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# DYNAMICS OF SELECTED APOPTOTIC AND HEAT SHOCK PROTEINS IN CRYOPRESERVED AND VITRIFIED SEMEN FROM NORMOZOOSPERMIC MEN

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**ABSTRACT**

Conventional slow freezing is the most widely used technique for sperm cryopreservation, however recently vitrification has begun to be used as an alternative. The present work aims to compare the efficiency of slow freezing and sperm vitrification in preventing changes in the levels of heat shock proteins 70 (HSP 70) and 90 (HSP 90) as well as apoptotic markers BAX, Bcl-2, caspase-3 and caspase-9 in human spermatozoa. Semen samples were obtained from 35 donors with a normal spermiogram and divided into three aliquots. The first aliquot represented the neat sample. The second fraction was used for slow freezing and the third fraction was subjected to vitrification. Each set of samples was subjected to motility assessment and protein extraction for western blot analysis. No significant differences were found in the recovered sperm motility when comparing slow cryopreservation and vitrification, however significantly lower sperm motility ($P<0.01$), progressive motility ($P<0.01$), progressive velocity ($P<0.05$) ad track speed ($P<0.01$) were found in the cryopreserved group in comparison to the neat sample. Both freezing techniques have led to a significant decrease of the HSP 70 protein levels in comparison to the neat state ($P<0.001$ in case of slow cryopreservation; $P<0.01$ with respect to vitrification). Furthermore, both techniques resulted in a significant decrease of the HSP 90 protein levels ($P<0.01$) when compared to neat specimens. No significant differences in HSP 90 levels were detected between the slow cryopreserved and vitrified group. In the meantime, vitrification led to significantly lower BAX ($P<0.05$) and caspase-9 ($P<0.0001$) levels in comparison to cryopreservation. Our results show that sperm vitrification is a technique that can be carried out with good post-thaw sperm vitality outcomes, primarily by providing protection to the mitochondrial system and preventing apoptosis.

**Keywords:** spermatozoa, cryopreservation, vitrification, heat shock proteins, apoptosis, caspases

## INTRODUCTION

The term “cryopreservation” defines techniques that are used to stabilize and preserve intact living cells and/or tissues using very low temperatures. The origin of cryopreservation can be traced back to 1776, when Spallanzani froze spermatozoa using snow and subsequently demonstrated their motility after rewarming (**Nagy *et al.*, 2019**). Since then, a significant progress in the evolution of freezing techniques and media has allowed sperm cryopreservation to overcome many space and time limitations, and thus to become an integral part of assisted reproduction technologies (ARTs) (**Hezavehei *et al.*, 2018; Nagy *et al.*, 2019**). Sperm cryopreservation, when properly performed, enables a long-term storage of male gametes in a state of metabolic arrest that prevents cellular aging while maintaining their viability and fertilizing potential, therefore it allows them to be used when and where needed (**Hezavehei *et al.*, 2018**). In humans, sperm freezing represents an effective strategy to preserve male reproductive capacity following cytotoxic or surgical treatment that may cause testicular or ejaculatory dysfunction. Cryopreservation may also be routinely used in men who are exposed to potentially toxic agents which may interfere with spermatogenesis, or who begin assisted reproduction treatment, and thus have a back-up sperm source (**Agarwal *et al.*, 2014; Sharma and Sharma, 2020**). In donor insemination programs, cryopreservation is necessary to have enough time to screen the donors for infectious diseases, before the frozen semen sample is used for clinical purposes (**Tvrdá *et al.*, 2018**).

Slow and rapid freezing are currently the cryopreservation methods of choice in practical andrology. During slow freezing, spermatozoa are cooled progressively over a period of 2-4 h in two or three steps, either manually or automatically, using a programmable machine (**Nagy *et al.*, 2017**). In case of the rapid freezing technique, spermatozoa are mixed with the cryoprotectant, the suspension is loaded into cryovials and subsequently exposed to a liquid nitrogen vapor phase for at least 10 min prior to being plunged into liquid nitrogen (**Riva *et al.*, 2018**). Recently, vitrification has emerged as an alternative freezing method without the need for permeable cryoprotectants. In this case, the sperm suspension is plunged directly into liquid nitrogen and the cells are cooled down in an ultra-rapid manner (**Tao *et al.*, 2020**). Independently of the procedure selected to preserve spermatozoa at low temperatures, the most important factors contributing to the resulting sperm vitality and fertilization ability is the sperm quality prior to the freezing process, and the changes male gametes must withstand during their exposure to low temperatures.

