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Open Access Original Article

Crescent Journal of Medical and Biological Sciences
Vol. 7, No. 3, July 2020, 382–388
eISSN 2148-9696

# Effect of Selenium and Electrical Stimulation on the Expression of Cation Channel Spermia 1 and 2 Genes in Spinal Cord Injury Rat Model

**Soheila Bani[1], Iraj Lotfinia[2], Parviz Shahabi[2], Abolfazl Barzegari[3], Jalal Abdolalizadeh[4*], Amir Vahedi[5], Meysam Ghorbani[2]**

**Abstract**

**Objectives:** Cation Channel Spermia (CatSper) 1-4 are considered as a unique family of "sperm cation channels" and play an important role in the motility of the sperm and male fertility. The aim of this study was to compare and evaluate the effects of selenium (Se) and electrical stimulation (Es) on the expression of CatSper 1 and 2 genes in the spinal cord injury (SCI) rat model.

**Materials and Methods:** The present experimental study was performed on 50 male Wistar rats. The animals were randomly divided into 5 groups of 10 which experienced SCI using an SCI impactor device. Forty-eight hours after injury, the Se group received Se for 6 consecutive days for 6 weeks, and Es was performed for 1 hour per day for 6 consecutive days during 6 weeks in the Es group. Finally, the real-time polymerase chain reaction (PCR) was performed for the expression of CatSper 1 and 2 genes, followed by using one-way ANOVA for the analysis of gene expression data. The results were considered statistically significant when $P < 0.05$.

**Results:** Se treatment showed an increase in the relative expression of CatSper 1 and 2 genes compared to the control group, but this increase was higher for CatSper 2. Eventually, Es did not affect the relative intensity of CatSper 1 and 2 gene expression after SCI.

**Conclusions:** Se, more than Es, could increase the relative intensity of CatSper1 and 2 expressions, and this increase was higher in CatSper2.

**Keywords:** Selenium, Electrical stimulation, CatSper 1 and 2 genes

## Introduction

Spermatogenesis is known as a procedure by which "spermatogonial cells" are converted into adult "spermatozoa" (sperm), and male fertility needs to be performed delicately. The spermatogenesis regulation relies on the "hypothalamus-pituitary-gonad" axis which is firmly arranged by the feedback control of the activity of the testis (1). Previous research has suggested that sperm count, motility, and morphology need to be assessed to determine the fertility power of the sperm (2).

Sperm ion channels have a significant function in fertilizing ability (3). In addition, asymmetric sperm motility requires the entry of $Ca^{2+}$ into the spermatozoa (4). The Cation Channel Spermia (CatSper) are the most widely studied $Ca^{2+}$ ion channel proteins in mammalian sperms (5). The CatSper channel has a heterotetrameric structure which is combined of four pore-forming α subunits (CatSpers 1–4) and supplementary auxiliary subunits viz such as "CatSper β, CatSper γ, and CatSper δ". The lack of a single subunit may lead to the degradation of the remaining CatSper proteins (6). Spinal cord injury (SCI) causes a significant decline in all parameters of the sperm and the expressions of CatSpers 1 and 2 (4).

Some researchers believe that a reduction in the percentage of the motile sperm may be due to the defective transcription of some gene-encoding proteins intricate in the motility of the sperm and other functions. A small part of the motion may consist of heterogeneous subunits in which various genes are not transcribed. Another statement for the differential dispensation of mRNAs between the high and low motility of the sperm might lie in the translation occurring in the sperm. There are some mechanisms related to enhancement in the overproduction of reactive oxygen species (ROS) and an effect on the DNA of the sperm (7).

