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Comparison between open and closed systems for vitrification of individual sperm: assessing morphometric measurements and chromatin integrity

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Abstract

Background Classic vitrification methods are not appropriate when there are minimal numbers of viable sperm, and the new methods emphasize the low semen volumes in these cases. The aim was to assess the efficacy of the cryotech as a device for freezing low sperm volume, through the two methods of open (OVS) and closed (CVS) vitrification systems.

Methods Testicular biopsy samples from 30 men with obstructive azoospermia (OA) were assigned to three groups fresh control (FC), OVS, and CVS. Testicular sperms were selected using an ICSI injection pipette and vitrified on the cryotech straws, containing one droplet of freezing medium. After warming, sperm head morphometric characterizations were evaluated with the MSOME technique. Sperm motility, membrane integrity, chromatin quality assessment including DNA fragmentation, Chromomycine A3 staining (CMA3), and Aniline Blue (AB) were assessed. Fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (FITC-PSA) was done to examine sperm acrosome integrity.

Results The mean sperm motility, viability, and sperm with intact acrosome reduced after vitrification, in both methods of CVS, and OVS, but the results were more promising in the closed method ($p < 0.05$). However, the variations were not significant between the two methods of cryopreservation, the OVS undergoes significant head dimensions changes compared to the control group ($p < 0.05$). The results also showed higher membrane, and chromatin abnormality after OVS ($p < 0.05$).

Conclusions The overall post-thaw recovery of human testicular sperm proposes that CVS is more efficient for single sperm cryopreservation, while higher sperm viability, and lower alterations in chromatin, acrosome, and sperm head morphometry were seen compared to OVS.

Keywords Sperm vitrification, Closed system, Open system, Morphometric measurements, Chromatin integrity

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Background

Approximately 40 to 50% of infertility in couples is male origin. Ten percent of this population belongs to azoospermic individuals. Sperm in these patients is obtained by various surgical methods [1]. Well-organized cryopreservation has some important advantages such as decreasing the risk of unnecessary ovulation induction, no need for synchronous oocyte retrieval, as well storing many vials for future use, especially in the case of the small testis, while damage from repeated testicular biopsies is avoided [2].

The sperm cryopreservation technique besides easily detectable damage, such as sperm motility, and morphology, can induce more subtle, sub-lethal sperm damage, following thawing. These damages can alter the composition of the spermatozoon's plasma, acrosomal, and mitochondrial membranes, as well as the spermatozoon's nucleus, chromatin decondensation, and induce DNA denaturation [3]. There is a concern about the proper cooling rate in these systems, especially regarding the preservation of the biopsied sperm from the testicles, due to the low number and greater vulnerability of these samples.

In oligozoospermia or azoospermia which are known as severe male factor infertility, if sperms can't be found in the ejaculation, they must be earned through testicular biopsy specimens or epididymal sperm aspiration. In some cases, repeated sperm retrieval is needed which has many side effects, such as the economic burden, psychological pressure, and testicular damage [4]. Therefore, it is necessary to upgrade and improve existing techniques for the cryopreservation of sensitive spermatozoa.

In this regard, some efforts have been made by the researchers. In 1997, and 1998, when the zona pellucida was used as a freezing carrier for the few sperm [5, 6]. A few years later, the cryoloop was used for testicular and epididymal sperm cryopreservation [7]. Vitrification is performed in two distinguishable methods, "open" like cryoloop, and "closed" systems such as straw-in-straw, Cryotip, high-security vitrification straw, Cryopette, VitriSafe [8]. While samples are exposed to liquid nitrogen in the open systems, this is eliminated in the closed systems. Open systems are unsafe because of the disease transmission risk, but closed systems are safe [9]. In this regard when sperm cryopreservation (oligoasthenoteratozoospermia) was done using droplets, open-pulled straws, and open-standard straws, the researchers concluded that all three methods are good in the aim of sperm freezing, but, the open-pulled straw and open straw methods were preferred because of the isolation from liquid nitrogen, with a maximum reduction of the potential risk of microbial contamination [10]. According to reports, the cooling rate in open systems is more than – 20,000 °C/min, while is below – 2000 °C/min in closed systems [11]. A high percentage of the sperm population is unable to withstand the temperature changes induced during cryopreservation [12]. A small number of spermatozoa also was vitrified successfully, in a closed straw system, using two different methods of sperm loading: a spontaneous capillary action (CA), and a polar body biopsy (PBB) pipette [13].

