DEPARTMENT OF OBSTETRICS, GYNAECOLOGY & REPRODUCTIVE MEDICINE

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The Impact of Cryopreservation on CatSper 2 and Tektin 2 in Human Spermatozoa

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Abstract

Due to deteriorating quality of spermatozoa after cryopreservation, it became so inevitable to find solutions to maintain its quality so it can fertilize the oocyte. Therefore, the researchers' focuses was directed toward the genes responsible for the spermatozoa movement and to assess whether or not they are sensitive to the cryopreservation process. CatSper 2 and Tektin 2 are defined as spermatozoa tail proteins which are involved in sperm motility. In fact, cryopreservation is known to cause damage on spermatozoa in many different aspects such as DNA, RNA, motility of spermatozoa and spermatozoa morphology. It was thus hypothesized in this study that CatSper 2 and Tektin 2 are genes responsible for spermatozoa motility and the possibility of being affected during the cryopreservation process is very high, which leads to a decrease in the spermatozoa ability to fertilize the oocyte. So, the purpose of my study was, hence, to investigate the levels of CatSper 2 and Tektin 2 proteins in human spermatozoa samples before and after exposure to liquid nitrogen (LN2) during cryopreservation process. In addition, to assess the CatSper 2 and Tektin 2 mRNA expression before and after cryopreservation stress. Finally, to determine the levels of cryodamage of CatSper 2 and Tektin 2 proteins in human spermatozoa during cryopreservation process in fertile and subfertile males. In total, 48 semen samples were collected and included in this study with a mean age of 36.75 ± 6.25 years.

The results showed a decrease in the expression levels of CatSper 2 and Tektin 2 mRNA in the cryopreserved samples compared to the fresh samples, and this downregulation ranged between a fold change of 2.04–11.95 and 1.11–62.68 in the CatSper 2 and Tektin 2 genes, respectively. The differences in expression levels of CatSper 2 and Tektin 2 were significant, with *P*-values of 0.0039 and 0.0166, respectively, in the cryopreserved samples compared to the fresh samples using RT-qPCR. In addition the levels of the CatSper 2 and Tektin 2 proteins were lower in spermatozoa of cryopreserved samples compared to fresh samples, with 0.44 ± 0.35 vs. 0.77 ± 0.25 ($P \leq 0.0001$) and 0.58 ± 0.24 vs. 0.76 ± 0.09 ($P \leq 0.0001$), respectively. On the other hand, these results showed an association between the decrease in the levels of these proteins and fertility status. In conclusion, these

findings may be used as markers to explain some of causes related with infertility problems. The findings of my study are remarkable because, according to the available information, this study is considered the first study to evaluate the effects of the cryopreservation process on the CatSper 2 and Tektin 2 mRNA and protein levels in human spermatozoa. Furthermore, the present study points to differences in these two proteins after cryopreservation in fertile and subfertile men.

Zusammenfassung

Um der bei Kryokonservierung auftretenden Qualitätsminderung der Spermatozoen entgegenzuwirken und damit eine Befruchtung der Oozyten zu ermöglichen, ist es notwendig eine Lösung zum Erhalt der Spermatozoenqualität zu finden. Aus diesem Grund beschäftigte sich die bisherige Forschung bereits mit den für die Spermatozoenbewegung verantwortlichen Gene, um zu beurteilen, ob sie vom Prozess der Kryokonservierung beeinflusst werden oder nicht. CatSper 2 und Tektin 2 sind bekannt als Spermatozoen-Schwanzproteine, die an der Spermienmotilität beteiligt sind. Tatsächlich führt die Kryokonservierung der Spermatozoen zu Schäden der DNA, RNA und zu Veränderungen der Motilität und Morphologie der Spermatozoen. Auf Grund dieser Studie wurde daher angenommen, dass CatSper 2 und Tektin 2 als essentielle Gene für die Spermatozoen-Motilität von dem Kryokonservierungsprozess stark betroffen seien. Dies führe zu einer Abnahme der Spermatozoenfähigkeit die Oozyte zu befruchten. Das Ziel meiner Studie war es daher, die Konzentration der Proteine CatSper 2 und Tektin 2 in menschlichen Spermatozoenproben vor und nach der Exposition mit flüssigem Stickstoff (LN2) im Rahmen des Kryokonservierungsprozesses zu untersuchen. Darüber hinaus wurde die mRNA-Expression der genannten Gene vor und nach dem Kryokonservierungsstress ermittelt. Abschließend wurde der während des Kryokonservierungsprozesses entstandene Schaden der CatSper 2- und Tektin 2-Proteine in menschlichen Spermatozoen bei fertilen und subfertilen Männern untersucht. Insgesamt wurden 48 Samenproben von Probanden mit einem mittleren Alter von 36,75 ± 6,25 Jahren gesammelt und in diese Studie aufgenommen.

Die Ergebnisse zeigten eine Abnahme des Expressionsniveaus der mRNA der Gene CatSper 2 und Tektin 2 in kryokonservierten Proben im Vergleich zu frischen Proben. Diese Herrunterregulierung lag zwischen einem Faktor von 2,04-11,95 und 1,11-62,68 für die Gene CatSper 2 und Tektin 2. Die Unterschiede in den Expressionsniveaus von CatSper 2 und Tektin 2 konnten signifikant mit P-Werten von 0,0039 bzw. 0,0166 in den kryokonservierten Proben im Vergleich zu den frischen Proben unter Verwendung der RT-qPCR nachgewiesen werden. Das Vorkommen der CatSper 2- und Tektin 2-Proteine war in den frischen Proben im

Vergleich zu den kryokonservierten Proben mit 0,44 ± 0,35 vs. 0,77 ± 0,25 ($P \leq 0,0001$) und 0,58 ± 0,24 gegenüber 0,76 ± 0,09 ($P \leq 0,0001$) niedriger. Abschließend Die Ergebnisse meiner Untersuchungen sind bemerkenswert, weil nach den vorliegenden Informationen die Wirkung des Kryokonservierung-Prozesses auf die CatSper 2 und Tektin 2 mRNA- und Protein-Ebenen in menschlichen Spermien erstmals untersucht wurde. Darüber hinaus zeigt die vorliegende Studie die Unterschiede zwischen diesen beiden Proteinen nach Kryokonservierung in fruchtbaren und subfertilen Männern auf.

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List of Abbreviations

APS	Ammonium Persulfate
AR	Acrosome Reaction
ART	Assisted Reproductive Techniques
ATP	Adenosine Triphosphate
bp	Base pair
BSA	Bovine Serum Albumin
Ca^2+	Voltage-dependent Calcium channels
CatSper	Cation Channels of Sperm
cDNA	complementary Deoxyribonucleic Acid
CNG	Cyclic Nucleotide-Gated Ion Channel
CPF	Computerized Program Freezer
Cryo	Cryopreserved
CT	Cycle threshold
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HCL	Hydrochloric Acid
HRP	Horseradish Peroxidase
HV1	Voltage Gated H+ Channel
ICSI	Intracytoplasmic Sperm Injection
IF	Intermediate Filament
IgG	Immunoglobulin G
IVF	In Vitro Fertilization
kDa	Kilodalton
KSper	K+ channel of Sperm
mA	Milliampere
Me OH	Methanol
Min	Minute
mM	Millimolar

mRNA	Messenger Ribonucleic acid
ND	Nanodrop
NL2	Liquid Nitrogen
Nm	Nanometer
nM	Nanomolar
PBS	Phosphate-Buffered Saline
PBST	Phosphate Buffered Saline with Tween20
PCR	Polymerase Chain Reaction
PRM1	Protamine 1
PRM2	Protamine 2
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
Rpm	Rounds Per Minute
RT-qPCR	Quantitative Reverse Transcription -Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Sec	Second
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween 20
TEKT	Tektin
TEMED	Tetramethylethylenediamine
TRP	TRP Transient Receptor Potential Channels
UV	Ultraviolet
V	Volt
v/v	Volume/Volume
VGCC	Voltage-Gated Cation Channels
w/v	Weight/Volume
WHO	World Health Organization
β-Met	β-Mercaptoethanol
Mg	Microgram

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1 Introduction

Infertility problems are observed in 15% of all couples of reproductive age, with male-specific factors contributing to about 50% of these cases (1). There are currently several techniques that can be used in assisted reproductive technology (ART) to help couples suffering from infertility problems that cannot achieve pregnancy through intercourse. In addition, due to the development of assisted reproductive techniques, the ability to store human spermatozoa has increased the reproductive potential of many men with low spermatozoa counts. This is also an option for men requiring chemotherapy or radiotherapy treatment, which can damage the existing spermatozoa and render them impotent. Although frozen spermatozoa can be stored for decades, it is recommended that their viability be monitored every 10 years during long-term freezing, as cryodamage may affect their viability and motility (2). In addition, the freezing and thawing procedures are generally harmful to the spermatozoa membrane, as temperature and osmotically induced changes can occur in the organization, permeability, and lipid composition of the spermatozoa membranes. Therefore, the freeze-thaw process itself can lead to spermatozoa with low motility and damaged membrane structures (3). As a consequence, the half-life of the spermatozoa in the female genital tract could be decreased, potentially reducing the fertilization rate (4). In addition, many other changes have been reported to occur during freeze-thawing, such as altered sperm motility, viability, reactive oxygen species (ROS), and adenosine triphosphate (ATP) production. These changes suggest that the parameters of spermatozoa are affected by the cryopreservation process (5, 6). The spermatozoa acquire their initial motility potential in the epididymis (7). They are capacitated in the female reproductive tract, where they acquire the hyperactivated motility and other attributes that enable fertilization (8). During the hyperactivation stage, the spermatozoon tail motion changes from symmetric, fast, and low amplitude (sinusoidal) motion to asymmetric, slow, and large amplitude motion (9, 10). The hyperactivation of spermatozoa is a critical stage, as it is required by the spermatozoon for the fertilization process. Furthermore, this stage provides the force needed to free the spermatozoon cells

from the oviduct reservoir, and to penetrate the cumulus and zona pellucida surrounding the oocyte (11, 12). Therefore, the motility of spermatozoa is considered to be one of the most important semen parameters affected by the cryopreservation process and the duration of storage. Spermatozoa deterioration during the freeze-thawing possess has a detrimental effect on the outcomes of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (13).

1.1 Proteins related to spermatozoa motility

The most frequent cause of reduced spermatozoa motility and impaired morphology in infertile men is dysfunctional spermatozoa maturation. Throughout spermatozoa maturation, there is increased expression of proteins involved in gamete generation, cell motility, energy metabolism, and oxidative phosphorylation, combined with decreased expression of proteins involved in protein biosynthesis, protein transport, protein ubiquitination, and response to oxidative stress processes. The characteristics of spermatozoa proteins differ, and understanding the structure and function of these proteins could provide insight into the biological processes that are activated or suppressed during spermatozoa maturation (14). Proteins related to spermatozoa motility belong to three different classes: (I) ion channels, (II) cytoskeletal proteins, and (III) cell signaling proteins and glycolytic enzymes (13). In order to evaluate spermatozoa quality and motility after freeze-thawing and storage, it is necessary to evaluate spermatozoa proteins, especially those related to spermatozoa motility, acrosome reaction, and capacitation processes, all of which are required for successful fertilization. The flagella of spermatozoa are important for sperm movement, and correctly formed flagella are required for sperm to reach and fertilize the oocyte. Therefore, any defect in spermatozoon flagellum molecules can result in an abnormality of the spermatozoa flagella proteins, leading to impaired spermatozoa motility (15). The core component of the spermatozoa flagella is the axoneme, composed of a central pair of microtubules, structural elements such as radial spokes, and nine sets of outer doublet microtubules. Each outer doublet microtubule is made up of proto-filaments, consisting of tubulin heterodimers, dyneins, and other microtubule-associated proteins. Dynein motor proteins present on the outer doublet microtubules generate the force for flagella propulsion (16-18). It

is important to understand the subcellular distribution and physiological functions of the spermatozoa tail components, as well as the structural complexity of the spermatozoa flagella. Wang *et al.* (2) found that some spermatozoa proteins act as biomarkers of cryodamage, with the Tektin and CatSper proteins demonstrated to play a critical role in the motility of human spermatozoa.

1.2 Spermatozoa ion channel proteins

Although the characteristics of spermatozoa ion channels are not completely understood, it is unlikely that all 40 different ion channel proteins present in spermatozoa play an important role in spermatozoa physiology, including motility, capacitation, and the acrosome reaction (19). Even though most spermatozoa channels are expressed as protein or mRNA in spermatogenic cells or spermatozoa, they are not always functionally active in the mature spermatozoa cells. For example, some ion channels are functionally expressed in spermatogenic cells but become quiescent in mature spermatozoa, such as voltage-gated Ca^{2+} (HV1) channels (20, 21). The regulation of spermatozoa activity by these channels varies between species, as some channels are active in one species but functionally absent in another. The dramatic difference in functional expression of the HV1 channel between mouse and human spermatozoa represents a good example of closely related species with different ion channel physiology (22). Many ion channels are distributed throughout the spermatozoon head, mid-piece, and flagella. Moreover, it has been demonstrated that the expression of spermatozoa ion channels are highly compartmentalized, and differ significantly between the head and the principal piece (21-24). Ion channels may be classified by their gating, the type of ions, or by their localization. These include cyclic nucleotide-gated (CNG) ion channels, which function in response to the binding of cyclic nucleotides. These CNG channels are non-selective cation channels found in the membrane of spermatozoa (25). Another type of gated ion channel, the K^+ channel of spermatozoa (KSper), sets the resting membrane potential. The KSper channel is expressed in the principal piece of the spermatozoon flagellum, as measured in fractionated spermatozoa by the patch-clamp technique (23). The resting motile cilia Ca^{2+} concentration (~170 nM) is only slightly elevated over the cytoplasmic level (~100 nM) at steady state. The Ca^{2+}

concentration is altered via the cation channels of spermatozoa (CatSper) that arise in the cytoplasm rapidly equilibrate in motile cilia (26). Calcium cations are required for hyperactivation, an important process associated with spermatozoa motility. Hyperactivation is characterized by numerous movements of the spermatozoa filaments, which are needed for the spermatozoa to move through the membrane of the oocyte at the time of fertilization (24) (**Figure 1.1**).

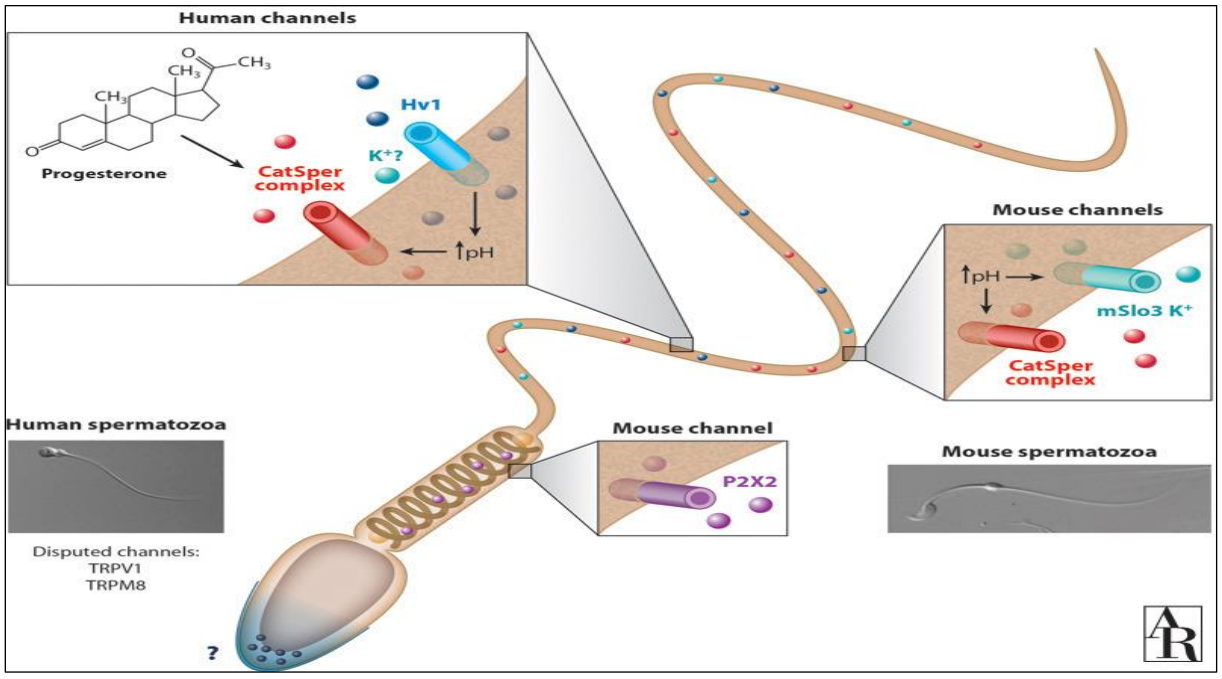

Figure 1.1 Localization of sperm ion channels. Adapted from (27).