Antioxidant enzymes have an important function in declining ROS levels. (8). Selenium (Se) is the specific function of selenoproteins in modulating the immune receptor of signaling pathways along with the $Ca^{2+}$ release, oxidative cascades, and other cellular activities

**Received** 6 August 2019, **Accepted** 1 October 2019, **Available online** 13 October 2019

[1]Pediatric Health Research Center, Nursing and Midwifery Faculty, Tabriz University of Medical Sciences, Tabriz, Iran. [2]Neurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. [3]Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran. [4]Drug Applied Research Center, Paramedical Faculty, Tabriz University of Medical Sciences, Tabriz, Iran. [5]Department of Pathology, Tabriz University of Medical Sciences, Tabriz, Iran.
***Corresponding Author:** Jalal Abdolalizadeh, Tel: +989141059313, Fax: +98 41 33361300, Email: jabdolalizadeh@gmail.com

(9). Further, Se is known to protect against some nervous system diseases and fertility (10).

Electrical stimulation (Es) can improve the movement of the upper/lower extremities, along with the loss of other parts of the body function (11). Previous evidence indicates that the cathode results in a decline in the cytodestructive effects of the innate calcium current of injury into injury axons, and thus leads to a marked diminish in the decadence of the axonal growth tip facing the cathode. However, it is not yet known how Es works at the molecular level (12).

SCI (about 25.5 million per year) is highly prevalent, particularly in males (80%) and antioxidants, especially Se have an effect on male fertility. Given that most previous studies regarding the effect of Se on the central nervous system focused on the brain, the present study was the first one to compare and evaluate the effects of Se and Es on the expression of CatSper 1 and 2 genes in the SCI rat model.

## Materials and Methods

### Animals

This experimental study was performed on 50 male Wistar rats weighing 250-300 g. All procedures of this study were in accordance with the rules and procedures of the Ethics Committee of Tabriz University of Medical Sciences and the Helsinki Declaration on the use and care of the animals. The rats were kept in controlled temperature (23˚C and 50% humidity) and well-lit light, food and water access, and a 12:12 hour light/dark cycle. To adapt animals to laboratory conditions, they were brought to the operating room 3 days before the surgery.

The male rats were randomly divided into 5 groups of 10. Group I (the control group) received no intervention. Group II (the sham group) underwent only laminectomy while group III (the SCI group) underwent laminectomy and SCI without any intervention. Moreover, group IV experienced SCI and was treated by Se whereas group V experienced SCI and was treated by Es.

### SCI Model

Except for the control group, the other groups underwent surgery (Figure 1). The sham group only underwent laminectomy and the opened area was stitched, but rats in groups III, IV, and V were exposed to SCI after laminectomy using a SCI model device in the laboratory of the Neuroscience Center. This device (NsrcImpactor Home-made code: 90778) induces a standard model of traumatic injury in the spinal cord with high accuracy and speed in laboratory animals and then calculates the physical parameters affecting trauma such as displacement, force, and the time with the least changes (13). For SCI in the studied groups, moderate injury (equivalent to 150-175 kilo dynes) was entered into the T10 space to the spinal cord. Spinal segments located in the ninth and tenth thoracic vertebrae are of particular interest. Additionally,

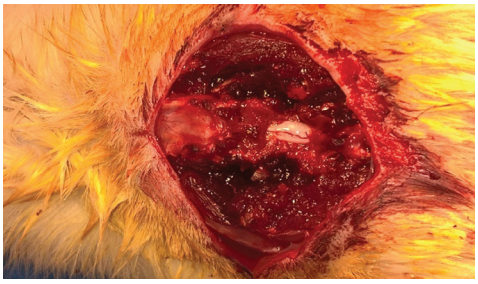

**Figure 1.** The Anesthetized Groups (except for the control group) Using Isoflurane 2.5% Inhalation.

these sequences are associated with the centers of the motion of lower extremity motions. Normally, the highest point of the spine is at the site of the last thoracic vertebra to the first lumbar spine (T13) and can be surgically determined to the number of the vertebrae (14). To limit postoperative pain, 5 mg/kg/d of Metacam was injected at the first 24-48 hours after injury. Before the intervention, each animal was individually kept in a cage with a thick straw in order to prevent non-surgical injuries of the recovery period. The pellet of the animals was soaked and poured into the cage to prevent the extra movement of the animal while eating and thus any possible damage. Bottles with long nozzles were used to supply water. Finally, the cage was replaced every 3 days and washed with alcohol 70% to minimize the risk of infection.