Sperm and testicular tissue cryopreservation is capable of preserving male fertility for years. On the other hand, sperm head pathology may reflect genetic defects, which are related to abnormal early embryonic development or early pregnancy loss [14], so, finding a reliable method, and suitable device, which are accompanied by the lowest sperm structure changes, are of paramount importance. However, a reliable and safe protocol that can be used as a common technique for clinical cryopreservation of the few spermatozoa still has not been introduced.

It was mentioned that, both the conventional method of cryopreservation and present vitrification techniques are less suitable in cases in which the initial quality of semen is low, such as; severe oligozoospermia, cryptozoospermia, epididymal or testicular samples. A very limited number of studies have focused on the subject of cryopreservation of the small number of single spermatozoa, and more novel works are required, to improve sperm recovery after freezing. there`s some concern regarding the difficulties of such freezing protocols, as well as the toxic effect of permeable CPAs or the risk of cross-contamination in an open freezing system, [13]. For the first time, in this study, we aimed to systematically compare the efficacy of open (OVS) and closed (CVS) vitrification systems for freezing low sperm volumes, and assessing sperm motility, viability, and morphometric and chromatin characteristics. This study provides more insights into the optimal approach for single sperm cryopreservation and contributes novel findings to the existing literature.

Materials and methods

Sperm samples

This study was conducted in the Andrology clinic, at our infertility center, on 30 men with obstructive azoospermia (OA), who were referred to for diagnostic and therapeutic purposes. All patients (20 to 55 years) signed an institutional informed consent document, prior to participation. The unilateral or bilateral biopsy technique (testicular sperm extraction) was done routinely, under local anesthesia. Study samples were collected between January 2021 and January 2022.

After excision, the testicular tissue biopsies were placed into HEPES-buffered medium supplemented with human serum albumin (HSA, 5%). All earned surgical tissue

samples were minced and dispersed using fine needles, in the Petry dish (10 cm). The tissue suspension was examined for the presence of sperm cells under high-power inverted microscopy. The testicular tissue and cellular suspensions were then centrifuged for 5 min at 300×*g*, then 2 ml of erythrocyte lysing buffer was added to the testicular sperm pellet, 10 min at room temperature, and then washed by adding 5 ml of HAMs F10 medium, and centrifuged for another 10 min at 500×*g*. Finally, the pellet product was re-suspended with 1.5 ml of culture media. If the sample contained the mature sperm sent for intra cytoplasmic sperm injection (ICSI), and the remaining went for vitrification.

For maturation induction, the testicular biopsied samples were cultured at 25°C for 24 h after preparation [15]. After that sperm motility, DFI, and morphology were evaluated on the earned sample, according to the WHO manual [16], and sperm viability was assessed via emission of a single laser shot, as described later.

Single sperm vitrification procedure

A droplet of the prepared sample (1–3 µL) was transferred to the 5 µL droplet of Hams F10 (5% HSA), in the ICSI dish (Falcon, 1006 dishes) (RT). Twenty-five motile or sluggish spermatozoa with normal morphology were isolated using a homemade microinjection needle.

In the vitrified groups, a droplet of 0.3 µl CPAs (Vitrolife, Sweden) was placed on the polypropylene strip of a cryotech (Kitazato, Tokyo, Japan), at room temperature (Fig. 1) [17, 18]. The ICSI needle was placed into the narrow end of the cryotech and selected spermatozoa were then transferred into the cryoprotectant droplet. Since the post-thawed sperm motility in testicular cases was greatly reduced, Pentoxifylline was added to the extender [19].

In the end, for vitrification two methods were used; open (OVS) and closed (CVS) systems. For this purpose, after loading spermatozoa on the straws, in OVS, straws were immersed into LN2, without recaption of the straw, and after the vitrification, straws were caped in the LN2, before storage. In the CVS group, however, the straws were worn with plastic caps and then inserted into the other protective straws (similar to the straw-in-straw method). The protective straws were sealed before plunging into LN_2. Subsequently, all the devices were exposed to LN_2 directly for storage, and stored until warming.