1.3 Cytoskeletal proteins in spermatozoa

The structure of the spermatozoa plasma membrane is of great importance for successful fertilization, capacitation, acrosome reaction, and sperm-egg fusion, all of which are membrane-associated events (28, 29). Actin has been identified in the spermatozoa head, neck, and flagellum of many mammalian species (30, 31). Together with other cytoskeletal proteins such as tubulin, actin appears to be implicated in the regulation of capacitation, the acrosome reaction, and sperm motility, which are essential for fertilization (32). In addition, other important cytoskeletal proteins called Tektins, found in sperm flagella, play a significant role in motility (33).

1.4 Cation channels of spermatozoa (CatSper) proteins

1.4.1 CatSper structure and location

Cation channels of sperm (CatSper) proteins are calcium ion channels found in the flagellum of sperm (**Figure 1.2**). These channels are related to two-pore channels, and distantly related to transient receptor potential (TRP) channels (24). This protein was discovered in 2001, and represents a new family of selective calcium ion channels. Before this time, it was thought that voltage-gated Ca^{2+} channels and voltage-gated cation channels (VGCC) were responsible for the change in Ca^{2+} levels (34). The CatSper channel is formed by four subunits, named CatSper 1–4 (22, 23), and at least three auxiliary subunits, CatSper β, CatSper γ, and CatSper δ (35-37). 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 (>30 minutes) after capacitation (24). CatSper 1 is expressed within the primary piece of sperm. The gene encoding CatSper 1 is located on chromosome 19 and encodes a protein of 686 amino acids. CatSper 1 plays an important role in Ca^{2+} entry and the regulation of hyperactivation in spermatozoa. CatSper 2 is localized to the spermatozoa tail, and is responsible for the regulation of hyperactivation. The gene encoding CatSper 2 is located on chromosome 2 and encodes a protein of 588 amino acids (23). CatSper 3 and CatSper 4 are found in both the testes and spermatozoa, and play important roles in the motility of hyperactivated spermatozoa (38). CatSper 3 is located on mouse chromosome 13 and encodes a protein of 395 amino acids, whereas CatSper 4 is mapped to mouse chromosome 4 and encodes a protein of 442 amino acids (39).

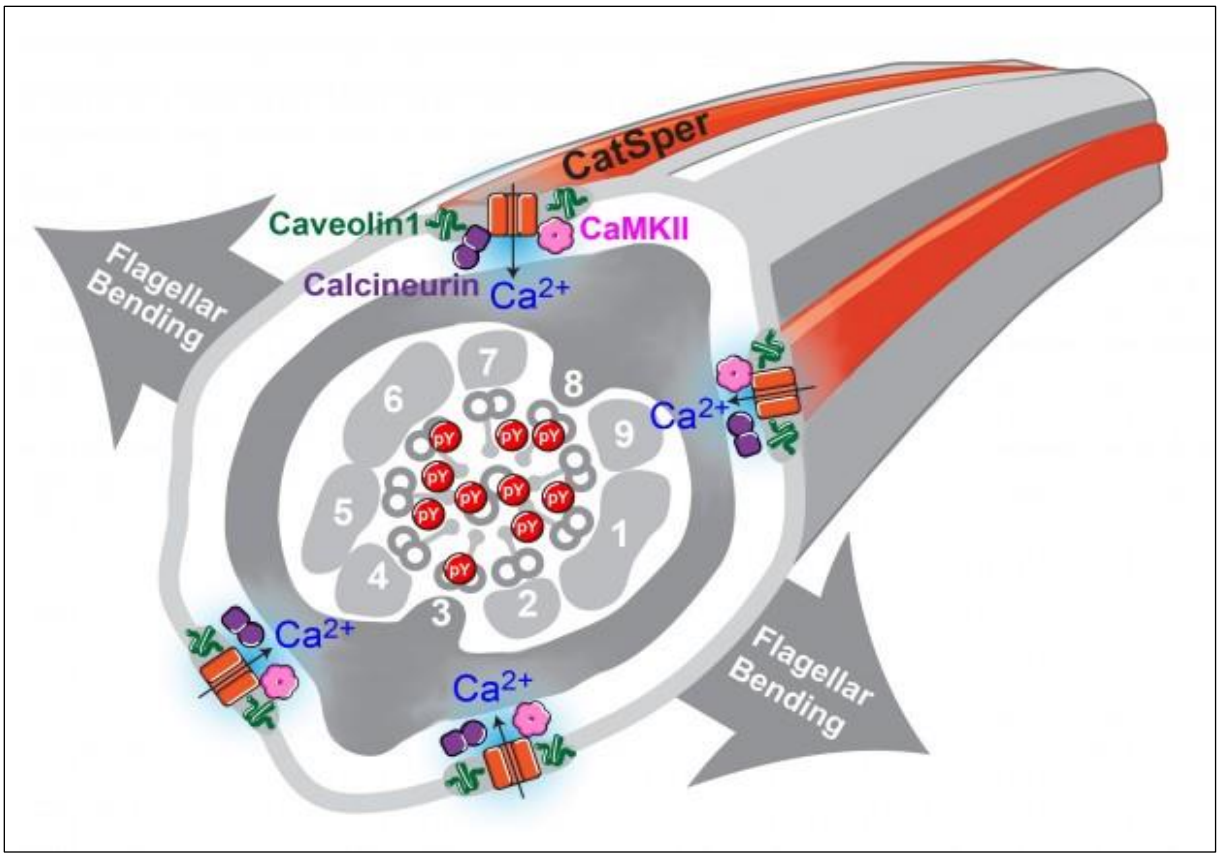

Figure 1.2 Location and function of CatSper proteins. Adapted from (40).

1.4.2 CatSper expression and function

CatSper is a sperm-specific, weakly voltage-dependent, Ca^{2+}-selective, pH-sensitive ion channel that controls the entry of positively charged calcium ions into spermatozoa, which is essential for spermatozoa hyperactivation and male fertility (41-43). The CatSper channel is exclusively expressed in the testis, and is localized to the principal piece of the spermatozoa tail (39). *In situ* hybridization studies of the CatSper subunits suggest that CatSper channels are differentially transcribed at the time of spermatogenesis. CatSper 1, CatSper 3, and CatSper 4 transcripts are restricted to late-stage germ line cells (spermatids) (34, 44, 45), while CatSper 2 is transcribed during the early stages of spermatogenesis (pachytene spermatocytes) (45). CatSper β, CatSper γ, and CatSper δ are expressed in the spermatocytes and spermatids of the testes, and are strictly localized to the principal piece of spermatozoon (46). Expression of CatSper has been reported to be low in subfertile 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 subfertile patients (47). Several previous studies have identified an absence of CatSper channels as one of the causes of infertility in mice (34, 35), primarily due to an inability of spermatozoa to become hyperactivated (48, 49). Other studies have investigated the CatSper-dependent mechanisms underlying this flagellar switch using super-resolution fluorescence microscopy and phosphoproteomics approaches, reporting that the CatSper channel is required for four linear calcium domains responsible for the organization of signaling proteins along the flagella. This unique structure focuses tyrosine phosphorylation in time and space as spermatozoa acquire the capacity to fertilize. In spermatozoa, CatSper channels are triggered by the altered ion concentration present in the increasingly alkaline environment of the female reproductive tract (40).

1.4.3 CatSper 2

The cation channel sperm-associated 2 protein (CatSper 2) gene is located on chromosome 15 (15q15.3), and belongs to a family of putative cation channels specific to spermatozoa, where they are localized to the flagellum. There are several other names for this gene: (I) cation channel sperm-associated protein 2, (II) cation channel, sperm associated 2, (III) CTSR2_HUMAN, and (IV) sperm ion channel (27).

1.4.4 CatSper 2 and spermatozoa motility and fertilization

The CatSper 2 protein is a weakly voltage-dependent, Ca^{2+}-selective, pH-sensitive ion channel, which plays an important role in the entry of positively charged calcium ions into sperm cells. This is essential for processes associated with successful fertilization, such as spermatozoa hyperactivation, acrosome reaction, and chemotaxis towards the oocyte (34, 43, 44). A number of other voltage-gated calcium ion channels have been identified in the testis, spermatocytes, and spermatozoa (50). The influx of Ca^{2+} ions leads to an increase in the intracellular calcium level of spermatozoa, which plays a critical role in their movement. Furthermore, this type of Ca^{2+} channel has been shown to be essential for hyperactivation of spermatozoa motility. Hyperactivated motility is fundamental for the release of spermatozoa from the isthmic reservoir so that they can reach the site of fertilization, and subsequently,

penetrate the zona pellucida of the oocyte (**Figure 1.3**). This channel functions in the detachment of spermatozoa from the epithelium of the female reproductive tract, oocyte coat penetration, and fertility (24, 51). In relation to the other ion channels and pumps present in spermatozoa, the CatSper channel facilitates the entry of calcium required for rapid changes in spermatozoa motility, thereby allowing the spermatozoa to navigate through the hurdles of the female reproductive tract in order to reach the oocyte, where it also functions to facilitate penetration through the outer layers of the oocyte (**Figure 1.4**) (34, 52, 53).

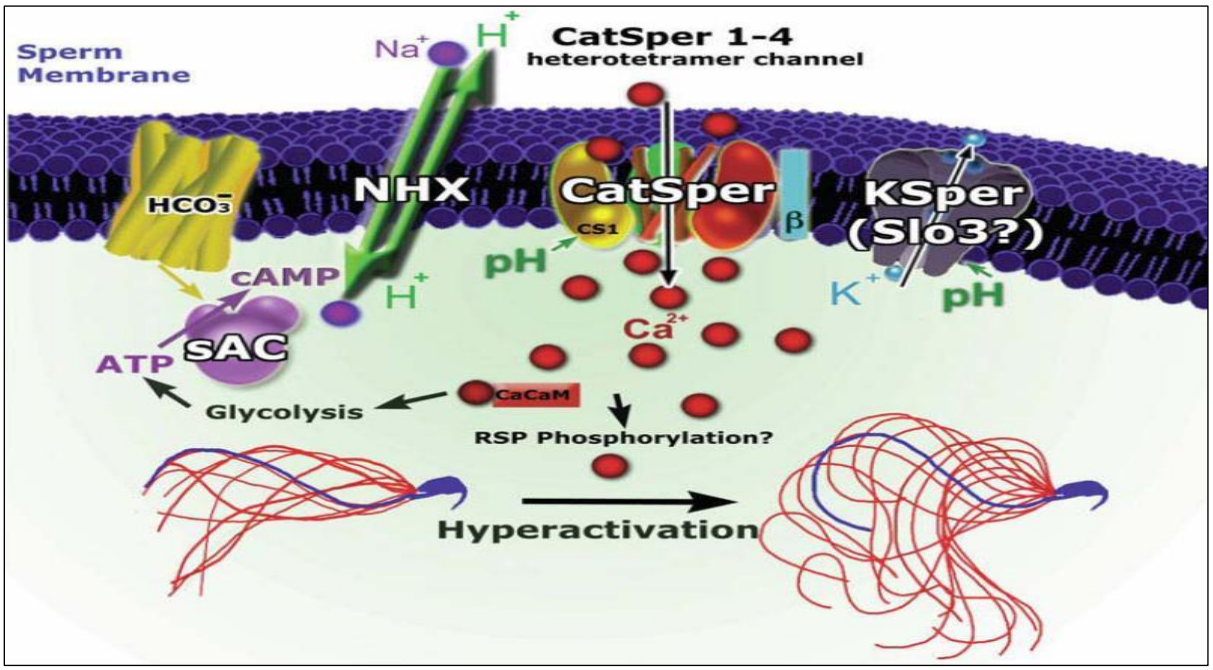

Figure 1.3 Different ion channels associated with spermatozoa activation and the regulation of calcium entry. Adapted from (48).

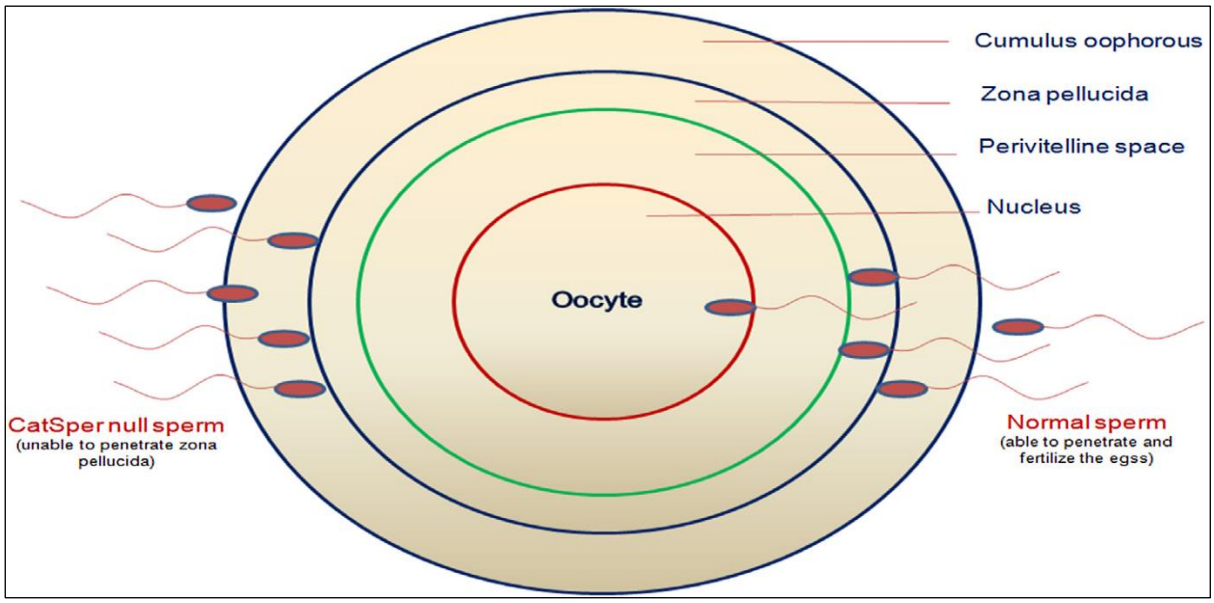

Figure 1.4 Functional significance of CatSper. The protective layers offer a challenge to the spermatozoa. CatSper assists the penetration of the zona pellucida layer, such that CatSper-null spermatozoa are unable to penetrate this layer, failing to fertilize the oocyte. Adapted from (53).