Unfortunately, the full potential of semen cryopreservation is not fully exploited yet, since a complete understanding of the sperm physiology prior to or during the freezing and thawing process mandatory to ensure maximal success is still lacking. A key factor in the sperm cryobiology is that male gametes are small cells with a relatively large surface (**Morris *et al.*, 2012**) which will affect the viscosity and transition temperature of the intracellular cytosol (**Hezavehei *et al.*, 2018**). In the absence of cryoprotective agents, cold shock, osmotic stress, and ice crystal formation may lead to the deterioration of organelles and molecules crucial to maintain sperm structural integrity and functional activity (**Hezavehei *et al.*, 2018; Sharma and Sharma, 2020**). Sperm freezing procedures are furthermore accompanied by an outburst of reactive oxygen species (ROS), supraphysiological levels of which may promote the loss of membrane fluidity, apoptosis, mitochondrial swelling, and DNA damage (**Peris-Frau *et al.*, 2020**). Within this context, vitrification seems to cause a lower degree of damage in comparison to conventional cryopreservation (primarily slow freezing) (**Ali Mohamed, 2015; Riva *et al.*, 2016**). More specifically, vitrified spermatozoa have been reported to maintain higher progressive motility, membrane and acrosome integrity (**Ali Mohamed, 2015; Cuevas-Uribe *et al.*, 2017**). Additionally, lower levels of phosphatidylserine, indicative of sperm cell death, have also been recorded (**Isachenko *et al.*, 2012**). Such favorable impact of vitrification on the post-thaw sperm motility coupled with low requirements for any specific cooling or freezing instrumentation suggests that vitrification could become a method of choice for long-term sperm preservation in the future. Nevertheless, the vitrification technique requires rigorous training and a precise execution of the freezing and thawing protocol (**Tvrdá *et al.*, 2021**). Moreover, its implementation into practice is hindered by the current absence of any commercially available vitrification and

devitrification media which are required in clinical practice by the European Union Tissues and Cells Directive 2004/23/EC (**European Commission, 2004**).

Our previous study has revealed that iatrogenic sperm DNA damage is lower and progresses more slowly following vitrification when compared to traditional cryopreservation (**Tvrdá *et al.*, 2021**). This study revisits this comparative approach, focusing on the levels of proteins that are involved in the cellular stress response and the apoptotic machinery both of which have been suggested as important contributors to the loss of viable spermatozoa following the freeze-thaw process. As such, the aim of this research was to assess changes in the levels of heat shock proteins 70 (HSP 70) and 90 (HSP 90) as well as apoptotic markers BAX, Bcl-2, caspase-3 and caspase-9 in human spermatozoa subjected to conventional cryopreservation or vitrification.

## MATERIAL AND METHODS

### Sample collection and processing

Semen samples for this study were obtained from 35 healthy donors (age of 33.4±3.9 years). The inclusion criteria were as follows: (1) normal semen quality according to the sixth edition of the World Health Organization Manual for the Laboratory Examination and Processing of Human Semen (**WHO, 2021**); and (2) no sexually transmitted infections. All donors provided an informed consent to use their samples for experimental purposes. All volunteers were informed about the aim and expected outcomes of the study and subsequently signed informed consents. All procedures complied with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The samples were collected following 2–3 days of abstinence and allowed to liquefy for 30 min at 37 °C.

Each specimen was divided into three aliquots. The first aliquot represented the neat (native) sample. The second aliquot was processed using a conventional cryopreservation protocol. The samples were centrifuged at 300 × g for 5 min, seminal plasma was discarded, and spermatozoa were resuspended in G–Gamete (Vitrolife; Gothenburg, Sweden). Subsequently, 70% SpermFreeze™ single step freezing medium (FertiPro; Beernem, Belgium) was added slowly to the sperm suspensions. Each sample was then placed at 4 °C for 15–20 followed by a 15–20 min exposure to nitrogen vapors. The cryovials were then submerged into and stored in liquid nitrogen. The samples were stored in liquid nitrogen for one month. In order to thaw the samples, each specimen was placed into a water bath at 37 °C for 10 s and washed with sperm washing medium (Irvine Scientific; Santa Ana, CA, USA) twice to eliminate the cryopreservation medium. The cells were resuspended in modified HAM-HEPES-F10 medium (Irvine Scientific; Santa Ana, CA, USA) without protein supplementation (**Tvrdá *et al.*, 2021**).

The third fraction was subjected to vitrification following the protocol of **Isachenko *et al.* (2008)**. Each ejaculate was centrifuged at 250 × g for 5 min, seminal plasma was discarded, and spermatozoa were diluted with G-Gamete with 0.5 M sucrose (Sigma Aldrich, St. Louis, MO, USA). Subsequently, small aliquots of the suspension were immediately dropped into liquid nitrogen, collected into cryovials and stored in liquid nitrogen for one month. For the devitrification procedure, frozen spermatozoa were treated with 2 mL of Sydney IVF Gamete Buffer (Irvine Scientific; Santa Ana, CA, USA) supplemented with 1% dextran (Sigma Aldrich, St. Louis, MO, USA), pre-warmed to 42 °C. Following devitrification, the samples were washed with sperm washing medium twice to eliminate the cryopreservation medium, and the cells were resuspended in modified HAM-HEPES-F10 medium without protein supplementation.

