Original Article

Expression of *TRPV1* as A Heat Sensitive Voltage-Dependent Ion Channel and Oxidative Stress in Sperm Samples of Infertile Men with Varicocele: A Case-Control Study

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Abstract

Objectives: Transient receptor potential vanilloid 1 (TRPV1) is a heat-activated nonselective cation channel that plays important role in the spermatogenesis, capacitation, acrosome reaction and sperm/oocyte fusion. Considering the high testicular temperature and oxidative stress in varicocele condition, we aimed to assess expression of TRPV1 in sperm of infertile men.

Materials and Methods: In this case-control study, twenty-five men with varicocele (grade II and III) as well as twenty-five fertile were recruited. Sperm parameters, protamine deficiency (Chromomycin A3), DNA damage (TUNEL), lipid peroxidation (BODIPY), *TRPV1* gene expression (real time polymerase chain reaction), TRPV1 protein (flowcytometry and immunocytochemical techniques), and acrosome reaction were assessed between fertile and varicocele groups.

Results: We observed a significant decrease in the sperm parameters, and also, an increased DNA damage, lipid peroxidation, and protamine deficiency in varicocele group. Although, the mRNA expression of *TRPV1* was similar between varicocele and fertile groups, its expression at the protein level was significantly decreased in the varicocele group in comparison with fertile group. Additionally, the TRPV1 localization was changed from the equatorial region to the acrosomal region of the head, especially in the acrosomal region, which was more significant in the fertile group than the varicocele group after inducing acrosome reaction.

Conclusion: In addition to the quality of sperm parameters, and chromatin integrity that were lower significantly in varicocele group, the expression of TRPV1 protein was also lower in varicocele condition that could be associated with reduced capacitation, acrosome reaction and sperm/oocyte fusion and thereby infertility.

Keywords: Acrosome Reaction, Capacitation, Semen Parameters, TRPV1, Varicocele

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Introduction

Varicocele is regarded as one of the main causes of male infertility which accounts for about 40% of primary male factor infertility and 80% of secondary male factor infertility (1, 2). Varicocele is characterized by the abnormally enlarged veins of the spermatic cord pampiniform plexus inside the scrotum which is proven to be associated with low sperm count, motility, and viability as well as high abnormal sperm morphology and DNA fragmentation (3).

Although, the exact underlying mechanisms of varicocele pathophysiology are not well-recognized, the induced hyperthermia following the reflux of abdominal warm blood to the pampiniform plexus due to incompetent valves have long been acknowledged to affect spermatogenesis and compromise the fertility in affected ones (2). Impaired or arrested spermatogenesis is among the observed consequences of heat stress due to the fact that germ cells and Sertoli cells are extremely sensitive to hyperthermia (3). As a temperature-sensitive procedure, spermatogenesis takes place at 35-37˚C which is 2-3˚C below the core body temperature, while the scrotum temperature rises by 2.6˚C higher than the optimal temperature in varicocele condition (2).

Elevated oxidative stress and reactive oxygen species (ROS), as well as apoptosis, are among the most destructive consequences of heat stress, which are likely to be induced a change in the composition of the protective or regulative proteins at the level of RNA, protein, post-translational modification and/or its location (2, 4).

Endocannabinoids (ECs) are involved in various physiological and pathological functions including addictive behavior, food intake, inflammation, immunomodulation, analgesia, cancer, epilepsy as well as in reproduction. In reproduction, ECs are considered as evolutionary check points. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are the two main components of ECs. Receptors of these components (CB1, CB2, and TRPV1) play a crucial role during

spermiogenesis as well as sperm capacitation, acrosome reaction, thermotaxis, and sperm/oocyte fusion. Recent studies, animal model and human, showed that TRPV1 receptor is affected by hyperthermia (1, 5-8).