1.5 Tektin

1.5.1 Tektin structure and location

Tektins are proteins that make up the microtubules in cilia, flagella, basal bodies, and centrioles (54-56). Genes encoding Tektin have been cloned in rodents, and five genes have been identified in humans (57) (58) including TEKT1, TEKT2, TEKT3, TEKT4, and TEKT5 (59). The Tektin monomers are typically proteins of around 45–60 kDa, representing a defined family of proteins that have been significantly conserved from protist to human. These monomers form coiled tails (60, 61) and have similar structural properties to the proteins that make up intermediate filaments (62); (63). In mice, TEKT1 is expressed in the centrosomes of round spermatids, then slowly disappears during spermatogenesis (55). Human TEKT2 (also named tektin-t or h-tekB1) is present in the principal piece of spermatozoa (64). The location of TEKT3 in spermatozoa of mice is unclear; however, mice with a deficiency in TEKT3 show reduced spermatozoa motility and forward progression (65). Mouse TEKT4 is

present in the outer dense fibers, and any deletion or mutation in the TEKT4 gene leads to a drastic reduction in the forward motility of spermatozoa resulting from poor coordination of flagellum beating (66). Rat TEKT5 is localized between the mitochondria and the outer dense fibers (67). Recent studies discovered a testis-specific Tektin protein not located in doublet microtubules, but rather on the surface of structures called dense fibers (66, 68). These fibers augment the elastic strength of the spermatozoa tails of many animals, including mammals (69).

1.5.2 Tektin expression and function

Tektins are necessary components of microtubules, as they are essential for their structural integrity. Mutations in the TEKT2 gene can cause defects in flagellar activity, which could have a detrimental effect on spermatozoa motility, leading to male infertility (54). Tektins are thought to be involved in the assembly of the basal body. The amino acid sequences of Tektins are well conserved, with significant similarities between mouse and human homologs (55).

1.5.3 Tektin 2

The gene encoding Tektin 2 in humans (TEKT2, Tektin-t, or h-tekB1) is located on chromosome 1 (1p34.3), and this gene product belongs to the Tektin family of proteins. Tektins comprise a family of filament-forming proteins that are co-assembled with tubulins to form ciliary and flagellar microtubules **(Figure 1.5)**. This gene is found throughout the animal kingdom, and Tektin 2 proteins are localized to the flagella of the spermatozoa, indicating that they may play a critical role in the formation and development of the cilia or flagella of spermatozoa (56, 70).

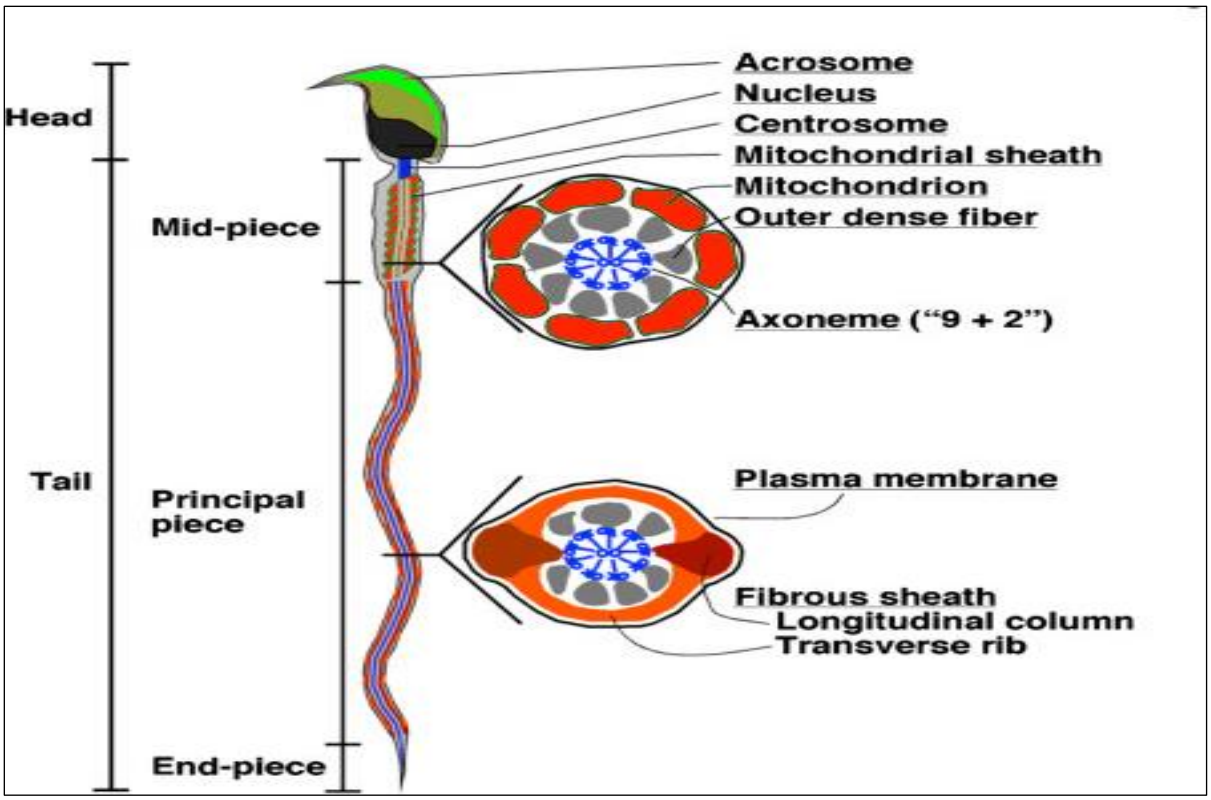

Figure 1.5 Diagram of spermatozoon showing the main structural domains (mid-piece, principal piece, and end piece) and key ultrastructural details (axoneme, outer dense fiber, fibrous sheath, mitochondria, and annulus). Adapted from (71).

1.5.4 Tektin 2 gene and sperm motility

Tektin 2, also known as Tektin-t, has been reported to be localized in the principal piece of human spermatozoa, with no detectable immunosignalling found in the middle or end pieces (54, 58). Tektin 2 is localized to the axoneme, and TEKT2-null male mice are infertile. Furthermore, TEKT2-null sperm display flagellar bending and reduced motility (64) due to disruption of the dynein inner arm. Several studies have demonstrated that Tektins, including Tektin 2, are critical for sperm motility (64, 65). In addition, another study showed that the level of Tektin 2 protein is positively associated with spermatozoa motility parameters, fertilization rate, embryo quality, and pregnancy rate (33).

1.6 RNAs and spermatozoa

Male infertility is a complex health condition, and to our knowledge, there are currently no molecular biomarkers. Spermatozoa RNA represents a potential biomarker for detecting spermatozoa abnormalities and viability at infertility clinics. However, the use of RNA for this purpose is hindered by its inconsistent quantity, quality, the multiple cell types present in semen, and the condensed spermatozoa structure (72). Two models of the biological roles of spermatozoa RNA have been proposed: (I) existing RNAs are residual material with no functional significance, and (II) spermatozoa RNAs have a vital biological role in maturation, fertilization, and/or post-fertilization events (73, 74). It is worth mentioning that the lack of high-quality RNA isolation techniques represents an obstacle for studies investigating spermatozoa RNA (75, 76).

1.7 Spermatozoa cryopreservation

Spermatozoa cryopreservation is a procedure used to preserve spermatozoa for a specific period of time (commonly called sperm banking). Cryopreservation is the freezing of cells or tissues to sub-zero temperatures, exactly -196ºC (boiling point of liquid nitrogen). Over 50 years ago, the first successful cryopreservation of spermatozoa was performed by Sherman and Bunge (77) who froze human spermatozoa equilibrated with 10% glycerol on dry ice, and reported a 67% survival rate. For human spermatozoa, the longest period of successful storage resulting in a live birth was reported in spermatozoa stored for 40 years (78). Currently, sperm banking is used to preserve fertility in a variety of men, including cancer patients prior to undergoing chemotherapy or radiation treatment, patients undergoing testicular surgery, patients with diabetes or multiple sclerosis, and in combination with other ART. Over the past century, the indications for sperm banking have become more frequently implemented as male reproductive health has deteriorated. It is now widely viewed as an effective method of fertility preservation for a variety of men (79). During cryopreservation, all biological activity of the spermatozoa is paused until it is thawed when needed. Cryoprotectant agents such as glycerol, ethylene glycol, and

dimethyl formamide are used to minimize the damage to the spermatozoa during the freeze-thawing cycle (80).

1.7.1 Cryopreservation factors and their effect on spermatozoa characteristics

The ability of frozen spermatozoa to successfully fertilize an oocyte is considerably reduced compared to fresh spermatozoa (81-83), as cryopreservation leads to a decreased number of motile spermatozoa and reduced velocity of those that remain motile (84). This is due to changes resulting from rupture of the plasma membrane by intracellular ice formation or by effects of the cryopreservant solutions (83). More than one factor is responsible for the loss of spermatozoa function during cryopreservation (85). Injury or damage to the spermatozoa during cryopreservation occurs due to a combination of four factors: osmotic stress/dehydration, formation of ROS, intracellular formation of ice crystals, and cryoprotectant toxicity (86) (**Figure 1.6**). These factors are responsible for 25–75% loss of sperm motility, decreased spermatozoa cryosurvival, and DNA fragmentation after thawing (87). The ROS concentration in seminal plasma is known to affect the quality of spermatozoa (88). Cryopreservation affects the acrosome integrity, the fluidity and permeability of the plasma membrane, and leads to the degradation of mRNAs and proteins (89) (**Figure 1.7**). Calcium is an important secondary messenger, and plays a major role in controlling spermatozoa motility (90) and the acrosome reaction (91). An elevated intracellular Ca^{2+} concentration may be a major factor underlying the suppressed motility of immature bovine spermatozoa (92). Semen contains a high concentration of calcium, and this becomes further concentrated as water is removed by ice formation. During cooling, 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 motility and fertility (85). Although semen cryopreservation has proven to be a valuable process, the quality of the frozen spermatozoa is reduced as the damage associated with cryopreservation includes a reduction in motility (4), cold shock, freezing-related

injury, oxidative stress, altered membrane composition, and osmotic stress (93). The detrimental effects on the spermatozoa include reduced metabolic activity, loss of cytoplasmic proteins, membrane-bound proteins, enzymes and other cellular components, and defects in spermatozoa proteins associated with sperm motility, fertilization, and early post-fertilization events (94).

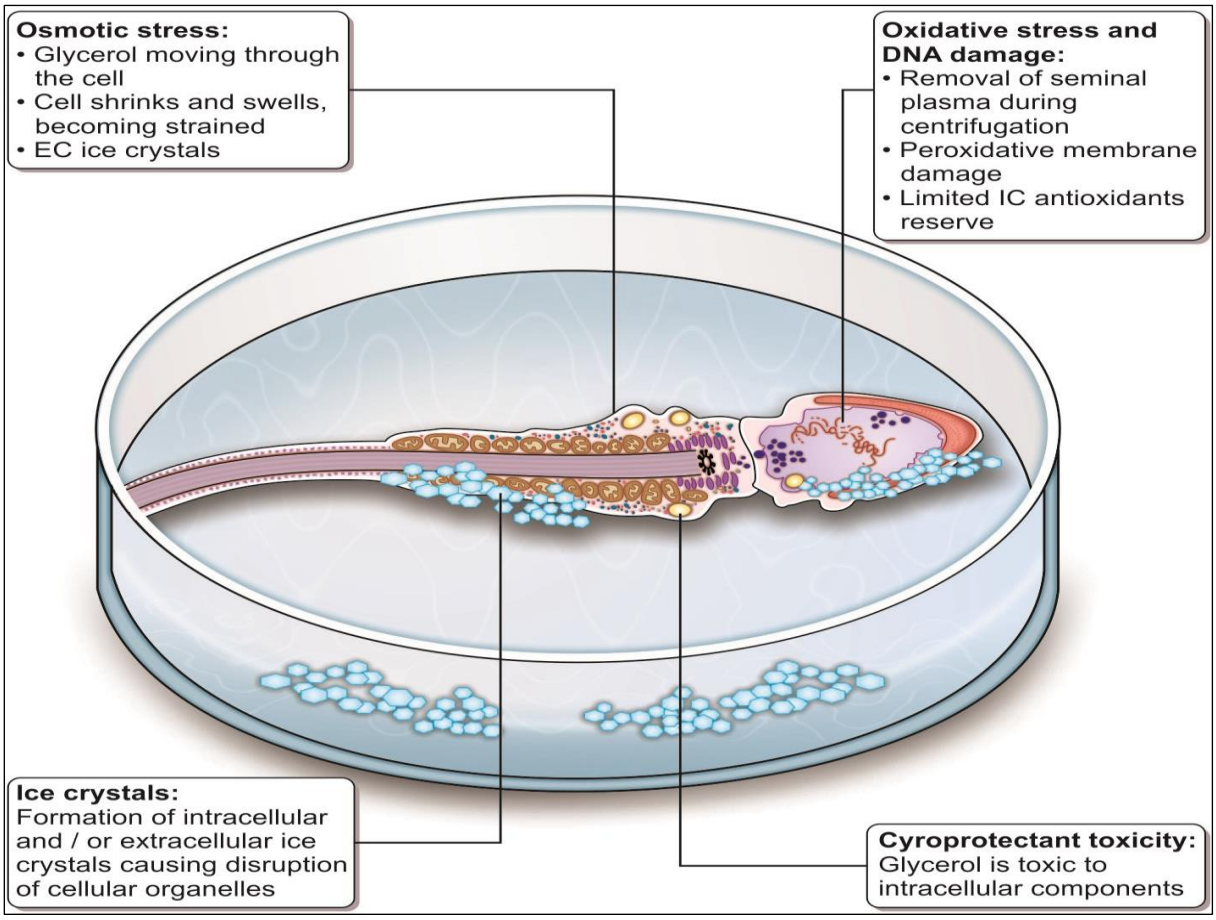

Figure 1.6 Potential factors responsible for sperm cryoinjury. Reprinted with permission from the Cleveland Clinic Center for Medical Art & Photography (2011). EC, extracellular; IC, intracellular (86).

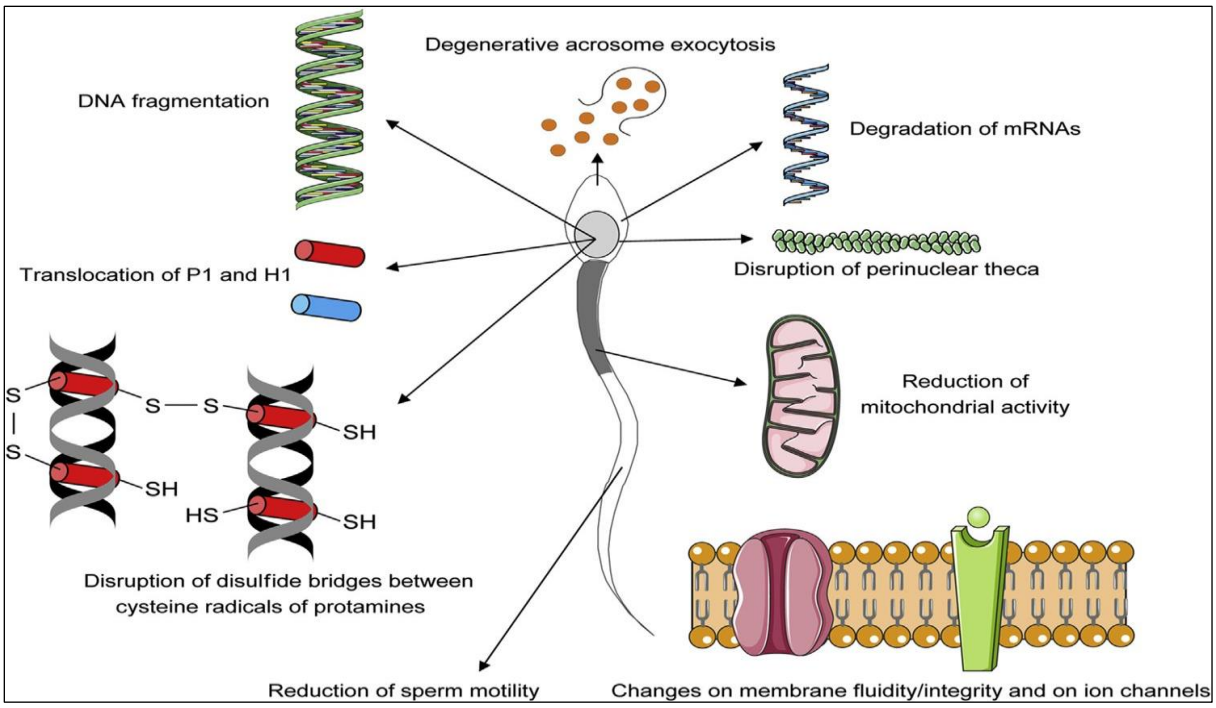

Figure 1.7 The main causes of damage on boar spermatozoa from freeze-thawing procedures. Cryopreservation affects acrosome integrity and the fluidity and permeability of the plasma membrane, and leads to the degradation of mRNAs and proteins (89).