### Treatment

Forty-eight hours after the injury, group IV received 0.2 mg/kg of Se (Sodium selenite, Sigma-Aldrich Company, USA) for 6 consecutive days for 6 weeks via gavage (15). At the same time, group V experienced the Es in which 2 dipole electrodes were planted in 2 rostral and caudal regions relative to the SCI segment. The artificial dura was placed in the spinal cord and sutured after stitching these electrodes in the paravertebral muscles. Then, Es was performed at a frequency of 100 Hz with the intensity of below threshold for 1 hour per day for 6 consecutive days for 6 weeks (16).

### Sperm Preparation

After treatment termination, the euthanasia method was used to kill the animals so that each animal was exposed to 100% $CO_2$ in a chamber. Each rat testicle was separately and immediately frozen in liquid nitrogen at -80˚C to extract protein and RNA. Next, sperm specimens were floated in a culture medium (TCM) at 45˚C, 5% $CO_2$ and 37 ˚C for 60 minutes to eliminate any potential of cellular contamination. The top slice (1/2) of sperm samples was separated by centrifugation at 1200 g for 5 minutes at 25˚C and washed twice in TCM and then used for RNA extraction (17). To confirm the SCI, the specimens of the spinal cord in the groups were checked by a pathologist (Figure 2).

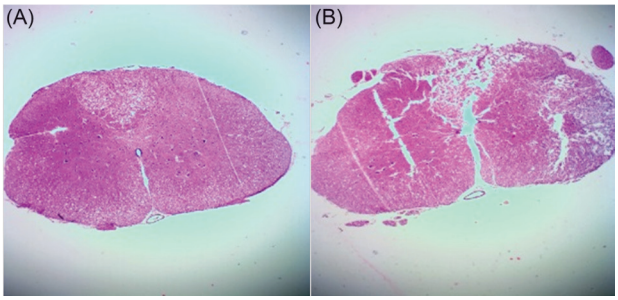


**Figure 2**. Histological Sample of Spinal Cord in Control (A) and SCI (B) Groups With a Force of 150 Kilo Dyne. *Note*. SCI: Spinal cord injury.

### Protein Extract Preparation

Radioimmunoprecipitation assay (RIPA) buffer was prepared as a sterile PBS1X (Sigma, Aldrich). Then, 50 µL of RIPA buffer was poured on each of the specimens, placed on the ice, and shaken for 45 minutes every 10 minutes. Next, a centrifuge was performed and 400-500 µL of supernatant were poured into new cryovials and used for dot blot (18). The Dot blot technique was used in this study. In addition, 10 µL of 100 µg/mL of primary antibody (Rabbit anti-rat CATSPER1 antibody and CATSPER2 biorbyt, LifeSpan BioSciences, Inc) and β-actin as an internal control were blotted onto the membrane, and it was ensured that the blots were dried completely. Then, the membrane was blocked with 5% skim milk in TBS-T for an hour at room temperature (RT). Next, it was shaken for half an hour and washed 3 times with TBS-T, and subsequently incubated with the primary antibody for 1 hour at RT in TBS-T. Further, the membrane was rinsed 3 times with TBS-T for 10 minutes each time, and the secondary antibody (HRP conjugated goat anti-rabbit IgG) coupled with HRP on the membrane was incubated for 30 minutes at RT, followed by rinsing it 3 times with TBS-T and TBS (5 minutes). Finally, ECL was incubated for 1 minute and imaged on 5, 10, and 15 minutes using the blot imaging system (19), the related data are illustrated in Figures 3A and 4A.