Vanilloid compounds like capsaicin and cations, as well as noxious stimuli such as protons and heat (>42˚C), are potential activators of TRPV1. TRPV1 showed a protective effect against apoptosis in the seminiferous tubules of high-temperature exposed mice (9). In a mouse model capsaicin, an agonist of TRPV1, proved to be a protective agent against apoptosis in the spermatogenic cells subsequent to scrotal hyperthermia (5). In several studies, the expression levels of proteins and enzymes of ECs and their components, such as AEA and 2-AG, have been assessed and significant difference has been observed between fertile and infertile men. Infertility in these cases has been attributed to distorted capacitation and acrosome reaction (10, 11). In this regard, Perruzza et al. (10) took the advantage of neural networking system. They assessed different components of ECs in the induced varicocele rats and showed that the only component of ECs that its expression had a predicate value, based on the mean number of new born following two consecutive mating was TRPV1. Therefore, based on these findings, we aimed to assess whether TRPV1, principal component of ECs, is differentially expressed in infertile men with grade II and III varicocele in comparison with fertile men and its relation to the different sperm parameters.

Materials and Methods

Design of Study

This case-control study was approved by the Institutional Review Board from the Royan Institute, Tehran, Iran (IR.ACECR.ROYAN.REC.1398.003). Informed written consent was provided by all the candidates. Fifty in two equal groups of men with varicocele affected and fertile male, who referred to Isfahan Fertility and Infertility Center (IFIC), Iran, were recruited in this study. These participants that made our varicocele and fertile groups, respectively, had less than 40 years old. All members of the fertile group were fertile men who were candidate for family balancing panel. The case group contains, infertile men who suffered from left varicocele of grade II or III. Diagnosis of varicocele grade was performed by a urologist. When vein was palpable during Valsalva maneuver, patient categorize in grade I, not a visible vein palpable at rest made, grade II, and visible, palpable at rest vein was considered as a grade III. Infertile men with grade I varicocele, leukocytospermia, abnormal hormonal profiles, fever nearly 90 days prior to the semen, azoospermia, and urogenital infections were excluded from this study. The fertile men who suffered of varicocele or showed any sign of infertility were excluded from the study. Also, they were excluded if in their semen samples possessed higher than 1 million per ml leukocytes, and/or low quality of sperm parameters and comprised an abnormal hormonal profile.

Semen collection and analysis

Semen samples were obtained in sterile containers through masturbation after 3-5 days of sexual abstinence. Sperm parameters were assessed within 30 minutes after ejaculation (12). Sperm concentration was evaluated using a sperm counting chamber (Sperm processor, Garkheda, Aurangabad, India). Sperm motility and morphology were assessed by computer-assisted semen analysis (CASA) software, and Diff quick staining (Hooshmand Fanavar, Tehran, Iran) based on the fifth version of World Health organization (WHO) protocol, respectively.

Sperm protamine assay

Chromomycin A3 (CMA3) staining was carried out for analyzing the replacement of histones by protamine during spermatogenesis process (13). Briefly, 40 µL of washed semen with phosphate-buffered saline (PBS, Sama Tashkhis, Iran) was smeared and fixed with Carnoy solution (3 methanol:1 glacial acetic acid, Cat No:1.00063, Merck, Darmstadt, Germany) at 4˚C for 5 minutes on slides and stained with 150 µL CMA3 solution (0.25 mg/ml, Cat No: C2659, Sigma-Aldrich, United States) for 20 minutes. Slides were subsequently rinsed with PBS and about 500 spermatozoa per slide were analyzed using an Olympus fluorescent microscope (BX51, Tokyo, Japan) for distinguishing the CMA3 positive spermatozoa from CMA3 negative ones which are stained bright yellow and dull yellow based on the level of protamine level which is reported as a percentage.

Evaluation of sperm DNA damage

In this study, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was applied to evaluate the DNA damage by fluorescence microscope as described previously (14). Briefly, the sperm sample was fixed using 4% paraformaldehyde (Cat No:158127; Sigma, USA) and permeabilized with 0.2% Triton X-100 (Cat No: 108643, Merck, Darmstadt, Germany). A DNA fragmentation detection kit (Cat No: G3250, Apoptosis Detection System Fluorescein, Promega, Mannheim, Germany) was used according to the manufacturer's instructions. DNA damage was evaluated in about 500 sperms using an Olympus fluorescent microscope (BX51, Tokyo, Japan) for distinguishing the positive TUNEL spermatozoa (with fragmented DNA) from negative TUNEL ones (with intact DNA) which are stained green and red, respectively.

