CryoLetters 43(4), 222 – 226 (2022)

https://doi.org/10.54680/fr22410110712

USE OF BIOLOGICAL AND SYNTHETIC POLYMERS FOR HUMAN SPERMATOZOA CRYOPRESERVATION

Maryna Petrushko[1,2] and Taisiia Yurchuk[1*]

[1]Institute for Problems of Cryobiology and Cryomedicine (IPC&C) of the National Academy of Sciences of Ukraine, 23, Pereyaslavska Str., Kharkiv 61016, Ukraine.
[2]ART-Clinic of Reproductive Medicine, 38B, Gagarina av., Kharkiv 61000, Ukraine.
Corresponding author's E-mail: taisiya.yur@gmail.com

Abstract

BACKGROUND: Spermatozoa cryopreservation is an integral part of the assisted reproductive technologies for treatment of infertility. It is also used to preserve the reproductive potential of men. However, using a standard freezing method with glycerol leads to a decrease in morphological and functional characteristics of spermatozoa in the case of oligoasthenoteratozoospermia (OAT). Therefore, it is relevant to develop effective methods of cryopreservation for such sperm. The use of various biopolymers can stabilize the membrane and bind excess water, which forms ice crystals in the medium that causes cell damage when temperature decreases. **OBJECTIVE:** To study the effectiveness of using cryoprotectant mixtures based on biological and synthetic polymers [serum albumin, polyvinylpyrrolidone (PVP) and insulin] for the cryopreservation of human spermatozoa with OAT. **MATERIALS AND METHODS:** Human spermatozoa with OAT were cryopreserved using different cryoprotectant media containing 10 % glycerol or 10 % PVP, 20 % albumin and 1 μg/mL human insulin. The viability, motility and mitochondrial membrane potential of spermatozoa were assessed after rewarming. **RESULTS:** A cryoprotectant solution containing 10 % PVP, 20 % human serum albumin and 1 μg/mL insulin enabled a similar level (%) of viable gametes compared with the standard method using glycerol, while the number of motile cells was significantly lower ($p < 0.008$). The membrane mitochondrial potential did not differ significantly from fresh sperm. **CONCLUSION:** The data obtained in this study show the effectiveness of a biopolymer mixture containing PVP, serum albumin and insulin for the cryopreservation of human OAT spermatozoa.

Keywords: cryopreservation; insulin; oligoasthenoteratozoospermia; polyvinylpyrrolidone; serum albumin; spermatozoa.

INTRODUCTION

Cryopreservation of cells, tissues and organs is an important part of modern biotechnology and transplant medicine (1). Low-temperature preservation has upgraded the effectiveness of infertility treatment programs to a new level and has become especially important in assisted reproductive technologies (ART). Modern methods of spermatozoa cryopreservation are based on the use of organic solvents such as glycerol, 1,2-propanediol or dimethylsulfoxide (DMSO) as cryoprotectants. However, these permeable cryoprotectants have cytotoxicity and, moreover, mutagenic activity (2). Therefore, they must be removed from the cells after warming by double centrifugation and change of the media. However, these manipulations negatively affect the morphology and functional spermatozoa characteristics (3).

Human spermatozoa with spermatogenesis defects such as oligoasthenoteratozoospermia (OAT) are particularly sensitive to the effects of cryopreservation and cryoprotectant removal (4). Therefore, developing alternative methods of cryopreservation of such cells is relevant.

We assumed that the most promising OAT spermatozoa freezing method should include non-removed cryoprotectant media. Therefore, the search for suitable cryoprotectants was aimed at non-permeable substances. Biological and synthetic polymers exhibit cryoprotective properties due to the synergistic effect by improving the stability of the amorphous state of solutions, suspending devitrification and forming a matrix around the cell membrane, and thus protect cells from osmotic stress (5). Such properties are found in serum albumin (SA), polyvinylpyrrolidone (PVP) and insulin, which, moreover, are widely used in ART. Therefore, the aim of our work was to determine the effectiveness of using cryoprotectant mixtures (CM) of biological and synthetic polymers for cryopreservation of spermatozoa of men with OAT.