After 7 days, for thawing, a 5-µL droplet of pre-warmed Hams F10 + 10% HSA was placed on a sterile ICSI dish. While the Cryotech was submerged in the LN_2, the cap of cryotech was removed. Then, the tip of the cryotech containing sperms was quickly and directly placed in a pre-warmed droplet of Hams' F10 medium supplemented with 5 mg/mL HSA. Thereafter the droplets containing sperm are covered by pre-warmed oil (Ovoil; Vitrolife, Sweden). Following 30 min incubation at 37 °C, the sperm cells were aspirated using the micropipette equipped with a micromanipulator on an inverted microscope and transferred to a new droplet of the basic medium [18].

Sperm parameters assessments

In the thawed sperm recovery evaluation, sperm motility and viability were assessed. For the motility assessment, under a phase-contrast microscope, any sign of sperm movement or twitching of the head or tail is considered as motile sperm and reported as total motility (%) rate. The Diff-Quik staining was used to assess the percentage of morphologically normal sperms (Fig. 2) (×1000 magnification). The scoring was according to what was described in the WHO laboratory manual for sperm analysis [16].

Sperm membrane integrity

As the spontaneous tail swellings take place after sperm cryopreservation, sperm viability was assessed using laser-assisted immotile sperm selection (LAISS).

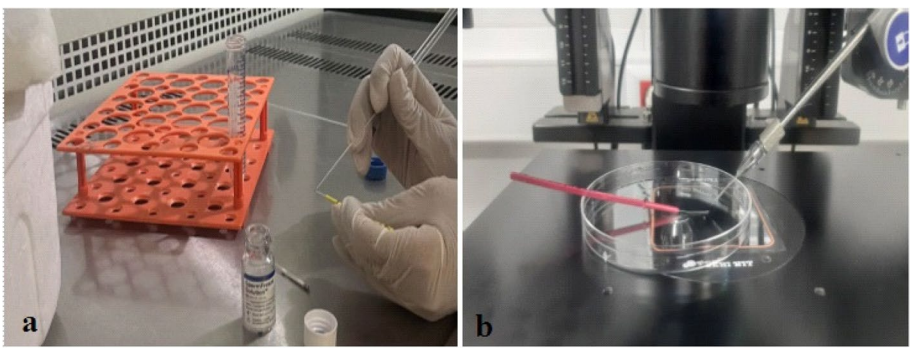

Fig. 1 **a** sperm freezing medium was loaded on the Cryotech device using a mouth pipet. **b** Testicular sperms were selected using an ICSI injection pipette and then transferred into the one droplet of sperm freezing medium

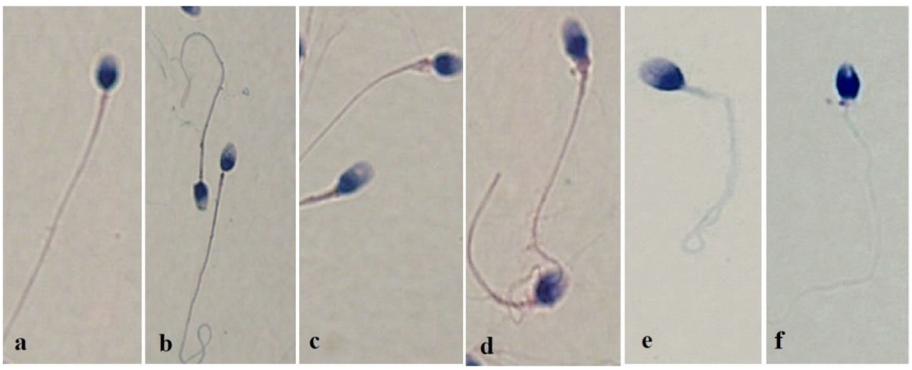

Fig. 2 Diff-quick staining, for sperm morphology scoring. **a** A normal shape sperm, **b** coiled tail after sperm warming, **c** sperm with misshaped head and excess residual cytoplasm (ERC), **d** sperm with amorphous head and broken tail in the weakened site, **e** proximal bent tail, **f** distal bent tail