### Sperm motion characteristics

The motility, progressive motility, path velocity (VAP), progressive velocity (VSL) and track speed (VCL) of fresh, frozen-thawed and devitrified spermatozoa was assessed using the computer assisted sperm analysis (CASA) system (Sperm Class Analyzer version 5.4 - SCA®; Microptic, S.L., Barcelona, Spain,) equipped with a Basler A312fc digital color camera (Microptic, S.L., Barcelona, Spain). At least 300 cells were scanned in each analysis (**Ayad et al., 2021**).

### Western blots

Native semen specimens and thawed sperm suspensions were centrifuged at 300 × g for 7 min, and the collected spermatozoa were exposed to RIPA lysis buffer (Merck, Darmstadt, Germany) containing a proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C to allow a complete cell lysis. After centrifugation at 13,000 × g for 30 min, the supernatant was aspirated, subjected to the determination of the protein concentration using the commercially available Pierce™ BCA protein assay kits (Thermo Fisher Scientific, Waltham, MA, USA) (**Cui *et al.*, 2016**).

Three randomly selected triplets of samples with a suitable protein concentration were selected for the Western blot analysis. All samples were adjusted using PBS reach a uniform concentration of 25 µg protein. The samples were then treated with 4× Laemli buffer (BioRad, Hercules, CA, USA) and β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and subsequently boiled at 95 °C for 10 min. The samples were loaded (20 µL) into readily available Mini-PROTEAN TGX stain-free polyacrylamide gels (BioRad, Hercules, CA, USA), together with 7 µL of Precision Plus Protein marker (BioRad, Hercules, CA, USA). SDS-polyacrylamide gel electrophoresis was run at 110 V for 1 h, and the gels were visualized with the ChemiDoc Imaging System (BioRad, Hercules, CA, USA) to confirm the loading uniformity. The gels were then transferred to polyvinylidene difluoride membranes (Trans-Blot Turbo Pack; BioRad, Hercules, CA, USA) using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA) at 18 V and 2.5 A, for 30 min. The resulting membranes were incubated for 3 × 10 min in Tris-buffered saline (TBS) (Sigma-Aldrich, St. Louis, MO, USA) and stained with 5% skim milk (Blotting grade blocker; BioRad, Hercules, CA, USA) in TBS containing 0.1% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Finally, the membranes were incubated with selected primary antibodies (listed in Table 1) in 5% skim milk in TBS/0.1% Tween-20 at 4 °C overnight (**Cui *et al.*, 2016, Tvrdá *et al.*, 2023**).

**Table 1** Primary antibodies used for the Western blot analysis

| Antibody | ID | Source | Characteristics | Dilution | Manufacturer |
|---|---|---|---|---|---|
| Anti-Hsp90 antibody [AC88] | ab13492 | mouse | monoclonal | 1:1 000 in 5% milk | Abcam (Cambridge, UK) |
| Anti-Hsp70 antibody [5A5] | ab2787 | mouse | monoclonal | 1:1 000 in 5% milk | Abcam (Cambridge, UK) |
| Anti-Bax antibody [E63] | ab32503 | rabbit | monoclonal | 1:1 000 in 5% milk | Abcam (Cambridge, UK) |
| Anti-Bcl-2 antibody [EPR17509] | ab182858 | rabbit | monoclonal | 1:1 000 in 5% milk | Abcam (Cambridge, UK) |
| Anti-Caspase-3 antibody [E87] | ab32351 | rabbit | monoclonal | 1:1 000 in 5% milk | Abcam (Cambridge, UK) |
| Anti-Caspase-9 antibody [E23] | ab32539 | rabbit | monoclonal | 1:1 000 in 5% milk | Abcam (Cambridge, UK) |

The next day, the membranes were washed for 5 × 10 min in 1% milk in TBS/0.2% Tween-20, and subsequently incubated with a secondary anti-mouse antibody (for HSP 70 and HSP 90) (GE Healthcare, Chicago, IL, USA) or anti-rabbit antibody (for BAX, Bcl-2, caspase-3 and caspase-9) (GE Healthcare, Chicago, IL, USA) diluted 1:15 000 in 1% milk in TBS/0.2% Tween-20 for 1 h. Following incubation, the membranes were washed for 3 × 10 min in TBS/0.2% Tween-20 at room temperature. To visualize the target protein, membranes were incubated with the ECL substrate (GE Healthcare, Chicago, IL, USA) in the dark for 5 min. Proteins were visualized with the ChemiDoc Imaging System and quantified using the ImageLab software (version 6.1; BioRad, Hercules, CA, USA) (**Tvrdá *et al.*, 2023**).

