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New method for cryoprotectant-free freezing of human oligoasthenoteratozoospremic spermatozoa with high-molecular polymer

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ABSTRACT

Data about cryoprotectant-free cryopreservation of human ICSI spermatozoa are limited. The aim of this investigation was to compare two technologies for cryopreservation of spermatozoa from men with oligoasthenoteratozoospermia: standard conventional freezing with 5% glycerol (freezing in glycerol) and cryoprotectant-free freezing with 5% high-molecular-weight (360 kDa) polyvinylpyrrolidone (PVP) (PVP-freezing). Capillaries with spermatozoa were cooled in vapor and then plunginged into liquid nitrogen. Head-, midpiece- and tail-abnormality of spermatozoa, mitochondrial membrane potential (MMP) and DNA fragmentation rates after cryopreservation were evaluated. After warming of spermatozoa, fertilization of oocytes (ICSI) was performed. It was detected the lower rate of morphological abnormalities of PVP-frozen spermatozoa in comparison with cells frozen with glycerol (34.6 ± 4.1% vs. 20.7 ± 4.7%, respectively) ($P < 0.05$). Quality of cells with high MMP after warming in spermatozoa frozen with glycerol was lower than in PVP-frozen spermatozoa (34.7 ± 4.2 vs. 54.5 ± 4.2%, respectively) ($P < 0.05$). It was established that the DNA fragmentation rate in PVP-frozen spermatozoa was significantly lower in comparison with spermatozoa frozen with glycerol (23.1 ± 2.5% vs. 38.8 ± 3.0%, respectively) ($P < 0.05$). After fertilization (ICSI) of oocytes, it was established that cleavage and blastulation rates were higher in oocytes after fertilization with PVP-frozen spermatozoa than with spermatozoa frozen with glycerol. Fertilization-, development to 8-blastomeres-, and blastocyst-rates were for PVP-frozen and spermatozoa frozen with glycerol, respectively: 94.4 ± 7.8 vs. 82.2 ± 6.2% ($P > 0.1$ with tendency to increasing), 90.0 ± 4.6 vs. 69.5 ± 5.1% ($P < 0.05$), and 45.4 ± 4.1% vs. 30.9 ± 3.3% ($P < 0.05$). It was concluded that permeable cryoprotectant-free freezing with 5% high-molecular-weight (360 kDa) polyvinylpyrrolidone can be applied successfully for cryopreservation of human oligoasthenoteratozoospremic spermatozoa.

1. Introduction

Whole assisted reproductive technology (ART) includes two important groups of methods: intracellular sperm injection (ICSI) for overcoming of infertility with pathology of spermatogenesis in men, and cryopreservation of spermatozoa [1].

Male gametes with abnormal spermatogenesis (oligoasthenoteratozoospremia) are extremely sensitive both to negative effect of permeable cryoprotectant and to cryopreservation in general [2,3]. Low quality of such spermatozoa can be explained by their not good morphological and kinetic characteristics, which are commonly associated with high DNA fragmentation [4]. Therefore, such spermatozoa require a special cryopreservation protocol. Along the routine methods for spermatozoa cryopreservation by conventional (slow) and rapid freezing, the vitrification method is used for accelerating of freezing process and avoiding of damages induced by ice crystals [5,6]. However, both conventional freezing and vitrification with permeable cryoprotectants presuppose removal of these cryoprotectants after warming to

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reduce their toxic effect. This step of manipulations with spermatozoa causes additional damage of spermatozoa [7]. Excluding of removal of these permeable cryoprotectants from the technology of cryopreservation can be characterized as very helpful.

The use of non-permeable cryoprotectants (mono-, di-, and trisaccharides as well as polymers with high molecular weight) does not suppose a removal of such cryoprotectants after warming.

The aim of this investigation was to compare two technologies for cryopreservation of spermatozoa in men with oligoasthenoteratozoospermia: standard conventional freezing with 5% glycerol and cryoprotectant-free freezing with 5% high-molecular-weight (360 kDa) polyvinylpyrrolidone (PVP), cooling in vapor of and then plunging of capillaries with spermatozoa into liquid nitrogen.

2. Materials and methods

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

All studies were performed in accordance with principles of the Helsinki Declaration of Human Rights, the European Union Convention on Human Rights and Biomedicine, ESHRE and ARSM recommendations, and agreed by the Institutional Bioethics Committee No 4, 2016 and No 1, 2021. Written informed consent was obtained from each participant.