MATERIALS AND METHODS

All studies were performed in accordance with the rules of biomedical ethics following the order of the 09.09.2013 Ministry of Health of Ukraine N° 787, the principles of the Helsinki Declaration of Human Rights, the Union Convention on Human Rights and Biomedicine, ESHRE and ARSM recommendations, and agreed by the Bioethics Committee of IPC&C (N° 1, 2021). Written informed consent was obtained from patients for spermatozoa handling.

Eighty-eight ejaculates of men diagnosed with OAT were used in the study. The active motile spermatozoa fraction was obtained using the «swim up» technique by centrifugation in a two-layer density gradient (Sperm Gradient Kit (Cook, USA)).

The spermatozoa obtained from semen were divided in groups depending on the composition of cryoprotectant mixtures, CM: group 1 - 10 % glycerol solution (Sigma-Aldrich, USA); group 2 - 10 % PVP solution (Cook, USA); group 3 - 10 % PVP solution and 20 % SA (Cooper Surgical, USA); group 4 - 10% PVP solution and 1 μg/mL human insulin (Sigma-Aldrich, USA); group 5 - 10 % PVP solution, 20 % SA and 1 μg/mL human insulin. Group 6 consisted of fresh spermatozoa.

Spermatozoa viability and morphology assessment

The viability of male gametes was determined by eosin-nigrosine staining (6).

Spermatozoa morphology was evaluated according to WHO recommendations (7). One-hundred cells were counted in each sample using a confocal scanning microscope Carl Zeiss LSM 510 Meta (Carl Zeiss, Germany) at x 6000 magnification. LSM Image Examiner and AxioVision Rel. 4.7 (Carl Zeiss, Germany) were used for image analysis.

Spermatozoa mitochondrial membrane potential measurement

Mitochondrial membrane potential (MMP) was determined using lipophilic cationic dye 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolecarbocyanine iodide (JC-1) according to manufacturer's instructions (Sigma, USA). Flow cytograms were obtained using FACS Calibur (BD Bioscience). The samples were excited by a laser with a wavelength of 488 nm, and the emitted light was collected using emission filters at 530 nm (green fluorescence: FL1, dye monomers) and at 585 nm (red fluorescence: FL2, dye aggregates). Data analysis was performed using WinMDI 2.9. Change in the relative number of cells with double fluorescence were evaluated.

Spermatozoa cryopreservation

Spermatozoa were frozen using a two-stage cooling method by adding CM to the suspension of sperm in a ratio of 1:1. After incubation for 10 min, the samples were placed at the distance of 15 cm over the nitrogen mirror for 15 min, followed by immersion into LN_2 (-196 ºC). Cryovials were stored in LN_2 from one month to one year. Rewarming of cells was carried out using a water-bath at 40 °C for 10 min until complete disappearance of the solid phase. Spermatozoa of group 1 were washed out twice by centrifugation after rewarming according to standard freezing procedure before fertilization by intracytoplasmic sperm injection (ICSI). Spermatozoa of groups 2-5 were assessed immediately after rewarming since they do not need to be washed before ICSI.

Statistical analysis

Analysis of experimental data was performed using the software package Origin 8.5 (OriginLab Corporation, USA). For parameters with a normal distribution, data were given as the mean ± SD, and the t-test was used for independent samples. For a larger number of groups, the hypothesis of equality of means was tested using one-way analysis of variance (ANOVA). If the distribution of the studied parameters differed from normal, the Mann-Whitney test (U-test) was used to compare the two independent groups; to compare a larger number of independent groups we used a nonparametric analogue of one-way analysis of variance, the Kruskal Wallis test (H test). In multiple comparisons of values of different groups, the Mann-Whitney test (U test) for pairwise comparisons was used taking with Bonferroni correction.

The critical significance level (p) for all statistical criteria was set at 0.05, except in the case of multiple comparisons. For the U test in the case of multiple comparisons, p was adjusted by Bonferroni correction to 0.05 / m, where m is the number of different groups involved in pairwise comparisons.

RESULTS

After incubation of OAT spermatozoa with CM, it was found that cell viability did not change compared to the control, while motility decreased significantly in all samples with PVP (Fig. 1). This can be explained by the high viscosity of these solutions.