### Statistics

GraphPad Prism (version 10.3.1 for Mac; GraphPad Software Incorporated, La Jolla, CA, USA) was used for the statistical analysis. The results are expressed as mean (±SD). All data were subjected to the Shapiro-Wilk normality test. Differences between the samples were analyzed by one-way ANOVA and Tukey multiple comparison test. Statistical significance was set at $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

## RESULTS AND DISCUSSION

Whilst sperm freezing and long-term preservation at low temperatures constitute an important pillar of the management of male infertility (**Hezavehei *et al.*, 2018**), currently employed cryo-techniques are still imperfect. Currently, most cryobiological studies on spermatozoa or routine clinical freezing are based on conventional, either programmable or standard vapor freezing. Nevertheless, because of the damage inflicted during the freezing procedure, the survival of cryopreserved spermatozoa following thawing may be significantly reduced with respect to its pre-freeze state. Post-thaw sperm quality may also be affected by actual thawing process, addition and removal of osmotically active cryoprotective agents or their chemical toxicity (**Ozimic *et al.*, 2023**). In the meantime, vitrification has emerged as a simpler and more straight-forward alternative to conventional cryopreservation, as it avoids the use of traditional permeable cryoprotectants, thus avoiding osmotic shock, intracellular ice formation or cytotoxic effects of high salt concentrations during the freeze-thaw process. As pioneers of the sperm vitrification technique, **Isachenko et al. (2004a,b, 2005, 2011)** have developed, optimized and introduced comprehensive vitrification and de-vitrification protocols, suggesting that an ultra-fast and cryoprotectant-free sperm freezing technique yields higher post-thaw sperm structural integrity and functional activity as opposed to conventional slow freezing methods. In this study, we strived to compare traditional slow cryopreservation and vitrification in terms

of their impact on the expression levels of proteins involved in the cellular response to stress as well as in the intracellular apoptotic machinery.

**Sperm motility**

CASA was used to assess the motility of spermatozoa prior to the selected freezing procedures, as well as in their post-thaw and devitrified state, and the collected data are displayed in Table 1. No significant differences were found in the recovered sperm motility when comparing the conventional slow cryopreservation and vitrification, however the post-cryopreserved samples presented a significantly lower sperm motility (P<0.01) in comparison to the neat sample. In the meantime, a significant decline in the sperm progressive motility was found in cryopreserved spermatozoa when compared both to neat (P<0.01) as well as vitrified semen (P<0.05).

Whilst no significant differences were observed amongst the groups in terms of VAP, a significantly decreased VSL was found in cryopreserved spermatozoa in comparison to neat semen (P<0.05). Moreover, a significant decline of VCL was observed in cryopreserved (P<0.01) as well as vitrified samples (P<0.05) when compared to the neat group.

**Table 1** Sperm motion characteristics in the pre-established groups

| Parameter | Neat semen | Cryopreserved semen | Vitrified semen |
|---|---|---|---|
| Motility (%) | 72.00±7.61 | 47.47±5.29$^{**N}$ | 59.07±7.90 |
| Progressive motility (%) | 57.62±8.04 | 34.01±4.70$^{**N}$ | 47.14±4.03$^{*C}$ |
| Path velocity (VAP) (μm/s) | 59.50±2.25 | 52.82±2.31 | 58.10±5.80 |
| Progressive velocity (VSL) (μm/s) | 51.90±4.30 | 42.57±4.70$^{*N}$ | 44.80±4.92 |
| Track speed (VCL) (μm/s) | 102.00±5.40 | 77.30±4.01$^{**N}$ | 88.52±6.12$^{*N}$ |

Neat semen – native, untreated semen; cryopreserved semen – spermatozoa frozen using the conventional slow freezing protocol; vitrified semen – spermatozoa frozen using the vitrification protocol. Mean±S.D. $^{N}$ – vs. neat semen; $^{C}$ – vs. cryopreserved semen. $^{*}$ P<0.05; $^{**}$ P<0.01.

Data collected from this study reveal that vitrification results in higher motility rates when compared to traditional slow cryopreservation, which agrees with previous comparative reports on slow and ultra-fast sperm freezing techniques (**Isachenko et al., 2004a,b; Oberoi et al., 2014; Riva et al., 2016; Aizpurua et al., 2017**). This is of great clinical importance as only motile spermatozoa will be preferably selected for ARTs. Additionally, the fact of having a greater quantity of motile spermatozoa may determine the type of assisted fertilization procedure to be performed since, for example, when there is a reduced number of spermatozoa, the technique to be indicated will be intracytoplasmic sperm injection (ICSI), which presents with several disadvantages in comparison to conventional in vitro fertilization (IVF) (**Dumoulin et al., 2000**).

The decrease of motility in spermatozoa exposed to low temperatures has been repeatedly attributed to mitochondrial damage. Compromised ATP production derived from mitochondrial cryo-damage may be transferred to the microtubules participating in motility and hence reduce their activity (**O'Connell *et al.*, 2002**). Correspondingly to this hypothesis, a notable improvement in the mitochondrial activity following vitrification as opposed to conventional cryopreservation was reported in earlier studies, suggesting a higher capability of the vitrification medium components to prevent mitochondrial uncoupling and the loss of metabolic activity (**Isachenko *et al.*, 2008, 2011**). At the same time, **Aizpurua et al. (2017)** observed that vitrification has a low impact of vitrification on the distribution patterns of alpha tubulin as opposed to conventional cryopreservation which may be associated with the weakening of the tail structure and disassembling of the microtubules as a result of an income of calcium ions during slow freezing (**Bhagwat *et al.*, 2014; Gomez-Torres *et al.*, 2017**).

