Influence of cryopreservation on the CATSPER2 and TEKT2 expression levels and protein levels in human spermatozoa

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1. Introduction

Spermatozoa cryopreservation is a procedure used to preserve spermatozoa for a specific period of time. Cryopreservation is the freezing of cells or tissues to sub-zero temperatures, exactly -196 °C. During cryopreservation, all biological activity of the spermatozoa is paused until it is thawed when needed. Cryoprotectant agents such as glycerol, ethylene glycol, Dimethyl sulfoxide (DMSO), and dimethylformamide are used to minimize the damage that may occur on the spermatozoa during the freeze-thawing cycle [1–3]. Several studies showed a reduction in the ability of frozen spermatozoa to successfully fertilize an oocyte compared to fresh spermatozoa [4–6], as cryopreservation leads to a decreased number of motile spermatozoa and reduced velocity of those that remain motile [7]. This is due to changes resulting from rupture of the plasma membrane by intracellular ice formation [5]. Moreover, there are several factors responsible for the loss of spermatozoa function during cryopreservation for example osmotic stress/dehydration, formation of reactive oxygen species (ROS), intracellular formation of ice crystals, and cryoprotectant toxicity [8,9]. These factors are responsible for 25–75% loss of sperm motility, decreased spermatozoa cryosurvival, and DNA fragmentation after thawing [10]. Calcium is an important secondary messenger, and plays a vital role in controlling of spermatozoa motility [11] and the acrosome reaction [12]. An elevated intracellular Ca²⁺ concentration may be a major factor underlying the suppressed motility of immature bovine spermatozoa [13]. Semen contains a high concentration of calcium, and this becomes further concentrated as water is removed by ice formation. During cryopreservation process, the architecture of the plasma membrane is perturbed by lipid crystallization, and the rate of metabolism decreases. Both of these factors reduce the ability of the cells to regulate their intracellular Ca²⁺ concentration. Therefore, it is likely that cryopreservation alters the intracellular Ca²⁺ concentration in spermatozoa, which may contribute to the observed changes in the level of certain proteins, sperm motility, and fertility.

Cation channels of sperm (CATSPER) proteins are calcium ion channels found in the flagellum of sperm. These channels are related to two-pore channels, and distantly related to transient receptor potential (TRP) channels [14]. The CatSper channel is formed by four subunits, named CATSPER 1–4 [15,16], and at least three auxiliary subunits, CATSPER β, CATSPER γ, and CATSPER δ [17–19]. Each of these are essential for its function, contributing to the development of hyper-activated spermatozoa motility, fertility in male mice, and required for spermatozoa motility at longer times after capacitation [14]. The gene encoding CATSPER2 is located on chromosome 2 and encodes a protein of 588 amino acids [15]. The CATSPER2 is transcribed during the early stages of spermatogenesis (pachytene spermatocytes) [20], and the expression of CATSPER has been reported to be low in sub-fertile men, characterized by reduced sperm motility (up to 3.5-fold difference).
compared to those with no motility defects. This suggests a possible correlation between lower CATSPER gene expression and defective spermatozoa motility in a proportion of sub-fertile patients [21]. Several previous studies have identified an absence of CATSPER channels as one of the causes of infertility in mice [19,22], primarily due to an inability of spermatozoa to become hyper-activated [23,24].

TEKTINS are proteins that make up the microtubules in cilia, flagella, basal bodies, and centrioles [25–27]. Genes encoding Tektin have been cloned in rodents, and five genes have been identified in humans [28,29] including Tekt1, Tekt2, Tekt3, Tekt4, and Tekt5 [30]. Human Tekt2 (also named Tektin-1 & h-tekB1) is present in the principal piece of spermatozoa [31] and play a critical role in the formation and development of the cilia or flagella of spermatozoa [27,32]. A previous study reported that the mutation in the TEKT2 gene can cause defects in flagella activity, which could have a detrimental effect on spermatozoa motility, leading to male infertility [26].

