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Cryoprotectant-free vitrification of human spermatozoa in new artificial seminal fluid

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SUMMARY

Vitrification is a new method that has been recently introduced in Assisted Reproduction Technique programs. The aim of this study was to design a new medium similar to normal human seminal fluid (SF), formulation artificial seminal fluid (ASF), and to compare the cryoprotective potency of this medium with SF and human tubal fluid (HTF) medium. Thirty normal ejaculates were processed with the swim-up technique and sperm suspensions were divided into four aliquots: (i) fresh sample (control); (ii) vitrification in HTF medium supplemented with 5 mg/mL human serum albumin and 0.25 mol sucrose (Vit HTF); (iii) vitrification with patients' SF (Vit SF); and (iv) vitrification in ASF (Vit ASF). After warming, sperm parameters of motility, viability, and morphology were analyzed using WHO criteria. Also, sperm pellets were fixed in 2.5% glutaraldehyde and processed for scanning electron microscopy and transmission electron microscopy observations. The results showed that progressive motility (46.09 ± 10.33 vs. 36.80 ± 13.75), grade A motility (36.59 ± 11.40 vs. 16.41 ± 11.24), and normal morphology (18.74 ± 8.35 vs. 11.85 ± 5.84) and viability (68.22 ± 10.83 vs. 60.86 ± 11.72) of spermatozoa were significantly higher in Vit ASF than in Vit HTF. All parameters were better in Vit ASF than in Vit SF, but only viability was significantly different ($p = 0.006$). After cryopreservation, deep invagination in cytoplasm and mechanically weak point sites and folded tail were commonly observed. But, this phenomenon was more significant in Vit HTF and Vit SF than in ASF ($p < 0.05$). In transmission electron microscopy evaluation, acrosome damage, plasma membrane loss, chromatin vacuolation, and disruption of mitochondria arrangement and structures were observed in all vitrified groups. Adherence of several tail sections together was also seen in all cryo groups. But this was seen more in Vit HTF and Vit SF than in ASF ($p < 0.05$). In conclusion, vitrification of human spermatozoa with ASF can effectively preserve the quality of sperm motility in comparison with Vit HTF.

INTRODUCTION

One of the main problems associated with sperm cryopreservation is reduction in vital parameters, such as motility and viability. Despite many investigations, further studies are necessary to select the best cryopreservation method and media for spermatozoa. Nawroth *et al.* (2002) showed the feasibility of vitrification without any permeable cryoprotectant (CPA). CPA-free vitrification is direct plunging of sperm suspension into liquid nitrogen. This technique avoids the use of the classic toxic CPA that may have lethal effects of osmotic shock. It also prevents lethal intracellular ice formation and the detrimental effects of high salt concentrations during freezing and thawing (Isachenko *et al.*, 2003). Another study compared the vapor phase and liquid phase for sperm cryopreservation, using the cryoloop. The findings suggest that CPA-free cryopreservation of spermatozoa could occur in a wide range of cooling rates (Isachenko *et al.*, 2004b). It was reported that use of sucrose as non-permeable CPA enhanced the rate of motility and mitochondrial function after sperm vitrification (Isachenko *et al.*, 2008). In our recent study, vitrification of normal spermatozoa showed results similar to rapid vapor freeze, with two advantages of absence of toxic effect of permeable CPA along with less DNA damage (Agha-Rahimi *et al.*, 2014). Techniques for aseptic cryoprotectant free vitrification include intrauterine insemination by 0.5 mL straw (Isachenko *et al.*, 2011) or a capillary straw for IVF or ICSI (Isachenko *et al.*, 2012). In these techniques, spermatozoa can be used immediately after warming without any additional preparation. Isachenko *et al.* (2012) reported a healthy baby born after intrauterine insemination with vitrified swim-up spermatozoa.

One controversial issue is about the presence or absence of seminal fluid during sperm cryopreservation. The protective nature of seminal plasma has been highlighted, because of the presence of zinc and antioxidants (Donnelly *et al.*, 2001; Saritha & Bongso, 2001). Also, seminal fluid may sometimes contain high levels of reactive oxygen species (ROS) (Aitken & Clarkson, 1988). In a previous study, we concluded that vitrification of neat ejaculates, particularly in abnormal semen, has several effects on sperm parameters (Khalili *et al.*, 2014). Therefore, sperm preparation should include selection of best spermatozoa, preventing ROS production by dead or damaged spermatozoa. The aim of our study was to test an artificial culture medium similar to seminal plasma, and survey its effect on fine structure of human spermatozoa post vitrification. In addition, a new design for explanation of cryo-damage with isosmotic condition was investigated.