1.7.2 Influence of cryopreservation on spermatozoa proteins

Studies utilizing protein screening methods have reported that the reduction in motility observed in bovine, boar, and human spermatozoa is associated with changes in the level of certain proteins (95). Additionally, Cao *et al.* observed that the reduced motility of boar and human spermatozoa following cryopreservation was associated with a decrease in the HSP90 protein during cooling (96). Other studies have shown that a decrease in protein abundance may be due either to degradation following freezing-thawing stress (94) or leakage of proteins from spermatozoa into the extracellular medium, as reported in humans (96, 97). In another study, an increase in some protein spots could be due to one or more post-translational modifications (phosphorylation, acetylation, glycation, etc.) following the cryopreservation procedure, as demonstrated in gilthead sea bream spermatozoa, or an effect of the freeze-thawing procedure and/or exposure to cryoprotectants on the regulation of mRNA translation (98).

1.8 Correlation between spermatozoa RNA and protein

Transcriptomics usually generates larger datasets than proteomics; however, spermatozoa represent a special case, as proteome-based studies have generated more relevant data. Spermatozoa are transcriptionally inactive, and contain only small amounts of RNA (99). Furthermore, a significant proportion of the differential gene expression in spermatogenesis corresponds to the upregulation of genes expressed early, including transition proteins and protamine-coding genes, which have long been claimed to be switched on during spermatogenesis. The regulation of protamine expression is unique, and includes several possible mechanisms which may be responsible for dysregulation of protamine expression and concurrent broad-spectrum defects in spermatogenesis (100). Carrel *et al.* suggested two hypotheses: (i) that abnormal protamine expression is indicative of a generalized defect in mRNA storage and/or translation which affects other mRNA transcripts, or (ii) that protamines act as a checkpoint for spermatogenesis (101).

1.9 Purpose of the study

Due to the deteriorated quality of spermatozoa after cryopreservation, it has become inevitable to find methods to maintain sperm quality so that it can fertilize the oocyte. Emphasis has been directed toward the genes responsible for spermatozoa movement, specifically to assess whether they are sensitive to the freezing process. CatSper 2 and Tektin 2 are two spermatozoa tail proteins involved in sperm motility. The main questions to be investigated in this study include: (I) Are the levels of CatSper 2 and Tektin 2 decreased or increased after the cryopreservation process? (III) Do the retrieved spermatozoa from fertile and subfertile males have the same level of tolerance to the freezing and thawing cycle? (III) Can CatSper 2 and Tektin 2 serve as markers for predicting the success rate of ART in infertile men? The purposes of this study are summarized in the following points:

1. To investigate the levels of CatSper 2 and Tektin 2 proteins in human spermatozoa samples before and after exposure to liquid nitrogen during the cryopreservation process.

2. To determine the relationship between CatSper 2 and Tektin 2 mRNA and protein levels by determining the expression before and after cryopreservation-induced stress.
3. To assess the levels of damage to the CatSper 2 and Tektin 2 proteins in human spermatozoa during the cryopreservation process in fertile and subfertile-males.

2 Materials and Methods

2.1 Subject recruitment

A cohort of selected couples underwent assisted reproduction techniques for infertility screening. In total, 48 semen samples were collected from all included males (aged 24–50 years). This study was approved by the Institutional Ethics Committee of Saarland University (195/11), and all patient were signed a written consent to be enrolled in this study. All of the samples were analyzed in the Department of Obstetrics; Gynecology & Assisted Reproduction Laboratory, University of the Saarland, Germany**.** Samples were analyzed according to standard operating procedures

2.2 Materials

2.2.1 Chemicals, reagents, antibodies, and kits

The chemicals, reagents, kits, and antibodies used in this study are shown in **Tables 2.1** and **2.2.** All chemicals were of analytical and molecular biology grade.

Table 2.1 1List of chemicals and reagents in alphabetical order

Chemical or reagent	Manufacturer/distributor
100X immersion oil	Sigma-Aldrich, Germany
Absolute ethanol	Sigma-Aldrich, Germany
Absolute isopropanol	Sigma-Aldrich, Germany
Absolute methanol	Sigma-Aldrich, Germany
Acetic acid	Fluka, Germany
Acetone	Merck, Germany
Acrylamide-stock solution: Rotiphorese Gel 30	Carl Roth GmbH, Germany
Ammonium persulphate (APS) solution 10% (w/v)	Thermo Fisher, Germany
Bradford protein assay kit	Bio-Rad, Germany
Coomassie™ dye protein gel stain	Thermo Fisher, Germany

CryoSperm medium	Origio, Denmark
Disodium hydrogen phosphate	Merck, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Germany
DNA molecular size marker (ladder), 100 bp	Thermo Fisher, Germany
Dry milk powder	J.M. Galber Saliter GmbH, Germany
ECL Prime western blotting detection reagent	Sigma-Aldrich, Germany
Eosin stain	Sigma-Aldrich, Germany
Glycerin	Fluka, Germany
Ham's F10 medium	PAN Biotech, Germany
Hydrochloric acid (HCl)	Sigma-Aldrich, Germany
PCR master mix	Qiagen, Germany
Penicillin G/streptomycin sulphate	PAN Biotech, Germany
Phosphate-buffered saline (PBS), pH 7.4	Sigma-Aldrich, Germany
Phosphate-buffered saline containing Tween-20 (PBST)	Sigma-Aldrich, Germany
Ponceau S solution 0.1% (w/v) in 5% acetic acid	Sigma-Aldrich, Germany
Pre-stained protein molecular weight marker	Thermo Fisher, Germany
Protease inhibitor cocktail	Sigma-Aldrich, Germany
PureSperm gradient 40/90	Nidacon International AB, Sweden
Polyvinylidene difluoride (PVDF) western blotting membrane	Roche, Germany
Running buffer (Tris-glycine-SDS buffer 10× concentrate)	Sigma-Aldrich, Germany
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Germany
ß-Mercaptoethanol	Merck, Germany
Tetramethylethylenediamine (TEMED)	Thermo Fisher, Germany
Transfer buffer (Tris-glycine buffer 10× concentrate)	Sigma-Aldrich, Germany
Tris(hydroxymethyl) aminomethane (Tris)	Sigma-Aldrich, Germany
Tris-glycine SDS buffer 10×	Sigma-Aldrich, Germany

Tris-HCl pH 8.8	Sigma-Aldrich, Germany
Triton X-100	Sigma-Aldrich, Germany
Universal IVF medium	Origio, Denmark
Western blotting filter paper	Thermo Fisher, Germany

Table 2.2 List of kits and antibodies.

Kit and antibody	Manufacturer/distributor
QuantiTect primer assay (200), GAPDH	Qiagen, Germany
QuantiTect primer assay (200), CATSPER 2	Qiagen, Germany
QuantiTect primer assay (200), TEKT2	Qiagen, Germany
miScript II reverse transcription kit (with HiFlex buffer)	Qiagen, Germany
miScript SYBR Green kits	Qiagen, Germany
miRNeasy mini kit	Qiagen, Germany
Mouse monoclonal Tektin 2 antibody (ab60918)	Abcam, UK
Rabbit polyclonal CatSper 2 antibody (ab150890)	Abcam, UK
Conjugated anti-mouse IgG H&L (HRP) (ab6728)	Abcam, UK
Conjugated anti-rabbit IgG H&L (HRP) (ab6721)	Abcam, UK
Monoclonal anti-β-actin (A2228)	Sigma-Aldrich, Germany

2.2.2 Disposables

The disposables used in this study are shown in Table 2.3. Disposables are certified to be free from RNase, DNase, human DNA, PCR inhibitors, and pyrogens.

Table 2.3 List of disposables in alphabetical order.

Consumables	Manufacturer/distributor
Cover slips, 10 × 10 mm	Marienfeld, Germany
Cryovials, 2 ml	Greiner Bio-One, Germany
Eppendorf PCR tubes, 0.2 ml	Eppendorf GmbH, Germany

Eppendorf PCR tubes, 1.5 ml	Eppendorf GmbH, Germany
Eppendorf PCR tubes, 2.0 ml	Eppendorf GmbH, Germany
MicroAmp Fast 8-tube strip, 0.1 ml	Thermo Fisher, Germany
MicroAmp Optical 8-cap strips	Thermo Fisher, Germany
QIAcube filter tips, 200 µl	Qiagen, Germany
QIAcube rotor adapter	Qiagen, Germany
QIAcube filter-tips, 1000 µl	Qiagen, Germany
QIAcube filter-tips, 200 µl	Qiagen, Germany
QIAgility conductive tips, 200 µl	Qiagen, Germany
QIAgility conductive tips, 50 µl	Qiagen, Germany
Microscope slides, 76 × 26 mm	Gerhard Menzel, Germany
Semi-micro cuvette, acrylic, 10 × 4 × 45 mm	Sarstedt, Germany
Sterile filter tips, 10 µl	Sorenson BioScience, Inc., USA
Sterile filter tips, 1000 µl	Sorenson BioScience, Inc., USA
Sterile filter tips, 20 µl	Sorenson BioScience, Inc., USA
Sterile filter tips, 200 µl	Sorenson BioScience, Inc., USA

2.2.3 Equipment

The equipment used in this study is shown in **Table 2.4**.

Table 2.4 The list of major equipment used listed in alphabetical order.

Instrument	Manufacturer/distributor
Automatic pipettes	Eppendorf GmbH, Germany
Bench-top centrifuge	Eppendorf GmbH, Germany
Bench-top refrigerated centrifuge	Eppendorf GmbH, Germany
Binocular light microscope	Olympus, Japan
Documentation system	Wealtec, USA

Freezer, -20ºC	Sanyo, Japan
Freezer, -70ºC	Sanyo, Japan
Hamilton syringe, 25 µl	Hamilton, USA
Heat block	Uniequip, Germany
Incubator	Memmert GmbH, Germany
Magnetic stir plate and bar (×2)	Uniequip, Germany
Makler counting chamber	Irvine Scientific, USA
Microplate reader, 3550-UV	Bio-Rad, Germany
Microwave	LG, Korea
Mighty Small™ SE250 Mini-Vertical Gel system	Hoefer Scientific Instruments, Germany
NanoDrop ND-2000c spectrophotometer	Thermo Fisher, Germany
Personal computer	DELL, USA
pH-meter	Carl Roth GmbH, Germany
Planer Kryo 10 Series iii	Planer, United Kingdom
Power supply	Amersham Bioscience, Germany
QIAcube automated isolation robot	Qiagen, Germany
QIAgility automated pipetting robot	Qiagen, Germany
Safety cabinet	Heraeus, Germany
Scale	Sartorius AG, Germany
Shaker	Heidolph Instruments GmbH, Germany
Simple beam photometer	Amersham Bioscience, Germany
StepOnePlus™ real-time PCR system	Thermo Fisher, Germany
T-Professional Basic gradient 96 thermocycler	Biometra GmbH, Germany
Ultrasonic Bath Transonic 460	Elma GmbH, Germany
Ultrosopec 2100 Pro UV/visible spectrophotometer	Amersham Bioscience, Germany
Vacuum concentrator with heater	Uniequip, Germany
Vortex mixer	Uniequip, Germany

Water bath	B Braun, Germany

2.2.4 Buffers and solutions

The buffers and solutions that were prepared in the laboratory for use in this study are shown in Table 2.5. All chemicals were of analytical and molecular biology grade. Unless stated in **Table 2.5**, buffers and solutions were purchased ready-to-use from Sigma-Aldrich, Germany.

Table 2.5 Buffers and solutions used in the study.

Buffer	Component
Gel solution A (for both separating and stacking gels)	Acrylamide 30% and bisacrylamide 0.8%. Stored at 4°C.
Gel solution B (for both separating and stacking gels)	SDS 4 g and Tris-HCl 181.5 g, pH 8.8. Dissolved in 1000 ml H_2O and stored at 4°C.
Gel solution C (for stacking gel)	SDS 4 g and Tris-HCl 60 g, pH 6.8. Dissolved in 1000 ml H_2O and stored at 4°C.
3X SDS sample loading buffer (Laemmli loading dye)	1 M Tris-HCl (pH 6.8) 2.4 ml, SDS (20%) 3 ml, Glycerol (100%) 3 ml, β-mercaptoethanol 1.6 ml, and bromophenol blue 0.006 g. Dissolved in H_2O and stored at room temperature.
Electrophoresis buffer, pH 8.8	Tris-glycine-SDS buffer 10X concentrate, dissolved in H_2O and stored at room temperature.
Ammonium persulfate (APS)	APS 1 g dissolved in 10 ml H_2O and stored at 4°C.
Blocking buffer, pH 7.4	Tween-20 0.1% and skimmed milk powder 5%. Dissolved in TBS and

	stored at 4°C.
Destaining buffer	Methanol 40% and acetic acid 10%.
Protein lysis buffer	SDS (2%) 400 µl containing 1 µl of protease inhibitor mixture.
Stripping buffer, pH 2.2	Glycine 0.19 M, SDS 0.003 M, and Tween-20 10 ml.
Transfer buffer, pH 8.3	Tris-glycine buffer 10X concentrate dissolved in H_2O and stored at 4°C.
Washing buffer, pH 7.4	Tween-20 0.1% in TBS, stored at 4°C.

2.3 Methods

2.3.1 Collection and processing of semen samples

Semen samples were obtained from the participants by masturbation after 3 days of sexual abstinence. Samples were allowed to liquefy at 37°C for 30 minutes, then processed immediately. All samples were analyzed for basic semen parameters including liquefaction time, volume, pH, viscosity, agglutination, motility, viability, count, and spermatozoa morphology, according to the 2010 World Health Organization guidelines (102). These parameters were used to classify the samples into two groups: fertile group (a sperm concentration of >15 × 10^6/ml, normal morphology of >4%, and motility of >40%) and sub fertile group according to any decrease in the mentioned parameters (WHO, 2010) (102). A Makler counting chamber (Sefi-Medica, Haifa, Israel) was used to determine the spermatozoa count.

2.3.2 Ejaculate and spermatozoa preparation

Semen samples were prepared using a discontinuous PureSperm gradient (Nidacon, Sweden) by layering 2 ml of 90% and 40% PureSperm solutions in a 15 ml Falcon® centrifuge tube, beginning at the bottom of the tube with the 90% density. The

procedure was then completed according to the manufacturer's recommendations, summarized below:

1. The loaded semen samples were centrifuged at 500 × *g* for 20 minutes at room temperature.
2. The pellet was washed twice with Ham-F10 medium supplemented with human serum albumin (5 mg/ml) and penicillin G/streptomycin sulphate (0.1 mg/ml; PAN Biotech), then carefully overlaid with 0.75 ml of the same medium.
3. Samples were then placed in an incubator at 37°C for 45 minutes. The upper layer (supernatant) was then aspirated from the lower layer (pellet).
4. The supernatant was divided into two parts: part one (n = 48) was processed immediately, labelled as "fresh"; and part two (n = 48) was cryopreserved with liquid nitrogen for 30 days, labelled as "cryo". The clinical characteristics of the study population are summarized in **Table 3.1.**
5. All samples were processed and analyzed in the *In Vitro* Fertilization laboratory and Biochemistry department of the University Hospital of Saarland. Samples were analyzed according to standard operating procedures. Semen samples from males with a known medical cause of infertility, including cryptorchidism, childhood disease, varicocele and hydrocele, and/or environmental exposure to radiation, smoking, and alcohol consumption, were excluded from the study.

