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The impact of cryopreservation on the morphology of spermatozoa in men with oligoasthenoteratozoospermia

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ABSTRACT

The cryopreservation of ejaculate can reduce the viability, motility, and morphological characteristics of the spermatozoa of infertile men. Oligoasthenoteratozoospermia (OAT) is the most common cause of male subfertility. The aim of this study was to evaluate the morphological characteristics and viability of progressive motile sperm fraction before and after cryopreservation, and to determine whether cryopreservation of progressive motile sperm fraction is effective in eliminating morphologically abnormal sperm in men with OAT. An increased proportion of spermatozoa with normal morphology in fresh progressive motile sperm fraction compared with fresh ejaculate has been observed. After cryopreservation, the motility was 65.5 ± 8.8%; the proportion of spermatozoa with normal morphology increased non-significantly compared with freshly prepared motile sperm fraction (35.6 ± 5.5%). Concurrently, the proportion of cryopreserved spermatozoa with head defects increased significantly by 1.7 times (to 38.4 ± 4.7%) and the proportion of almost all morphologically abnormal sperm cells, particularly spermatozoa with multiple abnormalities, was reduced significantly. These data appear to be a novel finding in the context of patients with OAT. Using such spermatozoa for in vitro fertilization leads to a significant decrease in both a number of embryos at the cleavage stage and the blastocysts formation rate. High-magnification sperm morphology examination and selection, IMSI, post-cryopreservation significantly increased the likelihood of successful oocyte fertilization and subsequent embryo development.

1. Introduction

Male infertility is one of the main global health issues [26,52] and oligoasthenoteratozoospermia (OAT) is the most common cause of male subfertility. A decrease in the total number of spermatozoa, and in their motility, and changes to their morphological characteristics cause impaired sperm fertility. In Assisted Reproductive Technology (ART) motile spermatozoa can be selected by different methods of sperm separation, namely, swim-up, glass wool filtration, glass bead column separation, migration-sedimentation, density gradient centrifugation [26,27]. The quality of sperm can be improved by the classical swim-up procedure, but not to the extent that using density gradient procedures indicate [2]. It has been reported previously that sperm chromatin maturity in normozoospermic differs from OAT after gradient centrifugation [15].

It is thought that sperm morphology influences fertilization capacity to some extent [12,33,34,42]. The links between defects of chromatin condensation and unusually large nuclear pockets in a sperm head, abnormal protamine ratios, and large sperm heads have been reported [6,11]. The incidence of sperm morphological abnormality, as well as of sex chromosomal aneuploidy, is high in patients with OAT [22] Attention should be given, therefore, to the head pathology of sperm during intracytoplasmic sperm injection (ICSI) as a marker of genetic defects that can lead to abnormal early embryonic development or to increased early pregnancy loss [7]. Since 1951, various classifications of the morphological forms of spermatozoa have been introduced in efforts to evaluate its potential capability to fertilize the oocyte [38,42,43]. With the development of ART, however, particularly with extensive usage of the ICSI procedure, the morphological evaluation of spermatozoa has become an important prognostic criterion approved by the WHO. The WHO's fifth edition of the *Examination and Processing of Human Semen* provides updated, standardized, evidence-based procedures and

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recommendations on the isolation and evaluation of human sperm [12, 58]. Recently, the classification of various morphological forms of spermatozoa in accordance with up-to-date knowledge of sperm's morphometric characteristics has been developed assessing the correspondence between the defects observed by conventional microscopy and transmission electron microscopy, and the standardized categories of the majority of sperm defects have been established with great precision [3,4].

Cryopreservation of spermatozoa is used widely in infertility treatment [1]. Cryopreservation can reduce the viability, motility, and morphological characteristics of spermatozoa, however, and lead to their DNA damage through oxidative stress [45]. Cryopreservation has deleterious effects on plasmalemma, on acrosomes, and on the tails of spermatozoa [45]. Furthermore, cryopreservation can induce subtle non-lethal damage to sperm that may be still motile after thawing, and even temperatures below the freezing point of the medium during cooling can induce stress to the spermatozoa [57]. Cryopreserving samples with very low semen number in OAT men has been recommended as a backup procedure for the day of oocyte retrieval [44]. An optimal strategy, however, to assist patients with OAT syndrome with regards to the impact of cryopreservation on sperm morphology has not been established as yet. In this regard, the impact should be ascertained of cryopreservation on the morphology of sperm in patients with OAT that has been separated using density gradient procedures. This study was designed to establish the proportion of normal and various abnormal morphological forms of spermatozoa using Auger's classification. The aim of the study was to evaluate viability and the morphological characteristics of fresh sperm (group I), progressive motile sperm fraction before (group II) and after cryopreservation (group III), and to determine if the cryopreservation of progressive motile sperm fraction is effective in eliminating morphologically abnormal sperm in OAT syndrome men for further use in ART procedures. To assess the overall effect of cryopreservation on the sperm in OAT patients, two in vitro fertilization approaches were used and laboratory outcomes were evaluated by comparing fertilization rate, number of cleavage stage embryos on day 3 and blastocysts on day 5.