MATERIALS AND METHODS

Thirty normal ejaculates were obtained from the andrology laboratory. Written informed consent was obtained from all the patients. Each semen sample was divided into three parts (Fig. 1):

1 For obtaining seminal plasma free of spermatozoa, one part of the semen was centrifuged (2000 ***g***), and the pellet was removed.
2 Semen swim-up with human tubal fluid (HTF) medium. The harvested sperm suspension was divided into three parts: Part a was considered as fresh HTF, Part b was mixed with 0.5 M sucrose solution (1 : 1), and prepared for the vitrification procedure (Isachenko *et al.*, 2008) as vitrified HTF (Vit HTF). Part c was centrifuged and the sperm pellet was added to the pure seminal fluid (SF). This group was named vitrified seminal fluid (Vit SF). Sperm warming in these two cryo-groups was performed in HTF medium lacking sucrose. The HTF medium in all stages was supplemented with 5 mg/mL human serum albumin (HSA).
3 Part III was the swim-up with artificial seminal fluid medium (ASF). The harvested sperm suspension was mixed 1 : 1 with ASF containing 0.8% methyl cellulose and prepared for vitrification (Vit ASF). Thawing was performed the in ASF medium without methyl cellulose.

Therefore, we had five groups – fresh HTF, fresh ASF, Vit HTF, Vit SF, and Vit ASF (Fig. 1). After vitrification and warming, the analysis of sperm parameters was performed by light microscopy. Also, samples were prepared for both scanning electron microscopy and transmission electron microscopy.

Artificial seminal fluid

For determination of the ASF formulation, we referred to a previous work (Owen & Katz, 2005) with slight modifications. The concentration of ions in the human SF was reviewed, and a mean earned for each ion. The following components comprised ASF: NaCl: 2.69 g/L, Sodium citrate dihydrate: 8.13 g/L, KCl: 0.432 g/L, K_2HPO_4: 1.91 g/L, Na. pyruvate: 0.374 g/L, Na. lactate: 0.779 g/L, glucose. 1 H_2O: 1.12 g/L, fructose: 2.72 g/L, Na. HCO_3: 2.1 g/L, urea: 0.72 g/L, urate: 0.07 g/L, ascorbic acid: 0. 1 g/L, $MgSO_4$: 0.54 g/L, $ZnSO_4{\cdot}7H_2O$: 0.5 g/L, $CaCl_2{\cdot}2H_2O$: 0.73 g/L, human serum albumin: 11 g/L, gentamycin: 40 mg/L. All materials were purchased from Sigma (St. Louis, MO, USA). For the creation of viscose medium similar to SF viscosity during vitrification, we made 0.8% methyl cellulose. After mixing with the sperm suspension, the final concentration was 0.4%. Addition of methyl cellulose did not change the osmolality of the medium. Therefore, sperm vitrification procedure was performed in the isosmotic condition.

The chemical contents of HTF media and ASF are presented in Table 1. The ratios of Mg, Ca, and glucose are more in ASF media. Also, fructose, citrate, Zn, urea, urate, and ascorbic acid are present in ASF, but lacking in the HTF medium.

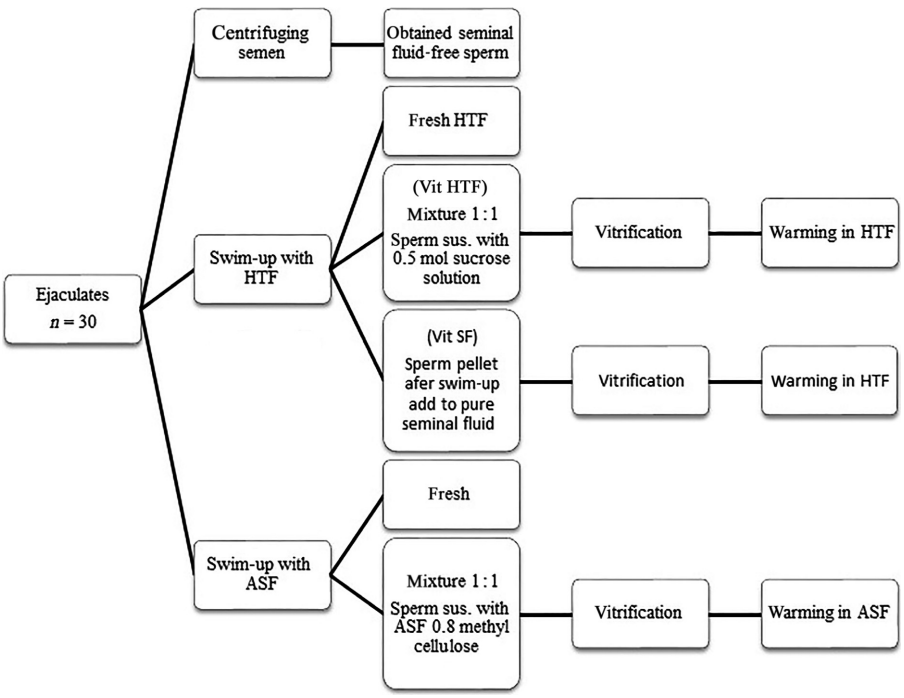


Figure 1 Schematic diagram for sample collection.

