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EFFECT OF THE CRYOPRESERVATION PROCESS ON THE CATSPER CHANNELS IN HUMAN SPERMATOZOA

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Abstract

Cryopreservation of spermatozoa is a routine procedure used in assisted reproductive technologies. However, there is limited data on its effect on the specific Ca^{2+} channels involved in the key processes of capacitation and hyperactivation of the male gametes. The influence of two different cryopreservation methods upon the CatSper index (CI) of human male gametes has been investigated. After initial evaluation of the ejaculates, they were separated into two groups and cryopreserved by slow freezing or vitrification and the CI index of the gametes was assessed. The results of the current investigation point out that the cryopreservation has an insignificant effect on the CatSper channels of spermatozoa, with the CI after freezing remaining within the normal range ($> 80\%$).

Key words: cryopreservation, CatSper channels, human spermatozoa

Introduction. The physiological changes that occur in spermatozoa in the female reproductive tract – hyperactivation, capacitation, and acrosome reaction – are crucial for successful fertilization and oocyte activation. Significant progress has been achieved in recent decades in our understanding of the molecular and cellular mechanisms underlying those processes. It has been shown that the Ca^{2+} ions have a key role in the preparation of gametes for fertilisation. Although it has been previously known that the influx of Ca^{2+}in spermatozoa is an important factor in the regulation of their motility and fertilisation capacity, the molecular

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structure of the Ca^{2+} transporters has not been identified up to 2001 [1]. CatSper (cation channel of sperm) are multiprotein complexes, which when activated by certain factors, allow the entering of cations in the tails of the gametes (Fig. 1). The protein complex consists of four main transmembrane subunits (CATSPER1–4) which shape the heterotetrameric pore, and auxiliary proteins, which form CatSper Ca^{2+} signalling nanodomains in mammalian spermatozoa [2, 3]. The pore is constructed in the time of the development of the spermatids and becomes fully functional during the transition of the gametes in the epididymis [4]. Data exists that mutations in the genes for the CatSper proteins lead to reduced motility and in-/subfertility [5].

An array of factors regulate the activity of the CatSper channels, such as temperature, pH, presence of certain hormones and cytokines. It has been shown that the channels are stimulated by temperature changes, but a specific threshold has not been defined yet in humans, in contrast with mouse gametes where it is estimated to be $\sim 33.5\,^{\circ}C$ [6]. The physiological increase, such as in the female reproductive tract, leads to an influx of Ca^{2+} and hyperactivation of the spermatozoa [7]. Some researchers show that the pH can influence the channels' activity in laboratory animal models [8]. Progesterone can also stimulate the CatSper permeability in human spermatozoa [9].

However, there is limited data on the effect of the cryopreservation process on the CatSper channel activity. Based on the fact that it may induce changes in