**Heat shock protein levels**

The Western blot analysis revealed that both freezing techniques have led to a significant decrease of the HSP 70 protein levels in comparison to the neat state (0.73±0.15 RQ) (P<0.001 in case of slow cryopreservation; P<0.01 with respect to vitrification). No significant differences were recorded between the slow cryopreservation (0.19±0.02 RQ) and vitrification (0.29±0.11 RQ), although slightly higher HSP 70 levels were detected in the devitrified group (Figure 1a).

Similarly to HSP 70, slow cryopreservation (0.62±0.02 RQ) as well as vitrification (0.67±0.02 RQ) led to a significant decrease of the HSP 90 protein levels (P<0.01) when compared to the neat sperm state (0.86±0.10 RQ). No significant differences in HSP 90 levels were detected between the cryopreserved and vitrified group (Figure 1b).

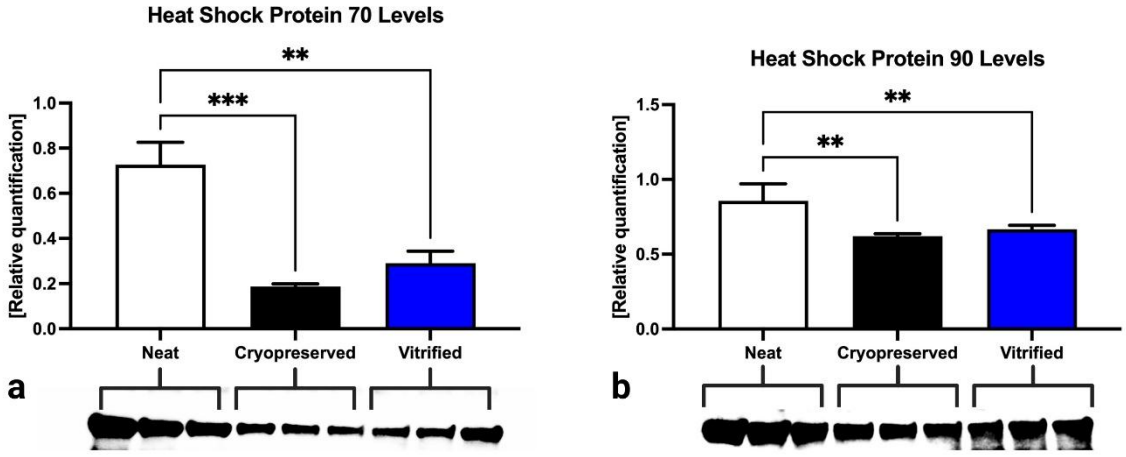


**Figure 1** Protein levels of the (a) Heat shock protein 70 and (b) Heat shock protein 90 in the pre-established groups. Neat – native, untreated semen; cryopreserved – spermatozoa frozen using the conventional slow freezing protocol; vitrified – spermatozoa frozen using the vitrification protocol. Mean±S.D. ** P<0.01; *** P<0.001.

It is well known that HSPs play intricate roles in stress attenuation and maintenance of a normal intracellular activity since changes in their levels have a direct effect on stress tolerance acquisition (**Shan *et al.*, 2020**). Besides, earlier studies have emphasized suggest HSP levels may be affected by thermal or cold stress situations (**Wang *et al.*, 2014; Zhang *et al.*, 2015a,b; Karabulut *et al.*, 2018**). Our collected data agree with previous reports on frozen-thawed mammalian spermatozoa (**Cao et al., 2003; Zhang et al., 2015a;b; Karabulut *et al.*, 2018; Parmar et al., 2021; Baňas et al., 2022**), indicating that both HSPs studied in our experiments might act as important players in the regulation of sperm motility, which may be altered by the freeze-thaw procedure. A significant reduction of HSPs in the in both groups processed by low temperatures in comparison to neat semen suggests that spermatozoa may respond to low temperatures by degrading HSPs located primarily in the plasma membrane (**Cao *et al.*, 2003**). A different hypothesis on the loss of HSPs was introduced by **Zhang *et al.* (2015a;b)**, suggesting that since frozen spermatozoa are inactive, hence unable to properly control HSP folding and activity, or both proteins might have been readily consumed to stabilize the folding or assembly of other proteins during the freeze-thaw process. Finally, sperm specimens exposed to low temperatures carry an increased number of already dead or dying cells unable to properly control the activity of their proteins (**Peris-Frau *et al.*, 2020**). Since HSPs intervene in the folding of membrane proteins and translocation of polypeptides across the plasma membrane (**Arispe *et al.*, 2002**), their low levels may lead to alterations in the conformation of membrane proteins and a compromised membrane fluidity (**Aboagla and Terada; 2003**). Moreover, it has been suggested that HSPs could protect biomolecules vital for the membrane integrity by preventing excessive oxidative insults caused by reactive oxygen species (ROS) overgeneration as a side-effect of cryodamage (**Baňas *et al.*, 2022**). As such, we may speculate that the decrease of HSPs in both groups exposed to low temperatures may lead to a lower resilience of the membrane integrity, and hence the sperm viability.

