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

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ABSTRACT

This study designed to assess the expression level of *CATSPER2 and TEKT2* and to evaluate the levels of CatSper2 and Tektin2 proteins in human spermatozoa before and after cryopreservation. One hundred and twenty semen samples were included in this study. All the samples were subjected to qPCR and Western blot analysis. The results showed a significant reduction in the expression levels of *CATSPER2* and *TEKT2* in the cryopreserved compared to the fresh samples (P = 0.0039 and P = 0.0166, respectively), and the results showed down-regulation in the expression level of *CATSPER2 and TEKT2* genes between the study groups. Moreover, the protein levels of the CatSper2 and Tektin2 were lower in cryopreserved samples compared to fresh samples (P = 0.0001). In conclusion, the reduction in the proteins level and expression level of the *CATSPER*2 and *TEKT*2 in cryopreserved samples could be used as an indicator of sperm motility loss.

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 Ca^{2+} 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 Ca^{2+} concentration. Therefore, it is likely that cryopreservation alters the intracellular Ca^{2+} 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 hyperactivated spermatozoa motility, fertility in male mice, and required for spermatozoa motility at longer times after capacitation [14]. The gene encoding *CATSPER*2 is located on chromosome 2 and encodes a protein of 588 amino acids [15]. The *CATSPER*2 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)

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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 *TEKT*1, *TEKT*2, *TEKT*3, *TEKT*4, and *TEKT*5 [30]. Human *TEKT2* (also named *Tektin-t* & *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 *TEKT*2 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 *CATSPER*2 and *TEKT*2 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 miRNeasy 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 Qiazol 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 C_t 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 10×10^6 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. Optimization 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].

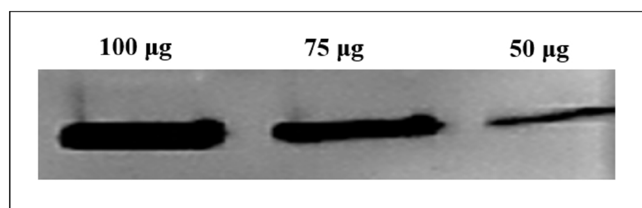

Fig. 1. The optimization process of protein concentration for the Western blot analysis, three protein concentrations were tested, 50, 75, and 100 µg.

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 (1:1000 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) against the Tektin2 and CatSper2 proteins, respectively, diluted 1:5000 (optimized dilution) in TBS-T. After the blots had been washed with TBS-T, detection of proteins was carried out using the Molecular Imager® Gel Doc™ XR + system with Image Lab™ software (Bio-Rad, Germany), and the signals were developed and visualized. For normalization, the same blot was stripped and re-probed with mouse monoclonal β-actin (1:100) antibody (Sigma-Aldrich, Germany). The membrane was first covered with stripping buffer (0.19 M glycine, 3 mM SDS, and 10 ml Tween 20; pH 2.2) and incubated at room temperature for 1 h, followed by three washes with PBS and one wash with PBS containing Tween-20 (PBST). The blots were blocked with non-fat dried milk and probed with an anti-β-actin antibody diluted 1:100 (optimized dilution) (Sigma-Aldrich, Germany), followed by the mouse secondary antibody. For the negative controls, parallel blots were incubated with TBS instead of the monoclonal and polyclonal 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% ($P \leq 0.05$). The relative RNA quantities in the "cry" versus "fresh" samples were calculated separately by the comparative ΔC_t method. The threshold cycle (C_t) reflects the cycle number at which the fluorescence curve generated within the reaction crossed the threshold for qPCR. The ΔC_t was calculated by subtracting the C_t values of *GAPDH* from the C_t values of the RNA of interest, where ΔC_t = (C_t RNA of interest) - (C_t *GAPDH*). The $\Delta\Delta C_t$ was then calculated by subtracting the ΔC_t of "cry" samples from the ΔC_t of the "fresh" samples, where $\Delta\Delta C_t$ = (ΔC_t fresh - ΔC_t cry). The fold-change for the RNAs was calculated by the $2^{-\Delta\Delta Ct}$ equation [36].

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.