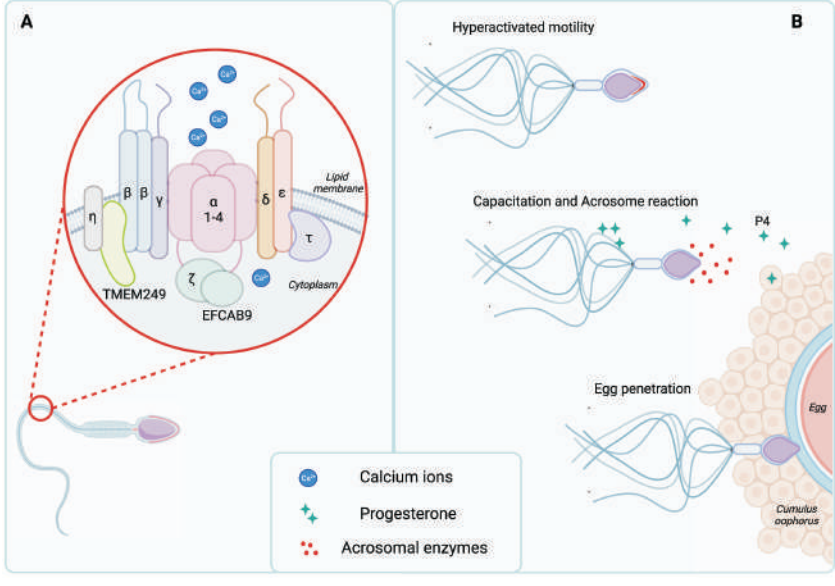


Fig. 1. A: Protein structure of the CatSper channels (transmembrane: α1–4; β; γ; δ; ε; η; TMEM249 and cytoplasmic subunits: ζ; τ; EFCAB9) and their localisation in the sperm membrane. B: Progesterone-induced CatSper-mediated capacitation and acrosome reaction on spermatozoa (P4 – progesterone) [10]

the membranes and in the structure of proteins [11, 12], it can be speculated that the cryopreservation might influence the Ca^{2+} influx in male gametes. The aim of the current report is to investigate the influence of different freezing methods on the CatSper channels in human spermatozoa.

Material and methods. ***Collection and initial evaluation of the semen samples.*** Donor semen has been used in the current investigation (after obtaining an informed consent from the donors). It was collected after 48–96 h of sexual abstinence. Post liquification, the semen was evaluated according to the uniformly accepted criteria (volume of the sample, pH, motility, morphology and concentration of spermatozoa) [13]. Only normozoospermic ejaculates have been used in the investigation. Each of them has been separated into three samples – control group (native semen), experimental group 1 (frozen on liquid nitrogen vapour), and experimental group 2 (frozen by vitrification).

Evaluation of the kinetic parameters of the gametes. Spermatozoa motility was investigated with computer assisted semen analysis (SCA – sperm class analyzer) in 4-well Leja chamber [14]. Apart from total motility, the following additional kinetic parameters have also been studied: curvilinear velocity (VCL), straight velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), beat/cross frequency (BCF).

Freezing on liquid nitrogen vapour. The sperm was diluted at room temperature with a cryoprotective medium (Sperm Freezing medium, FertiPro) in a ratio of 1:1. The diluted sperm was placed in 2.0 ml vials. After 20 min of equilibration at 4 °C, they were frozen on nitrogen vapour (3 cm above its surface, on a special holder) for a period of 30 min, and then they were transferred for storage in a liquid nitrogen container.

Vitrification. The semen was diluted 1:1 with flushing medium (Origio) enriched with 20 µg human serum albumin (FujiFilm) and packaged in 600 µm capillary tubes made of hydrophobic material (V-DEN-600, VitroMed). Freezing was performed by directly immersing them in liquid nitrogen.

Thawing. The samples have been stored for not less than a week and then thawed in 37 °C waterbath.

CatSper analysis. For this assay, we used the CatFlux kit (Truion), according to the protocol described by Young et al. [15]. The kit contains ready-to-use test and control solutions. Twenty µl of seminal fluid were added to each solution and the vials were incubated for 60 min at 37 °C. After that, we examined the motility of the spermatozoa in the experimental and control samples, analyzing a minimum of 200 spermatozoa. The percentage of motile spermatozoa was determined as the sum of spermatozoa from classes A and B. The CatSper index (CI) was calculated using the formula:

$$CI = \frac{\{Motility\ \ (A+B)\ \ Control - Motility\ \ (A+B)\, Test\}}{Motility\,(A+B)\, Control} \ \times\ 100.$$

A result below 80% is an indication of a CatSper defect.

Statistical analysis. The Prism 6 software was used to perform the statistical analysis. The results are reported as the mean ± the standard deviation (SD). The hypothesis testing is two-tailed and values of $p \leq 0.05$ were considered significant.

Results. ***Semen analysis.*** For the current investigation, 23 donor ejaculates from males aged 24–39 years have been used. The average semen volume was 2.8 ml (1.7–4.1 ml). The concentration of spermatozoa ranged from 31 to 97 mln/ml (mean 74.1 mln/ml). The average overall motility was 63%. After initial evaluation of the semen, according to the accepted criteria [13], computer assisted sperm analysis was performed, in order to achieve a more comprehensive analysis of the kinetic parameters of the spermatozoa (Fig. 2).