Vitrification and warming

Vitrification and warming was performed according to Isachenko *et al.* (2008). Twenty-five microliter aliquots of sperm suspension were dropped directly into a strainer that was immersed in liquid nitrogen using a micropipette. The solid spheres were packed in 1.8 mL cryotubes and stored for 24 h in liquid nitrogen. Warming was performed by quickly submerging spheres one by one into 5 mL pre-warmed medium to 37 °C accompanied by gentle vortexing for 5–10 sec. The post-thaw sperm suspension was centrifuged at 400 ***g*** for 5 min. The cell pellet was finally resuspended. The post-thaw sperm suspension was maintained at 37 °C/5% CO_2 for 30 min, and sperm analysis was carried out.

Sperm analysis

Sperm analysis was performed according to the World Health Organization (WHO) guideline (World Health Organization, 2010). Briefly, for motility analysis, we prepared a wet preparation with a volume of 10 μL of sperm suspension. Two hundred spermatozoa per replicate were assessed for the percentage of different motile categories. Grade A spermatozoa moved forward easily, and Grade B spermatozoa hardly moved forward in a shaky pattern. Also, the morphology of the 200 spermatozoa per sample was assessed using Papanicolaou staining. Sperm morphology was categorized into four types: normal, abnormal head, abnormal midpiece and tail, abnormal head + midpiece and tail. Also, 200 stained (dead) or unstained (vital) spermatozoa were counted per sample for assessment of vitality with eosin–nigrosin staining.

Table 1 Chemical contents of human tubal fluid (HTF) medium and artificial seminal fluid (ASF)

Components (mg/100 mL)	HTF medium	ASF
Na	340	378.67
Cl	387.92	236.43
K	190.21	108.3
Ca	8.1	20
Mg	0.49	11
Lactate	183.01	62
Pyruvate	2.8	29
HAS	5	11
Glucose	50	102
Fructose	–	272
Citrate	–	528
Zn	–	11.3
Urea	–	72
Urate	–	7.1
Ascorbic acid	–	10
Human serum albumin	500	1100

Electron microscopy

For transmission electron microscopy (TEM), the sperm suspension was fixed primarily in 4% glutaraldehyde and secondarily in osmium tetroxide 1% in the dark. The sperm pellet was transported to 1% gel agar. Dehydration was carried out with an alcohol series. Resin infiltration was done in propylene oxide : resin 1 : 1, 1 : 3, and pure resin. Samples were embedded in the beam capsule, then baked in 60 °C ovens for 48 h. Ultrathin sections at 60–90 nm thick were prepared and stained with uranyl acetate and lead citrate. Preparations were observed using a Zeiss EM 10 electron microscope operating at 80 kV.

For scanning electron microscopy (SEM), the sperm suspension was fixed primarily in 4% glutaraldehyde. Dehydration was carried out with the alcohol series. The final pellet was diluted with ethanol 100%. Small pieces of glass slides were prepared, and then the sperm suspension was smeared on them. Smears were dried in air for 24 h and coated with gold–palladium membranes with 4 nm thickness. Preparations were observed using TESCAN, VEGA 3. Spermatozoa were evaluated according to Visual Atlas (Sathananthan, 1996).

Statistical analysis

Comparison of sperm parameters before and after freezing was performed with the paired sample *t*-test. Comparisons of data between groups were performed with the ANOVA–Tukey test. Kruskal–Wallis was used for data with abnormal distribution. A difference with a *p*-value of <0.05 was regarded as a significant difference.

RESULTS

Comparisons of sperm parameters in fresh and vitrified groups

All parameters were similar in fresh HTF and ASF fresh groups. Sperm parameters including motility, viability, and normal morphology in all vitrified samples were significantly reduced when compared with fresh samples ($p < 0.0001$). Table 2 shows sperm parameters in vitrified samples. Briefly, sperm progressive motility, viability, and normal morphology were significantly higher in Vit ASF than in Vit HTF. All parameters were better in Vit ASF than in Vit SF, but only viability was significantly different ($p = 0.006$). Also, Grade A motility was more in Vit ASF and Vit SF (36.59 ± 11.40 and 31.13 ± 15.38) than in Vit HTF (16.41 ± 11.24) significantly ($p < 0.001$).

Also, the pattern of sperm abnormal morphology was changed after cryopreservation. In fresh samples, the most abnormal category was related to the head region. However, in all cryogroups, this category was reduced significantly and tail and head

Table 2 Spermatozoa parameters after vitrification in different solutions. Values are given as mean and standard deviation