2. Materials and methods

2.1. Study design

Ejaculated sperm samples obtained from OAT patients undertaking infertility treatment at the ART-Clinic for Human Reproduction, Kharkov, from 2011 to 2018 were analyzed. The morphology and functional characteristics of freshly ejaculated sperm and of progressive motile sperm fraction before and after cryopreservation were assessed by routine semen analysis with the written consent of patients. The study included 138 patients aged from 27 to 52 years (mean age 32 ± 3.4). The study was approved by the Biomedical Ethical Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, in line with the Law of the Ukraine "On Personal Data Protection" (2010). Informed consent forms were obtained from all participants in line with WHO regulation. All sample identifiers were encrypted [59].

2.2. Semen analysis

Patients with OAT were advised to produce a semen sample by means of masturbation. The men had an abstinence period of ≥3 days. The concentration of spermatozoa in oligoasthenoteratozoospermic samples were less than 15 million per ml. The motility of each spermatozoon from high rapid progressive to immobility was graded 'a', 'b', 'c', or 'd', in accordance with the WHO recommendation [58]. The motility of 100 spermatozoa from the two different locations on the slide from each sample was determined at 200 × (final magnification).

On average spermatozoa concentration was 7.3 ± 4.1 × 10^6/ml, the motile fraction (a+b) was 27.4 ± 5.8%. After ejaculation, semen samples were kept for 30 min at room temperature until liquefaction and divided into two parts. One part was used to separate progressive motile sperm fraction and the second part was kept as neat ejaculated sperm. Small aliquots (10 μl) from each part were used for assessment of viable sperm morphology of fresh and freeze-thawed samples using an eosin-nigrosin staining technique [59]. Abnormal morphology of spermatozoa was subdivided into three groups: head; midpiece; and tail defects. The morphology of 100 sperm from each of two different locations of the slide were analyzed at 400 × with bright field microscopy.

Classification of the spermatozoa head morphological forms by Auger et al. was based on measurements of the length and width of the head (dimensions, elongation and ellipticity) [4]. The head defect group was subdivided into several categories: tapered head - increased major axis length and normal minor axis length; thin head- normal major axis length and decreased minor axis length; microcephalic - decreased major and minor axes lengths; macrocephalic - increased major and minor axes lengths; multiple heads-more than one head per sperm cell; abnormal post-acrosomal region - any type of outline and/or size and/or texture defects of the posterior part of the head; abnormal acrosomal region-any type of outline and/or size and/or texture abnormality of the anterior part of the head; and abnormal post-acrosomal region: residual cytoplasmic area >30% of the head area [4]. To provide high contrast a 100 × oil objective with a 10 × ocular lens of the Olympus IX-71 microscope (Olympus, Japan) with equipped with Nomarski/differential interference contrast optics was used. A 5–10 μl drop of ejaculated semen or progressive motile sperm fraction was placed on a slide then covered with a glass-over slip to assess morphology of the sperm head, mid piece and the tail of 100 sperm from each of two different locations on the slide.

2.3. Freeze-thawing of progressive motile sperm fraction

To obtain progressive motile fraction from the ejaculate aliquot, categories (a) and (b), a Sperm Gradient Kit (Cook Medical, USA) was used. Sperm preparation was done through density-gradient separation performed according to the Sydney IVF, Cook Medical Manual followed by swim-up procedure.

A two-stage cryopreservation method was used. A progressive motile sperm fraction was diluted in a ratio of 1:1 with a cryoprotective medium and exposed for 10 min. The cryoprotective medium, which consisted of 15% glycerol (Sigma-Aldrich, USA), 20% human serum albumin (Life Global, USA) in a Sperm Preparation Medium (Cook Medical, USA) was added to an equal volume of semen, and then to the prepared progressive motile sperm fraction in a dropwise manner, gently mixed at room temperature, and allowed to sit finally for 10 min to allow for proper equilibration between the cells and the medium. Next, the samples were placed in 0.5 ml straws with an outer diameter of 2.5 mm, a length of 133 mm, and a volume of 0.47 ml (Cryo Bio System, France). Cooling was performed in a controlled rate freezer CL-8800 (Cryologic, Australia) from 23 °C to −35 °C at the rate of 1 °C/min and from −35 °C to -70 −70 °C at the rate of 15 °C/min. Then the samples were immerced into the liquid nitrogen. The straws were stored in liquid nitrogen for one to twelve months.

A water bath with a temperature of 37 °C was used for thawing. The samples were warmed until the solid phase (ice) disappeared completely. The removal of cryoprotectants was performed by dilution in Sperm Preparation Medium (Cook Medical, USA) and by centrifugation at 200*g* for 5 min. Then, the viability and the morphology of the spermatozoa were evaluated.

Every ejaculated sperm sample was divided into three aliquots that comprised study groups: group I - fresh ejaculated sperm; group II - progressive motile sperm fraction; and group III - cryopreserved progressive motile sperm fraction.