The present study was designed to investigate the influence of cryopreservation process on the expression level of the CATSPER2 and TEKT2 in human spermatozoa. Additionally, to evaluate the levels of damage on the Tektin2 and CatSper2 proteins in human spermatozoa during the cryopreservation process.

2. Materials and methods

2.1. Ethics statement

The present study was approved by the Institutional Ethics Committee of Saarland University (195/11), and written informed consent was obtained from all males enrolled in the study. All of the samples were analyzed by the Department of Obstetrics; Gynecology & Assisted Reproduction Laboratory, University of the Saarland, Germany. Samples were analyzed according to standard operating procedures.

2.2. Samples collection and sample criteria

The semen samples were collected from males underwent to assisted reproduction techniques for infertility treatment. In total, one hundred and twenty semen samples were collected from males by masturbation, directly in the next day of the 3-day of sexual abstinence. All of these samples were divided into two part: the first part as fresh samples and the other part of samples for cryopreservation (120 as a fresh semen sample and 120 semen samples were exposed to liquid nitrogen). Based on the medical records and questionnaire, males who had the following characteristics were excluded from this study: cryptorchidism, childhood disease, varicocele and hydrocele, and/or environmental exposure to radiation, smoking, and alcohol consumption, the presence of anti-sperm antibodies, Y chromosome microdeletions, abnormal hormonal parameters, and abnormal body mass index.

2.3. Sample preparation

The samples were allowed to liquefy at 37°C for 30 min and then processed immediately by using a Meckler counting chamber (Sefi-Medica, Haifa, Israel). The semen parameters were analyzed according to the World Health Organization guidelines [33]. Briefly, Semen samples were prepared using a discontinuous PureSperm gradient (Nidacon International) by layering 2 ml of 90% and 40% PureSperm solutions and then centrifuged at 500 × g for 20 min at room temperature. The pellet was washed twice with Ham-F10 medium, and then the samples were placed in an incubator at 37°C for 45 min. The upper layer (supernatant) was then aspirated from the lower layer (pellet). The supernatant for all samples was divided into two parts: part one (n = 120) was processed immediately as “fresh”, and part two (n = 120) was cryopreserved with liquid nitrogen at −196°C for 30 days as “cry”.

2.4. Spermatozoa cryopreservation using a computerized program freezer

Spermatozoa cryopreservation was performed using a programmed, slow machine–freezing method. The cryovials were placed vertically in the freezing chamber of a semi-programmable freezing machine (Planer kryo 10 series iii, United Kingdom). DeltaTV-6 software was used to achieve cooling from 20°C to −80°C at a rate of 1.5°C/min then 6°C/min for 12 min. Once the freezing process was complete, the straws were removed and the samples were stored in liquid nitrogen at −196°C. The cryopreservation procedure takes around 40 min.

2.5. RNA expression level study

2.5.1. Isolation of RNA from spermatozoa and reverse transcription

Total RNA was isolated from all spermatozoa samples (before and after cryopreservation) using the miNeasy Mini kit (Qiagen, Germany) with slight modifications. Briefly, 100 μl of culture medium (PAN Biotech, Germany) containing 10 × 106 spermatozoa was homogenized in 700 μl QiAozol lysis reagent (Qiagen, Germany) for 7 min to ensure complete lysis of the spermatozoa. Thereafter, the procedure was completed according to the manufacturer’s recommendations. The quantity and purity of extracted RNA were assessed by using a Nanodrop ND-2000 spectrophotometer (Thermo Scientific, USA) in order to ensure that the quality and quantity of extracted RNA was sufficient for qPCR analysis. Total RNA was converted into cDNA in a 25 μl reaction volume using miScript reverse transcription kit (Qiagen, Germany), all procedures were carried out according to the manufacturer’s recommendations.