3. Results

The present study was prepared to determine the effect of cryopreservation on the expression levels of *CATSPER*2 and *TEKT*2 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 level 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

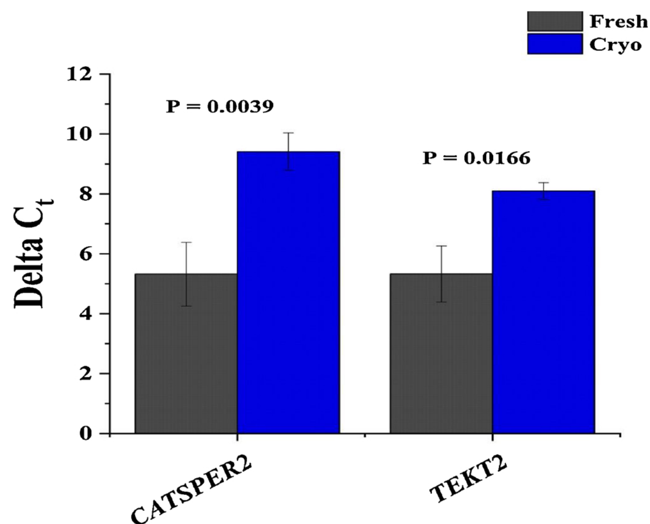

Fig. 2. RNA expression levels of *CATSPER2* and *TEKT2* in human spermatozoa. ΔC_t of the RNAs in the spermatozoa of cryopreserved (cryo) samples (n = 120) compared to the fresh samples (n = 120) of the same males as determined by qPCR. Data were analysed by the Mann–Whitney (U test), and P ≤ 0.05 was considered significant.

Table 2
Expression of *CATSPER2* and *TEKT2* genes in spermatozoa samples after cryopreservation samples compared to fresh samples.

		TEKT2	*CATSPER2*
Fresh samples	Mean C_t	34.18	33.45
	ΔC_t	4.59	4.75
Cryopreserved samples	Mean C_t	33.2	34.86
	ΔC_t	8.06	9.68
$\Delta\Delta C_t$		−3.47	−4.93
Fold change		11.08	30.48
Regulation		Down	Down

C_t: Cycle threshold.

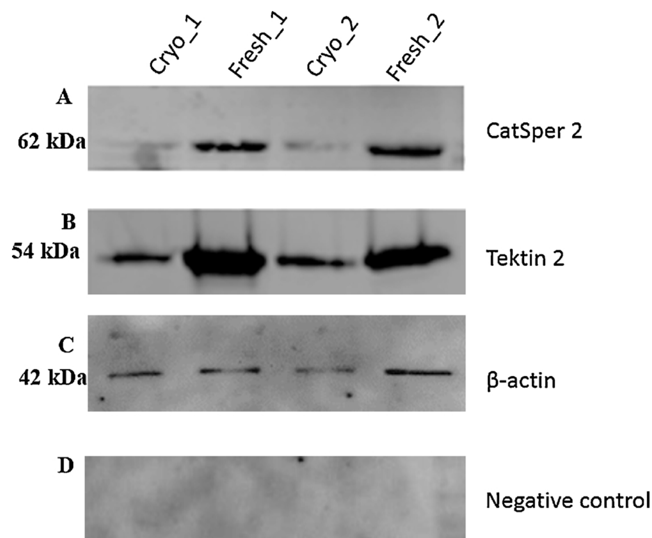

Fig. 3. Protein levels of CatSper2 and Tektin2 in human spermatozoa before and after exposure to cryopreservation, as determined by western blotting. The proteins were separated on a 12.5% SDS-polyacrylamide gel then transferred to a PVDF membrane. (A) CatSpar2, (B) Tektin2, and (C) β-actin bands were visualized with the appropriate antibodies on an ECL system. The CatSpar2 and Tektin2 proteins were 62 and 54 kDa, respectively. The loading control, β-actin (42 kDa), was used to confirm equal loading of protein in each lane. (D) The negative control was incubated without primary antibodies to check for non-specific binding. Pierce pre-stained protein molecular weight marker (Thermo Fisher, Germany) was used. Cryo: cryopreserved sample; Fresh: fresh sample.