### RNA Extraction for Gene Expression Study

Total RNA was isolated by RNX plus (Cinnagen, Iran) solution according to the manufacturer's guideline and 500 µL of RNX plus solution was added to the sample and then incubated at RT for 20-15 minutes to be lyzed, followed by adding 500 µL of chloroform to the solution and centrifugation. Subsequently, the supernatant was taken and transferred to the new microtube. In the next step, the cold isopropanol was added as much as 1.5 times the sample size and centrifuged accordingly. After discarding the supernatant, 500 µL of ethanol 85% was added to the RNA pellet, then stored at -20 ˚C for 30 minutes. It was centrifuged after 30 minutes. Then, the pellet was dried at RT. Eventually, 50 µL of diethylpyrocarbonate water was added to the microtube and stored at -70 ˚C. The quantity and quality of the extracted RNAs were investigated by spectrophotometry at 280 and 260 nm and 1% agarose gel electrophoresis (20).

### cDNA synthesis and Real-time Polymerase Chain Reaction (RT-PCR)

To this end, 1 µg of each sample was used to prepare the cDNA. During this step, a strand of DNA nucleotides was generated from RNA and amplified by the PCR technique using a virus-derived reverse transcriptase. A general primer was then used to construct DNA, followed by designing a pair of reverse and forward primers for each gene using Oligo7 software. The endogenous controls glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene was used for the normalization of mRNAs (Table 1). To produce the cDNA, several wing materials were added to a 0.2-mL vial as the total RNA with the calculated volume (1 µL), a hexamer primer (1 µL), DNTP (1 µL), and nuclease-free water up to a final volume of 12 µL. The vial was then transferred to a thermo cycler and set at a temperature of 65˚C for 5 minutes. Next, the vial containing the reaction mix was immediately placed on the ice and other compounds were added, including an RNase inhibitor (0.5 µL), RT buffer (4 µL), MMLV enzyme (1 µL), and DEPS water up to the final volume of 20 µL. Next, the vial was transferred to the thermo cycler at 42˚C for 1 hour. It was subsequently continued for 10 minutes at 70 ˚C to stop the reaction and immediately placed on the ice and stored at -20˚C. Finally, RT-PCR was performed to measure the expression levels of Catsper1 and 2 genes using a SYBR Master Mix and Bio-Rad IQ5 RT-PCR detection system (Bio-Rad, Hercules, CA, USA) according to the standard protocols (20).

### Sperm Analysis

Sperm analysis was performed according to the World Health Organization guidelines to show some affected CatSper controlled sperm functions (15).

### Statistical Analysis

All data were expressed as the mean ± standard deviation and analyzed by SPSS software, version 21 using One-way ANOVA and the Tukey test as a post hoc. The results were assumed significant when $P < 0.05$.

## Results

### Effect of Selenium and Electrical Stimulation on CatSper1 Gene Expression in Rats With Spinal Cord Injury

According to Figure 3, the intensity of CatSper1 gene expression in the Se-fed group was notably higher compared to the Es group (40.04 ± 4.00 vs. 0.87 ± 0.07, respectively). The comparative intensity of the CatSper gene expression in the Se-fed group was greater compared to the control (40.04 ± 4.00 vs. 2.16 ± 0.08, respectively), sham (40.04 ± 4.00 vs. 2.14 ± 0.12, respectively), and SCI (40.04 ± 4.00 vs. 0.86 ± 0.06, respectively) groups, but the intensity of gene expression in the Es group was