2.3.3 Assessment of spermatozoa vitality by eosin–nigrosin staining

Spermatozoa vitality was assessed by eosin–nigrosin staining at least 30 minutes after sample collection to allow liquefaction of the samples. The procedure was completed according to WHO guidelines (1), summarized as follows:

1. Semen samples were mixed well by swirling before aliquots were taken.
2. For each semen sample, 50 µl was mixed with an equal volume of eosin–nigrosin suspension for 30 s.
3. After 30 s, one drop of the mixture was transferred onto a microscope slide, smeared, and then left to air-dry.

4. Slides were evaluated under a bright-field optic microscope (magnification 1000X, oil immersion) by distinguishing between the dead spermatozoa (stained pink or red) and the live spermatozoa (unstained). A total of 200 spermatozoa were evaluated per slide.

2.3.4 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. 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 minutes, as illustrated in **Figure 2.1.**

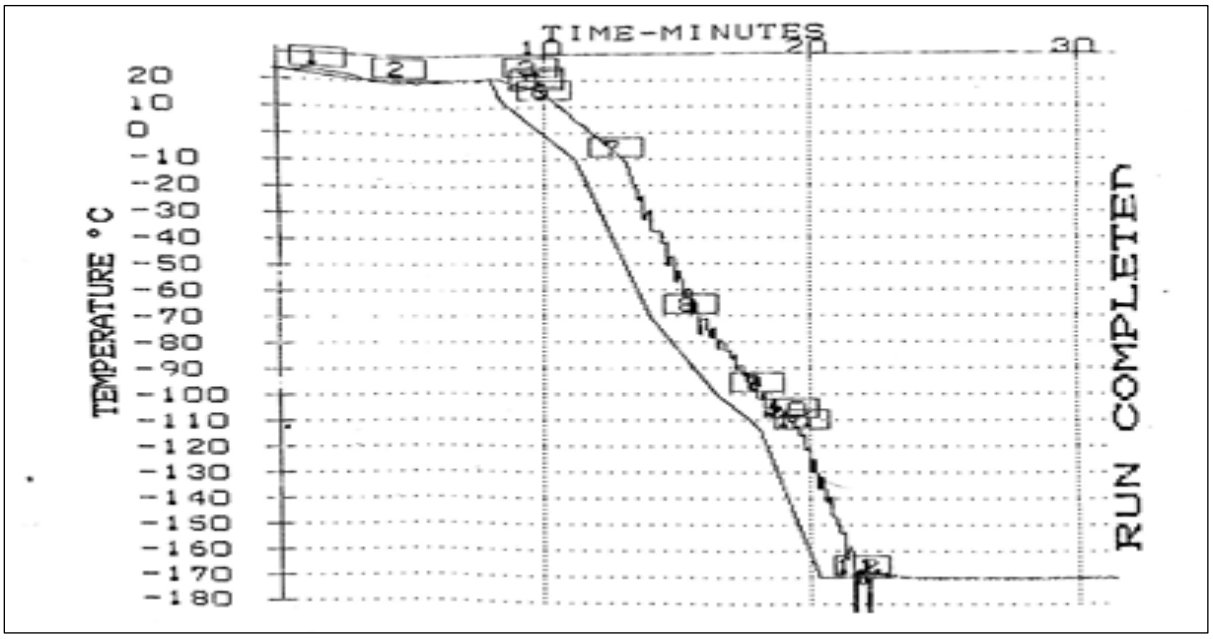

Figure 2.1 Computerized program freezing process used to achieve cooling from 20°C to −80°C at a rate of 1.5°C/min then 6°C/min to freeze the spermatozoa.

2.4 Cryopreservation and thawing

2.4.1 Spermatozoa cryopreservation

To preserve the spermatozoa samples for the desired time (30 days), samples were persevered according to the following steps:

1. The CryoSperm medium (Origio, Denmark) was pre-warmed at room temperature for a minimum of 2 h.
2. After liquefaction, the total volume of each ejaculate was measured and sperm analysis was performed.
3. The CryoSperm medium was added drop-by-drop into each sample at a ratio of 1:1 (v/v), and the solution was carefully mixed after each addition.
4. The mixture was left at room temperature for a minimum of 10 min.
5. The diluted sperm was loaded into cryovials and closed.
6. The cryovials were placed into the programmable freezing machine (Planer KRYO10). Cooling was initiated from 20°C with a freezing rate of 3°C/min, followed by 25 min of equilibration once the temperature reached 3°C. The freezing rate was then modified to 1°C/min to cool the cryovials down to -30°C, then later freezing rate was increased to 30°C/min from -30°C to -60°C.
7. The cryovials were removed from the freezing machine and plunged into liquid nitrogen, where they were stored at -196°C for 30 days.

2.4.2 Thawing of spermatozoa

Samples were thawed according to the following steps:

1. The cryovials containing the frozen spermatozoa were warmed at room temperature for 30 s.
2. The cryovials were placed in a warm water bath at 37°C for 1 min.
3. The cryovials were opened and the thawed sperm samples were transferred to a new sterile 50-ml Falcon tube.
4. The sperm samples were immediately used for the RNA and protein extraction procedures.

2.5 RNA analysis

2.5.1 Isolation of total RNA from spermatozoa samples

Total RNA was purified from randomly selected fresh (n = 10) and cryopreserved (n = 10) spermatozoa samples using QIAcube robot (Qiagen, Germany) using the miRNeasy Mini kit (Qiagen, Germany) with slight modifications. Briefly, 100 µl of culture medium (PAN Biotech, Germany) containing 10×10^6 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, summarized as follows:

1. The QIAzol lysis reagent (700 µl) was added to a 1.5-ml microcentrifuge tube containing 10×10^6 spermatozoa, then mixed gently by pipetting up and down several times.
2. The tube containing the homogenate was placed on the bench-top at room temperature (15–25°C) for 10 minutes. This step promoted the dissociation of nucleoprotein complexes.
3. Chloroform (140 µl) was added and the tube was closed securely and mixed vigorously by vortexing for 15 s.
4. The tube containing the homogenate was then placed on the bench-top at room temperature for 2–3 min.
5. The tube containing the homogenate was centrifuged at 12,000 × *g* at 4°C for 15 min.
6. After centrifugation, the samples were separated into three phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase.
7. The upper aqueous phase was transferred to an RNeasy Mini spin column in a 2-ml collection tube. The lid was closed gently and the column was centrifuged at 8000 × *g* at room temperature (15–25°C) for 15 s. The flow-through was discarded and the collection tube was reused in the next step. Using the QIAcube robot automated procedure, the upper aqueous phase was

transferred to the robot and samples were isolated according to the protocol used for miRNeasy Mini kit (Qiagen, Germany).

8. Step 7 was repeated using the remainder of the sample. The flow-through was discarded and the collection tube was reused in the next step.
9. The RWT buffer (700 µl) was added to the RNeasy Mini spin column. It was then centrifuged at ≥8000 × g for 15 s to wash the column, and the flow-through was discarded. The collection tube was used in the next step.
10. The RPE buffer (500 µl) was added to the RNeasy Mini spin column, the lid was closed gently and centrifuged at ≥8000 × g (≥10,000 rpm) for 15 s to wash the column. The flow-through was discarded and the collection tube was reused in the next step. Step 10 was repeated.
11. The RNeasy Mini spin column was placed into a new 2-ml collection tube, and the old collection tube was discarded with the flow-through. The column with the new collection tube was centrifuged in a microcentrifuge at full-speed for 2 min.
12. The long centrifugation step dried the spin column membrane. This ensured that no ethanol was carried over during RNA elution, as residual ethanol may interfere with downstream reactions.
13. The RNeasy Mini spin column was transferred to a new 1.5-ml collection tube.
14. RNase-free water (30 µl) was transferred directly onto the RNeasy Mini spin column membrane. The lid was closed gently and the column was centrifuged for 1 min at ≥8000 × g (≥10,000 rpm) to elute the RNA.
15. The total RNA was stored at -80°C until further analysis.

2.5.2 RNA concentration and purity analysis

Total RNA purity and yields were assessed to ensure that the material obtained was of sufficient quality and quantity for qRT-PCR analysis. The concentration and purity of RNA samples were determined using a Nanodrop ND-2000 spectrophotometer (Thermo Scientific, USA).

2.5.3 Reverse transcription of RNA

Total RNA was converted into cDNA in a 20-µl reaction volume using miScript reverse transcription kit (Qiagen, Germany) according to the manufacturer's recommendations, summarized as follows:

1. Total RNA (200 ng) was mixed with 4 µl of 5× miScript HiFlex buffer, 2 µl nucleic acid mix, and 2 µl miScript reverse transcriptase mix to make a total reaction volume of 20 µl.
2. Samples were incubated at 37°C for 60 min for the first-strand cDNA synthesis.
3. The reaction was inactivated by heating at 95°C for 15 min, and cDNA was stored at -20°C.

2.5.4 Real-time quantitative RT-PCR

Relative quantitative real-time PCR was performed to quantify the expression level of three genes, namely CatSper 2, TEKT2, and the housekeeping gene GAPDH, using a StepOnePlus™ System (Applied Biosystems, USA). In real-time PCR, binding of SYBR green to the amplified cDNA was used to measure increased fluorescence, as the dye binds to the increasing amounts of DNA in the reaction tube. SYBR Green I binds to all double-stranded DNA molecules, emitting a fluorescent signal of a defined wave-length upon binding. The excitation and emission maxima of SYBR Green I are 494 and 521 nm, respectively. The cDNA produced from the total RNA extracted from the samples served as the template for qRT-PCR analysis, which was performed using the QuantiTect primer assay (Qiagen, Germany) according to the manufacturer's recommendations, summarized as follows:

1. The PCR reaction contained 2 µl cDNA, 10 µl 2× miScript SYBR Green PCR master mix, 2 µl QuantiTect primer assay (for each of CatSper 2, Tektin 2, and the housekeeping gene GAPDH), and RNase-free water to make up to a total reaction volume of 20 µl. The reaction mixture was placed into the individual wells of a MicroAmp Fast 8-tube strip (0.1 ml).

2. Reactions were run with the following thermal cycling parameters: 50°C for 2 min; initial activation step of 95°C for 15 min; 40 cycles of 94°C for 15 s (denaturation), 55°C for 30 s (annealing), and 70°C for 30 s (extension).
3. A final dissociation curve (melting curve) was generated, and the PCR plates were kept at 4°C until they were removed from the PCR machine. The GAPDH QuantiTect primer assay was chosen as the housekeeping gene for normalization. In addition, a no template control (NTC) and no reverse transcriptase control (NRT) were included in each run.

Note: All qRT-PCR experiments were performed in triplicate and the resulting Ct values were normalized to GAPDH.

2.6 Protein analysis

2.6.1 Isolation of proteins from spermatozoa samples

Proteins were purified from the spermatozoa samples (n = 96 samples) 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. Thereafter, the procedure was completed as follows:

1. The pellet was re-suspended with protein lysis buffer (400 µl of 2% SDS containing 1 µl of protease inhibitor mixture) overnight at 4°C.
2. The samples were sonicated for 30 s with 50% pulses then centrifuged at 4000 × g for 30 min at 4°C.
3. The protein concentration in the supernatant was measured by Bradford protein assay (Bio-Rad, Germany).

2.6.2 Bradford assay

The Bradford assay was used to determine the concentration of solubilized protein. This assay involves the addition of an acidic dye to the protein solution, and subsequent measurement at 595 nm by spectrophotometry (Amersham Bioscience, Germany). Compared to a standard curve, the relative protein concentration can then be determined according to (103). The concentration of unknown samples are compared to known concentrations of bovine immunoglobulin (78). This step is used to measure the protein concentration, and also to ensure an equal amount of protein for the western blot analysis. The procedure was completed as follows:

1. The “blank” consisted of a mixture of 800 µl H_2O, 200 µl of Bio-Rad Bradford reagent (Bio-Rad, Germany), and 1 µl of extraction buffer.
2. In a separate tube, 1 µl of protein extract was added to 800 µl of H_2O and 200 µl Bradford reagent, then vortexed.

3. The reaction was incubated at room temperature for 5 min then measured against the blank.
4. The absorbance was measured at 595 nm in triplicate over time using spectrophotometry (Amersham Bioscience, Germany), then the mean was calculated.
5. The protein concentrations were calculated in µg/µl according to the prepared bovine IgG standard curve using the absorbance of each unknown sample.
6. The bovine IgG standard curve was created by measuring the absorbance at 595 nm of 1, 2, 3, 5, 7.5, 10, 15, and 20 µg bovine IgG by the Bradford method (**Figure 2.2**).

After measuring the absorbance of the different concentrations of bovine IgG, the standard curve was drawn with the bovine IgG concentrations presented on the X-axis and the absorbance (595 nm) on the Y-axis (**Table 2.6**).

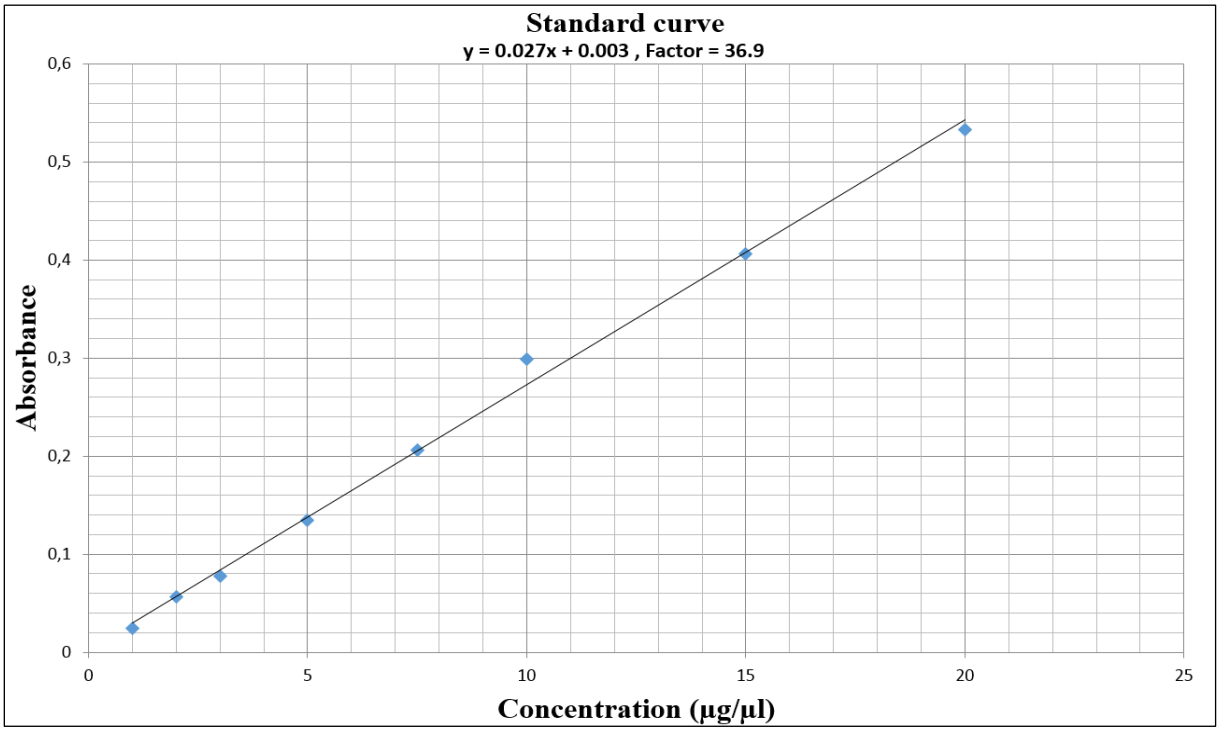

Figure 2.2 The bovine serum albumin standard curve.