Despite lower HSP 70 and HSP 90 levels in the cryopreserved as well as vitrified group, the decline of both proteins was less dramatic in sperm samples subjected to vitrification. This observation corroborates the outcomes of previous studies (**Isachenko *et al.*, 2008; Azipurua *et al.*, 2017**), according to which the sperm viability decrease was significantly higher in spermatozoa processed with conventional cryopreservation when compared to vitrified sperm. This phenomenon may be due to osmotic changes, which are accompanied by dehydration and rehydration resulting from the use of CPAs, intracellular crystal formation or ice recrystallization (**Morris, 2006; Chaytor *et al*., 2012**). It may be plausible to assume that the sperm vitality in vitrified specimens is less affected since only dehydration by means of non-permeable CPAs takes place and no water-CPA exchange is carried out (**Isachenko *et al.*, 2008**). Besides, the temperature drop typical for conventional slow cryopreservation may increase the levels of cytoplasmatic calcium, and induce premature capacitation (**Silva and Gadella, 2006**).

**Levels of apoptotic markers**

In case of the BAX protein, exposure of spermatozoa to low temperatures led to a significant increase of its levels when compared to the neat group (1.05±0.12 RQ) (P<0.0001 with respect to slow cryopreservation; P<0.01 in case of vitrification). Nevertheless, vitrification led to significantly lower BAX levels (1.41±0.08 RQ) in comparison to cryopreservation (2.06±0.13 RQ) (P<0.05) (Figure 2a).
In the meantime, lower levels of the Bcl-2 protein were observed in both cryopreserved (0.58±0.05 RQ) as well as vitrified (0.66±0.08 RQ) spermatozoa in comparison to their neat state (0.84±0.13 RQ). Nevertheless, a significant difference in the Bcl-2 protein levels was recorded amongst the neat and cryopreserved group (P<0.05) (Figure 2b).

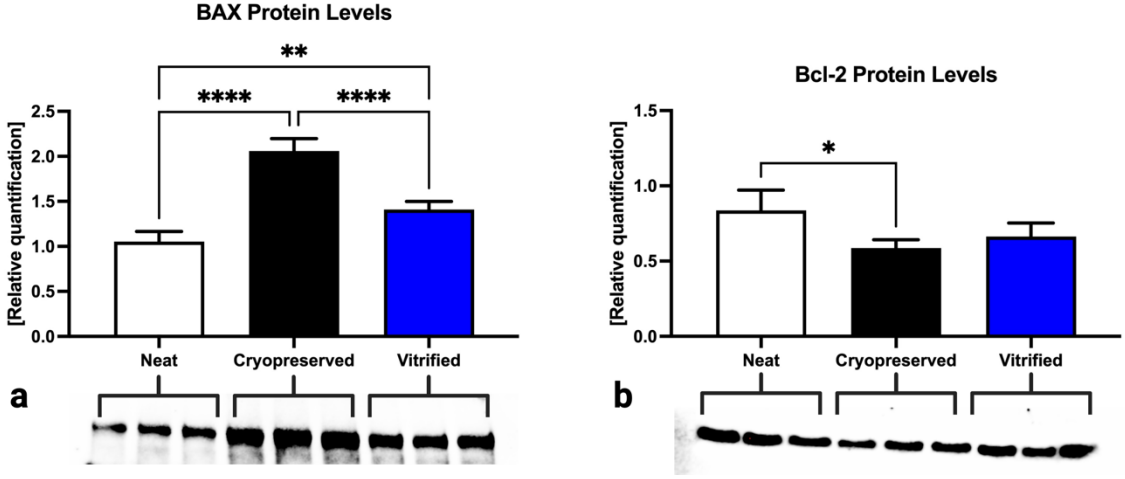


**Figure 2** Protein levels of the (a) BAX and (b) Bcl-2 protein in the pre-established groups. Neat – native, untreated semen; cryopreserved – spermatozoa frozen using the conventional slow freezing protocol; vitrified – spermatozoa frozen using the vitrification protocol. Mean±S.D. ** P<0.01; *** P<0.001; **** P<0.0001.