After rewarming, cell viability slightly decreased in all groups compared with the control (group 6), i.e., the fresh spermatozoa fraction (Fig. 2). At the same time, a significant decrease in motility was observed in the studied groups 1-5 compared to group 6: 24.5 ± 3.2; 43.3 ± 3.9; 44.1 ± 5.1; 43.9 ± 4.2; 45.2 ± 4.7; and 85.4 ± 6.9 %, respectively.

Thus, the results showed that using PVP in cryoprotectant media has advantages over the standard glycerol media in cryopreservation of spermatozoa of men with OAT, as maintaining a higher level of motile cells after rewarming was possible. However, it should be noted that, in these groups, the motility of the cells with PVP was significantly lower compared to fresh cells.

Because the proposed CM are characterized by high viscosity and they slow down the kinetic activity of sperm, their functional activity can be assessed by determining the level of MMP.

After isolation of the motile sperm fraction,

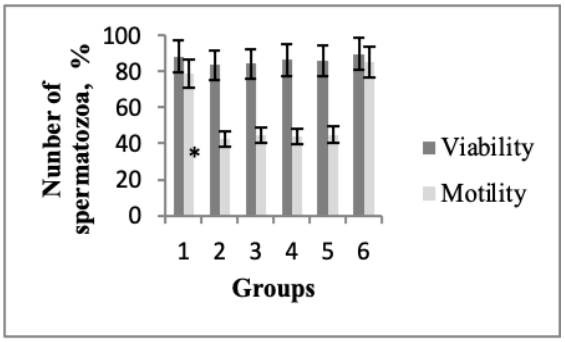

Figure 1. Viability and motility of spermatozoa after incubation with different cryoprotectant media. * – the difference is significant compared with the group 6 control (p < 0.008).

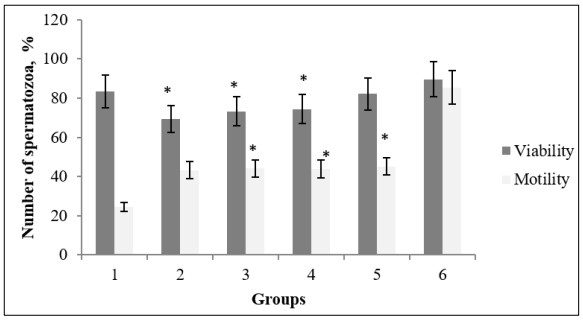

Figure 2. Viability and motility of spermatozoa after cryopreservation with different cryoprotectant media. * – the difference is significant compared to the group 6 control (p <0.008), # – the difference is significant compared to the motility of sperm in groups with PVP (p < 0.001).

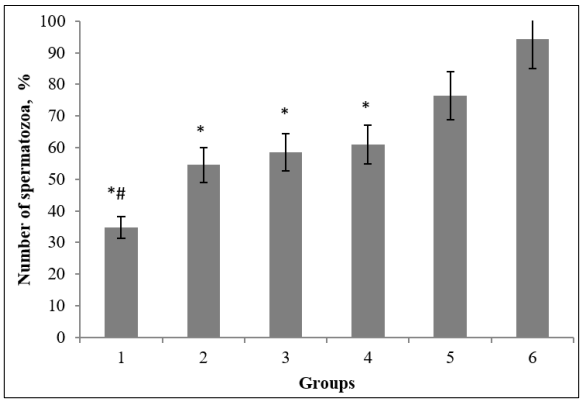

Figure 3. The effect of cryopreservation on the numbers of spermarozoa with high MMP. * – the difference is significant compared to the group 6 control (p < 0.008). # – the difference is significant compared to the motility of sperm in groups with PVP (p < 0.001).

the number of cells with high MMP was 94.9 ± 7.6 %, i.e., for the control group 6 (Fig. 3). The number of spermatozoa with high MMP decreased significantly after cryopreservation in groups 1-4 to 34.7 ± 4.2; 54.5 ± 4.2; 58.6 ± 2.3; 61.1 ± 3.1 %, respectively (Fig. 3). In group 5, this parameter did not differ from the control group and was 76.4 ± 6.7 %.