Table 2.6 Preparation of standard curve from bovine IgG.

Standard concentration (µg)	Volume of standard solution (µl)	Volume of H_2O (µl)	Volume of Bradford solution (µl)	Absorbance
1	0.7	799.3	200	0.024
2	1.5	798.5	200	0.057
3	2.2	797.8	200	0.078
5	3.6	796.4	200	0.135
7.5	5.4	794.6	200	0.206
10	7.9	792.1	200	0.299
15	10.9	789.1	200	0.406
20	14.5	785.5	200	0.533

2.6.3 Western blot

The protein samples were analyzed by western blot to evaluate the levels of Tektin 2 and CatSper 2 protein in sperm samples before and after cryopreservation (n = 96 samples). Western blotting was performed as previously described (104). Starting with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), the Bio-Rad method was used to separate proteins depending on their size and mobility in the electric field (103). The SDS denatured the proteins and caused the molecules to lose their original conformation by disrupting the non-covalent bonds. The samples were loaded into wells on the gel beside a molecular weight marker (protein ladder). Then, a current was passed through the gel to allow the proteins to move through the gel. Proteins with a small molecular weight move faster while the large ones lag behind. Upon completion of the sample run, the current was stopped and the protein was transferred onto a polyvinylidene fluoride (PVDF) membrane and analyzed by immunoblotting.

2.6.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer

The SDS-PAGE technique is commonly used for the separation of proteins by electrophoresis. The discontinuous polyacrylamide gel is used as a support medium and SDS to denature the proteins. SDS-PAGE gels were prepared by the following steps:

1. The Mighty Small™ SE250 Mini-Vertical gel system (Hoefer Scientific Instruments, Germany) was used for gel preparation. In this system, a lower part known as the “resolving gel” and an upper part known as the “stacking gel” (see Table 2.7) made up a single gel. The percentage of the stacking gel was 4%, and the resolving gel was 12.5%.
2. In the gel rack, white and transparent glass plates of 73 x 100 mm and 80 x 100 mm, respectively, and other Mighty Small™ multiple gel casters SE200 (Hoefer Scientific Instruments, Germany) were cleaned and assembled according to the manufacturer’s instructions. A spacer of 1 mm thickness was used between these plates.
3. The resolving constituents were gently mixed, then poured between the plates using a Pasteur pipette until the space was 3/4 filled.
4. A thin layer of isopropanol was added on top of the gel in each space to prevent the creation of air bubbles and to let the proteins run smoothly in the gel.
5. The stacking gel was made and layered on top of the separating gel after the isopropanol had been removed with filter paper. A well-comb was then placed into the solution between the plates. The gel was left to polymerize at room temperature.
6. The gel was gently removed from the gel rack after polymerization had occurred, then it was wrapped in a wet paper towel and stored at 4°C until use.

The gel solutions were prepared as follows:

Solution A

- Rotiphores Gel 30 (Roth, Germany).

Solution B

- Tris-base (1.5 M) 181.5 g was dissolved in 500 ml deionized water, and the pH was adjusted by adding HCl (pH = 8.8). To this solution, 4 g SDS (14 mM) was added, then made up to 1 L with deionized water.

Solution C

- Tris-base (495 mM) 60 g was dissolved in 500 ml deionized water, and the pH was adjusted by adding HCl (pH = 6.8). To this solution, 4 g SDS (14 mM) was added, then made up to 1 L with deionized water.

Note: All stock solutions were stored at 4°C until use.

Table 2.7 Content of separating and stacking gel solutions.

Separating gel	**12.5%**		**Stacking gel**	**4%**
Gel solution A	7.5		Gel solution A	1.1
Gel solution B	4.5		Gel solution C	1.9
Deionized H_2O	6		Deionized H_2O	4.5
APS (µl)	180		APS (µl)	75
TEMED (µl)	9		TEMED (µl)	7.5

2.6.5 Preparation of protein samples

Protein samples were prepared with 3X SDS sample loading buffer (Laemmli loading dye). This buffer plays an important role in protein preparation, summarized in the following points:

1. The β-mercaptoethanol in the loading buffer reduces the intra- and intermolecular disulphide bonds of the proteins, allowing separation by size rather than shape.
2. The SDS detergent binds to all positive charges of the proteins, which occur at a regular interval, giving each protein the same overall negative charge so that proteins will separate based on size and not by charge. The SDS also denatures the proteins and subunits to separate based on size. SDS binds to proteins at approximately 1.3 g of SDS/g of protein.
3. Bromophenol blue serves as an indicator dye and migration indicator, as the dye front that runs ahead of the proteins can be monitored. Bromophenol blue also makes it easier to see the sample during loading of the gel wells with the protein samples.
4. The glycerol present in the Laemmli buffer increases the density of the sample so it falls to the bottom of the well forming a layer, minimizing puffing or loss of protein sample into the buffer.
5. DTT is present in many formulations to reduce the formation of any disulphide bonds that could promote a secondary/tertiary structure and/or dimer formation.

The protein samples were prepared with SDS sample loading buffer as follows:

1. 75 µg of protein from each sample was centrifuged at 16,000 × *g* for 1 minute, then heated at 95°C for 5 minutes and centrifuged at 16,000 × *g* for 1 minute.
2. The chamber was filled with SDS-PAGE running buffer after the gel and the electrodes had been organized in the SDS-PAGE chamber.
3. A 25 µl Hamilton syringe was used to load the samples. In a separate well on the gel, a pre-stained molecular weight marker (Thermo Fisher, Germany) was

loaded. The samples were loaded into the wells, and the lid of the tank was replaced.

4. A constant voltage (25 V for one gel or 50 V for two gels) was applied to the tank for 1 to 2 h until the tracking dye had reached the bottom of the gel.
5. The proteins were then transferred to a PVDF membrane (Roche, Germany) and detected by immunoblotting.

2.6.6 Protein loading optimization

It is important to determine the protein load in each gel at the beginning of the western blot procedure. After determined of protein concentration according to (103) method, three different protein concentrations were tested for optimization (50, 75, and 100 µg) to determine the concentration that provided the best band appearance.

2.6.7 Coomassie blue staining

Coomassie dye protein gel stain (Thermo Fisher, Germany) was used to detect the proteins as follows:

1. The gel was placed in a container containing the Coomassie blue stain for 20 min.
2. The stained gel was removed and washed with distilled water to remove excess stain.
3. The protein bands were scanned and detected using a Bio-Rad system (**Figure 2.3**).

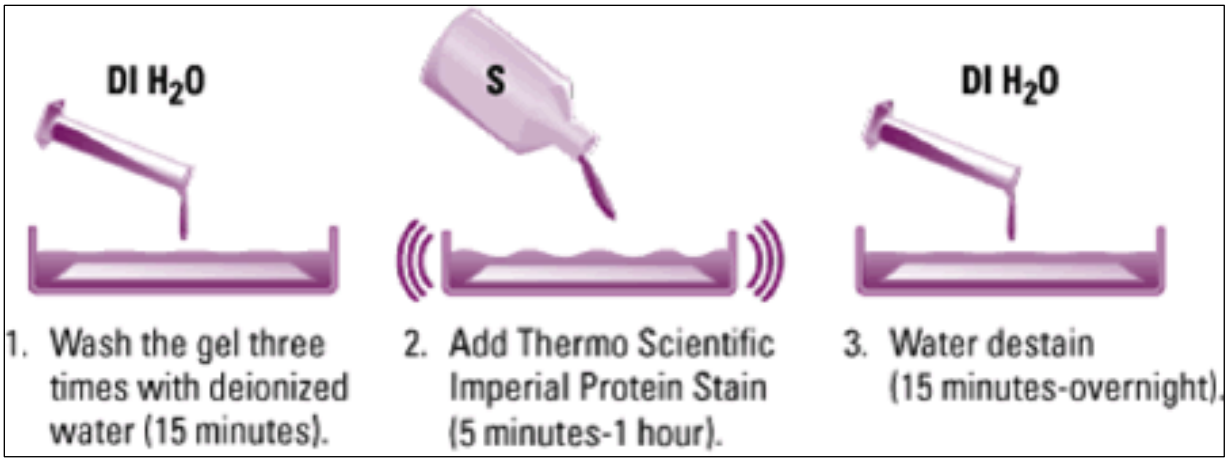

Figure 2.3 Coomassie blue staining protocol. Adapted from Thermo Fisher (Germany).

2.6.8 Transferring the protein from the gel to the membrane

After electrophoresis was complete, the proteins were transferred from the gel to a PVDF membrane with a pore size of 0.45 µm. The procedure was completed as follows:

1. The PVDF membrane was soaked in methanol at room temperature for 1 min for activation, then rinsed with transfer buffer.
2. Sponges and filter papers were placed in the transfer buffer prior to assembly of the transfer sandwich.
3. The gel and membrane were sandwiched between sponges and filter paper in the direction of electrical current (negative to positive) ordered as follows: sponge, three filter papers, gel containing the protein, PVDF membrane, three filter papers and then the final sponge. The layers were clamped tightly together after ensuring that no air bubbles had formed between the gel and membrane for smooth and accurate transfer **(Figure 2.4).**
4. The sandwich was then placed in the chamber.
5. The tank blot system was filled with transfer buffer and used to transfer the protein from the gel to the PVDF membrane by running the tank blot at 360 mA at room temperature for 1 h.

Figure 2.4 The structure of the gel and the membrane in the sandwich**.** Adapted from Abcam.uk.

2.6.9 Antibody incubation

After the protein transfer process was complete, immunoblotting was performed as follows:

1. 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 (TBST) until the background had become clear.
2. To block non-specific binding on the membrane, a solution of 5% non-fat dried milk powder in TBS containing 0.1% Tween-20 buffer was used at room temperature for 1 h with shaking.
3. The blots were incubated with mouse monoclonal Tektin 2 antibody (1:1000 diluted in TBS; 54 kDa; ab60918; Abcam, UK) and rabbit polyclonal CatSper 2 antibody (1:100 dilution in TBS; 62 kDa; ab150890, Abcam) (**Table 2.8)**. Incubations were performed overnight at 4°C with shaking.
4. The blots were washed with TBS buffer containing 0.1% Tween-20 (TBS-T) 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 Tektin 2 and CatSper 2 proteins, respectively, diluted 1:5000 (optimized dilution) in TBS.

5. After the blots had been washed with TBS-T, detection 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.
6. For normalization, the same blot was stripped and re-probed with mouse monoclonal β-actin (1:100) antibody (Sigma-Aldrich, Germany). The membrane was firstly 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.
7. For the negative controls, parallel blots were incubated with TBS instead of the monoclonal and polyclonal antibodies.

Table 2.8 Primary antibodies used for the western blot.

Antibody	**Dilution**	**Molecular weight**	**Species**	**Type**
Tektin 2	1:1000	54 kDa	Mouse	Monoclonal antibody
CatSper 2	1:100	62 kDa	Rabbit	Polyclonal antibody
β-actin	1:100	42 kDa	Mouse	Monoclonal antibody

2.6.10 Imaging and data analysis

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, as follows:

1. The excess wash buffer was drained from the washed membranes.
2. Detection solutions A and B were mixed in a ratio of 1:1, then this mixture was added to the membrane so that it covered the entire surface of the membrane for 2 min.
3. The membrane was placed on a sheet of plastic wrap with the protein side facing.

4. The chemiluminescent signals were captured using a CCD camera-based imager (Bio Rad-Germany).
5. The image was analyzed using a ChemiDoc XRS+ system software (Bio Rad-Germany) to read the band intensity of the target proteins.
6. The target protein levels were normalized to the loading control.

2.6.11 Protein band normalization

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 proteins of interest (PI), which were CatSper 2 and Tektin 2. The normalization steps can be summarized as follows:

1. The background-subtracted densities of the PI and the normalizing LC were calculated by ChemiDoc XRS+ system (Bio Rad-Germany).
2. A relative LC value was determined from dividing all LC values by the highest LC density value. Relative LC = LC values/highest LC value.
3. PI values were divided by the relative LC value for their respective lanes. Normalized value = PI values/relative LC (105).

After the normalized values were determined, the average, *P*-values, and fold-change were calculated, and graphs were produced.

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 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 mRNA quantities in the “cryo” versus “fresh” samples were

calculated separately by the comparative ΔCt method. The threshold cycle (Ct) reflects the cycle number at which the fluorescence curve generated within the reaction crossed the threshold for RT-qPCR. The ΔCt was calculated by subtracting the Ct values of GAPDH from the Ct values of the mRNA of interest, where ΔCt = (Ct mRNA of interest) - (Ct GAPDH). The ΔΔCt was then calculated by subtracting the ΔCt of "cryo" samples from the ΔCt of the "fresh" samples, where ΔΔCt = (ΔCt fresh - ΔCt cryo). The fold-change for the mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ equation (106). A Mann–Whitney U test was used for each mRNA of interest.

Note: all steps presented in this study were performed by myself. Only the statistical analysis step was done with contribution of Mr. Jakob Schöpe from Biomedical Statistician, Institute for Medical Biometry, Epidemiology and Medical Informatics (IMBEI), Saarland University School of Medicine, Homburg (Saar).

3 Results

Determining the effect of cryopreservation on the levels of CatSper 2 and Tektin 2 protein in the spermatozoa samples was the focus of this study. The age of males included in this study ranged between 24–50 years, with a mean age of 36.75 ± 6.25 years. The clinical characteristics of the study population are shown in **Table 3.1**.

Table 3.1 Clinical characteristics of the study population.

Variables	n = 48					
	Mean	**Median**	**SD**	**Minimum**	**Maximum**	**Range**
Age (year)	36.75	35.00	6.25	24.00	50.00	26.00
Sperm count (million/ml)	84.93	78.00	55.92	1.70	226.00	224.30
Percentage with total motility (%)	55.38	57.00	20.97	0.00	89.00	89.00
Percentage with progressive motility (%)	38.40	42.00	22.56	0.00	82.00	82.00
Percentage with non-progressive motility (%)	17.13	10.00	17.16	0.00	75.00	75.00
Vitality test (eosin test)	52.64	55.15	17.14	0.00	87.00	87.00

SD: Standard deviation

3.1 Optimization of protein concentration for western blot

In order to obtain a good signal, the protein concentration was optimized. Three different protein concentrations, 50, 75, and 100 µg, were tested to find the optimum concentration. As shown in Figure 3.1, the most defined band was observed when a concentration of 75 µg protein was used, therefore, this concentration was chosen for this study.

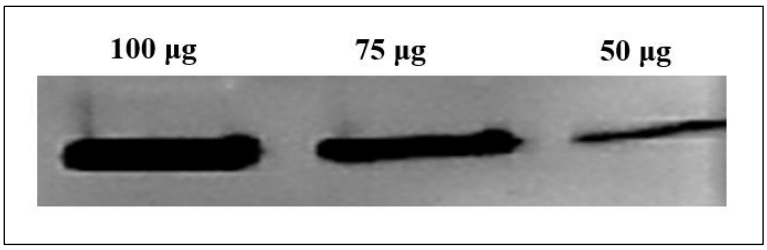

Figure 3.1 Optimization of Tektin 2 (54 kDa) protein concentration for the western blot analysis. Three protein concentrations were tested, 50, 75, and 100 µg.