Evaluation of sperm lipid peroxidation

We assessed the percentage of sperm membrane lipid peroxidation according to Aitken et al. (15). Briefly, 5 mM of BODIPY 581/591 C11 (D3861, Molecular Probes, United States) was incubated with 2×10^6 concentrations of spermatozoa at 37˚C for 30 minutes and lipid peroxidation was determined by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and reported as "percentage of lipid peroxidation.

Evaluation of TRPV1 protein by Flowcytometry and Immunocytochemical techniques

Briefly, 12-15×10^6 isolated spermatozoa were washed with PBS at 3000 rpm and fixed with 4% paraformaldehyde (Cat No:158127; Sigma, USA) for 30 minutes. The sample was rinsed twice with PBS (at 3000 rpm, for 5 minutes). The sperm pellet was re-suspended in 500 mL of PBS and treated with Triton X-100 (Cat No: 108643, Merck, Darmstadt, Germany) followed by twice washing with PBS. In the next step, the sample was divided into two portions of the test and control and incubated with 3% bovine serum albumin (BSA, Cat No: A3311, Sigma-Aldrich, USA) for blocking the non-specific binding sites. Following the centrifugation at 3000 rpm and removal of the supernatant, test and control tubes were incubated overnight at 4˚C with rabbit polyclonal anti-TRPV1 antibody (Cat No:ACC030 , Alomone labs, Jerusalem BioPark, Israel) and 1% BSA solution, respectively. After twice washing with PBS, test and control tubes were incubated with secondary antibody (Goat Anti-Rabbit-IgG- FITC, Cat: F1262, Sigma-Aldrich, USA) at 37˚C for 1 hour. After twice washing with PBS, 400 µL of the test and control samples were transferred to a flow cytometric tube and readout with FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A similar procedure was used to TRPV1 protein immunocytochemical assay in sperm on the slides. To detect the location of the TRPV1 protein, the microscopic analysis was performed using a fluorescence microscope (BX51, Tokyo, Japan) which about 400 sperm per slide were evaluated. In addition, TRPV1 protein was also assessed before and after the acrosome reaction by immunocytochemical technique.

Acrosome reaction induction

For acrosome reaction induction, semen samples (n=5 of each group) before and after induction of acrosome reaction by calcium sample were processed by density gradient centrifugation (DGC) procedure, and incubated with 10 µM calcium ionophore (Cat: A23187, Sigma-Aldrich, USA) diluted in Dimethylsulfoxide (DMSO, Cat No: D8418, Merck, Germany) at a final concentration for 30 minutes (16). Then, chlortetracycline (CTC, Cat No: 26430, Sigma-Aldrich, USA) staining (17) was used for assessment of capacitation ionophore. CTC method is based on the differential pattern of staining in sperm head for intact or non-capacitated (Non-Cap), capacitated (Cap) and acrosome reacted (AR), sperm.

Evaluation of *TRPV1* gene expression

Total RNA was extracted from washed sperms samples of our both groups using YTzol pure RNA (Cat No:YT9064, Yekta Tajhiz Azama, Iran) according to the manufacturer's protocol, followed by cDNA Synthesis using Yekta Tajhiz cDNA Synthesis Kit (Cat No: YT4500, Yekta Tajhiz Azama, Iran). Expression of *TRPV1* gene at the mRNA level in sperm cells was analyzed through real-time polymerase chain reaction (PCR) in StepOnePlus Real-Time PCR System (Model No: 4376357, Applied Biosystems, Foster City, USA) using YTA SYBR Green qPCR MasterMix 2X (Cat No: yt2551, Yekta Tajhiz Azama, Iran) according to the manufacturer's instruction. Specific primers were designed for *TRPV1*-

F: 5′GGCTGTCTTCATCATCCTGCT3′
R: 5′GTTCTTGCTCTCCTGTGCGATCTT3′ (accession number: NM_080705.4, NM_080704.4, NM_080706.3, NM_018727.5).

We also used F: 5′-CCACTCCTCCACCTTTGACG-3′ and R: 5`-CCACCACCCTGTTGCTGTAG-3′ primers for the *GAPDH* housekeeping gene as an internal control for normalization (accession number: NM_001357943.2, NM_001256799.3,NM_001289745.3,NM_001289746.2, NM_002046.7). The relative expression of *TRPV1* at the RNA level was calculated through the $^{2\text{-}\Delta\Delta ct}$ method.