2.4. Evaluation of spermatozoa mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm) was determined with lipophilic cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolcarbocyanine iodide (JC-1) in accordance with the manufacturer's instructions (Biomolecular Research Laboratory, PA, USA). Ejaculated sperm or the progressive motile sperm fraction was diluted with JC-1 work solution (20 μg/ml) in DMEM (Sigma, USA) without phenol red at 1:1 ratio and incubated for 15 min at 37 °C. Afterwards, the cells were analyzed using a confocal laser scanning microscope Axio Obzerver Z1 (Carl Zeiss, Germany). The excitation wave-length was 488 nm; emission was observed in the green zone of the spectrum at 520 nm, in the red zone at 590 nm. The number of cells with a high ΔΨm was counted in 100 spermatozoa in two different field locations on the slide from each sample at ×600 (final magnification). The viable population was defined as those motile cells with a high ΔΨm (Fig. 1).

2.5. Oocytes, ICSI and IMSI procedures

Ovarian stimulation was controlled using the follicle-stimulating hormone ("Fostimon", IBSA, Switzerland) started on the second day of the menstrual cycle, with the initial doses of 150–225 IU/day. Gonadotropin releasing hormone (GnRH) ("Cetrotide", Serono, Switzerland) was used as an antagonist (0.25 mg/day) with the dominant follicle size of 14 mm. Ovulation was induced by 0.4 mg GnRH agonist ("Diphereline", Beaufour Ipsen Internation, France), which caused the dominant follicle to reach the diameter of 20 mm, and the serum estradiol level corresponded to the required concentration. Oocytes were retrieved by a transvaginal aspiration using sonography-guided technique at 36 h after ovulation induction. The retrieved oocytes after hyaluronidase exposure (Sydney IVF Hyaluronidase, Cook Medical, Spencer, IA, USA) were denudated from the surrounding cumulus cells. Only the mature metaphase II oocytes were selected for either ICSI or the intracytoplasmic morphologically selected sperm injection (IMSI) based on sperm assessment under high power magnification (total 6400 ×). The procedure was performed 4 h after oocyte retrieval. In case of IMSI, spermatozoa morphology was additionally classified into four groups according to the presence or size of vacuoles: Grade I: normal form and no vacuoles; Grade II: normal form with less than or equal to two small vacuoles; Grade III: normal form with more than two small vacuoles or at least one large vacuole; Grade IV: large vacuole and abnormal head shapes or other abnormalities. The spermatozoa with a normal morphology with grade I or II were used for injection into the oocytes as described by Vanderzwalmen et al. [55].

2.6. Embryo culture

All injected oocytes were cultured in Global medium (Life Global, USA) under mineral oil at 37 °C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Fertilization was evaluated 16–18 h post injection by the presence of two pronuclei. The number of cleavage stage embryos on day 3 and blastocyst in extended 5 day in vitro cultured were evaluated.

2.7. Statistics

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

3. Results

A significant increase in the proportion of spermatozoa with normal morphology in prepared progressive motile sperm fraction (30.7 ± 1.5%, group II) compared to the fresh ejaculate (16.6 ± 1.6%, group I; 46% increase, $P < 0.001$) was observed.

The proportion of sperm with head defects and the proportion with tail defects after the isolation of progressive motile sperm fraction prior to cryopreservation were similar to those of the fresh ejaculated sperm, 23.2 ± 1.1% *vs* 22.2 ± 4.1% and 19.3 ± 0.9% *vs* 17.7 ± 1.3%, respectively; the results were not significantly different (Fig. 2). Furthermore, the percentage of spermatozoa with midpiece defects in group II had decreased significantly compared to group I (10.6 ± 2.1% vs 16.8 ± 0.9%; decreased by 1.6 times, $P < 0.01$).

The percentage of cells with multiple morphological defects in all study groups also was analyzed. The proportion was 24.0 ± 1.2% in fresh ejaculated sperm. The percentage of cells with multiple morphological defects in progressive motile sperm fraction decreased significantly to 18.8 ± 1.6% (reduction by 1.3 times, $P < 0.01$) (Fig. 2).

The data obtained demonstrated that the decrease in the proportion of morphologically abnormal sperm occurred mainly as a result of eliminating cells with multiple morphological defects during progressive motile sperm fraction isolation from the fresh ejaculated sperm in OAT men.

The viability rate of progressive motile sperm fraction ('viability')