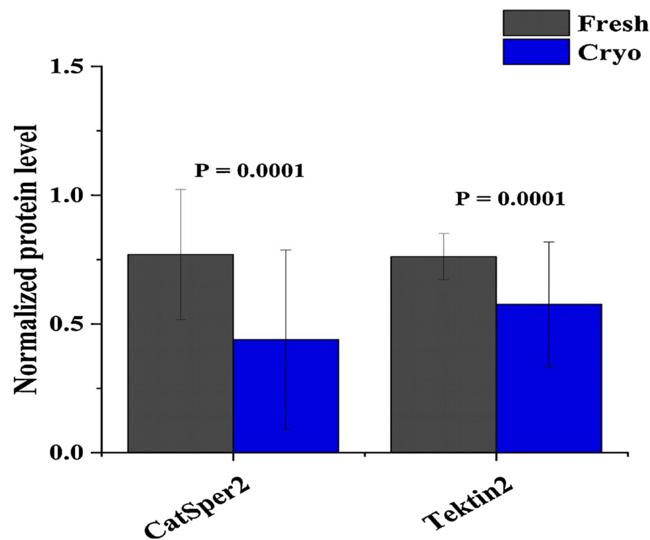

Fig. 4. CatSper2 and Tektin2 protein levels in human spermatozoa of cryopreserved samples compared to fresh samples.
Cryo: cryopreserved sample; Fresh: fresh sample,
P ≤ 0.05 considered significant.

preserve the biological function of spermatozoa. During the cryopreservation process, the 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 the 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 *CATSPER*2 and *CATSPER3* mRNAs in high-motility spermatozoa than in the low-motility fraction [44,45]. The low *CATSPER*2 and *TEKTIN*2 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 *CATSPER*2 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 spermatozoon 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 *CATSPER*2 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 RNA 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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References