DISCUSSION

Cryopreservation of reproductive cells is an important tool in modern biotechnology and medicine. The traditional cryopreservation methods are slow cooling and fast freezing but these lead to a decrease in motility and an increase in peroxidation and vacuolization, and DNA fragmentation rates in OAT spermatozoa (8).

Recently vitrification was identified as new cryopreservation effective method for OAT spermatozoa (9). Nevertheless, this method includes removal of the cryopreservation media with sucrose prior to the fertilization procedure, which can cause additional damage to the gametes.

The cryopreservation methodological approach for sperm involves the addition of significant amounts of organic solvents as cryoprotectants. The key cause of cell death after cryopreservation is recrystallization, which mainly occurs during warming (10). It has been shown that adding polymers of both synthetic and biological origin, which inhibit ice recrystallization, to solutions containing low concentrations of permeable cryoprotectants, increases survival of cells up to 75 % (11). Biological and synthetic polymers can inactivate the growth of ice crystals in the extracellular space, and limit their size (12). This mechanism of protection likely explains the results observed in our experiments. The addition of polymers to aqueous solutions significantly affects not only the formation of ice but also the inhibition of its recrystallization (13).

The intracellular cryoprotectants such as glycerol, DMSO, and 1,2-propanediol have become widely used in clinical practice, and the cryoprotective properties of polymers have been reported in many recent scientific papers (14). The use of polymers can reduce the concentration of permeable cryoprotectants, in particular glycerol (15).

PVP belongs to the class of artificial polymers and is a product of polymerization of N1-vinylpyrrolidone and acetylene. The PVP molecule has hydrophilic-hydrophobic properties. In aqueous solutions, these molecules have a chaotic spiral configuration, which allows them to hydrate a sufficient number of H_2O molecules (16). Due to the high hydration properties of PVP, the nature of freezing solutions changes. The crystallization process occurs in a lower temperature range (17). PVP solutions have high adsorption properties to water and to various substances and the ability to form complexes with medical preparations, toxins, dyes and salts.

Also, PVP was chosen as a cryoprotectant agent due to the positive results obtained during cryopreservation of some cell types: peripheral blood lymphocytes, bone marrow cells of experimental animals and other biological objects (18). The presence of this polymeric substance in the cryopreservation medium contributes to the stable state of the cell membrane, which prevents the elimination of cell contents through the plasma membrane even under conditions of osmotic stress (19, 20).

In our studies, we first used a solution of PVP with a molecular weight of 360 kDa as a cryoprotectant medium for OAT men gametes. This biopolymer is widely used in the embryological stage of ART to facilitate micromanipulation with spermatozoa for immobilization during ICSI into the oocyte, which confirms the biosafety of its use for gametes. The CM used in the work contained PVP, SA and recombinant human insulin which are natural polymers and can positively affect the cryotolerance of spermatozoa.

SA is a water-soluble globular protein. The main physiological function of SA is the transport of proteins, metal ions, lipids and other metabolites through plasma (20). SA is thought to act as an extracellular cryoprotective compound that protects and binds to plasma membranes, particularly with phospholipid groups. Most freezing and warming solutions used for spermatozoa vitrification contain SA (21). J. Riel et al. reported a short-storage method of spermatozoa using electrolyte-free medium with glucose and SA (22). They suggested that SA provided cells with energy substrates during storage.

Adding insulin to the media for spermatozoa cryopreservation leads to protective effects. The use of this biopolymer can be

recommended for further clinical applications. Insulin at 1000 ng/ml increased spermatozoa motility and significantly reduces DNA fragmentation (23).

In our study, the number of spermatozoa with membrane integrity was almost identical to the number of gametes with high MMP.

It has previously been shown that mitochondrial activity and viability are equally sensitive to the damage caused by cryopreservation (24). However, there are no reports of spermatozoa cryopreservation with PVP and of the effect of this cryoprotectant on gamete MMP. Due to the immobilization effect of PVP on spermatozoa, it was important to assess the MMP of the cells after their cryopreservation with PVP. We have shown that the inclusion of PVP in the cryoprotectant medium has a visible protective effect on spermatozoa membrane integrity and MMP.