2.5.2. Quantitative PCR (qPCR-Screening study)

Quantitative PCR (qPCR) was performed for all fresh and cryopreservation samples to quantify the expression level of three genes, namely CATSPER2, TEKT2, and the housekeeping gene GAPDH as a reference gene (Qiagen, Germany), using a StepOnePlus™ System (Applied Biosystems 7500Fast, USA). The cDNA served as the template for qPCR analysis, which was performed using the QuantiTect primer assay (Qiagen, Germany) according to the manufacturer’s recommendations. In addition, a no template control (NTC) and no reverse transcriptase control (NRT) were included in each run. Note: All qPCR experiments were performed in triplicate and the resulting Ct values were normalized to GAPDH.

2.6. Protein level study

2.6.1. Isolation of proteins from spermatozoa samples

Proteins were isolated from all spermatozoa samples (before and after cryopreservation) using lysis buffer (400 μl of 2% SDS containing 1 μl of protease inhibitor mixture) for the Western blot analysis. Briefly, 200 μl culture media (PAN Biotech, Germany) contained 10 × 106 spermatozoa. Spermatozoa samples were washed twice with 400 μl phosphate-buffered saline (PBS) by centrifugation at 4000 × g for 5 min to create a pellet. The protein concentration in the supernatant was measured by Bradford protein assay (Bio-Rad, Germany).

2.6.2. Optimisation the concentration of extracted protein and Western blot analysis

In order to obtain a good signal of protein, three different concentrations of protein (50, 75, and 100 μg) were used to find the optimum concentration. As shown in Fig. 1, the most defined band was observed when a concentration of 75 μg protein was used, therefore, this concentration was chosen for this study. All protein samples that extracted from the spermatozoa were analyzed by using Western blot to evaluate the levels of CatSper2 and Tektin2 protein in sperm samples before and after cryopreservation (fresh, n = 120 & cryopreserved, n = 120). Western blotting was performed as previously described in the study of Abid and his colleagues [34].
2.6.3. Incubation of antibodies and detection of proteins

After the protein transfer process was complete, immunoblotting was performed as follows: The PVDF membrane was rinsed with water then stained for 1 h with Ponceau S solution (Sigma-Aldrich, Germany) to detect the proteins. The stain was rinsed off with three washes of Tris-buffered saline containing 0.1% Tween-20 (TBS-T) until the background had become clear. To block non-specific binding to the membrane, a solution of 5% non-fat dried milk powder in TBS-T buffer was used. The blots were incubated with mouse monoclonal Tektin2 antibody (1:100 diluted in TBS; 54 kDa; ab60918, Abcam, UK) and rabbit polyclonal CatSper2 antibody (1:100 dilution in TBS; 62 kDa; ab150890, Abcam) overnight at 4°C with shaking. The blots were washed with TBS-T buffer, then incubated at room temperature for 1 h with anti-rabbit and anti-mouse IgG H&L horseradish peroxidase (HRP)-conjugated antibodies (ab6728 and ab6721; Abcam) according to the manufacturer’s recommendations. No bands were present in the negative control, confirming the specificity of the antibodies.

2.6.4. Imaging and normalization of protein bands

The Amersham ECL Prime Western blotting detection reagent (Sigma-Aldrich, Germany) containing a chemiluminescent substrate was applied to the blot according to the manufacturer’s recommendations. To control for variability during the protein loading step in the Western blot, β-actin was used as a loading control (LC) for normalization of the CatSper2 and Tektin2 proteins. The normalization steps were conducted as previously described in the study of Liu and his colleagues [35].