Parameters (%)	Fresh HTF	Vit HTF	Fresh ASF	Vit ASF	Vit SF
Progressive motility	83.68 ± 4.94^{a}	$36.80 \pm 13.75^{b,c}$	86.09 ± 4.44^{a}	46.09 ± 10.33^{d}	41.70 ± 12.70^{b}
Total motility	94.53 ± 3.70^{a}	53.70 ± 11.16^{b}	95.25 ± 2.99^{a}	52.34 ± 10.65^{b}	47.73 ± 10.67^{b}
Viability	94.84 ± 3.01^{a}	$60.86 \pm 11.72^{b,f}$	95.62 ± 2.67^{a}	$68.22 \pm 10.83^{b,g}$	$59.65 \pm 14.55^{b,h}$
Normal morphology	21.51 ± 7.16^{a}	$11.85 \pm 5.84^{b,i}$	23.44 ± 6.35^{a}	$18.74 \pm 8.35^{b,j}$	14.00 ± 6.84^{b}

Fresh HTF, sperm suspension after swim-up in human tubal fluid; Fresh ASF, sperm suspension after swim-up in artificial seminal fluid; Vit HTF, sperm vitrification in HTF supplemented with human serum albumin and sucrose; Vit ASF, sperm vitrification in artificial seminal fluid; Vit SF, sperm vitrification in patient seminal fluid. All parameters significantly reduced after vitrification than fresh samples; also, parameters with superscripts are significantly different: (a, b: $p < 0.001$), c, d: $p = 0.003$, f, g: $p = 0.03$, g, h: $p = 0.006$, i, j: $p = 0.004$.

abnormalities were increased significantly ($p < 0.001$). This increase was more in both VIT HTF and Vit SF than in Vit ASF.

Recovery rates with sperm parameters after vitrification in different solutions

Recovery rates were defined (sperm parameter before cryopreservation)/(sperm parameters after cryopreservation) × 100. Results of sperm recovery rates are presented in Table 3. Viability and normal morphology recovery rates were more in Vit ASF than in other vitrified samples ($p < 0.05$). Progressive motility recovery rates were higher in Vit HTF than in Vit ASF ($p = 0.03$). All parameter recovery rates were similar between cryo-HTF and SF (Table 3).

Scanning electron microscopy analysis

Figure 2 shows SEM micrographs of fresh and vitrified spermatozoa. Our data revealed that the intact head and tail was significantly decreased in Vit groups than in the fresh sample (Fig. 3). There were deep invaginations in the head, especially acrosomal spaces that could be involved in acrosomal discharge (Fig. 3B). In addition, mechanically weak sites were observed in the spermatozoa midpiece and tail in a single or multiple manner that probably was broken mechanically in these regions. The rough surface of the membrane was another finding, especially in mid-piece segments. The tail defects were one of the other damages in the cryo-groups. Folded tail was also commonly observed. However, this phenomenon was more frequent in Vit HTF and Vit SF than in ASF (Figs 3 & 2C,D). Other damages were visualization of loose head and tail fragmentation along with irregular thickness of connection between the head and the midpiece (Fig. 2).

Transmission electron microscopy analysis

Figure 4 shows TEM micrographs of fresh and vitrified spermatozoa. In fresh samples, the majority of spermatozoa showed intact cell membranes and normal acrosomes. The inner membrane of the acrosome was close to the nuclear membrane. The nucleus was shown to be condensed, sometimes with small vacuoles. The neck region was intact, and mitochondria arranged regularly along the midpiece. Their cristae were numerous, arranged irregularly, the internal cristae space was homogenous and moderately electron dense. After vitrification, however, several damages were observed in all cryo-groups. The acrosome was the most sensitive organelle and various damages, such as complete or partial loss of acrosome, swollen or vesiculate, heterogeneous content, and sub-acrosomal swelling were observed after vitrification. The plasma membranes showed several defects including swelling, wrinkle, rupture, or loss. In addition, nucleus injury included granular and less dense electron nucleus with single or larger vacuoles. These vacuoles were white holes without membrane, usually located in the center of the nucleus. Mitochondria were round and their cristae were not sharp and visible. The damaged acrosome and chromatin were increasingly significant in all vitrification groups than in the fresh sample, but between groups these damages differed insignificantly (Fig. 5). The arrangement of mitochondria was disturbed in some cells. Also, in Vit HTF and Vit SF, the presence of two or more sections of the tail adherent together (aggregated tails) was observed more significantly than in Vit ASF. These sections probably related to folded or coiled tails (Fig. 5). Degradation of cells including lack of acrosome, disruption of neck, and degraded karyoplasm was observed after vitrification (Fig. 4).

DISCUSSION

Seminal fluid contains high levels of fructose as the main energy source and metal ions such as zinc that contribute to maintaining the integrity of sperm membranes during cryopreservation (Saritha & Bongso, 2001). It was concluded that SF contains some materials with cryoprotectant properties. Therefore, some reports concluded that sperm freezing with SF improved post-thaw motility and DNA integrity (Donnelly *et al.*, 2001; Saritha & Bongso, 2001). On the other hand, oxidative stress is a common phenomenon seen in approximately half of infertile men. ROS, defined as including oxygen ions, free radicals, and peroxides are generated by the spermatozoa and seminal leukocytes within the semen (Aitken & Clarkson, 1988; Tremellen, 2008). Therefore, in these patients, the presence of SF may not have an effective role in spermatozoa protection during cryopreservation. We formulated a new culture medium with a formulation similar to the SF. It was based on the review proposed by Owen & Katz (2005). Our medium is bicarbonate base system, similar to other body fluids. It also has all energy sources including glucose, fructose, lactate, and pyruvate that are naturally present in SF. We increased the concentration of Na and Cl in our media, to reach the osmolality of 320 ± 10. The other property of ASF is the presence of three most important antioxidants, ascorbic acid, urate, and albumin (Smith *et al.*, 1996; Gavella *et al.*, 1997). Previous studies confirmed that antioxidant supplementation of the freezing media improved sperm motility (Askari *et al.*, 1994; Bilodeau *et al.*, 2000; Pena *et al.*, 2003).