CONCLUSION

The results showed that the use of a cryoprotectant solution containing 10 % PVP, 20 % SA and 1 μg/mL insulin for the cryopreservation of spermatozoa from OAT men allows the retention of viability and the number of cells with high mitochondrial membrane potential at the level of fresh gametes ($p < 0.008$). The data obtained showed the effectiveness of biopolymer mixtures (PVP, SA and insulin) for the cryopreservation of OAT spermatozoa.

Acknowledgments: The authors are grateful to Dr Elena Pavlovich and Dr Anna Gapon (IPC&C) for the help with the research.

REFERENCES

1. Buriak I, Fleck RA, Goltsev AM, Shevchenko N, Petrushko M, Yurchuk T, Puhovkin A, Rozanova S, Guibert EE, Robert MC, de Paz LJ, Powell-Palm MaJ & Fuller B (2020) *Med Devices* **14**, 010801.
2. Kopeika J, Thornhill A & Khalaf Y (2015) *Hum Reprod Update* **21**, 209-27.
3. Pavlovych O, Hapon H, Yurchuk T, Repin M, Marchenko L, Govorukha T & Petrushko M (2020) *Probl Cryobiol Cryomed* **30**, 24–33.
4. Yurchuk T, Petrushko M, Gapon A, Piniaiev V & Kuleshova L (2021) *Cryobiology* S0011-2240 (21) 00059-6.
5. Deller RC, Vatish M, Mitchell Dam & Gibson MI (2014) *Nat Commun* **5**, 3244.
6. Björndahl L, Söderlund I & Kvist U (2003) *Hum Reprod* **18**, 813-816.
7. WHO (2010) WHO *Laboratory Manual for the Examination and Processing of Human Semen* (5th ed.), WHO Press, Geneva, 287 pp.
8. Yurchuk TO, Pavlovich OV, Gapon G O, Pugovkin AY & Petrushko MP (2021) *Ukr Biochem J* **93**, 24-29.
9. Isachenko V, Maettner R, Petrunkina AM, Sterzik K, Mallmann P, Rahimi G, Sanchez R, Risopatron J, Damjanoski I & Isachenko, E (2012) *J Androl* **33**, 462-468.
10. John Morris G, Acton E, Murray BJ & Fonseca F (2012) *Cryobiology* **64**, 71-80.
11. Gibson MI (2016) *Biomater Sci* **4**, 1079-1084.
12. Salian SR, Kalthur G, Uppangala S, Kumar P & Adiga SK (2012) *Fertil Steril* **98**, 321-325.
13. Biggs CI, Bailey TL, Ben Graham, Stubbs C, Fayter A & Gibson MI (2017) *Nat Commun* **8**, 1546.
14. Rajan R & Matsumura K (2018) *Adv Exp Med Biol* **1081**, 339-354.
15. Tekin K & Daşkın A (2019) *Cryobiology* **89**, 60-67.
16. Koczkur KM, Mourdikoudis S, Polavarapu L & Skrabalak SE (2015) *Dalton Trans* **44**, 17883-17905.
17. Chauhan H, Mohapatra S, Munt DJ, Chandratre S & Dash An (2016) *AAPS PharmSciTech* **17**, 640–651.
18. Li J, Hua TC, Gu XL, Ding Y, Luo M, Xiao HH, Wu ZJ, Meng LR, Gao QR & Chen J (2005) *CryoLetters* **26**, 193–200.
19. Skaer HB, Franks F, Asquith MH & Echlin P (1977) *J Microsc* **110(3)**, 257–270.
20. Francis GL (2010) *Cytotechnology* **62**, 1–16.
21. Agha-Rahimi A, Khalili MA, Nottola SA, Miglietta S & Moradi A (2016) *Andrology* **4**, 1037-1044.
22. Riel JM, Yamauchi Y, Huang TT, Grove J & Ward MA (2011) *Biol Reprod* **85**, 536-547.
23. Shokri S, Ebrahimi SM, Ziaeipour S & Nejatbakhsh R (2019) *Cryobiology* **87**, 68-73.
24. O'Connell M, McClure N & Lewis SE (2002) *Hum Reprod* **17**, 704–9.