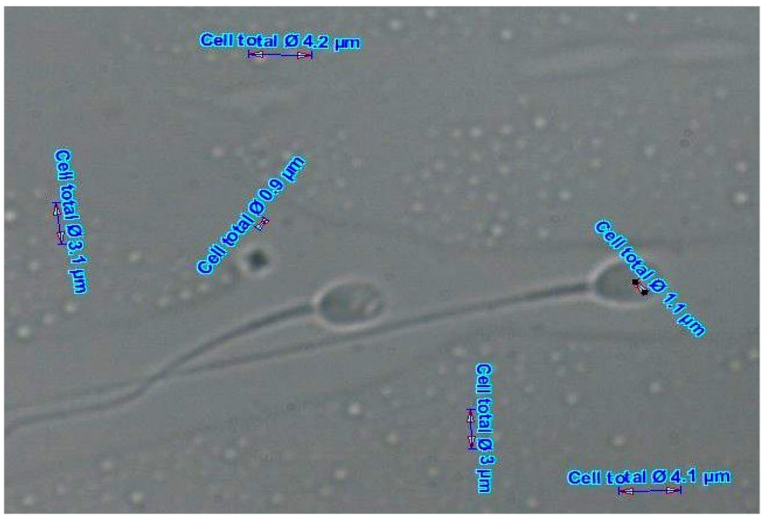

Fig. 3 Measurements of sperm head length and head width were performed using the MSOME

This system identifies the viable motile sperm via emission of a single laser shot of 129 µJ of approximately 1.2 Ms. Briefly if the tail coiled in response to the laser shot (OCTAX Laser Shot®, MTG, Germany), it would be considered viable. Besides this method is fast, simple, repeatable, and safe, there is no need for any chemical compounds for motility induction or flagellum curling [20].

Morphometric assessments

Knowing sperm characteristics and morphometric description can be effective in understanding the impact of cryopreservation on sperm cells (sperm cryo-tolerance), and is a useful method in the prediction of fertility. Measurements of the sperm head length and head width were performed using the MSOME (TE300; Nikon, Tokyo, Japan) and OCTAX software manually. The total viewing magnification of the spermatozoa on the computer monitor was×6600 (Fig. 3). For each sperm head, the ellipticity, elongation, roughness, and regularity were calculated according to Table 1 [21, 22].

Table 1 Parameters assessed for the morphometric characterization of sperm heads

Variable	Formula
Length (µm)	L
Width (µm)	W
Perimeter (µm)	P
Area (μm^2)	A
Ellipticity	L/W
Elongation	(L−W)/(L+W)
Roughness	4π (A/P2)
Regularity	(π*L*W/4*A)

Sperm chromatin assessment

Chromomycine A3 (CMA3) staining was performed as follows: recovered sperms were transferred on a glass slide and air-dried. Samples were fixed in Carnoy's solution, stained by CMA3 solution for 10 min in the dark, and analyzed by fluorescence microscopy [23]. Bright yellow stained cells (chromatin packaging abnormal) were considered as $CMA3^{+}$; while yellowish-green stained sperms (normal chromatin packaging) were considered as CMA3- (Fig. 4).

The sperm histone status was studied by aniline blue (AB) staining. Slides were fixed in a solution of 3% buffered glutaraldehyde stained with the AB stain for 5 min and assayed under a light microscope (Olympus, Tokyo, Japan). Unstained or pale-blue spermatozoa were considered normal mature, and dark-blue stained as abnormal spermatozoa (Fig. 5) [23].

Sperm DNA fragmentation assessment

For the evaluation of the extent of DNA fragmentation, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was done using the In-Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. Spermatozoa with DNA fragmentation display a spectrum of green fluorescence and are considered TUNEL positive (Fig. 6) [24].

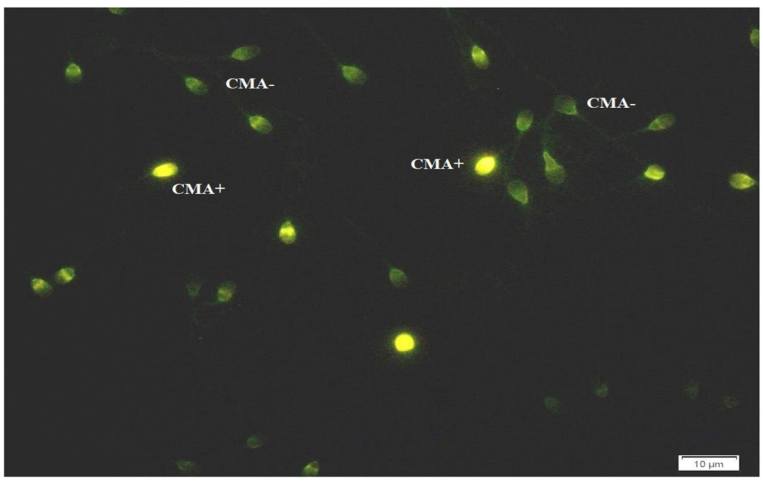

Fig. 4 Sperm Chromomycine A3 staining (CMA3) staining, bright yellow stained cells (chromatin packaging abnormal) were considered as $CMA3^{+}$; while yellowish-green stained sperms (normal chromatin packaging) were considered as CMA3-