Our findings presented a significant reduction in sperm motility, viability, and morphology after freezing in all the cryo-groups. Different motility recovery rates up to 60 and 20% were reported after thawing in normospermic and oligoasthenoteratozoospermic patients, respectively (Isachenko *et al.*, 2012). Progressive motility significantly higher in Vit ASF. It is reported that osmotic shock produced with CPAs caused reduction of viability and motility (Critser *et al.*, 1988; Gao *et al.*, 1995; Gao & Critser,

Table 3 Recovery rates of sperm parameters in different vitrified groups. Values are given as means and standard deviations (with the range in parentheses)

Recovery rate (%)	Vit HTF	Vit ASF	Vit SF
Progressive motility	44.02 ± 16.37[a] (11.11–70.00)	53.54 ± 11.65[b] (31–74)	49.97 ± 15.85 (17–68)
Total motility	56.82 ± 11.38 (37.8–78.57)	54.98 ± 11.25 (32.63–72.73)	50.18 ± 11.35 (28.72–73.86)
Viability	64.14 ± 11.99[c] (40.00–87.10)	71.32 ± 10.81[d] (40.00–91.40)	62.66 ± 15.62[e] (19.57–86.02)
Normal morphology	55.49 ± 22.69[f]	77.14 ± 25.86[g]	61.40 ± 21.00[h]

Fresh HTF, sperm suspension after swim-up in human tubal fluid; Fresh ASF, sperm suspension after swim-up in artificial seminal fluid; Vit HTF, sperm vitrification in HTF supplemented with human serum albumin and sucrose; Vit ASF, sperm vitrification in artificial seminal fluid; Vit SF, sperm vitrification in patient seminal fluid. Parameters with superscripts are significantly different: a, b: $p = 0.03$, c, d: $p = 0.01$, d, e: $p = 0.02$, f, g: $p = 0.004$, g, h: $p = 0.04$.

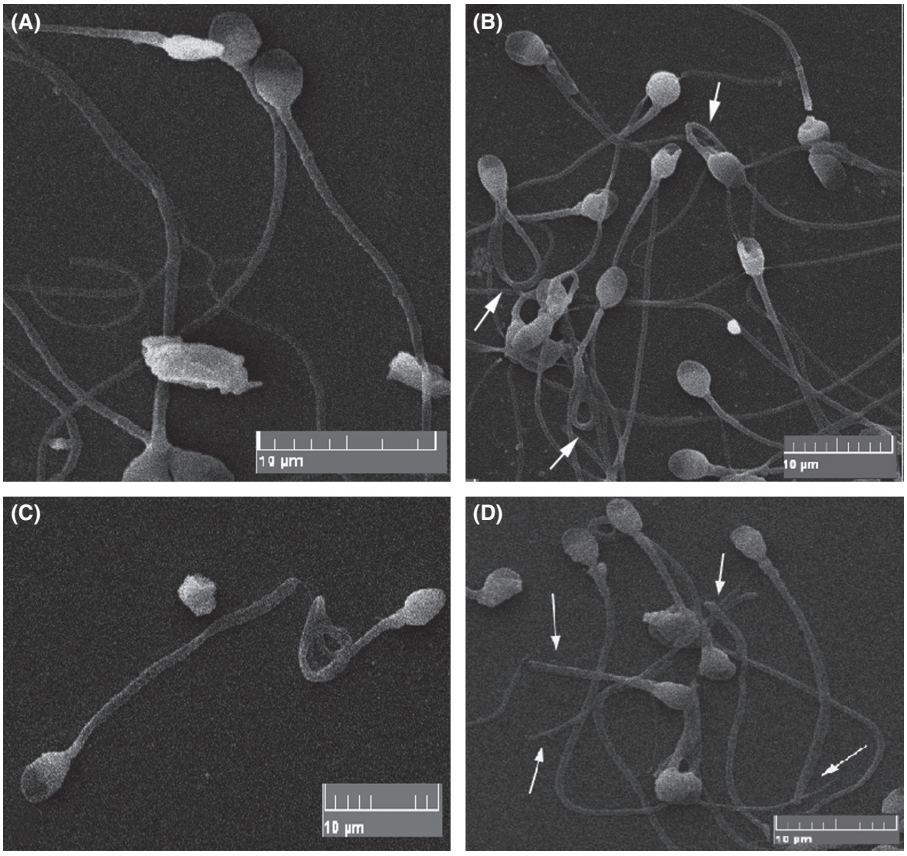
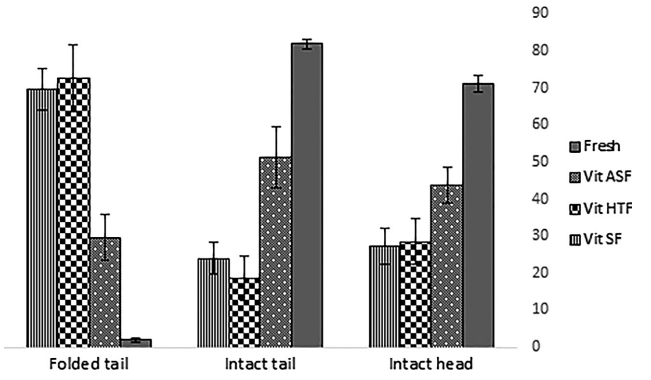