[1] R.A. Forero-Gonzalez, E.C. Celeghini, C.F. Raphael, A.F. Andrade, F.F. Bressan, R.P. Arruda, Effects of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes, Andrologia 44 (Suppl 1) (2012) 154–159.
[2] P.V. Dludla, B. Jack, A. Viraragavan, C. Pheiffer, R. Johnson, J. Louw, C.J. Muller, A dose-dependent effect of dimethyl sulfoxide on lipid content, cell viability and oxidative stress in 3T3-L1 adipocytes, Toxicol. Rep. 5 (2018) 1014–1020.
[3] S. Erdal, B.J. McCarthy, N. Gurule, M. Berwick, E. Gonzales, J. Byrd, K. Flores, J. Shimek, D. Il'yasova, F. Ali-Osman, D.D. Bigner, Application of mutagen sensitivity assay in a glioma case-control study, Toxicol. Rep. 5 (2018) 183–188.
[4] B.L. Bordson, E. Ricci, R.P. Dickey, H. Dunaway, S.N. Taylor, D.N. Curole, Comparison of fecundability with fresh and frozen semen in therapeutic donor insemination, Fertil. Steril. 46 (1986) 466–469.
[5] P. Watson, Artificial insemination and the preservation of semen, Marshall's Physiol. Reprod. 2 (1990) 747–869.
[6] P.F. Watson, The causes of reduced fertility with cryopreserved semen, Anim. Reprod. Sci. 60-61 (2000) 481–492.
[7] E.A. McLaughlin, W.C. Ford, M.G. Hull, Motility characteristics and membrane integrity of cryopreserved human spermatozoa, J. Reprod. Fertil. 95 (1992) 527–534.
[8] E.A. McLaughlin, W.C. Ford, Effects of cryopreservation on the intracellular calcium concentration of human spermatozoa and its response to progesterone, Mol. Reprod. Dev. 37 (1994) 241–246.
[9] M.W. Alaa Hamada, Sajal Gupta, Ashok Agarwal, Sperm Banking: Indications and Regulations, (2014).
[10] J.K. Critser, A.R. Huse-Benda, D.V. Aaker, B.W. Arneson, G.D. Ball, Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility, Fertil. Steril. 50 (1988) 314–320.
[11] C.B. Lindemann, K.S. Kanous, Regulation of mammalian sperm motility, Arch. Androl. 23 (1989) 1–22.
[12] D.L. Garbers, Molecular basis of fertilization, Annu. Rev. Biochem. 58 (1989) 719–742.
[13] S. Vijayaraghavan, D.D. Hoskins, Changes in the mitochondrial calcium influx and efflux properties are responsible for the decline in sperm calcium during epididymal maturation, Mol. Reprod. Dev. 25 (1990) 186–194.
[14] H. Qi, M.M. Moran, B. Navarro, J.A. Chong, G. Krapivinsky, L. Krapivinsky, Y. Kirichok, I.S. Ramsey, T.A. Quill, D.E. Clapham, All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 1219–1223.
[15] B. Navarro, Y. Kirichok, J.J. Chung, D.E. Clapham, Ion channels that control fertility in mammalian spermatozoa, Int. J. Dev. Biol. 52 (2008) 607–613.
[16] P.V. Lishko, I.L. Botchkina, A. Fedorenko, Y. Kirichok, Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel, Cell 140 (2010) 327–337.
[17] J. Liu, J. Xia, K.H. Cho, D.E. Clapham, D. Ren, CatSperbeta, a novel transmembrane protein in the CatSper channel complex, J. Biol. Chem. 282 (2007) 18945–18952.
[18] H. Wang, J. Liu, K.H. Cho, D. Ren, A novel, single, transmembrane protein CATSPERG is associated with CATSPER1 channel protein, Biol. Reprod. 81 (2009) 539–544.
[19] J.J. Chung, B. Navarro, G. Krapivinsky, L. Krapivinsky, D.E. Clapham, A novel gene required for male fertility and functional CATSPER channel formation in spermatozoa, Nat. Commun. 2 (2011) 153.
[20] N. Schultz, F.K. Hamra, D.L. Garbers, A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 12201–12206.
[21] P. Nikpoor, S.J. Mowla, M. Movahedin, S.A. Ziaee, T. Tiraihi, CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility, Hum. Reprod. 19 (2004) 124–128.
[22] D. Ren, B. Navarro, G. Perez, A.C. Jackson, S. Hsu, Q. Shi, J.L. Tilly, D.E. Clapham, A sperm ion channel required for sperm motility and male fertility, Nature 413 (2001) 603–609.
[23] A.E. Carlson, R.E. Westenbroek, T. Quill, D. Ren, D.E. Clapham, B. Hille, D.L. Garbers, D.F. Babcock, CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 14864–14868.
[24] A.E. Carlson, T.A. Quill, R.E. Westenbroek, S.M. Schuh, B. Hille, D.F. Babcock, Identical phenotypes of CatSper1 and CatSper2 null sperm, J. Biol. Chem. 280 (2005) 32238–32244.
[25] M. Larsson, J. Norrander, S. Graslund, E. Brundell, R. Linck, S. Stahl, C. Hoog, The spatial and temporal expression of Tekt1, a mouse tektin C homologue, during spermatogenesis suggest that it is involved in the development of the sperm tail basal body and axoneme, Eur. J. Cell Biol. 79 (2000) 718–725.
[26] N. Iguchi, H. Tanaka, Y. Nakamura, M. Nozaki, T. Fujiwara, Y. Nishimune, Cloning and characterization of the human tektin-t gene, Mol. Hum. Reprod. 8 (2002) 525–530.
[27] T. Matsuyama, Y. Honda, M. Doiguchi, H. Iida, Molecular cloning of a new member of TEKTIN family, Tektin4, located to the flagella of rat spermatozoa, Mol. Reprod. Dev. 72 (2005) 120–128.
[28] M. Xu, Z. Zhou, C. Cheng, W. Zhao, R. Tang, Y. Huang, W. Wang, J. Xu, L. Zeng, Y. Xie, Y. Mao, Cloning and characterization of a novel human TEKTIN1 gene, Int. J. Biochem. Cell Biol. 33 (2001) 1172–1182.
[29] M.J. Wolkowicz, S. Naaby-Hansen, A.R. Gamble, P.P. Reddi, C.J. Flickinger, J.C. Herr, Tektin B1 demonstrates flagellar localization in human sperm, Biol. Reprod. 66 (2002) 241–250.
[30] M.A. Pirner, R.W. Linck, Tektins are heterodimeric polymers in flagellar microtubules with axial periodicities matching the tubulin lattice, J. Biol. Chem. 269 (1994) 31800–31806.
[31] H. Tanaka, N. Iguchi, Y. Toyama, K. Kitamura, T. Takahashi, K. Kaseda, M. Maekawa, Y. Nishimune, Mice deficient in the axonemal protein Tektin-t exhibit male infertility and immotile-cilium syndrome due to impaired inner arm dynein function, Mol. Cell. Biol. 24 (2004) 7958–7964.
[32] P.W. Setter, E. Malvey-Dorn, W. Steffen, R.E. Stephens, R.W. Linck, Tektin interactions and a model for molecular functions, Exp. Cell Res. 312 (2006) 2880–2896.
[33] World Health Organization, WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th ed., WHO, Geneva, Switzerland, 2010.
[34] S. Abid, J. Gokral, A. Maitra, P. Meherji, S. Kadam, E. Pires, D. Modi, Altered expression of progesterone receptors in testis of infertile men, Reprod. Biomed. Online 17 (2008) 175–184.
[35] N.K. Liu, X.M. Xu, β-tubulin is a more suitable internal control than β-actin in western blot analysis of spinal cord tissues after traumatic injury, J. Neurotrauma 23 (2006) 1794–1801.
[36] K. Livak, T. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.
[37] G.M. Centola, R.F. Raubertas, J.H. Mattox, Cryopreservation of human semen. Comparison of cryopreservatives, sources of variability, and prediction of post-thaw survival, J. Androl. 13 (1992) 283–288.
[38] R.M. Turner, Moving to the beat: a review of mammalian sperm motility regulation, Reprod. Fertil. Dev. 18 (2006) 25–38.
[39] J.L. Jin, A.M. O'Doherty, S. Wang, H. Zheng, K.M. Sanders, W. Yan, Catsper3 and