Eighty eight ejaculates of men with a diagnosis of severe oligoasthenoteratozoospremia were object of this study. Sperm Gradient Kit (Cook Medical LLC, Bloomington, IN, USA) was used to obtain progressive motile fractions from aliquots of ejaculates. Sperm preparation was carried out by density-gradient separation according to the Sydney IVF, Cook Medical Manual followed by swim-up procedure.

2.1. Spermatozoa cryopreservation

Spermatozoa of each sample were divided into two aliquots, which were cryopreserved in different cryopreservation solutions: with 5% glycerol (cryoprotectant-included freezing) and with 5% polyvinylpyrrolidone (PVP) (cryoprotectant-free freezing).

Cryoprotectant-included as well as cryoprotectant-free freezing of spermatozoa were performed by two-stages method. Cryoprotectant medium (in the case of cryoprotectant-included freezing) was added to the 30 μl of progressive motile sperm fraction in a ratio of 1:1 with 5% of the glycerol end concentration. For cryoprotectant-free freezing, 10% PVP was added to the 30 μl of spermatozoa fraction in a ratio of 1:1 with 5% of end concentration of PVP.

Then polycarbonate capillaries for denudation of oocytes with diameter 170 μm (Cook Medical) was filled with 60 μl of obtained spermatozoa suspension using oocyte stripper (Cook Medical). Then capillaries were disconnected from stripper, and both free ends of capillaries were sealed with medical clay (Becton Dickinson Co., Franklin Lakes, NJ, USA). Then capillaries were packed into 5 ml cryovials (TermoFisher Sci., Schwerte, Germany) (5 capillaries per cryovial) and after 10 min exposure at room temperature these cryovials were placed at 15 cm above liquid nitrogen for 10 min. Then cryo-vials were plunged into liquid nitrogen.

For warming of spermatozoa, cryovials were opened in liquid nitrogen and capillaries were expelled and plunged into 40°C water bath for 30 s. Then both seal ends of capillaries were cut and spermatozoa suspension was expelled into 1.5 ml microtube (Eppendorf, Wesseling-Berzdorf, Germany).

Removal of cryoprotectant (glycerol) was performed by gradual addition of 60 μl Sperm Preparation Medium (Cook Medical) to the spermatozoa suspension with subsequent double centrifugation at 380g for 10 min. Then supernatant was removed and pellets were resuspended in the 50 μl of Sperm Preparation Medium for further analyse and fertilization of oocytes. Vitrified spermatozoa were used after warming without removal of cryoprotectant because no permeable cryoprotectants were used.

2.2. Spermatozoa evaluation

Spermatozoa characteristics were estimated by CASA system (Hamilton Thorne, Beverly, MA, USA) (Fig. 1). It was calculated spermatozoa concentration, total number and motility of cells: immotile, curvilinear velocity (VCL <5 μm/s), locally motile (VCL = 5–25 μm/s) and motile (VCL> 25 μm/s).

Spermatozoa viability and morphologically normal and abnormal forms were evaluated by eosin-nigrosin-staining (200 cells in each smear at 400 × with bright field microscopy (Fig. 2).

DNA fragmentation rate in samples after cryopreservation was assessed by chromatin dispersion technique using Halosperm® G2 test kit (Halotech, Madrid, Spain) and DNA Fragmentation software within the CASA II motility program (Hamilton Thorne). Cells with large (a halo width is equal to or more than a minor diameter of the core) and medium (a halo size is less than diameter of a minor core but greater than 30% of diameter of a minor core) halo were considered as cells without DNA fragmentation. Cells with no halo or small halo (a halo width is equal to or less than 30% of a minor diameter of core) were considered as cells with DNA fragmentation.

It was earlier considering that the functional activity of spermatozoa mitochondria, is correlates with cell motility [8]. For evaluation of this mitochondrial activity, it was measured changes in the mitochondrial membrane potential (MMP) using a lipophilic cationic dye 5.5′, 6, 6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolecarbocyanine iodide, commonly known as JC-1 [9]. This test was performed as per the manufacturer instructions for Mitochondrial Permeability Detection Kit AK-116 (BIOMOL International LP, Plymouth Meeting, PA, USA). The MMP was determined after incubation of 0.5×10^6 spermatozoa with 2.5 ml JC-1 solution for 15 min at 37°C in 5% CO_2. Then, the cell suspension was resuspended in 750 ml human tubal fluid (HTF) and centrifuged at 400g for 5 min at room temperature. After removal of supernatant, spermatozoa were analyzed using flow cytometry (BD™ LSR II flow cytometer, Franklin Lakes, NJ, USA).