Figure 2 Scanning electron microscopy micrographs. (A) Fresh sample, the entire tails are without defects and folded tail. (B) Vit ASF, arrows show folded tails with U shape. But, some tails are without loops. Some heads show deep invaginations in acrosomal site. (C) Vit HTF, folded tail, similar to the short tail is created. (D) Vit SF, arrows show the folded tail.

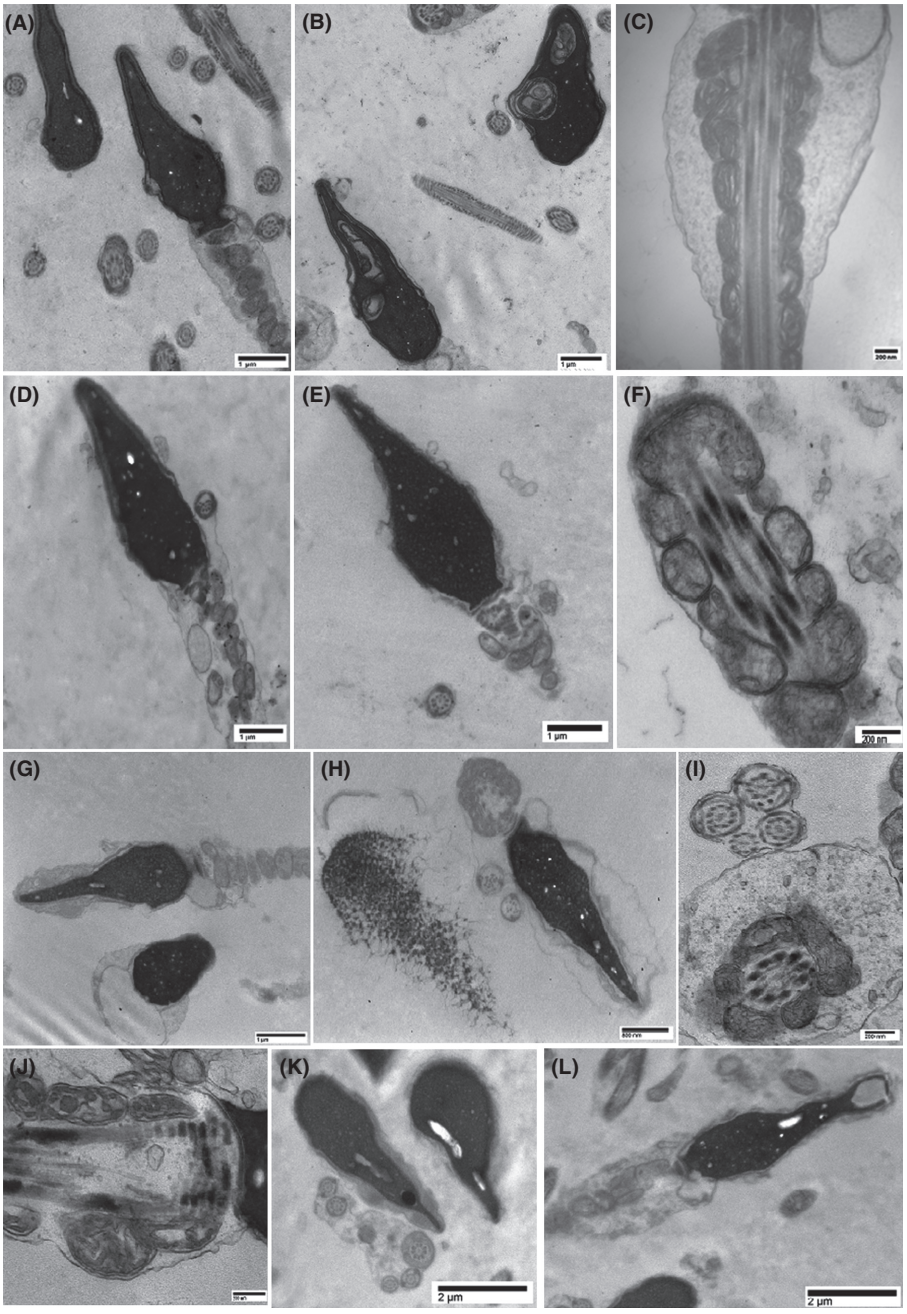
Figure 3 Comparison of intact head, intact tail, and folded tail in fresh and vitrified groups. In all groups, intact head and tail was decreased significantly and folded tail was increased significantly after vitrification ($p < 0.05$). Folded tail was significantly more in both Vit HTF and Vit SF than in Vit ASF ($p < 0.05$). Values are mean and standard error.