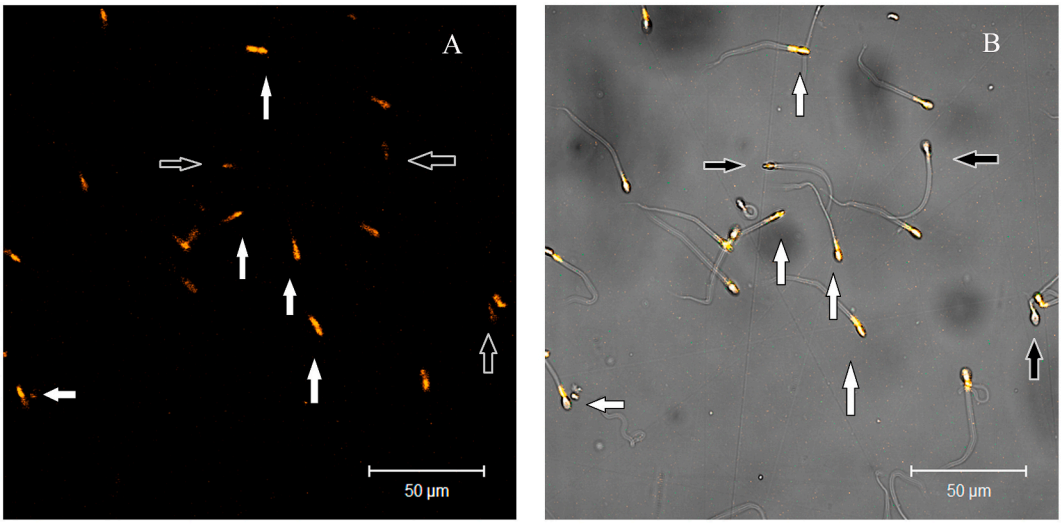

Fig. 1. Progressive motile sperm fraction with high and low mitochondrial membrane potential (ΔΨm, JC-1 staining, 200 ×). A – Fluorescent image, B- merged image; white arrows – cells with high ΔΨm, black arrows – cells with low ΔΨm.

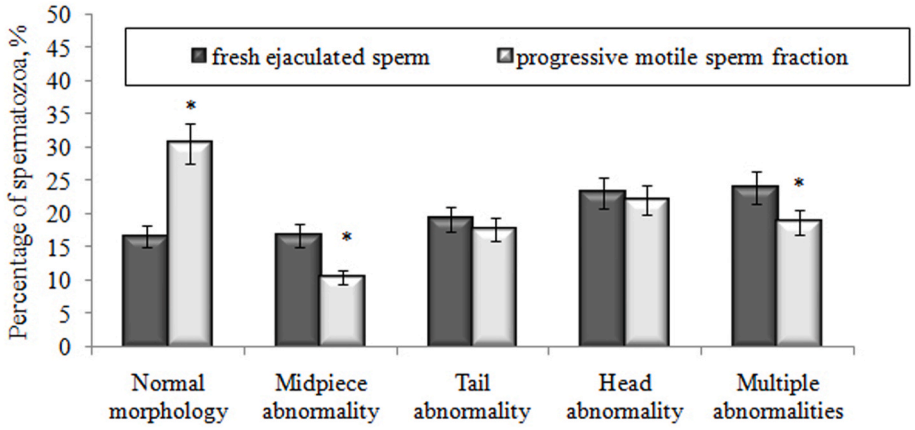

Fig. 2. The proportion of various types of spermatozoa morphological abnormalities in fresh ejaculated sperm (group I) and progressive motile sperm fraction (group II). A statistically significant difference was observed between the study groups marked with an asterisk (*), ($P < 0.05$).

estimated by ΔΨm correlated positively with the sperm's motility. This tendency remained after cryopreservation. Both the viability and motility were decreased after cryopreservation in the same way compared to the fresh prepared fraction (72.3 ± 6.7% *vs* 96.2 ± 1.8%; decreased by 1.3 times, $P < 0.05$) (Fig. 3).

A significant difference in the percentage of almost all morphological types of spermatozoa in progressive motile sperm fraction post-cryopreservation (group III) was observed compared to that of group II (Fig. 4). The proportion of spermatozoa with normal morphology non-significantly increased compared to freshly prepared motile sperm fraction (35.6 ± 5.5 *vs* 30.7 ± 1.5%; increased by 1.2 times). The proportion of spermatozoa with midpiece, tail, and multiple morphological defects decreased up to 10.2 ± 2.9%, 7.3 ± 2.5%, and 8.6 ± 1.9%, respectively. By contrast, the proportion of spermatozoa with head defects in the surviving population increased significantly to 38.35 ± 4.7% (increased by 1.7 times, $P < 0.05$), which appears to be a novel finding not reported elsewhere in the scientific literature.

Further study aimed to identify the predominant forms of head defects of spermatozoa in progressive motile sperm fraction post-cryopreservation. The percentage of spermatozoa with thin head and microcephalic head prior to cryopreservation was small, i.e. 13.2 ± 2.4% and 11.3 ± 1.9%, respectively. By contrast, the main subpopulations of sperm with morphological head defects in progressive motile sperm fraction after cryopreservation were spermatozoa with thin head and microcephalic head, which amounted to 21.5 ± 1.3% and 27.5 ± 3.8% or increased by 1.6 and 2.4 times ($P < 0.001$) as compared to the same subpopulation of group II, respectively (Fig. 5 A, B). The percentage of sperm with other head defects such as an abnormal acrosomal region decreased (22.1 ± 3.2% *vs* 18 ± 2.2%) (reduction by 1.2 times; $P < 0.05$). Sperm with abnormal post-acrosomal regions (14 ± 2.7%), tapered head (7 ± 1.2%), macrocephalic head (6 ± 0.9%) and multiple heads (6 ± 1.3%) were among the smallest subpopulations after cryopreservation. A drastic reduction in the proportion of sperm with macrocephalic head defects (decreased by 4.5 times) was found after cryopreservation.