2.7. Statistical analysis

All data obtained from the Western blot and qPCR were analyzed using IBM SPSS for Windows software package version 24.0 (SPSS Inc., USA). To avoid any statistical errors during data analysis the type of data distribution was detected through the SPSS program by using the following tests: skewness test, Kurtosis test, Z-value, and Shapiro test. The results of these tests showed that the samples included in this study were not normally distributed (non-parametric). The Mann–Whitney U test (Mann–Whitney test) was used to compare quantitative variables. The results were accepted as statistically significant when the P-value was less than or equal to 5% (P ≤ 0.05). The relative RNA quantities in the “cry” versus “fresh” samples were calculated separately by the comparative ΔΔCt method. The threshold cycle (Ct) reflects the cycle number at which the fluorescence curve generated within the reaction crossed the threshold for qPCR. The ΔCt was calculated by subtracting the Ct values of GAPDH from the Ct values of the RNA of interest, where ΔCt = (Ct RNA of interest) - (Ct GAPDH). The ΔΔCt was then calculated by subtracting the Δ Ct of “cry” samples from the ΔCt of the “fresh” samples, where ΔΔCt = (ΔCt fresh - ΔCt cry). The fold-change for the RNAs was calculated by the 2−ΔΔCt equation [36].

3. Results

The present study was prepared to determine the effect of cryopreservation on the expression levels of CATSPER2 and TEKT2 and protein level in the human spermatozoa. The age of males included in this study ranged between 24–50 years, with a mean age of 36.8 ± 6.3 years. The clinical characteristics of the study population are shown in Table 1.

3.1. Expression level of CATSPER2 and TEKT2 gene in spermatozoa samples before and after cryopreservation

In the qPCR study, 120 sample from the fresh and 120 sample from the cryopreserved group were used, to determine whether there was any change in the expression levels of CATSPER2 and TEKT2 genes in spermatozoa before and after cryopreservation. The results of this study showed a significant decrease in the expression levels of CATSPER2 and TEKT2 in the cryopreserved samples compared to the fresh samples (P = 0.0039 and P = 0.0166, respectively) (Fig. 2). Also the present results showed down-regulation in the expression level of TEKT2 and CATSPER2 genes between fresh samples and cry samples with fold change 11.08 and 30.48, respectively (Table 2).

3.2. Protein levels of CatSper2 and Tektin2 in human spermatozoa before and after cryopreservation

The level of CatSper2 and Tektin2 protein in the human spermatozoa before and after cryopreservation was evaluated using Western blotting. The results of this study have been shown a single band at 62 and 54 kDa, the expected sizes of the CatSper2 and Tektin2 proteins, respectively, were observed more clearly in the fresh samples compared to cryopreserved samples (Fig. 3). Beta-actin was used as a loading control to determine if the samples had been loaded equally across all wells, and to confirm protein transfer during the Western blot protocol. No bands were present in the negative control, confirming the specificity of the bands. The levels of the CatSper2 and Tektin2 proteins were lower in spermatozoa of cryopreserved samples compared to fresh samples, with 0.44 ± 0.35 vs. 0.77 ± 0.25 (P = 0.0001) and 0.58 ± 0.24 vs. 0.76 ± 0.09 (P = 0.0001), respectively; (Fig. 4).

4. Discussion

Cryopreservation of spermatozoa is a widely used technique to

Table 1

| Clinical characteristics of the study population (n = 120). |
|-----------------|--------|--------|--------|--------|--------|
| Variables       | Median | Mean   | SD     | Min    | Max    | Range  |
| Age (year)      | 35.00  | 36.8   | 6.3    | 24.00  | 50.00  | 26.00  |
| Sperm count (million/ml) | 78.00 | 84.93  | 55.92  | 1.70   | 226.00 | 224.30 |
| Percentage of sperm total motility | 57.00 | 55.38  | 20.97  | 0.00   | 89.00  | 89.00  |
| Percentage of sperm progressive motility | 42.00 | 38.40  | 22.56  | 0.00   | 82.00  | 82.00  |
| Percentage of sperm non-progressive motility | 10.00 | 10.00  | 17.16  | 0.00   | 75.00  | 75.00  |
| Sperm vitality test (Eosin test) | 55.15 | 55.15  | 17.14  | 0.00   | 87.00  | 87.00  |