2000). In our study, there was a significant correlation between motility and viability in all groups (data not reported). This means that loss of viability is the main cause for the drop in motility. ASF medium is the only group for which we tried to create an isosmotic condition during cryopreservation. Addition and removal of sucrose before and after vitrification caused osmotic shock, even when sucrose was added to 1/2 X HTF. In a recent study, Chen *et al.* compared sperm vitrification by cryotop in the presence or in the absence of sucrose. Cryotop is a special vitrification container consisting of a fine, thin film strip attached to a hard plastic handle. The authors reported that post-thawed spermatozoa cryopreserved without sucrose had a higher viability and lower damage to sperm chromatin and DNA than those cryopreserved with sucrose. Despite the non-toxicity of sucrose for the human sperm, the osmotic damage caused by this still exists (Chen *et al.*, 2015).

A significant reduction in normal sperm morphology was demonstrated after thawing in all groups of cryopreservation. Abnormalities in tail and mid-pice were more common. It is reported that tail curling had taken place after exposure of sperm to hypo-osmotic media. Even when the sperm was exposed to a hyperosmotic media, it returned to isosmotic; curling took place. In addition, sperm motility dropped significantly when osmolality was >50 above or below isotonic (Gao *et al.*, 1995). Others have also reported an increasing trend toward sperm abnormal morphology, especially in the tail region (Hammadeh *et al.*, 2001a,b; Ozkavukcu *et al.*, 2008; Satirapod *et al.*, 2012). Recovery of normal morphology, especially in the mid and tail regions, was mostly noticed in Vit ASF and less in Vit HTF. The change of tail structure is an indicator of osmotic shock that was significantly more in Vit HTF than in Vit ASF. Therefore, creation of isosmotic condition improved the results in cryopreservation. The pattern of the coiling tail was different in cryo-groups. In HTF, we noticed a folded loop that was at first imagined to be a short or cutting tail, but we recognized a folded tail causing this phenomenon after SEM. In ASF, a mild U-shaped loop was observed. This means that despite the isosmotic condition, osmotic shock occurred during vitrification in this group. According to the Morris theory, the rapidly cooled samples during warming revealed extensive ice recrystallization around the cell during the warming phase. These were subjected to osmotic

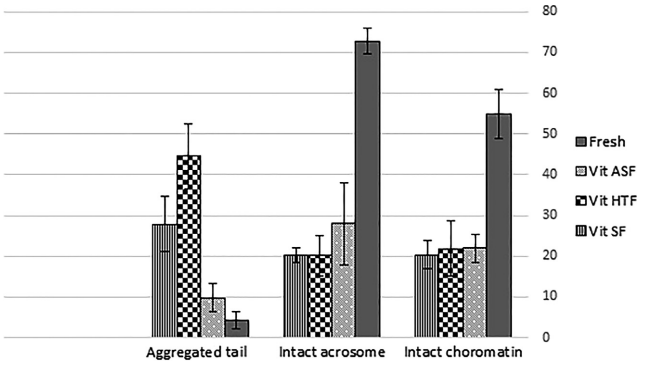
Figure 4 Ultramicrograph of human spermatozoa. (A) Fresh sample. With dense, homogenous and intact acrosome and condensed nucleus without large vacuole. (B) Fresh sperm with head vacuole with membrane and including membrane apparatuses. (C) Sperm mitochondria in fresh sample with numerous cristae and irregular arrangement. The internal space is limited. (D) Vit ASF, approximately intact acrosome and chromatin. (E) Vit ASF, acrosome with reacted view, chromatin is slightly granular, sperm is denuded from plasma membrane. (F) Sperm mitochondria in Vit ASF, the cristae of mitochondria are not sharp with expanded white space and mitochondria are swollen. (G) Vit HTF group, upper sperm shows acrosome swelling and loss of membrane in neck and mid piece, and decondensed chromatin; redundant nuclear envelope in both spermatozoa is swollen. (H) Vit HTF, left sperm shows necrotic cell with disrupted plasma membrane and degraded chromatin; right sperm shows disrupted and discharged acrosome, subacrosomal swelling, wrinkling plasma membrane, and partially decondensed chromatin with small vacuoles. Also two tail sections adhere to the head with abnormal tubulin arrangement; swelling of plasma membrane on the mid piece is also seen. (I) Vit HTF, adherence of several sections of tail together (agregated tail) shows folded tail. (J) Sperm mitochondria in HTF cryo-group, the crista of mitochondria is vesiculated and is not recognizable with expanded white space, it is a sign of swelling mitochondria. (K) Vit SF, two sperm heads with expanded white space; adherence of several sections of tail together shows folded tail. (L) Vit SF, sperm head with expanded white space, acrosome discharge, and swollen redundant nuclear envelope.