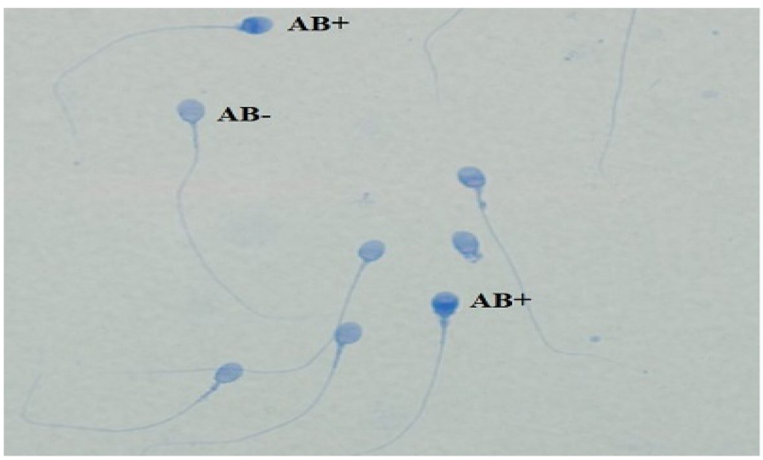

Fig. 5 The sperm histone status by aniline blue (AB) staining. Unstained or pale-blue spermatozoa were considered normal mature, and dark-blue stained as abnormal spermatozoa

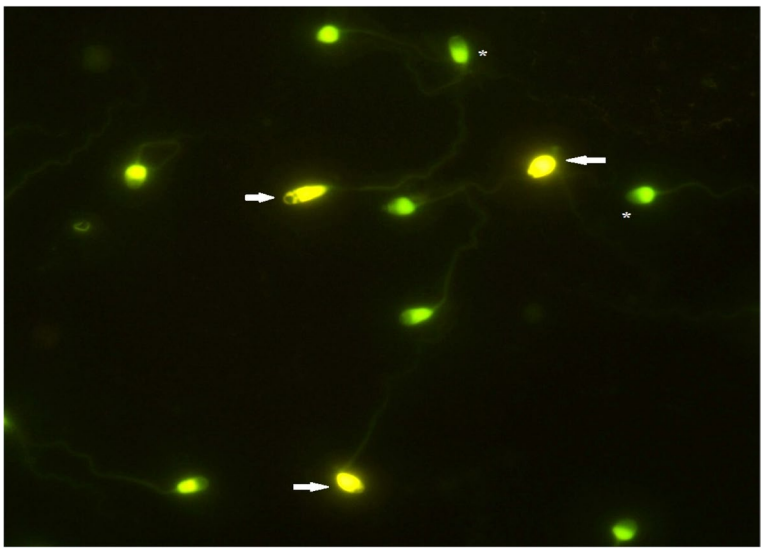

Fig. 6 Extent of DNA fragmentation, (TUNEL) assay. Spermatozoa with DNA fragmentation showed yellow–red fluorescence (arrow), and sperm with intact DNA display green fluorescence (star)

Acrosome integrity assessment

Briefly, spermatozoa were fixed with ethanol incubated with FITC-PSA (Sigma Co, USA), and evaluated using a fluorescent microscope. When over half of the sperm heads were bright and uniformly fluorescing, they were considered acrosome-intact. However, when the fluorescence band was found in the equatorial segment or any fluorescence staining in the acrosome area was considered acrosome-reacted (Fig. 7) [25].

Statistical analysis

SPSS software was used for the evaluation of the effect of vitrification on sperm head morphometric dimensions. The data were analyzed by one-way analysis of variance (ANOVA) and expressed as means ± SD. The normal distribution of data was determined by the Kolmogorov–Smirnov normality test. The significance of differences between the groups was evaluated using the Tukey test and $P < 0.05$ was considered significant.