catsper4 encode two cation channel-like proteins exclusively expressed in the testis, Biol. Reprod. 73 (2005) 1235–1242.
[40] M.S. Hildebrand, M.R. Avenarius, M. Fellous, Y. Zhang, N.C. Meyer, J. Auer, C. Serres, K. Kahrizi, H. Najmabadi, J.S. Beckmann, R.J. Smith, Genetic male infertility and mutation of CATSPER ion channels, Eur. J. Hum. Genet. 18 (2010) 1178–1184.
[41] S. Petyim, R. Choavaratana, S. Kunathikom, Freezing effect on post-thawed sperm characteristics especially sperm DNA integrity comparing between liquid nitrogen vapour and computerized program freezer, Siriraj Med. J. 59 (2017) 298–302.
[42] P. Stanic, M. Tandara, Z. Sonicki, V. Simunic, B. Radakovic, E. Suchanek, Comparison of protective media and freezing techniques for cryopreservation of human semen, Eur. J. Obstet. Gynecol. Reprod. Biol. 91 (2000) 65–70.
[43] E. Flores, L. Ramio-Lluch, D. Bucci, J.M. Fernandez-Novell, A. Pena, J.E. Rodriguez-Gil, Freezing-thawing induces alterations in histone H1-DNA binding and the breaking of protein-DNA disulfide bonds in boar sperm, Theriogenology 76 (2011) 1450–1464.
[44] H.G. Li, X.F. Ding, A.H. Liao, X.B. Kong, C.L. Xiong, Expression of CatSper family transcripts in the mouse testis during post-natal development and human ejaculated spermatozoa: relationship to sperm motility, Mol. Hum. Reprod. 13 (2007) 299–306.
[45] X. Chen, Y. Wang, H. Zhu, H. Hao, X. Zhao, T. Qin, D. Wang, Comparative transcript profiling of gene expression of fresh and frozen–thawed bull sperm, Theriogenology 83 (2015) 504–511.
[46] D.G. Valcarce, F. Carton-Garcia, M.P. Herraez, V. Robles, Effect of cryopreservation on human sperm messenger RNAs crucial for fertilization and early embryo development, Cryobiology 67 (2013) 84–90.
[47] R.H. Hammerstedt, J.K. Graham, J.P. Nolan, Cryopreservation of mammalian sperm: what we ask them to survive, J. Androl. 11 (1990) 73–88.
[48] B.A. Ball, A. Vo, Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential, J. Androl. 22 (2001) 1061–1069.
[49] S. Wang, W. Wang, Y. Xu, M. Tang, J. Fang, H. Sun, Y. Sun, M. Gu, Z. Liu, Z. Zhang, F. Lin, T. Wu, N. Song, Z. Wang, W. Zhang, C. Yin, Proteomic characteristics of human sperm cryopreservation, Proteomics 14 (2014) 298–310.
[50] S.J. Yoon, M.S. Rahman, W.S. Kwon, D.Y. Ryu, Y.J. Park, M.G. Pang, Proteomic identification of cryostress in epididymal spermatozoa, J. Anim. Sci. Biotechnol. 7 (2016) 67.
[51] S. Collin, M.A. Sirard, M. Dufour, J.L. Bailey, Sperm calcium levels and chlortetracycline fluorescence patterns are related to the in vivo fertility of cryopreserved bovine semen, J. Androl. 21 (2000) 938–943.