shock at low temperatures, causing the damage, especially in the plasmalemma (John Morris *et al.*, 2012). A recent study reported that warming at 42 °C for de-vitrification of human spermatozoa is the optimum temperature required for enhancing sperm viability and motility significantly (Mansilla *et al.*, 2016).

Under all cooling rates, spermatozoa do not contain intracellular ice. This means that vitrification of the intracellular compartment always occurs (Morris *et al.*, 2007). Therefore, the cryopreservation system should be designed in such a way that it removes extracellular ice without osmotic shock, resulting in

Figure 5 Comparison of intact chromatin, intact acrosome, and aggregated tail in fresh and vitrified groups. In all groups, intact chromatin and intact acrosome decreased significantly and aggregated tail increased significantly after vitrification ($p < 0.05$). Aggregated tail was significantly higher in Vit HTF and Vit SF than in Vit ASF ($p < 0.05$).



less significant cryo-damage to the sperm. Also, we supplemented the medium with methyl cellulose to have a viscose media similar to SF. In unpublished data, we concluded that addition of methyl cellulose to vitrification media improved the sperm viability rate. Probably, these materials capsulated cells and protected them from extracellular ice damage.

Deep invagination was seen in the SEM images that is an indication of cell rupture. The presence of these ruptures in the acrosome caused acrosome discharge. Different ultrastructural abnormalities after sperm cryopreservation were noted, such as decomposition of the plasmalemma, acrosomal damage, as well as early acrosomal reaction, and chromatin condensation abnormalities (Ozkavukcu *et al.*, 2008; Aydin *et al.*, 2013). We did notice acrosome damage abundantly in all cryo groups. It is proposed that acrosomal loss because of cryopreservation is associated with cell death (Cross & Hanks, 1991). We reported changes in mitochondrial structure similar to Woolley & Richardson (1978). The order of mitochondria was disrupted in some cells. This phenomenon has been reported in SEM evaluation with the raphe midpiece or bubble formation in the midpiece. Isachenko *et al.* (2008) reported a decrease in the function of mitochondria after vitrification. It is probable that ATP production would be reduced contributing to the drop in motility (Watson, 1995). Maybe with the enrichment of media with a high concentration of energy source, the ATP production with mitochondria becomes feasible. This condition is provided in ASF media, probably helping quality of motility after vitrification than in HTF.

The other finding was the presence of the ruptured plasma membrane and its loss in the neck region. In SEM evaluation, we detected the connection between head and neck becoming thin in some cells, probably because of the loss of plasma. Ice formation and cryopreservation sub-lethally modify the sperm membrane to become more reactive, a state similar to that observed during the capacitation state after thawing (Buhr *et al.*, 1994). Both capacitation and acrosome reaction increased after cryopreservation (Bailey *et al.*, 2000; Isachenko *et al.*, 2008, 2011). Thus, sperm survival is limited, because capacitated spermatozoa do not have a prolonged survival phase. Cryopreserved spermatozoa display motility changes, more likely because of injury than due to hyperactivity (Watson, 1995).

The chromatin structure was changed in sperm after vitrification in our study. Chromatin in fresh samples was more condensed with few 'nuclear holes'. The number of nuclear holes increased after vitrification in some cells. In some spermatozoa, a vacuole may expand and cause chromatin to marginalize. However, we are unaware whether these chromatin shapes caused increasing DNA damage or not. Insignificant differences have been noticed in sperm DNA integrity after vitrification (Isachenko *et al.*, 2004a; Agha-Rahimi *et al.*, 2014). Several tail section adherents together with fused membrane were seen significantly in Vit HTF and Vit SF, suggesting that ultrastructure damage of the tail is more pronounced in these groups. Probably, a more stable osmotic condition in Vit ASF prevented the formation of a severe folded tail; therefore, creating this phenomenon in this group. In addition, we observed very different responses to cryopreservation in all cryo-groups. It is believed that the plasma membrane affects sperm recovery after cooling and warming (Watson, 1995; Giraud *et al.*, 2000). In Vit SF, a wide range of differences was noticed in all parameters. Lately, it has been shown that variation in freezing resilience of ram spermatozoa is related to the source and seminal plasma composition (Rickard *et al.*, 2014).

CONCLUSION

Seminal fluid in normal semen can act as cryo-protectant. Human sperm vitrification in ASF media can effectively preserve these spermatozoa in comparison with vitrification in HTF supplemented with human serum albumin and sucrose.

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