Statistical analysis

Data analysis was performed by statistical package for the social sciences for windows, version 26 (SPSS, Inc., Chicago, IL, USA) software using Independent T-test and Two-tailed Pearson correlation. The graphs are plotted using 8.4.3 (GraphPad Software, Inc., San Diego, CA). Real-time PCR data were evaluated using the $2^{-\Delta\Delta ct}$ method. Data were presented as mean± standard error of the mean (SEM), and $P<0.05$ was assumed as significant.

Results

Sperm parameters, sperm DNA damage, sperm lipid peroxidation, and sperm protamine deficiency

As shown in Table 1, the mean sperm concentration and sperm motility were significantly lower in the varicocele group than the fertile group. Additionally, the mean percentage of sperm DNA damage, membrane lipid peroxidation, and protamine deficiency were significantly higher in the varicocele group than the fertile group.

TRPV1 expression analysis

As shown in Figure 1A, the percentage of sperm TRPV1 protein in men with varicocele (31.00 ± 3.54) was significantly lower than fertile individuals (60.61 ± 3.45, P=0.001) which are presented in Figure 1B and C in more detail, however, comparison of *TRPV1* at the RNA level between fertile and varicocele groups showed no significant difference (Fig.1D). Also, immunofluorescence staining revealed the presence of the TRPV1 protein in the neck, flagellum, and different areas of the sperm head (equatorial and post acrosome regions) (Fig.1E).

Table 1: Comparison of conventional sperm parameters

Parameters	Fertile (n=25)	Varicocele (n=25)	P value
Sperm concentration (10^6/mL)	83.46 ± 7.36	51.74 ± 5.56	0.002
Sperm motility (%)	55.83 ± 2.7	44.61 ± 2.68	0.008
Abnormal sperm morphology (%)	96.36 ± 0.27	97.20 ± 0.17	0.013
Sperm DNA damage (%)	4.64 ± 0.53	9.62 ± 1.19	0.002
Sperm lipid peroxidation (%)	18.23 ± 2.20	43.80 ± 2.75	0.001
Sperm Protamine deficiency (%)	14.12 ± 1.05	27.70 ± 1.75	0.001

Values are expressed as mean ± standard error of the mean (SEM).