Metaphase II stage oocytes were retrieved after ovarian stimulation using follicle-stimulating hormone (Gonal F®, Merck Serono Int., Genf, Switzerland) started on the second day of the menstrual cycle, gonadotropin releasing hormone (GnRH) (Cetrotide®, Merck Serono Int.) as an antagonist and GnRH agonist (Diphereline®, Beaufour Ipsen Internation, Paris, France) as ovulation inductor. Fertilization of oocytes was performed by intracellular spermatozoa injection (ICSI).

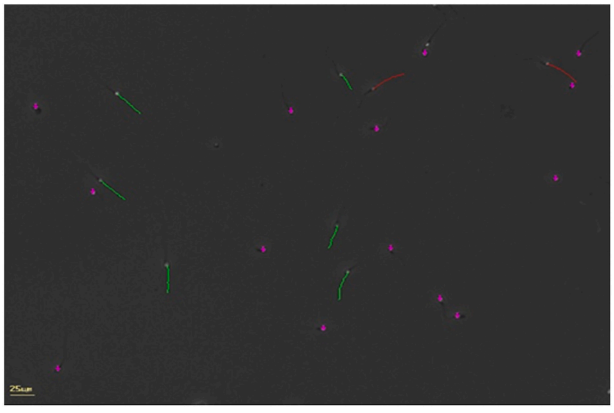

Fig. 1. Sperm tracks generated by CASA.
(red tracks) motile spermatozoa,
(green tracks) locally motile spermatozoa,
(violet points) immotile spermatozoa. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

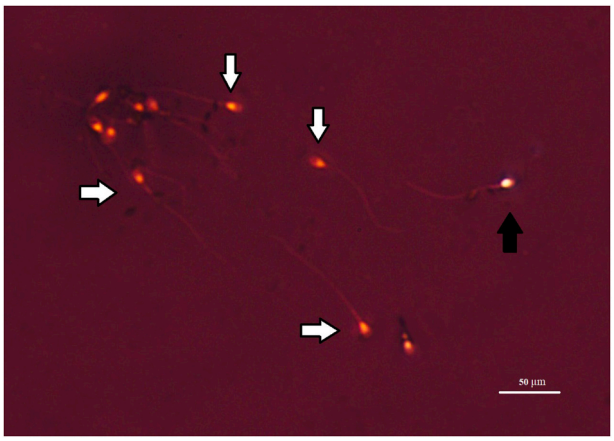

Fig. 2. Example of eosin-nigrosine staining of spermatozoa.
(white arrows) dead cells,
(black arrows) alive cells.

Zygote were cultured *in vitro* for 5 days until the blastocyst stage in LifeGlobal® culture medium (Cooper Surgical, Los Angeles, CA, USA) in four-well Nunc® plates (Merck KgaA, Darmstadt, Germany).

Statistical analysis was performed using Past Statistics v/3/01 (Sweden). The differences between the data were evaluated by Student's t-test for the parametric data distribution and by the Mann Whitney *U* Test for non-parametric data distribution. The difference was considered significant at $P < 0.05$.

3. Results

It was detected the lower rate of morphological abnormalities of spermatozoa frozen with PVP in comparison with cells frozen with glycerol: 34.6 ± 4.1% vs 20.7 ± 4.7%, respectively (Table 1).

Spermatozoa with one large or several small vacuoles [10] were the majority among all possible variants of head pathologies in spermatozoa frozen with glycerol. The differences of spermatozoa midpiece and tail abnormalities in spermatozoa frozen with glycerol and PVP were insignificant: 13.0 ± 0.9% and 13.4 ± 2.1%, as well as 5.3 ± 1.7% and 6.4 ± 1.8%, respectively ($P < 0.1$). The multiple defect of head, midpiece and tail abnormalities were significantly lower in PVP-frozen in comparison with spermatozoa frozen with glycerol: 26.3 ± 2.6% and 35.7 ± 3.6% ($P < 0.05$) (Table 1).

After warming, 78.8 ± 6.6% of spermatozoa frozen with glycerol and 41.4 ± 8.1% of PVP-frozen spermatozoa were motile ($P < 0.05$). The cell viability was 92.1 ± 8.6% and 89.6 ± 8.6% after freezing with glycerol and freezing with PVP, respectively ($P > 0.1$). When samples contained 5% permeable cryoprotectant glycerol, this cryoprotectant needs to be removed after warming. Therefore, it was re-evaluated the cell motility after this procedure. Despite the high survival rate of spermatozoa frozen with glycerol, after centrifugation and cryoprotectant removal, the number of motile spermatozoa was reduced to 27.3 ± 4.8% ($P < 0.05$). The use of non-permeable cryoprotectants does not require their removal, thus their motility is maintained at the same level.