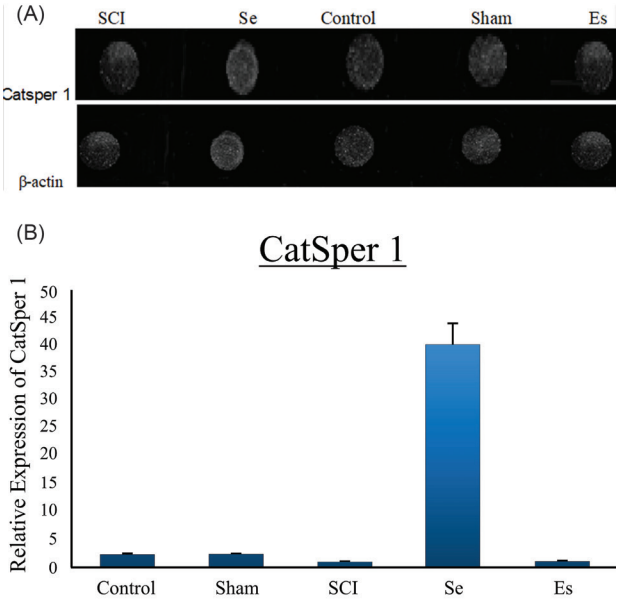


**Figure 3.** (A) The Expression of CatSper1 Proteins as Se > Control ≥ Sham > SCI > Es.(B) Comparison Between the Relative Gene Expressions of CatSper1 in the Studied Groups.
*Note*. SCI: Spinal cord injury; Se: Selenium; Es: Electrical stimulation; CatSper: Cation channel spermia. Values are shown as the mean ± SD. The experiments were replicated at least 3 times.

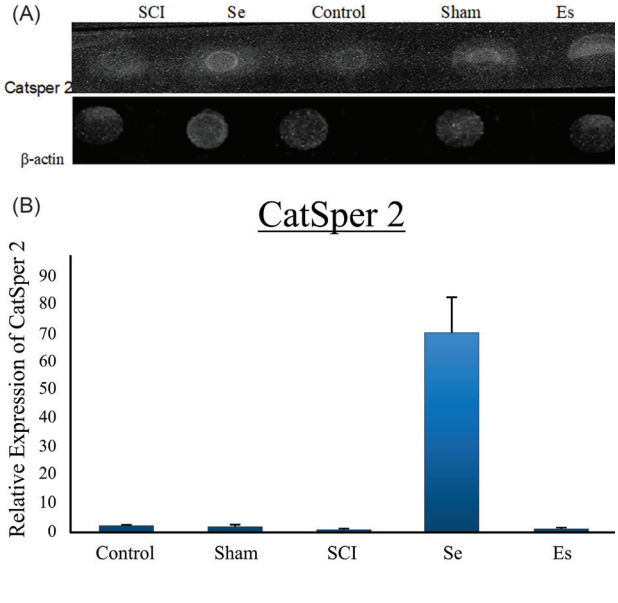


**Figure 4.** (A) The Expression of CatSper2 Proteins as Se > Control ≥ Sham > SCI > Es.(B) Comparison Between the Relative Gene Expressions of CatSper2in the Studied Groups.
*Note*. SCI: Spinal cord injury; Se: Selenium; Es: Electrical stimulation; CatSper: Cation channel spermia. Values are shown as the mean ± SD. The experiments were replicated at least 3 times.

equal to that of the SCI group (0.87 ± 0.07 vs. 0.86 ± 0.06, respectively) while less than the control ( 0.87 ± 0.07 vs. 2.16 ± 0.08, respectively) and sham (0.87 ± 0.07 vs. 2.14± 0.12, respectively) groups (Figure 3B).

### Effect of Se and Electrical Stimulation on CatSper2 Gene Expression in Rats With Spinal Cord Damage

Based on the data in Figure 4, the intensity of CatSper2 gene expression in the Se-fed group was remarkably higher compared to the Es group (70.11 ± 11.90 vs. 0.91 ± 0.50, respectively). In addition, the intensity of CatSper2 gene expression in the Se-fed group was greater compared to the control (70.11 ± 11.90 vs. 2.34 ± 0.09, respectively), sham (70.11 ± 11.90 vs. 2.13 ± 0.12, respectively), and SCI (70.11 ± 11.90 vs. 0.93 ± 0.08, respectively) groups. Moreover, the intensity of gene expression in the Es group was almost equal to that of the SCI group (0.91 ± 0.50 vs. 0.93 ± 0.08, respectively) but less than that of the control (0.91 ± 0.50 vs. 2.34 ± 0.09, respectively) and sham (0.91 ± 0.50 vs. 2.13 ± 0.12, respectively) groups (Figure 4B).

