni, C., Human oocyte cryopreservation: new perspectives regardin oocyte survival, Hum. Reprod., 2001, 16, 411-416
13. Ghasem Saki, Fatemeh Ghalambor Dezfuly, Vitrification of human oocyte using cryoloop, Iranian Journal of Reproductive Medicine, 2005, 3, 1, 19-24
14. Moore Karen, Aline Quadros Bonilla, Cryopreservation of Mammalian Embryos: The State of the Art, ARBS Annual Review of Biomedical Sciences, 2006
15. Larman Mark, G., Courtney B Sheehan and David K Gardner, Calcium-free vitrification reduces cryoprotectant-induced zona, pellucida hardening and increases fertilization rates in mouse, oocytes, Society for Reproduction and Fertility, 2006, 1741–7899

16. Shell, A., Shelton, J.N., Szell, K., Osmotic characteristics of sheep and cattle embryos. Cryobiology; 1989, 26, 297-301
17. Valdez, C.A., Abas Mazni, O., Takahashi, Y., Fujikawa, S., Kanagawa, H., Successful cryopreservation of mouse blastocysts using a new vitrification solution, J Reprod Fertil; 1992, 96,793-802
18. Van der Elst, J,, Van den Abbel, E., Nerinckx, S., Parthenogenetic activation pattern and microtubular organization of the mouse oocyte after exposure to 1, 2-propanedior. Cryobiology; 1992, 29,549–562
19. Barbara Gajda, Factors and methods of pig oocyte and embryo quality improvement and their application in reproductive biotechnology, Reproductive biology, 2008, 9, 2
20. Vajta, G., Zsolt Péter Nagy, Are programmable freezers still needed in the embryo laboratory, Reprod Biomed., 2006, 12, 779-96
21. Van Wagtendonk de Leew A. M., den Daas J. H. G., Rall W. F., Field trial to compare pregnancy rates of bovine embryo criopreservation methods: vitrfication one step dilution verses slow freezing and three step dilution. Theriogenology, 1997, 48, 1071-1084
22. Fosas, N., Marina, F., Torres, P. J.; Jove, I., Martin, P., Perez, N.; Arnedo N., Marina S., The Births Of Five Spanish Babies From Cryopreserved Donated Oocytes, Human reproduction, 2003, 18, 7, 1417-1421
23. Dessolle, L., De Larouziere, V., Ravel, C., Berthaut I.; Antoinej-M., Mandelbaum J., Congélation lente et vitrification des ovocytes humains matures et immatures, Gynécologie obstétrique & fertilité, 2009, 37, 9, 712-719
24. Kasai, M., Mukaida, T., Cryopreservation of animal and human embryos by vitrification. Reprod Biomed Online, 2004, 9,164-70
25. Hettig Andrea, Vasile Miclea, Marius Zăhan, Iulian Roman, Florin Varo-Ghiuru, Alexandru Rusu, Results Concerning Immature Swine Oocytes Vitrification using Different Concentration of Cryoprotectant, Bulletin UASVM Animal Science and Biotechnologies, 2010, 67(1-2), 486
26. Saeed Zavareh, D.V.M., Mojdeh Salehnia, Adel Saberivand, Comparison of Different Vitrification Procedures on Developmental Competence of Mouse Germinal Vesicle Oocytes in the Presence or Absence of Cumulus Cells, International Journal of Fertility and Sterility, 2009, 3/3:111-118;
27. Singh Neeta, Suneeta Mital, Cryopreservation of Oocytes and Ovarian Tisuue, JK Science, 2009, 11(1)
28. Vincent, C., Pruliere, G., Pajot-Augy, E., Effects of cryoprotectants on actin filaments during the cryopreservation of one-cell rabbit embryos. Cryobiology, 1990, 27:9–23
29. Wang, W., Yan, Z. J., Cai, L. B., Zhonghua Yi Xue Za Zhi, Clinical effects of oocyte cryopreservation in assisted reproduction technology, 2008, 88(39), 2755-58
30. Garg, N., Purohit, G. N., Effect of different cryoprotectant concentrations for ultrarapid freezing of immature goat follicular oocytes on their subsequent maturation and fertilization in vitro, Anim. Reprod., 2007, 4(3/4) ,113-118
31. Tao, T., si Del Valle, A., Human oocyte and ovarian tissue cryopreservation and its application. J Assist Reprod Genet.; 2008, 25(7), 287-96
32. Haidari Kamran, Mojdeh Salehnia, Mojtaba Rezazadeh Valoujerdi, Ultrastructural Characteristics of Mouse Preantral Follicles Isolated from Vitrified and Non Vitrified Ovaries after In Vitro Maturation, Yakhteh Medical Journal, 2007, 9/3
33. Siqueira-Pyles E. S. C., Landim-Alvarenga, F. C., Effect of meiotic stage on viability and developmental competence of bovine oocytes vitrified in TCM-199 and bovine follicular fluid, Anim. Reprod., 2005, 2/1, 63-69