Due to the fact that spermatozoa suspensions containing glycerol and PVP have a high viscosity, the study of spermatozoa motility in these solutions cannot reflect their real functional state. In the same time, we underline that the study of spermatozoa MMP may objectively reflect their functionality. It was detected that the number of cells with high MMP after warming was 34.7 ± 4.2% and 54.5 ± 4.2% for spermatozoa frozen with glycerol and for PVP-frozen spermatozoa, respectively (Fig. 3).

Analysis of DNA fragmentation shown that this rate was 38.8 ± 3.0% and 23.1 ± 2.5% in frozen with glycerol and in PVP spermatozoa, respectively ($P < 0.05$) (Fig. 4).

After ovarian stimulation, 356 Metaphase II stage oocytes were retrieved from 49 patients of age 30.8 ± 3.5 years (7.3 ± 1.2 oocytes per patient). Oocytes were fertilized by frozen in glycerol and in PVP spermatozoa: 230 from 32 patients (age 31.1 ± 3.2 years) and 126 from 17 patients (30.4 ± 3.9 years), respectively. Pronuclear oocytes ($n = 308$) were cultured *in vitro* for 5 days until the blastocyst stage (Table 2, Fig. 5).

Morphokinetic analysis of embryos shown that about 30% of embryos obtained after oocyte fertilization with spermatozoa frozen with glycerol were arrested in their development at 8-cells stage. Such embryos had also an increased level of blastomere fragmentation, which in some cases reached to 100%. Only about 10% of embryos arrested at 8-cells stage were detected after fertilization with PVP-frozen spermatozoa ($P < 0.05$). The difference in blastocyst formation rate was noted: 30.9 ± 3.3% and 45.4 ± 4.1% after using of spermatozoa frozen with glycerol and PVP-frozen spermatozoa, respectively ($P < 0.05$) (Table 2, Fig. 5).

4. Discussion

Technology for cryoprotectant-free cryopreservation of human spermatozoa has 120 years history [11]. The first data regarding this technology was published in 1897 by Davennort [12]. However, this interesting data were forgotten for 40 years. In 1938, 1940 (Shettle, cited by Parkes), 1942, 1945 an interest to this technology was increased [13,14,15] but only for relatively short time (during 17 years). Probably it can be explained by the beginning of new era in cryobiology, era of permeable cryoprotectants.

It is known however, that cryopreservation with permeable cryoprotectants leads to cell injury that instead intracellular and extracellular ice crystal formation can be explained by osmotic damages [16,17,

Table 1
Morphological characteristics of spermatozoa, frozen in the presence and absence of cryoprotectants.

Rates of morphological abnormalities	Cryoprotectant-included freezing	Cryoprotectant-free freezing
Head abnormality, %	26.0 ± 2.7*	19.2 ± 2.7*
Midpiece abnormality, %	13.0 ± 1.0	13.4 ± 2.1
Tail abnormality, %	5.3 ± 1.7	6.4 ± 1.8
Multiple abnormalities, %	35.7 ± 3.6*	26.3 ± 2.6*
Normal forms, %	20.7 ± 4.7*	34.6 ± 4.1*

* Statistically significant respective difference, $P < 0.05$.

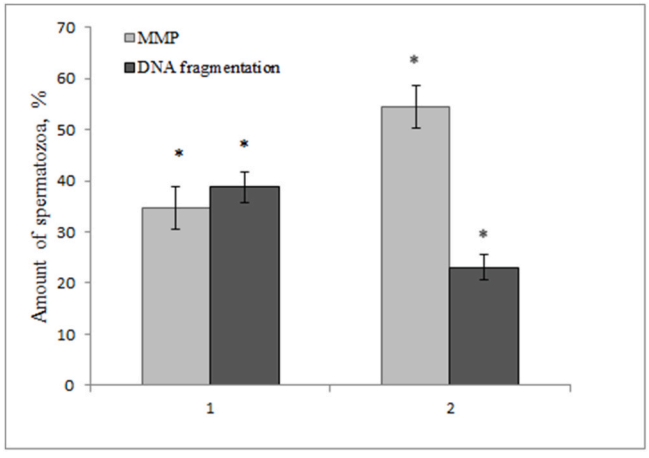

Fig. 3. High mitochondrial membrane potential (MMP) and DNA fragmentation rates of spermatozoa frozen in the presence or absence of cryoprotectant.
(1) Spermatozoa frozen in the presence of cryoprotectant.
(2) Spermatozoa frozen in the absence of cryoprotectant.
* Statistically significant respective differences, $P < 0.05$.