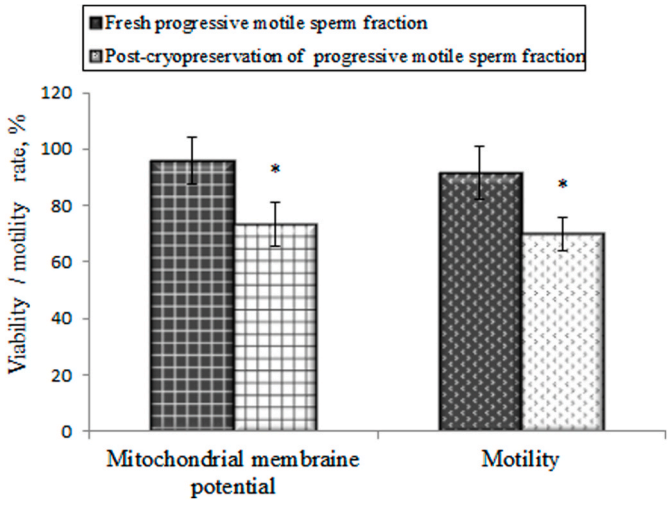

Fig. 3. Mitochondrial membrane potential and motility of progressive motile sperm fraction before (group II) and after cryopreservation (group III). Columns with checkered pattern correspond to study on mitochondrial membrane potential ΔΨm and columns with small waves correspond to study on motility. Asterisks indicate statistically significant differences of mitochondrial membrane potential or motility in the study groups (*), ($P < 0.05$).

Samples of progressive motile sperm fraction were divided into three groups to evaluate fertilization outcomes: fresh progressive motile sperm fraction of OAT men used for ICSI (group A), cryopreserved progressive motile sperm fraction used for ICSI (group B), and cryopreserved progressive motile sperm fraction used for IMSI (group C). Oocytes were obtained from fertile women of couples with only male factor of infertility to exclude oocyte quality impact on fertilization outcomes.

The fertilization rates in groups A and B were 82.5 ± 5.5% and 71.7 ± 5.6% respectively and had no statistical difference (Fig. 6). There was lower number of cleavage stage embryos in the group that used ICSI with the frozen/thawed progressive motile sperm fraction (59.9 ± 4.9%) compared with group that used ICSI with the fresh progressive motile sperm fraction (71.5 ± 5.6%) indicating negative impact of cryopreservation on sperm in combination with limitations of ICSI (Fig. 6 A, B) ($P < 0.05$). Cryopreservation of sperm resulted in significant reduction of the blastocyst formation rate (24.1 ± 9.1% vs 45.8 ± 5.1%) given that the fertilization technique in groups A and B was the same (Fig. 6 A, B) ($P < 0.05$).

The fertilization rate was significantly different between group B (71.7 ± 5.6) and C (87.4 ± 3.9%). Frozen-thawed progressive motile sperm fraction was used for oocyte fertilization in both groups. There was also significantly higher number of cleavage stage embryos in group C (85.1 ± 4.3%) compared with group B (59.9 ± 4.9%) due the use of higher magnification, the IMSI technique, in group C. There was lower number of cleavage stage embryos on day 3 after ICSI with fresh sperm, group A, (71.5 ± 5.6%) compared IMSI with the frozen/thawed sperm, group C, (85.1 ± 4.3%) but the difference was non-significant. Further observation showed that the blastocysts stage reached only 45.8 ± 5.1%; 24.1 ± 9.1% and 61.6 ± 6.4% in groups A, B, C respectively. Blastocyst formation rate was particularly low after ICSI with frozen/thawed sperm, 24.1 ± 9.1%, since many embryos were arrested after prolonged

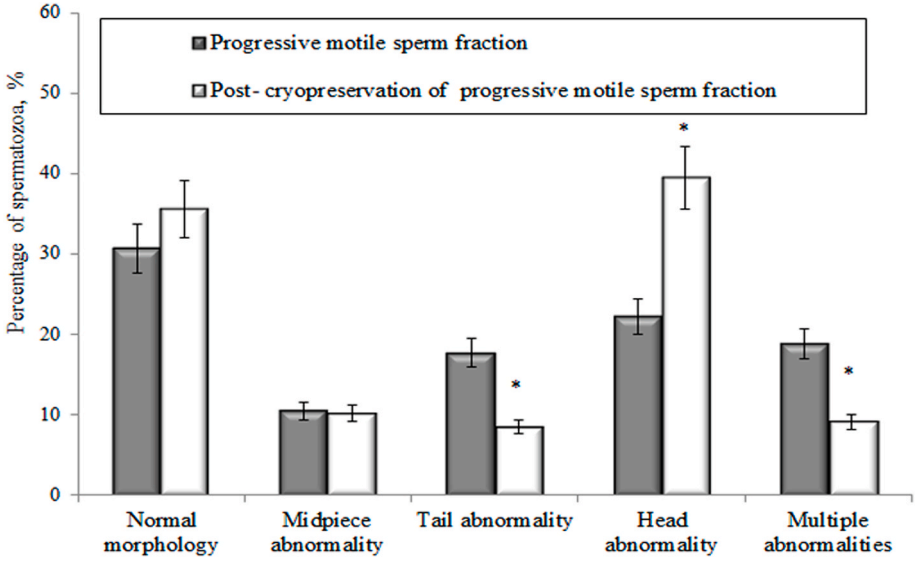

Fig. 4. The proportion of spermatozoa with various types of morphological abnormalities in progressive motile sperm fraction before (group II) and after cryopreservation (group III). A statistically significant difference was observed between the study groups marked with an asterisk (*), ($P < 0.05$).