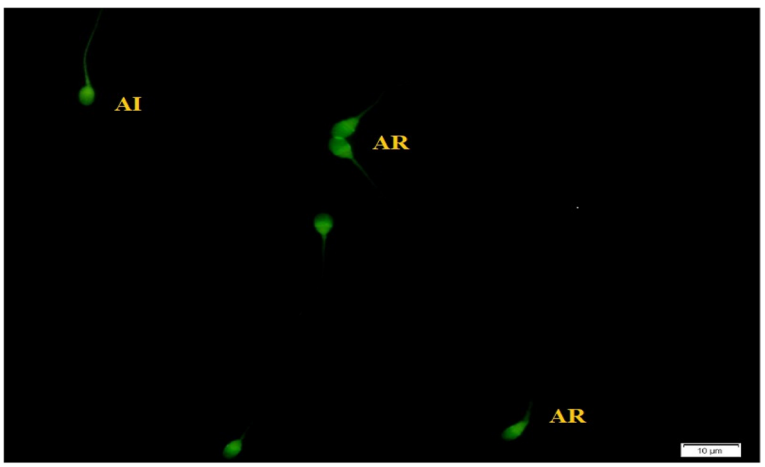

Fig. 7 Acrosome reaction test. When over half of the sperm heads were bright and uniformly fluorescing, they were considered as acrosome-intact. But when the fluorescence band was found at the equatorial segment or any fluorescence staining in the acrosome area was considered acrosome-reacted

Results

Post-vitrification sperm characterizations

Overall, 1500 sperms were frozen on 60 straws (two straws, for each case). Fresh testicular samples (after 24 h culture) used in this study had the following characteristics: motility (76.3 ± 10.5), viability (76.8 ± 6.6%), and the other sperm parameters showed that the cryopreservation process greatly affected the sperm head characterizations, sperm DNA fragmentation, acrosome integrity and other morpho-kinematic parameters (Tables 2 and 3).

The sperm head dimensions in vitrified samples were smaller than those obtained in the fresh samples. Also, significant variations in width, perimeter, roughness, and regularity between fresh and post-thawing spermatozoa in the OVS group were detected ($p < 0.05$) (Table 2). Within analysis variation, the measurements for head length, ellipticity, and elongation were insignificant.

Table 2 The effect of vitrification systems of closed (CVS) and open (OVS) on the mean sperm morphometric parameters

Variables	FC	CVS	OVS	*p* value
Length (μm)	5.56 ± 0.5	5.5 ± 0.43	5.26 ± 0.5	0.14
Width (μm)	3.8 ± 0.25	3.6 ± 0.23[a]	3.63 ± 0.2[a]	0.00
Area (μm^2)	14.6 ± 1	13.65 ± 1	12.44 ± 1	0.02
Perimeter	16.8 ± 1.9	15.8 ± 1.4	15.07 ± 2[a]	0.012
Ellipticity	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	0.30
Elongation	0.17 ± 0.05	0.2 ± 0.05	0.18 ± 0.5	0.31
Roughness	0.66 ± 0.11	0.73 ± 0.1	0.8 ± 0.17[a]	0.01
Regularity	1.1 ± 0.06	1 ± 0.05	1 ± 0.07[a]	0.00

Values are expressed as mean ± SD

FC Control, *CVS* Closed vitrification system, *OVS* Open vitrification system

[a] A significant difference between Controls vs. vitrified groups

Motility, viability

The movement of biopsied spermatozoa before freezing was slow and sluggish (13.22 ± 3.6), even observed as twitching or wavy flagellar movement. Since the Post-thawed sperm motility in testicular cases was greatly reduced, Pentoxifylline was added to the thawing medium. The mean motility rate improved significantly and reached 76.3% in fresh spermatozoa. The sperm motility rate decreased significantly in both vitrified groups compared with the control group (16.14% and 6.06%, in CVS and OSV, respectively ($p < 0.05$)). The motility rate was higher in the closed compared with the open system ($P = 0.0001$).

Sperm viability revealed a mean value of 76.8% prior to vitrification. This value diminished to 54.8% in CVS and 47.1% in OVS after thawing (Table 3).