It is now evident that mitochondria may act as initiators of cell death by apoptosis, primarily through the BAX and Bcl-2 dynamics (**Mostafa *et al.*, 2014**). The primary role of the anti-apoptotic Bcl-2 protein is to prevent the activation of the pro-apoptotic protein BAX responsible for the formation of pores in the mitochondrial system (**Llambi *et al.*, 2011**). As such, Bcl-2 and BAX ratio will decide on the fate of the cell where Bcl-2 inhibits and BAX promotes cell death (**Bagci *et al*., 2006**).
Our study suggests that as with the plasma membrane, the mitochondrial membranes are necessary to maintain a proper sperm vitality whilst being equally susceptible to cryodamage. At the same time, the data collected from the western blot assay strongly suggest that cryoinjury to mitochondria sets an apoptosis-like mechanism in motion, which may lead to the loss of sperm function, as observed in previous reports (**Critser *et al.*, 1987; Leffler and Walters, 1996**). According to **Isachenko *et al.* (2008)**, mature ejaculated spermatozoa are incapable of undergoing apoptosis as they lack the necessary assemble of enzymes and intracellular pathways possessed by cells capable of undergoing this process (**Lachaud *et al*., 2004**), however we may disagree with this hypothesis. Our data reinforce previous observations regarding oscillations of mRNA or protein levels in mature spermatozoa (**Vardiyan *et al.*, 2020; Yan *et al.*, 2020**). I must be remembered that is difficult to accomplish active transcription in spermatozoa whose genome is highly packaged. We may exclude de novo synthesis, since condensed mature spermatozoa have a significantly reduced cytoplasm, and lack ribosomes that would support de novo translation. As such, we may speculate that protein synthesis in mature spermatozoa could be mainly driven by mitochondria from paused transcripts waiting for activation (**Gur and Breitbart, 2008**). If the theory that mitochondria may be partially responsible for changes in the levels of proteins prior to of following cryopreservation is correct, changes in BAX and Bcl-2 levels may be primarily caused by the mitochondria-induced apoptotic machinery. This would make sense, since both BAX and Bcl-2 are mitochondria-bound, hence if the sperm mitochondria are active following the freeze-thaw process, their mitoribosomal complex could be held accountable for changes in the BAX/Bcl-2 ratio in the sperm cell.
When it comes to a comparative analysis between cryopreservation and vitrification, previous reports have suggested that that currently used conventional freezing techniques for human sperm cryopreservation are good enough to preserve the mitochondrial structures (**O'Connell *et al*., 2002; Meseguer *et al*., 2004**). These suggestions stem from the fact, that the mitochondrial function was previously assessed through functional tests, hypothesizing that the mitochondria are most probably uncoupled rather than membrane damaged. Significant changes in the markers of early apoptosis nevertheless suggest that active structural damage to the mitochondria may in fact be occurring. Whilst no functional changes may have been observed earlier, our data suggest that vitrification leads to a lower extent of mitochondrial damage and associated early onset of cell death. The exact mechanism is not clear yet, however we may speculate that similarly to the plasma membrane, the mitochondrial structures may be compromised to a lesser extend during vitrification, primarily due to the absence of rehydration of CPAs or intracellular crystal formation (**Morris, 2006; Chaytor *et al*., 2012**).

**Caspase levels**

In case of caspase-3, the analysis revealed a significant rise in its levels in the cryopreserved group (3.63±0.88 RQ) when compared to the neat group (0.97±0.09 RQ) (P<0.05). Whilst vitrification increased caspase-3 levels (2.21±0.07 RQ), no significant differences were recorded neither in comparison with the neat groups nor with the cryopreserved group (Figure 3a).
On the contratry, significantly higher caspase-9 levels were observed both in cryopreserved (11.91±0.75 RQ) and vitrified spermatozoa (5.82±0.81 RQ) in comparison to the neat group (2.21±0.51 RQ) (P<0.0001 with respect to cryopreservation; P<0.001 in case of vitrification). At the same time, devitrified spermatozoa presented with significantly lower caspase-9 levels when compared to the cryopreserved group (P<0.0001) (Figure 3b).
Apoptosis, also known as "programmed cell death" has been suggested to play an intricate role in the cryodamage to the sperm cell, as the freeze-thaw process has been linked to the post-thaw activation of specifically caspase-1, -3, -8 and -9 in a variety of mammalian gametes (**Anzar *et al*., 2002**; **Paasch *et al.*, 2004; Wündrich *et al.*, 2008; Karabulut *et al.*, 2018**; **Kaur and Atreja, 2018**).
Our collected data agree with early reports on caspase activation in ejaculated spermatozoa aaccording to which the mitochondrial (type II) mediated apoptotic signaling may play a predominant role in the loss of viable spermatozoa following cryopreservation (**Paasch *et al.*, 2003a,b**). While caspase-9 is an initiator caspase in the intrinsic apoptosis pathway, caspase-3 acts as the ultimate executor of apoptotic death by directly cleaving a variety of essential structural and functional proteins (**Dirican *et al.*, 2023**). Our data strongly indicate a rise in the levels of both caspases following sperm exposure to low temperatures corroborating previous studies on human spermatozoa (**Grunewald *et al.* 2001; Paasch *et al.*, 2003a;2004; Wündrich *et al*., 2008**) and supporting the existence of a higher sensitivity to mitochondria derived apoptosis in spermatozoa due to cryopreservation. All studies strongly emphasize that exposure of spermatozoa to low temperatures may lead to various structural and functional alterations of the plasma membrane (**Glander and Schaller, 1999; Duru *et al.*, 2001; Anzar *et al.*, 2002**), which may compromise the stability of the lipid bilayer (**Glander *et al*., 2002**) and result in sublethal sperm cryodamage (Alvarez and Storey 1993). The loss of membrane integrity as observed in previous studies (**O'Connell *et al*., 2002;**

**Isachenko *et al.*, 2008; Azipurua *et al.*, 2017**) may then initiate the caspase cascade (**Bratton *et al.*, 2000**).