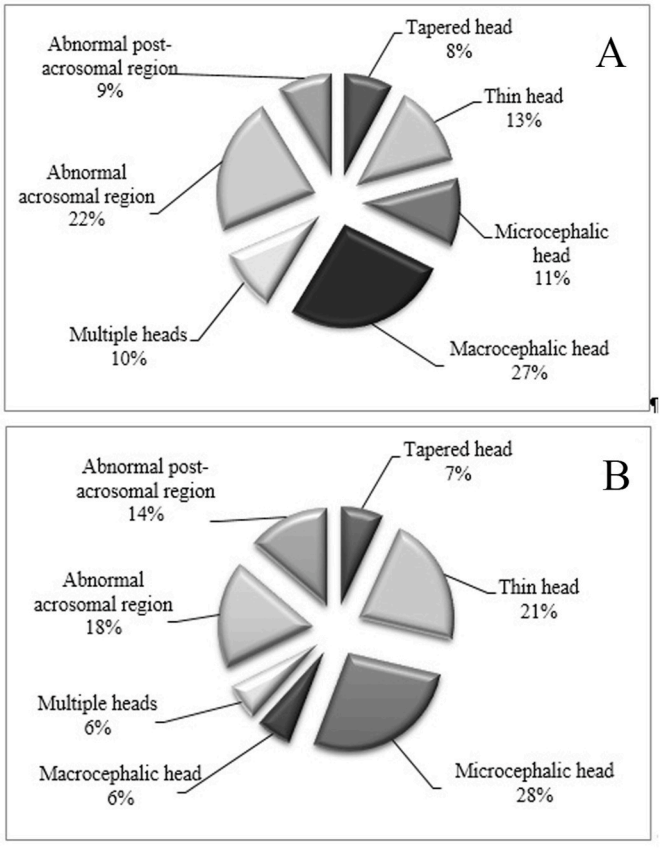

Fig. 5. The percentage of spermatozoa with different types of morphological head defects in progressive motile sperm fraction. A - before cryopreservation (group II); B - post-cryopreservation (group III). Statistically significant difference was observed between the study groups marked with an asterisk (*), ($P < 0.05$).

5-day embryo culture (Fig. 6 B).

Thus, the data suggests that the accumulation of abnormal forms of spermatozoa post-cryopreservation causes a significant decrease in number of embryos at the cleavage stage and a significant decrease in the blastocysts formation rate (Fig. 6 A, B). The use of the IMSI method for oocyte fertilization with such spermatozoa can increase the fertilization efficacy of post-cryopreserved sperm in OAT men (Fig. 6C).

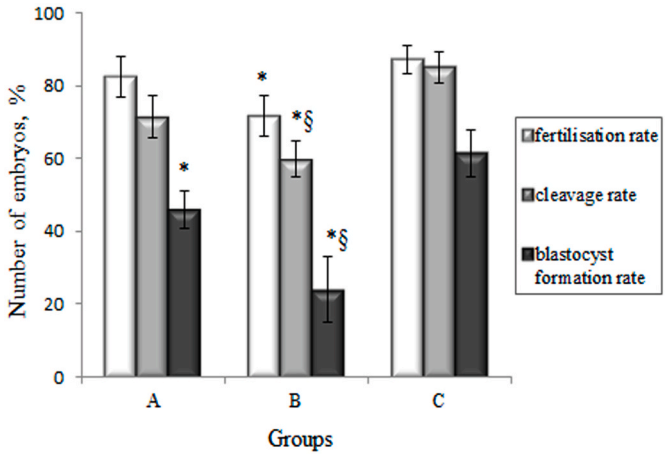

Fig. 6. Fertilization rate and embryo development characteristics after either ICSI or IMSI with fresh or frozen –thawed OAT spermatozoa. Group A – fresh progressive motile sperm fraction used for ICSI, group B – progressive motile sperm fraction after cryopreservation used for ICSI, group C – for IMSI. Statistically significant difference between groups A and C, as well as B and C, at corresponding time points is marked with an asterisk (*), ($P < 0.05$). Statistically significant difference between groups A and B at corresponding time points is marked with a section sign (§), ($P < 0.05$).