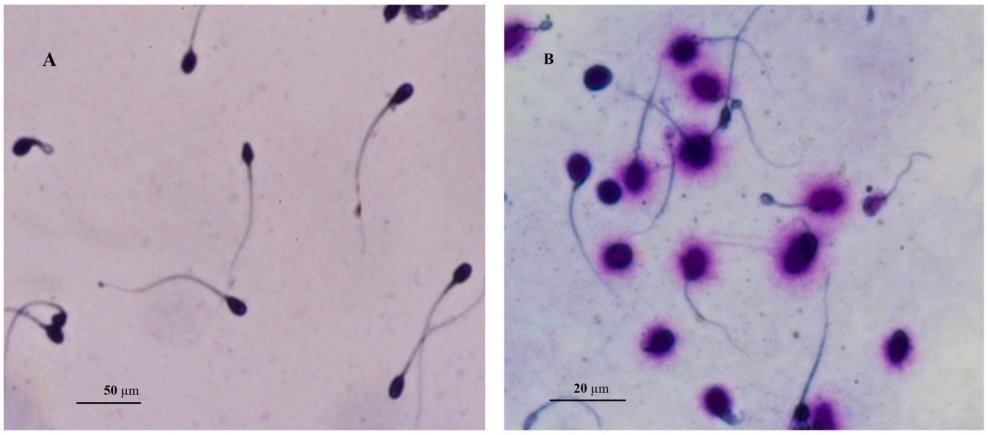

Fig. 4. DNA fragmentation of spermatozoa frozen in the presence or absence of cryoprotectants.
(A) spermatozoa with no halo after cryoprotectant-included freezing,
(B) spermatozoa with halo after cryoprotectant-free freezing.

Table 2
Developmental rate of oocytes after fertilization with spermatozoa frozen at the presence and absence of cryoprotectants.

Developmental rates	Cryoprotectant-included freezing	Cryoprotectant-free freezing
Fertilization rate, %	82.2± 6.2	94.4 ± 7.8
8-blastomeres rate, %	69.5 ± 5.1*	90.0 ± 4.6*
Blastocyst rate, %	30.9 ± 3.3*	45.4±4.1*

* Statistically significant respective differences, $P < 0.05$.

18,19] and chemical toxicity of cryoprotectants [20–22,23,24,25]. Negative effect of permeable cryoprotectants can also be reflected in chromatin damage and emergence of chromatin abnormalities [26].

Sixty years after publication of Hoagland and Pincus in 1942 [13], it was published the results of investigations aimed to compare the effectiveness of three methods of cryopreservation of human spermatozoa: conventional freezing with permeable cryoprotectants, cryoprotectant-free vitrification as well as vitrification with permeable cryoprotectant [27]. The test for evaluation of dead and acrosome reacted spermatozoa as well as sperm morphology was performed. Also, it was evaluated the ability of fresh (control) and vitrified without cryoprotectant spermatozoa to fertilize oocytes. It was concluded that cryoprotectant-free vitrification of human spermatozoa is a perspective direction of cryopreservation [27].

The prototype of the freezing technology tested here, was the technology for cryoprotectant-free vitrification of human spermatozoa in capillary with sucrose [28,29,30,31].

For technology described here instead sucrose it was decided to use polyvinylpyrrolidone (PVP).

PVP was first synthesized in 1939 for using in medicine. It belongs to the class of artificial polymers and it is a product of polymerization of vinylpyrrolidone. In aqueous solutions, the molecules of this substance have a chaotic spiral configuration which allows them to hydrate a sufficient number of water molecules. Nature of a role which PVP plays during cooling of cells can be explain by well-developed hydration properties of PVP: a crystallization process is shifted to a lower temperature region [32]. The presence of this polymeric substance in the cryopreservation media contributes to the stable state of a cell membrane. It is because PVP molecules prevent an elimination of intracellular components during ice formation (osmotic stress) [33].