### The Effects of Se and Es on CatSper1 and 2 Proteins

The detection of CatSper1 and 2 proteins was performed using the dot blot method on the rat sperm. The expression of the proteins was compared to the changes in the β-actin expression in each sample as an internal control. As shown in Figure 4, the intensity of CatSper1 protein expression in the Se-fed group was higher than compared to Es, SCI, sham, and control groups but the expression proteins in the Es group were equal to that of the SCI group while less than the control and sham groups. Regarding the expression of Catsper2 proteins, the results showed that the expression of CatSper2 proteins in the Se-fed group was higher than that of the Es, control, sham, and SCI groups. Moreover, the expression of proteins in the Es group was more compared to SCI, control, and sham groups. Generally, dot blot results demonstrated that an increase in the expression of Catsper2 protein was greater than that of Catsper1 protein (Figures 3A and 4A).

### Sperm Analysis

To show that CatSpers control some sperm functions, sperm analysis was conducted after SCI and interventions. The results of analyzing sperm parameters are reported in Table 2.

As shown in Table 2, the concentration, motility,

**Table 1.** The Sequence of the Designed Primers Used for RT-PCR

| Gene | Reverse Primer | Forward Primers |
|---|---|---|
| CatSper1 | 5´-TCATGTTTACCTGTCTCTTCC-3´ | 5´-TCTATGTAGATGAGGGACCAG-3´ |
| CatSper2 | 5´-GGTTCTTGACAGTTCTATCTTC-3´ | 5´-CTATTTCGACCATCAGCAC-3´ |
| GAPDH | 5´-GTAGGTGAACCTGCGGAAG-3´ | 5´-TCCTCCGCTTATTGATATGC-3´ |

Note. RT-PCR: Real-time polymerase chain reaction; CatSper: Cation channel spermia; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

**Table 2.** Sperm Parameters in SCI Rat Model Under the Influence of Selenium and Wlectrical Stimulation

| Sperm Parameters | Control (Mean±SD) | Sham (Mean±SD) | SCI (Mean±SD) | Selenium (Mean±SD) | Electrical Stimulation (Mean±SD) |
|---|---|---|---|---|---|
| Concentration (Sperm/rat×$10^6$) | 43.66±6.53[c] | 36.00±4.15[c] | 18.50±7.02[ab] | 38.66±4.81[c] | 26.00±3.42[a] |
| PMS (%) | 83.33±6.05 | 70.00±20.00 | 8.33±1.69[b] | 55.00±3.82[c] | 60.00±6.32[c] |
| NPMS (%) | 13.33±6.05 | 22.5±8.37 | 28.33±4.83 | 24.16±4.28 | 27.00±6.70 |
| NMS (%) | 5.00±1.47 | 7.5±1.58 | 46.66±8.29 | 20.83±4.11 | 11.00±5.47 |
| Viability (%) | 86.66±8.16 | 85.00±6.32 | 19.16±1.68[ab] | 81.66±8.64[c] | 70.83±9.70[ac] |
| Morphology (Normal %) | 87.50±8.80 | 90.00±5.47 | 61.66±4.71[ab] | 78.33±1.25 | 70.00±10.95[b] |

*Note*. SCI: Spinal cord injury; SD: Standard deviation; a: Significant difference with the control group in the same row ($P$<0.05); b: Significant difference with the Sham group in the same row ($P$<0.05); c: Significant difference with the SCI group in the same row ($P$<0.05); PMS: Progressive movement of the sperm; NPMS: Non-progressive movement of the sperm; NMS: Non-movement of the sperm.

viability, and normal morphology of the sperms were lower in the SCI group as compared to the control and sham groups ($P<0.05$). Eventually, Se and Es improved these variables but Se improved these variables more than Es ($P<0.05$).

## Discussion

The intracellular $Ca^{2+}$ level has an important action in sperm motility (21). The evolvement of "CatSper channels" enhances the intracellular $Ca^{2+}$ level, and as a result, hyper-activates sperm (22). The finding of the present research revealed that Se treatment increases the expression of CatSper1 and 2 genes compared with SCI and control groups, but this increase was higher for CatSper2.