Sperm DNA integrity

The results for chromatin integrity (CMA3 +) showed that there was a significant reduction in normal intact chromatin structure after vitrification (29.25% in FC, vs. 32.37% in CVS, and 40.13% in OVS groups. On the other hand, significant impairment of sperm chromatin status was seen after the freeze and thawing procedure, with a high positive response to Aniline blue staining, in the vitrified samples.

In comparison to the fresh semen sample. Also, CVS has better results in post-thawed spermatozoa groups (AB +; 30.37%, 37.92%, and 43.37%, respectively) (Table 3).

The sperm DNA fragmentation by TUNEL was 15.25% in the fresh control group and increased to 20.51% in the closed, and 23.17% in the open system group, after the freeze and thaw process ($P = 0.0001$) (Table 3).

Table 3 The testicular sperm quality after vitrification in two systems (closed and opened) compare to fresh control group

Sperm variable (%)	Control	Closed system	Open system	*P* value*
Motility after biopsy	13.22 ± 3.6			
Motility after 24 h in vitro culturing	76.3 ± 10.5	16.14 ± 5.2	6.06 ± 3.5	0.0001
Viability (LAISS)	76.8 ± 6.6	54.8 ± 4.6	47.1 ± 4.7	0.0001
Protamine deficiency (CMA3)	29.25 ± 3.6	32.37 ± 3.8	40.13 ± 3.5	0.001
Histone excess (AB)	30.37 ± 3.3	37.92 ± 2.6	43.37 ± 3.0	0.0001
DNA fragmentation after biopsy	13.4 ± 3.1			
DNA fragmentation after 24 h in vitro culturing	15.25 ± 1.8	20.51 ± 4.8	23.17 ± 5.4	0.0001
Intact acrosome	63.18 ± 7.1	58.03 ± 6.0	50 ± 5.4	0.016
Morphology after biopsy	1.5 ± 0.6			
Morphology after 24 h in vitro culturing	2.9 ± 0.7	2.5 ± 0.6	2 ± 0.6	0.075

*One-way ANOVA. data are expressed as mean ± SD

Acrosome integrity

The results showed among the two cryopreservation methods OVS systems greatly affected acrosome integrity compared to the fresh control group (63.18% vs. 50.0% in the fresh group) (Table 3).

Sperm morphology

However, sperm morphology decreased during the freeze and thaw process, and the results were better in the closed system ($p > 0.05$) (Table 3).

Discussion

Sperm cryopreservation is an important procedure that is used in ART labs for different purposes [26]. Many studies have tried to find a safe method, containers, or better medium to increase sperm cryo-resistance, and the challenge of post-thaw sperm loss would be greater, when there is a low sperm count for freezing (such as severe oligozoospermia, or azoospermia), in the aim to increase ICSI success rate. During sperm freezing, the cell temperature decreases to − 196 °C and then will return to the body temperature during thawing. So, the cold and warm shocks can loss of normal sperm structure and functions.

The cryodamage in mammal spermatozoa is associated with head dimensions alteration [27]. In the current study, we also used the head's measurements to evaluate which method of vitrification is associated with lower cryodamage, in the case of low semen volume; open, or closed system.

The results of this study showed more sperm head morphometry alteration, following vitrification with the OVS system, compared to the fresh control group. Since cryopreservation can cause sperm head alteration [28–30], it can be concluded that the close system (CVS) is a more optimal method, preserving more sperm head structure. Numerous mechanisms during cryopreservation may underlie the decrease in sperm head dimension, including osmotic changes [31], alterations of cell compartments [32], damage of the acrosome [33, 34], condensation of nuclear chromatin [35, 36], and sperm dehydration [37], while large sperm heads may be related to chromatin condensation defects, large nuclear pockets, and abnormal protamine ratios [14]. It was mentioned that sperm head morphology affects sperm fertilization capacity [38], and sperm head alteration is accompanied by a lower successful pregnancy after artificial insemination [29]. So, a safe and efficient sperm freezing method will be needed in the cases of OAT, which could guarantee sperm normality.