Effect of the cryopreservation process on the motility of the male gametes. The obtained results in the CASA analysis showed that the process of cryopreservation leads to a reduced overall motility of spermatozoa. At the same time, a trend toward better motility preservation is observed after vitrification compared to liquid nitrogen vapour freezing.

Effect of the cryopreservation process on the CatSper index (CI). We have investigated two methods for cryopreservation – on the liquid nitrogen vapour and vitrification, both of which induced changes in the CI in the experimental groups compared to the control. A statistically significant difference between the two experimental groups has not been observed. All of the samples showed CI within the reference values ($> 80\%$) (Table 1).

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CatSper analysis of native and cryopreserved semen ($n = 23$); $^{*}p \leq 0.05$ compared to the control

Cryopreservation method	Initial motility	Motility (A+B) of the control sample in the CatSper assay	Motility (A+B) of the test sample in the CatSper assay	CI
Native semen (control group)	63.11 (±7.23)	57.42% (±6.87)	1.50% (±0.80)	97.38% (±3.20)
Liquid nitrogen vapour freezing	43.20 (±7.1)	40.97% (±7.10)*	6.37% (±2.10)*	84.45% (±6.40)*
Vitrification	47.18 (±6.93)	44.18% (±6.80)*	5.51% (±1.90)*	87.52% (±5.90)*

Discussion. Advances in assisted reproductive technologies (ART) have markedly expanded the clinical application of freezing of human spermatozoa. Cryopreservation represents a well-established strategy for long-term storage of male gametes and the establishment of sperm banks, enabling preservation of male fertility potential for future clinical use. This approach is particularly relevant for:

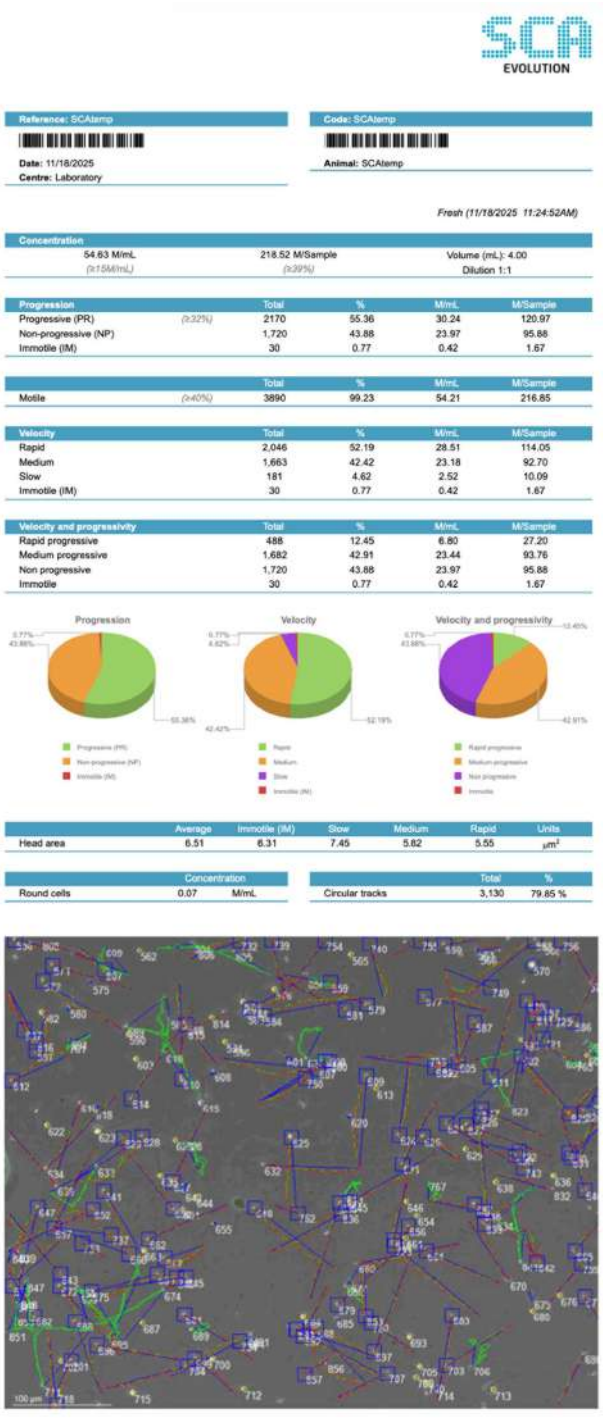


Fig. 2. CASA (computer-assisted semen analysis) of native semen (representative assay of 1 ejaculate). The green line signifies the curvilinear velocity (VCL), the red – average path velocity (VAP), and the blue – straight velocity (VSL) (representative analysis of one ejaculate)