Standard 10% PVP with molecular weight 360 kDa is routinely using in artificial reproductive technologies (ART) for performing of ICSI (for mechanical reduction of spermatozoa motility) [34,35]. PVP has been shown to be neutral for cells and does not lead to irreversible changes in cell structure. It has been shown that PVP did not affect the integrity of genetic apparatus of future embryo [36]. All these features of PVP make it promising to use as a cryoprotectant for freezing of spermatozoa with reduced morphokinetic characteristics [37].

PVP is approved for use in medical practice by European Medicines Agency (EMA, U.K.) and by Food and Drug Administration (FDA, USA). Just it was a ground why we have used PVP in our experiments.

Since morphological criteria for assessing spermatozoa is a main for the fresh gametes analysis, we have used morphological method to compare the effectiveness of spermatozoa cryopreservation. Earlier it has been shown that number of cells with head pathology is increased

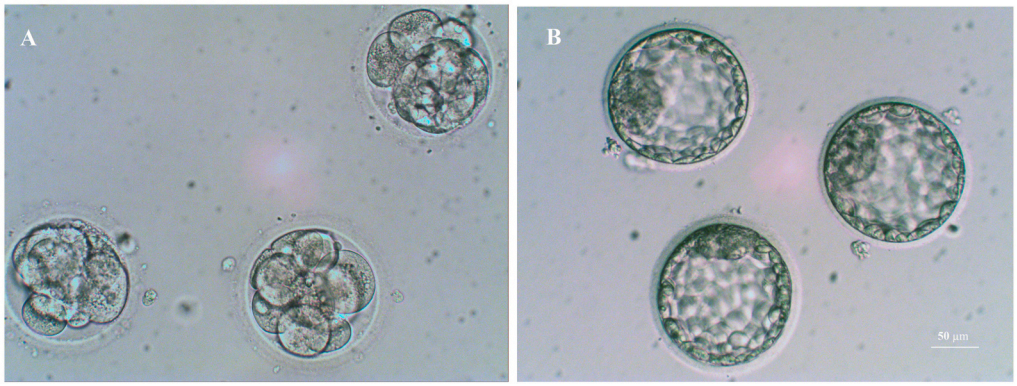

Fig. 5. Day 5 embryos obtained after oocyte fertilization with spermatozoa frozen with glycerol (A) and PVP (B).

after spermatozoa cryopreservation with glycerol in contrast with using of PVP [37]. In addition, the number of cells with vacuoles has increased among the pathological forms in frozen spermatozoa. It was reported that the number of vacuoles, their size and shape reflect defects at the level of sperm nucleus compaction [37]. Embryos obtained after fertilization of oocytes by such spermatozoa, stop their development at the early stages [38,10,39].

Technology, which we offer for the use in medical practice, has no stage "centrifugation of spermatozoa" for removal of permeable cryoprotectant. It is important peculiarity of described technology. Just by this fact we can explain the reduced motility of spermatozoa cryopreserved with the presence of glycerol.

In our case, it was especially important to determine the number of cells with high MMP to assess the effect of cryopreservation on the spermatozoa functional state [40,41]. It was found that vitrification with PVP leads to preservation of a significantly higher number of oligoasthenoteratozoospremic spermatozoa with high MMP than the routine cryopreservation with glycerol.

It was previously shown that the sperm head morphology correlates with an increasing of chromosomal abnormalities [42]. Considering that embryos culturing to blastocyst stage avoid a presence of abnormal parental genome [43], we decided to analyse the blastocyst formation rate. Results of our experiments confirm the better preservation of spermatozoa genome after freezing with PVP.

Analysis of the embryo *in vitro* development showed that embryos obtained after fertilization of oocytes more often shown arrest of their development at 8-cells stage in comparison with embryos after ICSI by vitrified spermatozoa.

For short, our results evidence about the following:

The use of 5% high molecular weight PVP allows to preserve cells with normal morphological characteristics, high MMP and intact DNA in comparison with standard glycerol-included freezing. Fertilization of oocytes with spermatozoa frozen with PVP reduces the number of arrested embryos at the cleavage stage, and the blastocyst formation rate is significantly higher than with standard freezing with glycerol. The described method of freezing with PVP is more simple than standard freezing method with glycerol because it is not necessary to remove this permeable cryoprotectant after thawing.

In conclusion, permeable cryoprotectant-free freezing with 5% high-molecular-weight (360 kDa) polyvinylpyrrolidone can be applied successfully for cryopreservation of human oligoasthenoteratozoospremic spermatozoa.

Declaration of competing interest

All authors declare that there is no conflict of interest.

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