SD: Standard deviation, Min: minimum, Max: Maximum.
preserve the biological function of spermatozoa. During the cryopreservation process, spermatozoa undergo a dramatic transformation in their chemical and physical characteristics as the temperature drops from +37 to -196°C, thus risking cryo-damage. The speed of cooling and thawing is a critical step, and inappropriate cooling or thawing rates are negatively correlated with spermatozoa survival [37]. Several proteins identified in human spermatozoa tails have been implicated in the regulation of motility, and these belong to diverse protein classes including ion channels, cytoskeletal proteins, cell signaling proteins, and glycolytic enzymes [38]. CatSper2, a Cation ion channel that regulates Ca^{2+}, and Tektin2, a membrane protein responsible for spermatozoa flagella movement, were the targets of this study. Previous studies have reported that CatSper and Tektin are related to male infertility problems, as they play an important role in sperm motility [14,39]. Furthermore, Hildebrand and his colleagues identified sperm motility defects due to the loss of CatSper channels, which are expressed in the principal piece of the spermatozoa flagellum [40]. Spermatozoa with a deficiency in CatSper channels can move through the extracellular matrix of the oocyte, but cannot penetrate the zona pellucida due to failure to achieve Ca^{2+}-dependent hyper-activated motility. In contrast, other study reported that Tektin2 is required for dynein arm integrity in spermatozoa flagella, and the deficiency in Tektin2 causes male infertility due to impaired sperm motility [31,41].

During the cryopreservation process, spermatozoa are subjected to detrimental chemical and physical effects such as intracellular ice crystal formation and dissolution, altered membrane permeability, cellular dehydration, and osmotic injury [42]. The effects of freezing and thawing can harm the spermatozoa, influencing their fertilization capacity by damaging their cell membrane, DNA, and acrosomes, and by severely impairing sperm motility [37]. The results of the present study show decreased RNA expression of CATSPER2 and TEKT2 in human spermatozoa of cryopreserved samples compared to fresh samples. This finding is in agreement with another study that found that cryopreservation affects the mRNA–protein interaction and makes mRNA molecules more susceptible to degradation [43]. Besides that, other studies found significantly higher levels of CATSPER2 and CATSPER3 mRNAs in high-motility spermatozoa than in the low-motility fraction [44,45]. The low CATSPER2 and TEKTIN2 mRNA expression observed in the
cryopreserved spermatozoa samples of the present study suggest that the reduced motility reported after freezing may result from impaired transcription of these genes in some spermatozoa, related to sperm motility. Valcarce and his colleagues studied the effect of cryopreservation on human spermatozoa mRNA expression and found a significant effect on fertilization and early embryo development [46]. In the present study, the levels of the Tektin2 protein were lower in the spermatozoa of cryopreserved samples compared to fresh samples. This result is in agreement with previous studies which reported that membrane cytoskeletal components are sensitive to temperature, causing damage to spermatozoa surface proteins [6,47]. Furthermore, cryoprotectant toxicity can induce alterations in the spermatozoa membrane components [48]. On the other hand, the result of the present study showed down-regulation in the expression level of CATSPER2 and TEKT2 gene in cryopreserved samples compared to fresh sample and these results in matching with previous studies that identified four down-regulated proteins (TEKTIN1, VIM, ACO2, AND ENO1) that were putatively involved in sperm motility, viability, acrosome integrity, ATP, mitochondrial membrane potential, capacitation, acrosome reaction, and intracellular calcium concentration. These marked differences strongly suggest that dysfunctional spermatozoa after cryopreservation may be due to protein degradation and protein phosphorylation [6,49–51].

5. Conclusion

The results of the current study showed a significant reduction in the expression level of the CATSPER2 and TEKT2 gene (down-regulation) and in the level of CatSper2 and Tektin2 proteins in cryopreserved samples compared to fresh samples. The reduction in the mRNA expression level and protein levels of CatSper2 and Tektin2 may be used as markers to explain the causes of motility loss in the spermatozoa after cryopreservation process in the males who underwent to assisted reproductive technology.

Declaration of Competing Interest

Authors do not have any potential conflict of interest.

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