In terms of a direct comparison amongst slow crzopreservation and vitrification, it seems apparent that the ultra-fast method of sperm freezing may lead to a lower extent of apoptotic damage to male gametes. **Wündrich *et al.* (2008)** hypothesize that the cryoprotectants, in addition to their cryoprotective properties, may be a trigger of the programmed cell death via direct toxic effects particularly on the sperm mitochondria, since cytotoxic stress involves mitochondrial perturbations which may be followed by DNA fragmentation (**Riva et al., 2014; Tvrdá et al., 2021**). This hypothesis would therefore corroborate higher BAX and lower Bcl-2 levels that we detected in spermatozoa processed by slow cryopreservation as opposed to vitrification. All in all it seems that the inadeuqate use of CPAs may be the key in unraveling the prime reason why slow cryopreservation may present with more risks to the post-thaw sperm survival. With an increase of CPAs concentration or their incorrect combination, their toxic effect may predominate the desired cell protection. At the same time, mitochondria, more than the plasma membrane may become the prime target for damage inflicted by the sperm freezing techniques. The molecular machinery underlying this phenomenon and subsequent measures to prevent or mitigate such damage are subjects to future research.

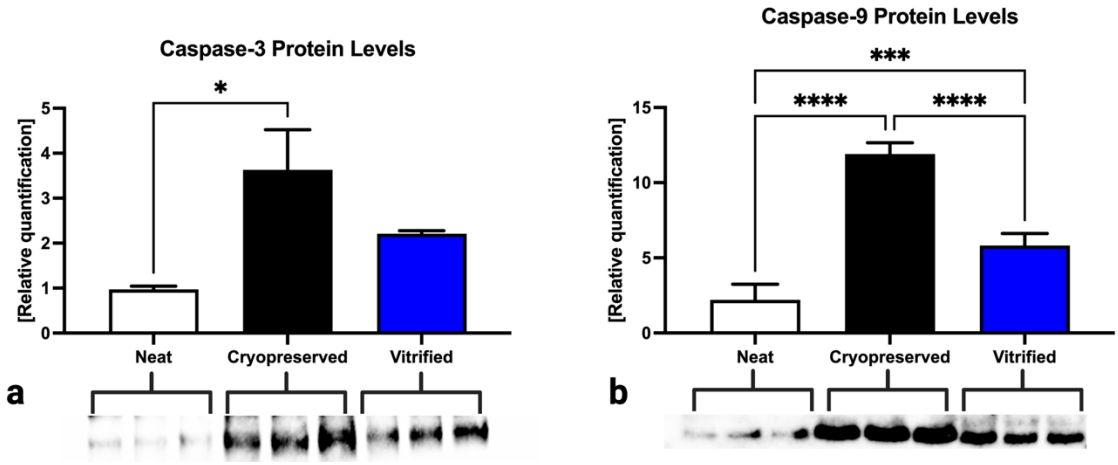


**Figure 3** Protein levels of (a) caspase-3 and (b) caspase-9 in the pre-established groups. Neat – native, untreated semen; cryopreserved – spermatozoa frozen using the conventional slow freezing protocol; vitrified – spermatozoa frozen using the vitrification protocol. Mean±S.D. * P<0.05; *** P<0.001; **** P<0.0001.

An important limitation that must be disclosed, is a relatively low sample size for our experiments. Nevertheless, taking into cosideration that significant differences were obtained in the three cohorts pre-established for the experimental procedures, we may speculate that an increase in the number of donors may not significantly impact the obtained data. The use of only normozoospermic males could be considered as another drawback of this study. As such, we must emphasize on future studies with samples collected from patients presenting with substandard semen quality (oligozoospermia, asthenozoospermia, teratozoospermia, etc.), and the methodology needs to be verified in clinical ART settings in terms of pregnancy or fertilization rate in IVF/ICSI cycles before recommending it for clinical use.

## CONCLUSION

In conclusion, this study shows that the sperm vitrification protocol applied in our experiment allows superior results on all parameters studied when compared to conventional slow cryopreservation and hence, this strategy could represent an efficient and reliable alternative to conventional freezing methods. Whilst exposure to cryogenic temperatures will compromise the sperm structural integrity and functional activity no matter which freezing technique is employed, the vitrification procedure led to a lower incidence of sperm apoptosis and mitochondrial damage as opposed to conventional cryopreservation which may ultimately lead to a higher sperm motion activity.

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