Catsper1 and 2 genes are specific flagellar proteins that have a more important role compared to Catsper3 and 4 in the sperm (23). In a study, notable down-regulation of CatSper1 and 2 genes was observed by 4 weeks after damage (4). Even though all CatSper subunits action in consonance with each other, CatSper1 and 2 mutations in humans with infertility have been found to be associated with asthenoteratozoospermia (24,25). The expression of CatSper1 is related to CatSper2 expression and contrariwise although the gene expressions of CatSper3 and 4 are not associated with the expression of CatSper1 and 2 genes (26-29).

The prevention of the overgeneration of ROS as a momentous issue has become the invitation of the scientists in the last years for realizing the mechanism of the function of antioxidants (8).

Se is a fundamental cofactor in enzymes intricate in the antioxidant defense system (30). The task of Se in keep safe versus oxidative damage via Se-dependent "glutathione peroxidases" (Se-GPx) in the mammals is well appointed (31,32). This fact was approved in a former study, indicating that Se could barricade secondary pathological events in traumatically damaged spinal cords and diminished functional deduction via its antioxidant properties (33).

In this study, the expression level of CatSper2 gene was higher compared to CatSper1. The CatSper2 protein resembles the "voltage-gated potassium channel" (34). This ion channel is located on the sperm flagellum, indicating its function in the arrangement of sperm motility. CatSper2 also mainly resembles another sperm-specific putative cation channel, namely, CatSper2 (34,35).

Based on the findings of our study, Es did not affect the relative intensity of CatSper1 and 2 gene expression after SCI. Brosenitsch and Katz found that calcium channels can straightly relate phasic membrane depolarization to the expression of the gene. Additionally, their results indicated phasic and chronic depolarization works through separate mechanisms in inducing gene expression in the neurons (36). These results suggested that Es might improve some of the functions in the late stage of SCI. The stringent mechanisms related to the increasing regeneration of axons in response to applied voltage inclination are not yet fully known. The development of axons with the usage of the electrical field has been suggested to intercede via "membrane-bound receptors" and some secondary messengers such as "adenyl cyclase" and interplay with other physiological neurotrophins which are located in the central nervous system (37).

Other feasible mechanisms contain a decline in the count of astrocytes within the damage site (38) and changes to post-traumatic spinal cord blood flow (39).

In another study, it was shown that the electric current of 0.25-3 Vrms for 4 minutes at 50 Hz had the least harmful effect on sperm motility (40). The results of this study revealed that higher doses of Es can have harmful effects. In our research, ES was performed at a frequency of 100 Hz with the intensity of the below threshold. According to previous studies, it is possible that different frequencies of ES have different results. The researcher did not find any available article regarding the direct impact of Es on the expression level of CatSper genes in this study, and Se acted better than Es in this study.

In addition, our research results on the effect of SCI on CatSper-controlling parameters showed that intervention with Se and Es were able to improve sperm parameters after SCI. As previously explained, this can be mediated by CatSper channels. Eventually, Se reduces ROS results in reduced DNA fragmentation and results in the increased

expression of the coat genes.

### Limitations of the Study

It was not possible to evaluate DNA damage using the TUNEL method and the Makler Counting Chamber and the lack of complete urination and the urinary tract infection that prevented the study from continuing. Therefore, the results of this study cannot be immediately generalized to humans.

### Suggestions for Further Research

- Using the combinations of these antioxidant supplements for this purpose;
- Using human samples to generalize the results to human models.

## Conclusions

In summary, the present study showed that Se, unlike the Es, increased the relative intensity of CatSper 1 and 2 expressions and this increase was higher in CatSper2.

## Acknowledgments

The authors of this article are grateful to the financial support of the Research Center for Neurosciences and "Drug Applied Research Center", "Tabriz University of Medical Sciences", and all those who helped us with this research.

## Conflicts of Interests

The authors declare that there is no conflict of interests.

## Ethical Issues

This article is part of a Ph.D. thesis registered in the Neurosciences Research Center and approved by the ethical committee of Tabriz University of Medical Sciences with the code of ethics IR.TBZMED.REC.1395.65.

## Financial Support

The Research Center for Neurosciences and Drug Applied Research Center, Tabriz University of Medical Sciences.

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