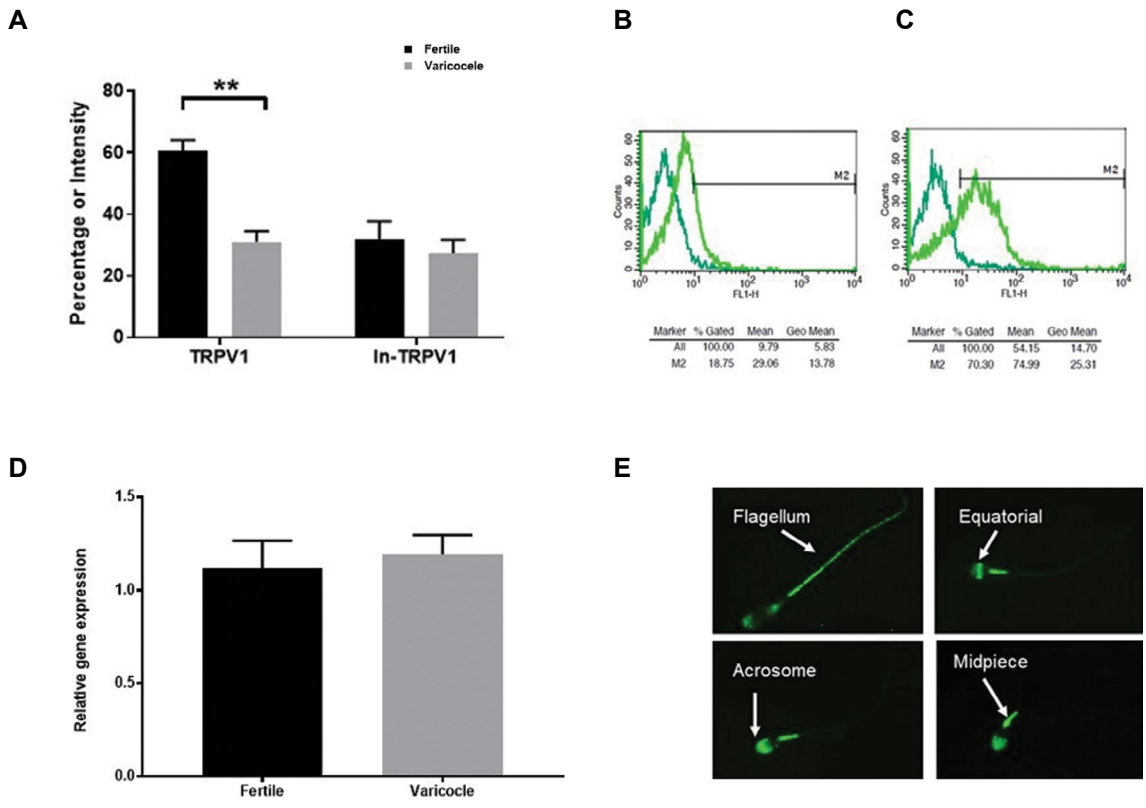

Fig.1: Assessment of sperm TRPV1 at RNA and protein levels. **A.** Comparison of mean percentage (P=0.001) and intensity of TRPV1 (In-TRPV1) proteins between varicocele (n=25) and fertile groups (n=25). In-TRPV1 show relative fluorescence intensity of TRPV1 in sperm sample. **B.** Flow cytometric plot of TRPV1 protein in a fertile man and **C.** An infertile man with varicocele. **D.** Comparison of gene expression analysis of the *TRPV1* gene relative to *GAPDH* gene (housekeeping gene as an internal control) between fertile and varicocele groups. **E.** Localization of TRPV1 protein in the sperms of fertile individuals. Values are expressed as mean ± standard error of the mean (SEM). The significant difference is presented as ** P<0.01.

Acrosome reaction and capacitation rate

Acrosome reaction and capacitation analysis in varicocele and fertile groups before and after of induced acrosome reaction are presented in Figure 2. While acrosome reaction and capacitation were not significantly different between the two groups, the percentage of sperm with reacted acrosome was significantly lower in the varicocele group (56.48 ± 2.06) than fertile ones (67.32 ± 2.52, P=0.006). Additionally, the percentage of non-capacitated (20.94 ± 1.57, P=0.02) and capacitated (22.64 ± 1.12, P=0.03) sperms were significantly higher in the varicocele group.

TRPV1 Localization before and after induction of acrosome reaction

Using immunochemistry, the percentage of TRPV1 positive sperm was evaluated before and after induction of acrosome reaction. The after induction of acrosome reaction results indicated that, the mean percentages of TRPV1 positive sperm in the acrosomal region (Fig.3A) were significantly higher than equatorial region (Fig.3B) in both groups. Also, it was significantly higher than "before" state in our groups. Comparison of the degree of localization

changes is significantly lower in the varicocele group in comparison with the fertile group before induction of acrosome reaction.

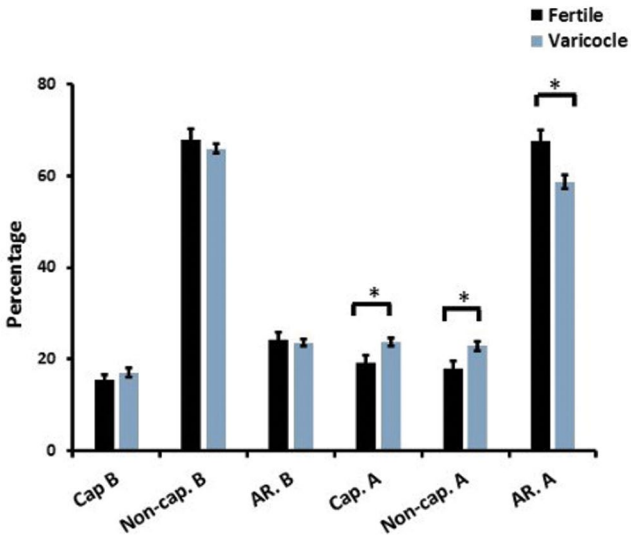

Fig.2: Comparison of the percentage of capacitated (Cap) and not capacitated (Non-Cap) sperms, as well as percentage of acrosome reacted sperms (AR) between fertile and varicocele groups before (B) and after (A) induction of acrosome reaction with calcium ionophore. Values are expressed as mean ± standard error of the mean (SEM). The significant difference is presented as *; P<0.05.