4. Discussion

The results of cryopreservation of cells in suspension, particularly human sperm, depend significantly on the initial cell concentration, on the morphological characteristics, and on the functional characteristics of the sample. Cryopreservation of semen with morphological defects and low concentration of gametes is inefficient. The highly effective methods of cryopreservation of human sperm in normospermic individuals [17,29] cannot be used in the case of ejaculated sperm obtained from OAT patients. The presence of leukocytes, bacteria, and cell debris in the ejaculate negatively affects sperm concentration, motility

and other conditions associated with infertility [5,35,46]. Therefore, removing them prior to the cryopreservation stage can improve survival rates [18] and can improve the quality of the sample particularly in infertile men, a finding that is also in line with our results [54].

Given that most sperm defects are less frequent in fertile than in infertile men [4], reduction of the percentage of abnormal spermatozoa may be considered to be a good indicator of the high efficiency of eliminating morphologically abnormal spermatozoa at the stage of preparation (Fig. 2).

A decrease in the percentage of spermatozoa with shape and nucleus size abnormalities after centrifugation in density gradient has been observed previously [24,25], a finding which is also in line with our results. There are several reports demonstrating advantages of separation methods [51,53]. As well as beneficial effects of antioxidant supplementation in combination with sperm selection by density gradient centrifugation before cryopreservation in OAT patients has been shown [9]. Preparation by different gradient centrifugation protocols is more effective than swim-up in selecting sperm with normal morphology, but it is the opposite in terms of motility [2,49].

Here, it has been demonstrated that the proportion of spermatozoa with multiple defects after preparation is consistently reduced while the proportion of spermatozoa with normal morphology occurred due to the elimination of cells that have multiple morphological defects (Fig. 2). Cryopreservation of progressive motile sperm fraction in men with OAT might be helpful to some extent in the further selection process for better fertilization outcomes. The fertilization rate correlated positively with mitochondrial membrane potential and motility of spermatozoa [39]. In this study, the viability and motility after cryopreservation decreased similarly as compared to freshly prepared sperm (Fig. 3). The cell morphology, the ratio of surface area to volume, and the osmotically inactive intracellular volume of water, are all critical parameters that affect directly the coefficient of water permeability and the probability of intracellular ice formation [31].

Developing optimal cryopreservation protocols is beneficial and it is also necessary to take into account both the structural and functional characteristics to maintain the spermatozoa's ability to achieve normal fertilization [52]. Considerable decrease was found in the proportion of normal spermatozoa during post-thawing using electron- and light microscopic studies [45]; in that study, swelling of the cytoplasm in the acrosome zone of the sperm with a tapered shape was demonstrated, which confirms our findings.

Four main hypotheses have been proposed to explain the reasons for the head dimension decrease that occurred post-cryopreservation in the sperm derived from a variety of species. These include osmotic changes, alterations of some cell compartments, damage or loss of the sperm acrosome, and over-condensation of sperm nuclear chromatin [13,28,40]. A significant reduction in sperm head dimensions as a result of cryopreservation of fresh sperm in all mammalians studied has been reported [40]. This reduction in sperm head dimension is reflected not only in the size of the sperm head, but also in its shape. Further reduction in sperm dimensions was observed for those semen samples with initial poor semen quality [28]. The sperm head dimension decrease after cryopreservation of human sperm reported here, perhaps is attributable to the poor quality of sperm of patients with OAT syndrome.

There is a change in the proportion of spermatozoa with a small head (Fig. 5 A, B) due to the elimination of spermatozoa with a large head post-cryopreservation and due to the fact that macrocephalic spermatozoa and spermatozoa with large acrosome regions undergo changes in volume, i.e. they undergo a reduction of volume post-cryopreservation under the influence of osmotic factors. The proportion of sperm subpopulation with thin and microcephalic heads increased due to non-lethal cryopreservation-related changes possibly linked to sperm membrane in OAT patients, particularly the known mechanisms that mediate changes in membrane permeability and in cell volume regulation [17]. Furthermore, high cryoresistance of the original sperm forms with microcephalic heads contributes significantly to increase the proportion of this subpopulation post-cryopreservation (Fig. 5 B). The water content of sperm with thin and microscopic heads is small; hence, the damage that ice crystals can cause during freezing is low compared to large macrocephalic sperm. It will be also the minimum change in the cells' osmotic volume in the case of low intracellular water content [31].

Spermatozoa with an abnormal acrosomal region also constitute a reasonably large subpopulation among sperm with head abnormalities post-cryopreservation (group III) in OAT patients (Fig. 5 B). Rapid changes in osmolality usually occur during freeze-thawing, which cause deformation of the membrane structures [20]. The acrosome area is vulnerable to potential damage during freeze-thawing [31]. Most likely, this kind of selection is due to insufficient dehydration of the acrosomal regions. As a result of damage to the acrosome membrane, spermatozoa survive either with reduced content or with swelling of this zone, most likely due to insufficient dehydration of the acrosomal regions. Probably, an increase of incubation time in a cryoprotective solution based on glycerol or addition to its composition of non-permeable cryoprotectant such as sucrose could reduce damage in the acrosomal cap and possibly increase the number of spermatozoa with a normal head [46]. It appears that the determining factor for sperm survival during cryopreservation is the membrane integrity of the sperm head [19,20]. Thus, cryopreservation acts as selective factor for the preservation of sperm with a certain head shape in OAT patients, which correlates with the state of chromatin condensation and ultimately affects the fertilization rate and the further development of the embryo [36,56].