- patients undergoing chemotherapy and/or radiotherapy for malignant diseases;
- adolescents scheduled for gonadotoxic treatments for autoimmune disorders;
- individuals with severe oligoasthenoteratozoospermia (OAT) or azoospermia;
- men unable to provide a fresh semen sample on the day of assisted ART procedures;
- healthy sperm donors [16, 17], etc.

Cryopreservation techniques are mainly categorized as slow (programmable) freezing (SPF) or vitrification (VIT). SPF minimizes ice formation through gradual cooling, whereas VIT employs ultra-rapid cooling to achieve glass-like solidification without crystallization of the water molecules. Both methods depend on cryoprotective agents that stabilize membranes and mitigate osmotic and thermal stress.

A large number of reports exist examining the influence of the cryopreservation process on the motility, morphology, DNA-fragmentation, hyaluronan-binding ability, as well as molecular and epigenetic status of the spermatozoa [18, 19]. In respect of the CatSper channels'activity, still we were not able to find enough data addressing the aforementioned effect in the available literature. Therefore, in the current investigation, we aimed to evaluate how the two main widely-used approaches for cryopreservation – slow freezing and vitrification will affect the activity of the Ca^{2+} channels. Both technologies showed a reduction in the overall motility, with a tendency for the vitrification to retain it better, compared to liquid nitrogen vapour freezing (Table 1). Those results are in concordance with reports from other authors, detecting decreased motility after cryopreservation of male gametes [14].

Some authors, however, have found downregulation of the mRNA and protein levels of CatSper2 and Tektin2 (flagellar protein) [10], which can potentially explain the reduced motility. In addition, the possible membrane damage during cryopreservation may lead to a disruption in related ion channels and permeability, affecting indirectly the CatSper channels. In our study, we established that both cryopreservation methods indeed lead to a reduction in the CI, with the results showing 84.40% in the slow freezing group and 87.52% in the vitrification one, compared to 97.38% in the control group. Irrespective of those findings, the CI remains within the reference limits for intact semen samples ($> 80\%$), which shows that the frozen-thawed spermatozoa can be used for fertilisation.

The regulation of the CatSper channels' activity is an interesting point of discussion. They can be stimulated by a number of factors, like progesterone, cytokines, pH, and temperature. The latter can be influenced by the cryopreservation process especially during thawing, where temperature gradient is achieved. The channels' premature activation may lead to limited sperm cell survival and subfertility, therefore the male reproductive system has a number of different

mechanisms in function to prevent overheating [6]. The cryopreservation process may as well alter the pH gradient across the sperm membrane due to change in its permeability for ions [20] and the osmotic pressure, therefore further elucidation of its effect on the CatSper channels may contribute to the improvement of the freezing techniques in ART.

It is important to note that while performing the CatSper test, we used semen immediately post thawing without removing the cryoprotectors. It is possible that the presence of glycerol in the slow-freezing medium may have an additional effect on the spermatozoa during the 60-minute incubation period specified in the test protocol.

Conclusion. The results obtained in the current investigation point out that the cryopreservation process of semen does not significantly affect the activity of CatSper channels of spermatozoa. Regardless of the observed decrease, the CatSper index after freezing remains within the reference values for intact semen samples ($> 80\%$).

In summary, we believe that the application of the CatSper test after freeze-thawing can provide additional information about the functional status of the male gametes. It might prove to be a valuable tool in the laboratories for assisted reproduction and can be used together with other methods for evaluation of the effects of the cryopreservation process.

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