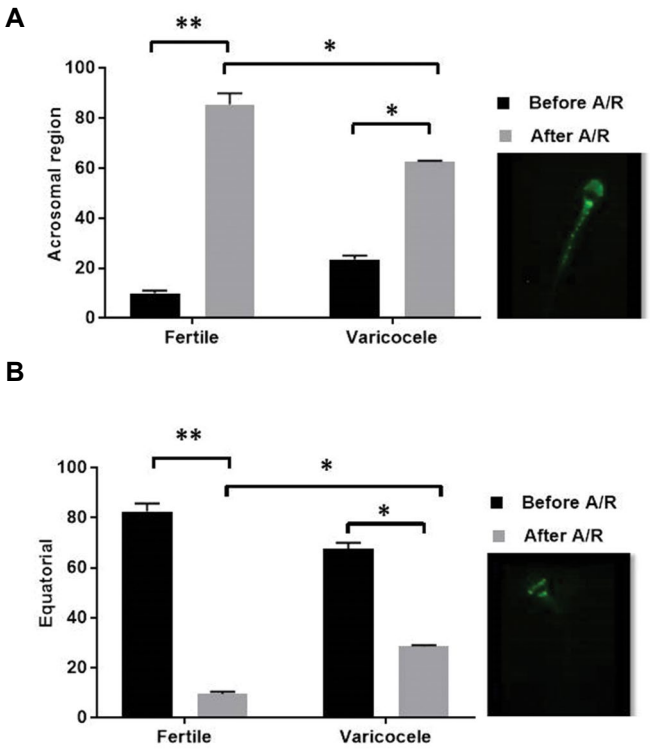

Fig.3: Comparison of TRPV1 protein localization between fertile and varicocele groups, before and after induction of acrosome reaction using the calcium ionophore at **A.** Acrosomal and **B.** Equatorial region. Values are expressed as mean ± standard error of the mean (SEM). The significant difference is presented as *; P<0.05 and **; P<0.01. A/R; Acrosom reaction.

Discussion

TRPV1 is a voltage-dependent ion channel that is sensitive to noxious stimuli like heat and oxidative stress, which are two main features of varicocele pathogenesis (10). *TRPV1* expression at the mRNA level in all male genital tissues has been reported previously (18). Similar to Lewis et al. (1) study in infertile men, we observed a significantly reduced level of TRPV1 protein in the varicocele group, however, we detected no differences at mRNA level compared to the fertile group. The lack of significant difference in the *TRPV1* expression at the RNA level between our groups may be due to post-transcription modification that might be induced by varicocele state (19, 20). In line with our results, the presence of TRPV1 in the human, bull, and boar spermatozoa, as well as rat Sertoli cells, were reported in previous studies, specifically at acrosomal and post-acrosomal parts of sperm cells (6, 21-25). According to Francavilla et al. (7) study, TRPV1 localization is restricted to the post-acrosomal region in the human sperm head and also, Chen et al. (26) reported TRPV1 receptor expression in the tail and neck regions of zebrafish spermatozoa. We also observed the presence of TRPV1 protein in the tail, equatorial, acrosome, and neck regions in sperm samples of varicocele and fertile groups.

Unlike the capacitation process that was significantly higher in the varicocele group, the mean of sperm reacted acrosome was significantly lower in the varicocele group in comparison with the fertile group.

In addition, the mean percentage of TRPV1 positive sperm in the acrosome region of sperm head significantly reduced following induction of acrosome and remained in the equatorial region of the sperm head in the varicocele group. Therefore, it can be concluded that the physiological role of TRPV1 protein in the acrosomal reaction has faded significantly in the varicocele group due to temperature stress and oxidative stress.