Increased levels of sperm DNA fragmentation correlated positively to the incidence of macrocephalic heads, amorphous heads, and short flagella in men with teratozoospermia [8,14,47]. Oligoasthenoteratozoospermic men showed abnormal chromatin/DNA integrity, and a higher global DNA methylation rate [50]. Thus one may assume that the reduced percentage of sperm with macrocephalic heads after cryopreservation may reduce the level of DNA fragmentation of the sperm after warming among the surviving cells; yet, it has been shown recently that there is an increase in the level of fragmented DNA after cryopreservation sperm from patients with teratozoospermia [30]. Similar effect has been also demonstrated by us for the OAT patients in cohort study 2011–2017 [48].

Considering the decrease in sperm fertilization capacity induced by cryopreservation and poor sperm quality in OAT patients, the use of such sperm affects the embryo development and may result in their arrest on Day 3 in groups associated with ICSI-only procedure (Fig. 6 A, B). Moreover, it can be easily understood that such post-cryopreserved spermatozoa result in a lower cleavage and blastocysts rates compared with insemination with fresh sperm (Fig. 6 A, B). Severe ultrastructure defects are associated with abnormal morphology and aneuploidy in infertal patients [10]. The use of higher magnification that allows one to select morphologically normal spermatozoa with predominantly vacuole-free head enhances efficiency which is reflected in an increase in the number of developing embryos (Fig. 6C). In the current study focused on cryopreserved sperm, the cleavage rate increased significantly to 85% after 3-day embryo culture (Fig. 6C). There was a tendency toward a higher percentage of cleavage stage embryos in the IMSI with the cryopreserved sperm group than ICSI with the fresh sperm group (Fig. 6 A, C). Although, it was not statistically significant, the difference of about 14% is clinically important.

This study established a clearer view on the benefits of IMSI in couples with male infertility, specifically OAT male patients. It became evident from this study that despite a variety of stresses on sperm imposed by any cryopreservation further outcomes can be very encouraging (Fig. 6C). IMSI is a time-consuming procedure that represents a more sophisticated way of ICSI. Our study on sperm cryopreservation showed improved embryo development during a prolonged 5-day embryo culture after the IMSI procedure reflected in a higher number of blastocysts (Fig. 6C). Similarly, Knez et al. demonstrated that application of the IMSI technique with fresh poor quality sperm resulted in a high number of blastocysts [32]. Furthermore, it has been shown

that use of the IMSI technique, based on sperm assessment under high power magnification, can improve embryo development and the clinical outcomes in couples with previous ICSI failures associated with male infertility that is consistent with our results [23].

Others reported that IMSI did not significantly improve the clinical outcomes, in couples with two previous ICSI failures [21]. Another study which was focused on fresh normal sperm reported not only similar fertilization rates, 79% and 77% after IMSI and ICSI respectively, but also embryo development in both groups up to day 5 of preimplantation development [16]. Furthermore, clinical pregnancy were equally distributed over transfers with embryos from IMSI or ICSI procedures [16]. Considering detrimental effects induced by cryopreservation in context of the current study, there is a likelihood of less encouraging results if cryopreserved sperm has to be used for future ICSI attempts in OAT syndrome patients. Although this study indicates that both ICSI and IMSI are able to overcome a reduced ability to repair cryoinjuries of spermatozoa at the fertilization stage to some extent, yet, IMSI is preferable as the IMSI group was characterized by a higher number of both blastocysts and cleavage stage embryos than the ICSI group. In contrast, it was observed for fresh sperm, similar rates up to day 3 of embryo development irrespectively of procedures, the IMSI or ICSI, while higher blastocyst development rates were only attributed to IMSI group [37]. Another plausible suggestion is to apply combination of ICSI with preimplantation genetic diagnosis prior to embryo transfers as such combination resulted in similar live birth and miscarriage rates in OAT syndrome and normal sperm couples [41].

In summary, cryopreservation of progressive motile sperm fraction is not entirely effective in eliminating morphologically abnormal sperm in infertile men with OAT. The impact of cryopreservation on sperm of OAT patients become apparent at earlier stage as compared to fresh sperm. Our results show that:

- there was the large proportion of spermatozoa with abnormal head morphology (38.4 ± 4.7%) after cryopreservation in OAT men,
- the morphological characteristics of spermatozoa impair embryo development, and
- high-magnification sperm morphology examination post-cryopreservation significantly increased the number of embryos at the cleavage stage and the blastocysts formation rate.

Considering these findings, the use of detailed intracytoplasmic morphological assessment and the selection of sperm is the only sensible option in the case of using cryopreserved sperm in OAT men undergoing ART therapy.

Declaration of competing interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2021.02.009.

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Authors' contribution

PM, PV, and Yu T performed the research, KL and PM analyzed the data and wrote the manuscript, PM and KL designed the research study. All authors had full access to all data in the study, take responsibility for the integrity and accuracy of the data analysis, and approved the submitted version.

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