3.2 Determination of RNA expression level of CATSPER2 and TEKT2 in spermatozoa samples before and after cryopreservation by RT-qPCR

To determine whether there was any change in the expression level of CATSPER2 and TEKT2 genes in spermatozoa before and after cryopreservation, 10 samples from the fresh (n=10) and cryopreserved (n=10) groups were tested. Using RT-qPCR, the difference in gene expression levels between fresh and cryopreserved samples (ΔCT) was determined. The results showed a decrease in the expression levels of CATSPER2 and TEKT2 in the cryopreserved samples compared to the fresh samples, and this downregulation ranged between a fold change of 2.04–11.95 and 1.11–62.68 in the CATSPER2 and TEKT2 genes, respectively (**Table 3.2**). The differences in expression levels of CATSPER2 and TEKT2 were significant, with *P*-values of 0.0039 and 0.0166, respectively, in the cryopreserved samples compared to the fresh samples (**Figure 3.2**).

Table 3.2 Expression of CATSPER2 and TEKT2 genes in spermatozoa after cryopreservation compared to fresh samples. (Ct) cycle threshold

Fresh samples				Cryopreserved samples				ΔΔCT	Fold change	Regulation
Sample	Gene name	CT	ΔCT	Sample	Gene name	CT	ΔCT			
Fresh 1	CATSPER2	29.3	3.85	Cryo 1	CATSPER2	35.41	10.39	- 6.54	92.73	Down
Fresh 1	TEKT2	37.2	11.75	Cryo 1	TEKT2	33.54	8.52	3.23	9.41	Up
Fresh 2	CATSPER2	37.08	11.28	Cryo 2	CATSPER2	38.22	13.2	- 1.93	3.81	Down
Fresh 2	TEKT2	33.56	7.76	Cryo 2	TEKT2	32.93	7.91	- 0.16	1.11	Down
Fresh 3	CATSPER2	36.4	4.86	Cryo 3	CATSPER2	34.12	9.59	- 4.73	26.54	Down
Fresh 3	TEKT2	34.91	3.37	Cryo 3	TEKT2	33.88	9.34	- 5.97	62.68	Down
Fresh 4	CATSPER2	34	1.52	Cryo 4	CATSPER2	33.82	6.27	- 4.75	26.9	Down
Fresh 4	TEKT2	36.22	3.74	Cryo 4	TEKT2	36.41	8.86	- 5.12	34.78	Down
Fresh 5	CATSPER2	30.58	1.73	Cryo 5	CATSPER2	33.37	8.77	- 7.04	131.59	Down
Fresh 5	TEKT2	35.45	3.14	Cryo 5	TEKT2	32.19	7.59	- 4.45	22.62	Down
Fresh 6	CATSPER2	30.95	4.09	Cryo 6	CATSPER2	35.33	10.84	- 6.75	107.34	Down
Fresh 6	TEKT2	36.36	1.31	Cryo 6	TEKT2	31.75	7.26	- 5.95	61.79	Down
Fresh 7	CATSPER2	36.13	7.59	Cryo 7	CATSPER2	34.04	8.61	- 1.03	2.04	Down
Fresh 7	TEKT2	32.8	4.26	Cryo 7	TEKT2	33.59	8.16	- 3.91	15.03	Down
Fresh 8	CATSPER2	33.93	10.43	Cryo 8	CATSPER2	31.43	6.99	3.45	10.92	Up
Fresh 8	TEKT2	29.69	6.19	Cryo 8	TEKT2	30.82	6.38	- 0.19	1.14	Down
Fresh 9	CATSPER2	30.96	3.46	Cryo 9	CATSPER2	35.42	10.38	- 6.93	121.94	Down
Fresh 9	TEKT2	34.48	6.97	Cryo 9	TEKT2	34.01	8.97	- 2.00	4	Down
Fresh 10	CATSPER2	35.65	4.39	Cryo 10	CATSPER2	33.99	9.08	- 4.69	25.82	Down

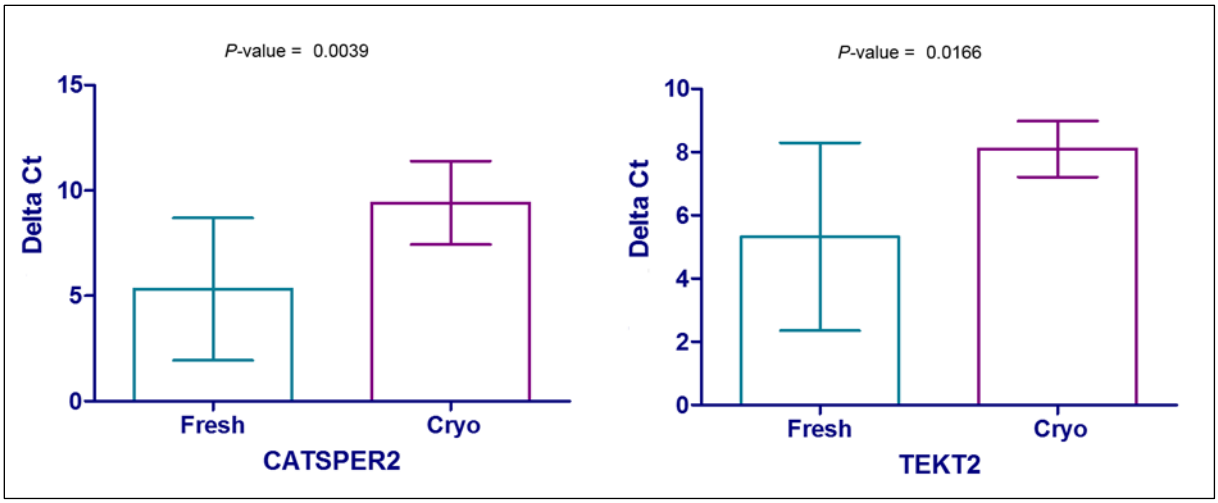

Figure 3.2 RNA expression levels of CATSPER2 and TEKT2 in spermatozoa. ΔCT of the mRNAs in the spermatozoa of cryopreserved (cryo) samples (n = 10) compared to the fresh samples (n = 10) of the same males as determined by RT-qPCR. Data are expressed the relative expression level of Delta Ct normalized to GAPDH as the endogenous housekeeping gene. Data were analyzed by Mann–Whitney U test, and $P \leq 0.05$ was considered significant.

3.3 CatSper 2 and Tektin 2 protein levels in spermatozoa before and after cryopreservation evaluated by western blot analysis

To determine if there were any changes in the protein levels of CatSper 2 and Tektin 2 in spermatozoa before and after cryopreservation, the samples of 48 males were tested by western blot (fresh, n = 48; cryopreserved, n = 48). The presence and concentration of CatSper 2 and Tektin 2 in the spermatozoa before and after cryopreservation were verified by western blotting, in addition to the quality of the antibodies used. Single bands at 62 and 54 kDa, the expected sizes of the CatSper 2 and Tektin 2 proteins, respectively, were observed more clearly in the fresh samples compared to cryopreserved samples (**Figure 3.3**). 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. The CatSper 2 and Tektin 2 protein levels were lower in spermatozoa of cryopreserved samples compared to fresh samples, with 0.44 ± 0.35 vs. 0.77 ± 0.25 ($P \leq 0.0001$) and 0.58 ± 0.24 vs. 0.76 ± 0.09 ($P \leq 0.0001$), respectively (**Figure 3.4**).

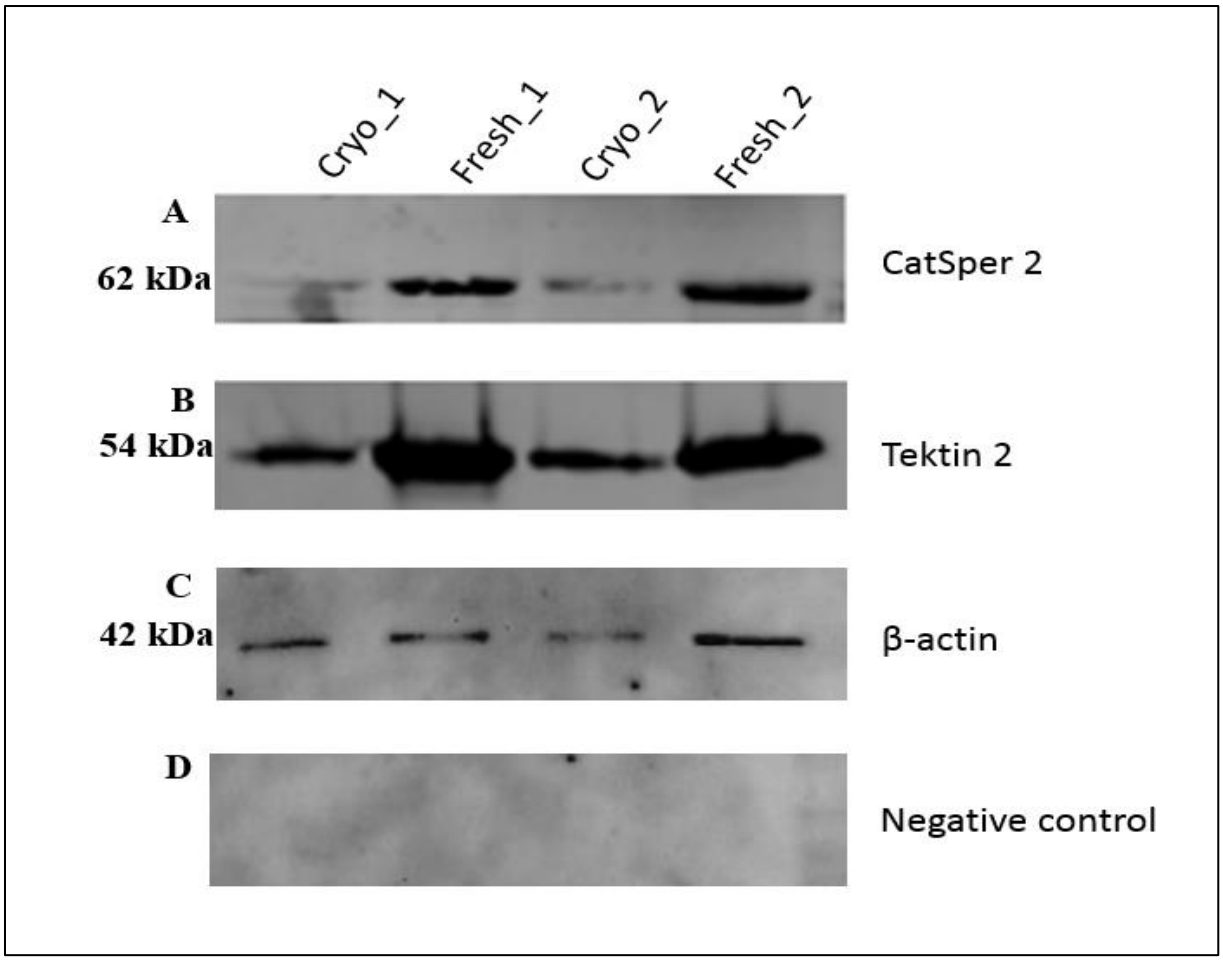

Figure 3.2 Protein levels of CatSper 2 and Tektin 2 in spermatozoa before and after exposure to cryopreservation, as determined by western blotting. The proteins (75 µg whole cell extract) were separated on a 12.5% SDS-polyacrylamide gel then transferred to a PVDF membrane. (A) CatSpar 2, (B) Tektin 2, and (C) β-actin bands were visualized with the appropriate antibodies on an ECL system. The CatSpar 2 and Tektin 2 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. C, cryopreserved sample; F, fresh sample.

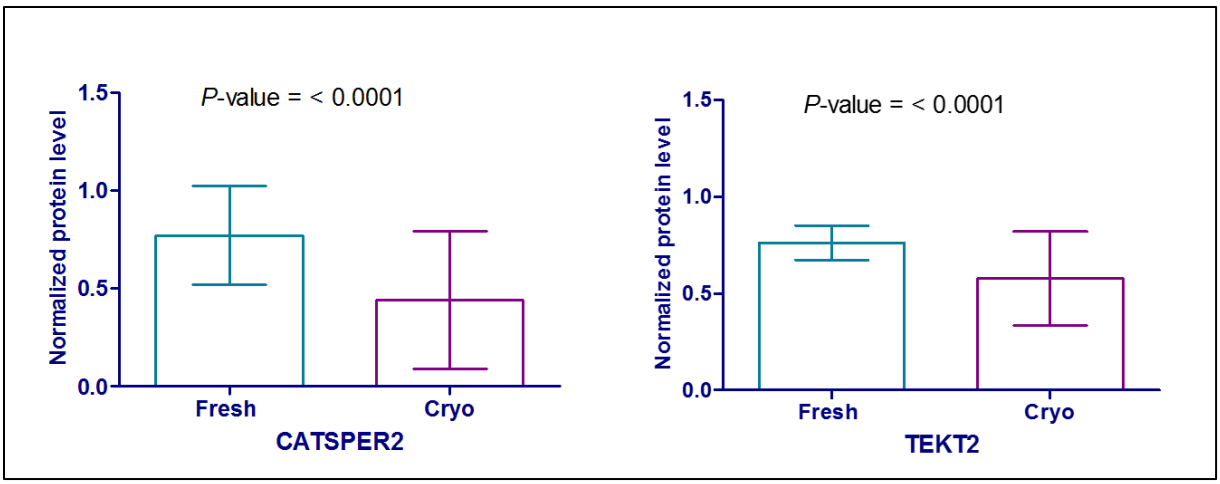

Figure 3.3 CatSper2 and TEKT2 protein levels in spermatozoa of cryopreserved samples compared to fresh samples**.** The spermatozoa were lysed and the extracted proteins were analyzed by western blot. The proteins (75 µg whole cell extract) were separated on a 12.5% SDS-polyacrylamide gel then transferred to a PVDF membrane. The levels of CatSper 2 and Tektin 2 proteins were visualized using appropriate antibodies. The bar diagram shows the density of the bands after normalizing to β-actin. Statistical analysis was conducted by Mann–Whitney U test, with $P \leq 0.05$ considered significant. Cryo, cryopreserved.

3.4 Levels of CatSper 2 and Tektin 2 proteins in spermatozoa after cryopreservation according to male fertility status

To determine whether the levels of CatSper 2 and Tektin 2 protein have an impact on the spermatozoa of fertile and subfertile males before and after cryopreservation, the fresh and cryopreserved samples from 48 males were studied. The descriptive characteristics of the study population according to fertility status are presented in **Table 3.3**.The fertile group consisted of 78 samples from 39 males (fresh, n = 39 samples; cryopreserved, n = 39) with a mean age of 37.33 ± 5.72 years. The subfertile group consisted of 18 samples from 9 males (fresh, n = 9; cryopreserved, n = 9) with a mean age of 34.22 ± 7.88 years. In the fertile group, there was a significantly lower level of the CatSper 2 and Tektin 2 proteins in the spermatozoa of the cryopreserved samples compared to fresh samples, with values of 0.45 ± 0.35 vs. 0.77 ± 0.27 (P ≤ 0.0001) and 0.59 ± 0.25 vs. 0.75 ± 0.09 (P ≤ 0.0001), respectively (Figures 3.5 and 3.6). Similarly, in the subfertile group, the level of CatSper 2 and Tektin 2 proteins in the spermatozoa of cryopreserved samples were significantly

lower than fresh samples, with 0.38 ± 0.36 vs. 0.75 ± 0.17 (P ≤ 0.0315) and 0.50 ± 0.22 vs. 0.80 ± 0.07 (P ≤ 0.008), respectively

Table 3.3 Descriptive characteristics of the study population according to fertility status.