TRPV1 also plays a role in managing temperature-oriented sperm motility in the fallopian tube, known as thermotaxis, through which spermatozoa sense the slight temperature increase from the reservoir to the probable site of fertilization as a guide for moving toward the oocyte via managing calcium trafficking (7, 21). Thermotaxis lays a foundation for selecting sperms with higher TRPV1 expression. Moreover, sperm exposure to the rising temperature drastically affects the structure and fluidity of the plasma membrane, preceding to capacitation and acrosome reaction. At the same time, capacitation intensifies the sperm response to the temperature gradient (21). Of note, sperm motility increases during capacitation and, changes from progressive to a hyperactivated pattern with more bending in flagella with an asymmetric "8" shaped movements in addition to the intense lateral head movements. These features are requisite for penetrating of sperm to the cumulus cells before fertilization and this change in motility pattern is exploited through TRPV1

via calcium influx, membrane depolarization, downstream cAMP and PKA signaling pathway, and subsequent production of ATP by activating mitochondrial machinery (1, 21, 27). Kumar et al. (27) reported the reduced progressive motility following the treatment of bull spermatozoa with Capsazepine, while sperm motility was not affected in *trpv1*$^{-/-}$ zebrafish. We also demonstrated that mean of sperm motility in the varicocele group was significantly lower than the fertile individuals which is likely associated with a decrease in the TRPV1 protein level. In this regard, we recently showed that treatment of varicocelized rats with Capsaicin could recover sperm concentration and motility through the reduction of oxidative stress level (28).

The sensitivity of spermatocytes to heat stress is a pathogenic feature of varicocele, in this case, the TRPV1, a heat transducer, gains more importance (9). Several lines of evidence have identified apoptosis as a major mechanism in removing the damaged, unwanted cells involved in the various diseases (5). TRPV1 activation is proven to play a role in settling the fate of non-neuronal cells, including sperm cells during spermatogenesis by choosing between death and survival of spermatozoa and a decreased level of TRPV1 could result in oligospermia (1, 9). In addition, TRPV1 is involved in the apoptosis or protection of cells at the gonads considering the more drastic depletion of germ cells after hyperthermia in *Trpv1*$^{-/-}$ mouse models (9, 10). In this context, it was found that capsaicin prevented apoptosis of gonadal cells induced by heat shock through antioxidative action by activating TRPV1 (5). Perruzza et al. (10) reported a significant downregulation of the *TRPV1* gene among other components of the endocannabinoid (EC) system in the rat model of experimental varicocele, which correlated positively with fertility status implying the pivotal role of this channel in varicocele. This downregulation could lead to the deprivation of testis from a defensive mechanism in a deleterious milieu like heat and oxidative stress in the varicocele state. In this study, we compared the TRPV1 expression between the sperm of varicocele and fertile groups and we showed unlike RNA and protein expression level are reduced in the varicocele group. Considering the role of TRPV1 in inhibiting premature capacitation and acrosome reaction (11), we observed that despite of an increase in the capacitated sperm, the mean of sperm intact acrosome is reduced in this varicocele group. This result indicates the reduced level of TRPV1 may prone sperm to premature capacitation before sperm reaching the site of fertilization and this may be of the reason for reduced fertility in the varicocele state. Therefore, capsaicin treatment, an activator of TRPV1, of varicocele affected, may have potential therapeutic value (28).

Conclusion

TRPV1 is a voltage-dependent ion channel sensitive to heat and oxidative stress, which plays important roles in the physiological phenomenon such as capacitation, acrosome reaction, sperm-oocyte fusion. In infertile men with varicocele compared to fertile men, the mean of TRPV1 protein, as well as sperm parameters (concentration, motility, morphology), were significantly lower and these reductions were associated with increased oxidative stress and DNA fragmentation, and protamine deficiency. These results could be associated with the high level of heat and oxidative stress in the testis of these individuals.

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Authors' Contributions

S.S.; Patients management, preparation tests, samples collection, and analysis of data. F.A.; Analysis of data and writing the manuscript. M.H.N.-E., M.T.; Study conception and design, data analysis and interpretation. S.M.S.; Data analysis and interpretation. All authors read and approved the final manuscript.

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