The results also showed a significant reduction in both sperm motility and viability, after vitrification. It is by other studies tried to vitrify single sperm in cases of severe oligozoospermia and azoospermia [39]. In this regard, better sperm motility and viability were earned, after the closed system compared to the open vitrification method. To the best of our knowledge, there has not been any study that investigated the cryo-tech device, in the two methods of open and closed system, for sperm freezing but there are some other studies that tried to cryopreserve of a small number of single spermatozoa in the open system [40, 41], or closed systems [17, 42, 43]. One of the main theories of these studies was the probability of microbial contamination with the open systems, and their results of using closed systems were satisfactory. Besides, some of the advantages of the closed devices, such as cryolock, and cryo-tech, are that allow for loading a very small volume of sperm (1–3 µL), and thawing the sperm samples directly into the ICSI dish, so there is no need for post-thawing washing and centrifugation [44].

More protamine deficiency and histone excess were observed in the OVS compared to CVS. Sperm is extraordinarily sensitive to rapid temperature reduction, which is called cold shock [45]. The protamine-histone replacement is a level of sperm nucleus organization that leads to condensation and compaction [46, 47]. The unbalanced protamine 1-to-protamine 2 ratios cause low chromatin compaction and higher DNA instability. These significantly enhance the likelihood of sperm DNA fragmentation [48].

The sperm DNA fragmentation increased after 24 h incubation, and also after the vitrification, especially in the open system. Other studies also showed that DNA fragmentation will be increased by the incubation and cryopreservation of the testicular spermatozoa [49], so for sperm cryopreservation, the method that is accompanied by minimal sperm DNA fragmentation should be used.

Some destructive events related to cold shock are intracellular enzymes and lipids releasing [50], also acrosomal and mitochondria membrane alterations (ion distribution change) may take place because of both osmotic and cold shock [51]. In this study, more intact acrosome was found following closed compared to open system. For successful fertilization, sperm must have a healthy plasma membrane, as well as acrosomes intact until insemination, for zona penetration and oocyte fusion [52]. So finding a reliable method for cryopreservation, which could guarantee the plasma and acrosome membrane, could increase the fertility rate of the warmed sperms.

Sperm morphology is the other parameter that declined after freezing [53]. However, the results were not significant, the morphological results were better in the closed system.

The osmolality and the tonicity of the sperm surrounding the medium are two main factors that influence

sperm. During cryopreservation, the sperm membrane may change due to osmotic gradients [54], which can lead to the reduction of viability [55]. The reaction of spermatozoa to osmotic challenge and their ability to regulate cell volume can determine sperm cryopreservability [56]. Almost all components of the membrane suffer from the freeze and thaw process. The rate of these changes is different among species and depends on the arrangement and biophysical properties of the plasma membrane [57]. A decrease in sperm viability after thawing, especially in OVS, shows that the membrane has undergone noticeable changes.

In addition, our observation elucidates the loss of acrosome during the vitrification process. According to a hypothesis, the cytoplasmatic Ca^{+2} levels can increase along with lowering temperature which ends up in a capacitated-like reaction, ion leakage, and exocytosis of acrosomal contents [58]. The decline of intact acrosome after cryopreservation may be the reason for the reduced reproductive potential of human spermatozoa. Also, an increase in the size of sperm components, as well as sperm head elongation is associated with better motility [59]. According to this theory, for more energy sources, the spermatozoa with larger heads swim faster. The decrease in sperm motility occurred following the vitrification. Perhaps, one of the causes of this lesion is the morphological changes in the sperm head.

In the CVS state with the falling of the cooling rate, the sperm alterations were less, and the samples were not in direct contact with liquid nitrogen. According to the other previous studies, it was shown that vitrification using a closed system does not compromise sperm motility, while in the double straw design, the specimen is not exposed to LN_2 [60].

Conclusion

The recovered testicular spermatozoa from CVS showed much higher sperm viability and fewer alterations in the chromatin, acrosome, and sperm head morphometry than OVS. As sperm head pathology, as a marker of genetic defects, increased after cryopreservation, the current findings are behind the safety of CVS with the lower head parameter changes.

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Authors' contributions
M A Khalili contributed to research design and manuscript drafting and approval. M Vatanparast contributed to manuscript revision and approval. E Mangoli and Saeid Ghasmi Esmaeilabadi performed the research. M Moshrefi contributed to data collection and analysis. Akram Hosseini interpreted the data and helped revise and approve the manuscript. All authors read and approve the final manuscript.

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Availability of data and materials
Derived data supporting the findings of this study are available from https://doi.org/10.7910/DVN/EPX623

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Yazd Reproductive Sciences Institute, which follows the Helsinki Declaration of 1975 (Ir.ssu.rsi.rec.1394.30).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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