Variables	Fertile group (n = 78)		Subfertile group (n = 18)	
	Mean	SD	Mean	SD
Age (year)	37.33	5.72	34.22	7.88
Spermatozoa count (million/ml)	95.35	53.33	39.76	43.94
Percentage of total motility (%)	63.13	12.26	21.78	17.44
Percentage of progressive motility (%)	44.21	19.68	13.22	16.25
Percentage of non-progressive motility (%)	19.10	18.30	8.56	5.84
Vitality test (eosin test)	55.21	16.42	41.50	16.08
Normalized of CatSper 2 protein (F)	0.77	0.27	0.75	0.17
Normalized of CatSper 2 protein (C)	0.45	0.35	0.38	0.36
Normalized of Tektin 2 protein (F)	0.75	0.09	0.80	0.07
Normalized of Tektin 2 protein (C)	0.59	0.25	0.50	0.22

All values are expressed as mean ± standard deviation (SD). F, fresh; C, cryopreserved.

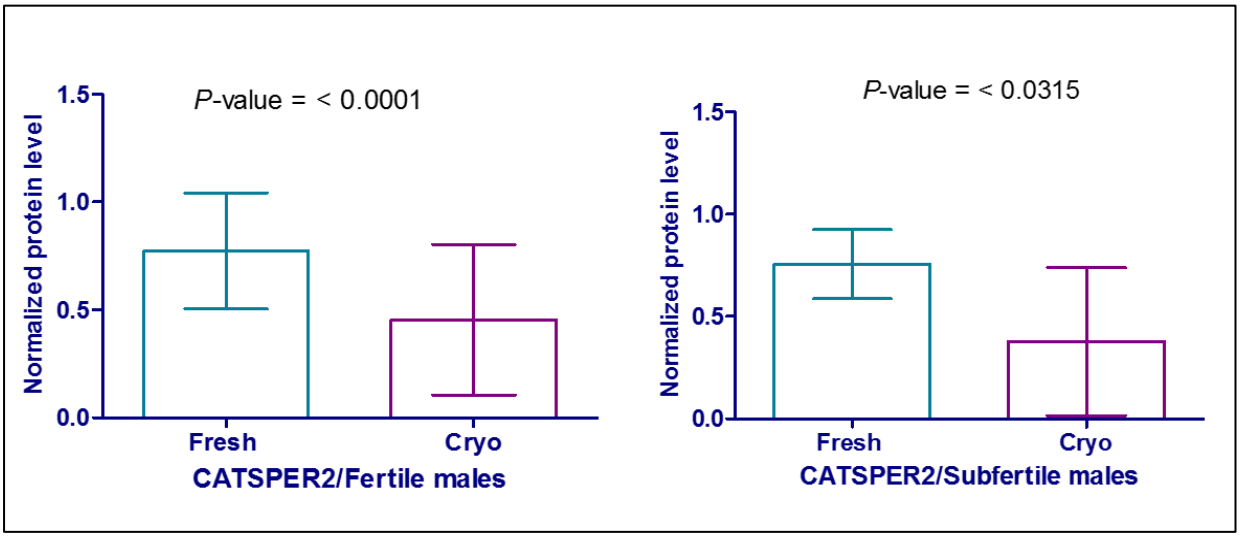

Figure 3.4 Level of CatSper2 protein in spermatozoa according to fertility status**.** The level of the CatSper2 protein in the spermatozoa of cryopreserved samples was compared to fresh samples of fertile and subfertile males. The spermatozoa were lysed and the extracted proteins were analyzed by western blot. The proteins (75 µg whole cell extract) were separated on a 12.5% SDS-polyacrylamide gel then transferred to a PVDF membrane. CatSper2 levels were visualized with an appropriate antibody. The bar diagram shows the density of the bands normalized to β-actin. Statistical analysis was conducted using Mann–Whitney U test, with $P \leq 0.05$ considered significant. Cryo, cryopreserved.

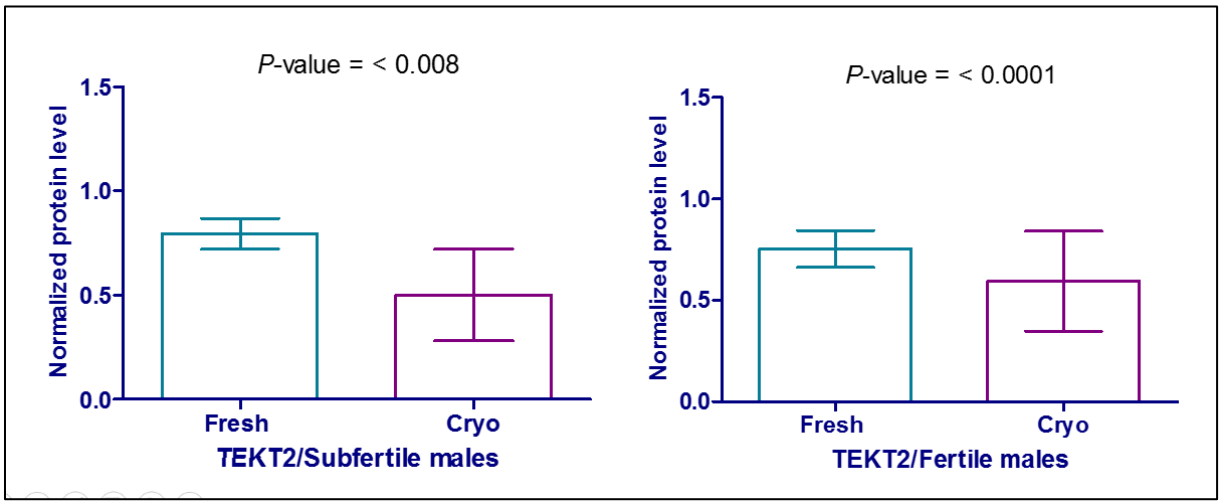

Figure 3.5 Level of TEKT2 protein in spermatozoa according to fertility status. The level of Tektin 2 in the spermatozoa of cryopreserved samples was compared to fresh samples of fertile and subfertile males. The spermatozoa were lysed and the extracted proteins were analyzed by western blot. The proteins (75 µg whole cell extract) were separated on a 12.5% SDS-polyacrylamide gel then transferred to a

PVDF membrane. The Tektin 2 levels were visualized with an appropriate antibody. The bar diagram shows the density of the bands normalized to β-actin. Statistical analysis was conducted by Mann–Whitney U test, with $P \leq 0.05$ considered significant. Cryo, cryopreserved.

4 Discussion

Cryopreservation of spermatozoa is a widely used technique to preserve the fertility of males. During the cryopreservation process, treated cells and tissue undergo a dramatic transformation in their chemical and physical characteristics as the temperature drops from +37 to -196°C, thus risking cryodamage(107). The speed of cooling and warming is a critical step, and inappropriate cooling or thawing rates are negatively correlated with spermatozoa survival. Several proteins identified in 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 (13). CatSper 2, a cation ion channel that regulates Ca^{2+}, and Tektin 2, a membrane protein responsible for spermatozoa flagella movement, were the targets of this study, which aimed to determine the effect of cryopreservation on these proteins and their correlation with male fertility status. Previous studies have reported that CatSper and Tektin are related to male infertility problems, as they play an important role in sperm motility (24, 44). In other studies, a genetic disruption in any of the four sperm-specific CatSper channels (CatSper 1, 2, 3, or 4) leads to male infertility by impairing sperm motility (24, 44). Furthermore, Hildebrand *et al*. (108) identified sperm motility defects due to the loss of CatSper channels, which are expressed in the principal piece of the spermatozoa flagellum. 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 hyperactivated motility. In contrast, Tanaka *et al*., reported that Tektin 2 is required for dynein arm integrity in spermatozoa flagella, and a deficiency in Tektin 2 causes male infertility due to impaired sperm motility (64). Moreover, another study found that Tektin 2 variants are probably one of the risk genetic factors related to idiopathy (109).

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 (62). 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 (110). Several studies have compared the computerized program freezer (CPF) method to liquid nitrogen vapor (LNV) after 6 months of freezing, and these studies showed no significant difference in the percentage of spermatozoa with normal morphology, despite the fact that the slow CPF provides significantly superior results in regard to the DNA integrity, sperm motility, and vitality of the thawed spermatozoa compared to LNV (111). In contrast, another study showed that the slow CPF method was superior to LNV freezing and fast CPF methods for spermatozoa cryopreservation. While none of these freezing methods had any discernible effects on spermatozoa morphology, the motility of spermatozoa was decreased due to exposure to the cryomedium (112). Furthermore, Hammadeh *et al.* (113) demonstrated that chromatin packaging and spermatozoa with normal morphology were decreased when LNV was used compared to the slow CPF method. As a result of these previous studies, the freezing method chosen for the current study was slow CPF in order to minimize cryodamage during the cryopreservation process.

The results of the present study show decreased RNA expression of CatSper 2 and Tektin 2 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. Cryopreservation has also been reported to affect the nucleoprotein structure in spermatozoa DNA (114).

Li *et al*. (46) found significantly higher levels of CatSper 2 and CatSper 3 mRNAs in high-motility spermatozoa than in the low-motility fraction. Cryodamage is a major problem for spermatozoa cryopreservation because it causes changes to spermatozoa transcripts that may influence spermatozoa function and motility (115). It is possible that spermatozoa cryopreservation decreases the level of transcripts through RNA degradation. The changes in mRNA expression as a result of cryopreservation may explain the differences in viability between the fresh and cryopreserved spermatozoa. 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 *et al*. (116) studied the effect of cryopreservation on human spermatozoa mRNA expression and found a significant effect on fertilization and early embryo development. In their study, protamine 1 (PRM1) and protamine 2 (PRM2) were analyzed in normozoospermic samples using two different methods, cryopreservation and vitrification. Transcripts from fresh semen samples showed an earlier threshold cycle (Ct) than cryopreserved samples, indicating a clear decrease in the transcript level after the cryopreservation process. Furthermore, the results indicate that the molecular status of the spermatozoa after freezing is poorer than that of fresh spermatoza, regardless of the method used.

Walson *et al*. (115) identified a few genes which were upregulated prior to cryopreservation (RPL31, GCLC, and PRKCE*)*. One of the proteins making up the ribosome complex, RPL31, was detected in the nuclei of human sperm, and might play an important role in spermatogenesis and fertilization. The glutamate–cysteine ligase catalytic (GCLC) subunit is a part of the glutathione metabolic pathway. Therefore, GCLC upregulation in freeze-thawed sperm may be a protective response of sperm to cold shock and oxidation stress. Also, protein kinase C epsilon (PRKCE), which plays an important role in several signal transduction pathways, was identified in mammalian sperm where it was expressed within the principal piece of the tail. Upregulation of these genes in freeze-thawed sperm may be a result of cryopreservation stress, which may have induced the differential expression. It is difficult to make conclusions regarding the effect of cryopreservation on the expression of these genes as the related function remains unclear, and this requires further investigation.

In the present study, the expression levels of the Tektin 2 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 (117, 118). Furthermore, cryoprotectant toxicity can induce alterations in the spermatozoa membrane components (119). Wang *et al*. (2) investigated the

characteristics of different proteins between cryopreserved and fresh spermatozoa samples of nine normozoospermic donors through proteomic analysis, and found altered in the levels of 27 proteins between the two groups. This study also identified four downregulated proteins (Tektin 1, 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 (2). In contrast, Tektin 3 and Tektin 4 (structural component of ciliary and flagellar microtubules) were upregulated after the cryopreservation process. Tektin 2, located in the principal piece of spermatozoa, was downregulated similar to that observed for Tektin 1, which is located in spermatozoa centrosomes. These movement-related proteins may be responsible for the reduced motility in freeze-thawed spermatozoa. The findings of the present study are in agreement with the results from a study that examined the effect of cryopreservation on spermatozoa, which showed that the spermatozoa proteins were affected by cold storage, and consequently, the metabolic processes were affected (118).

A previous study by Yoon *et al*. (120) found a reduced percentage of spermatozoa viability, percentage of sperm motility, average path velocity (VAP), amplitude of lateral head displacement (ALH), and capacitated spermatozoa, while also reporting an increase in the straightness and the acrosome reaction (AR) after cryopreservation. They also reported nine differentially expressed proteins (two proteins decreased and seven increased) before and after cryopreservation. The decreased proteins were ODF2 and LOC616410, and the increased proteins were SOD2, CAPZB, NDUFV2, NDPK, TPI, F1-ATPase, and AKAP. Of the proteins that were increased, ODF2 is a cytoskeletal structure protein localized in spermatozoa flagella, also functioning in spermatozoa structure and movement, while the function of LOC616410 is not yet clear (120). Human spermatozoa with absence of CatSper showed decrease in penetration when the spermatozoa were stimulated with progesterone and importantly there was failed fertilization at IVF (121).

Carlson *et al*. (48) found that CatSper 2-null spermatozoon were unable to stimulate Ca^{2+} entry and failed to enter the hyperactivation stage. Cryopreservation is known to deactivate the sperm plasma membrane, which may result from increased internal calcium levels after thawing (122). Considering the previous results and the results of the current study, the low level of CatSper 2 expression in cryopreserved spermatozoa could be due to absent or disruption of gene expression resulting in increased internal calcium levels, leading to failure to hyperactivate, and consequently, failure to fertilize the oocyte.

In the present study, cryopreservation was shown to have an effect on RNA and protein levels; therefore, it was important to investigate the impact of cryopreservation on CatSper 2 and Tektin 2 proteins in fertile and subfertile males. In the fertile group, significantly lower expression was observed for CatSper 2 and Tektin 2 in the cryopreserved samples compared to the fresh samples. Likewise, in the subfertile group, a significantly lower expression was also observed in the CatSper 2 and Tektin 2 protein levels between cryopreserved and fresh samples. The findings of this study are similar to a study by Moskovtsev *et al.* (107) which reported that the spermatozoa from fertile men appear to be more tolerant to damage induced by freezing and thawing compared to samples from subfertile men, which might be due to an increased presence of antioxidants that protect against cryodamage in the spermatozoa of fertile men. Furthermore, Bhilawadikar *et al.* (33) found that a reduction the levels of CatSper 2 and Tektin 2 proteins appeared to be associated with reduced spermatozoa motility and fertilization rates in subfertile men compared to fertile men, in addition to poor embryo quality and low pregnancy rates. The expression levels were also found to be lower in spermatozoa derived from oligoasthenozoospermic men compared to normozoospermic men. This suggests that CatSper 2 and Tektin 2 may serve as markers to clarify some reasons that related to male infertility problems. Moreover, the gene expression of CatSper in subfertile men, characterized by a loss in spermatozoa motility, was low (up to 3.5-fold difference) compared to spermatozoa with no motility defects (47). According to these studies, in addition to the findings of the current study, there may be a relationship between lower CatSper gene expression and defective spermatozoa motility in a proportion of subfertile patients.

5 Conclusion

In conclusion, the results of the present study demonstrate decreased expression of the CatSper 2 and Tektin 2 mRNA and protein levels of the spermatozoa tail in cryopreserved samples compared to fresh samples, as evaluated by RT-qPCR and western blot analyses. The reduced levels of these proteins appear to be associated with fertility status. Hence, these proteins may be used as markers for explained some of causes related to infertility problems. The reduced motility of spermatozoa may be treated by cryoprotectant supplements that stimulate the relevant signaling pathways or by genetic therapy.

Although few studies have been carried out to evaluate the effect of cryopreservation on spermatozoa tail proteins, this study confirms the reduced expression of two proteins associated with reduced fertility rates. It would be interesting to discover in the future a new cryoprotectant components that could decrease the cryodamage associated with CatSper 2 and Tektin 2. Another point to be considered is reducing the storage period of spermatozoa to minimize cryodamage. Furthermore, it will be necessary to conduct large-scale studies to understand the biological effect of cryopreservation on spermatozoa, and to discover new biomarker candidates that might be helpful for improving prognostic, diagnostic, and therapeutic aspects in the fertility field.

The results of this study are remarkable because, to the best of our knowledge, this is the first study that correlates the cryopreservation effect with CatSper 2 and Tektin 2 levels in spermatozoa. Furthermore, this study highlighted the differences in these two proteins after cryopreservation in fertile and subfertile men.

6 References

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