The Correlation of Gene Expression of Inflammasome Indicators and Impaired Fertility in Rat Model of Spinal Cord Injury: A Time Course Study

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Purpose: Expression assessment of the inflammasome genes in the acute and the chronic phases of Spinal cord injury (SCI) on adult rat testis and examination of associations between inflammasome complex expression and sperm parameters.

Materials and methods: In this study, 25 adult male rats were randomly divided into 5 groups. SCI surgery was performed at T10-T11 level of rats' spinal cord in four groups (SCI1, SCI3, SCI7, and SCI56). They were sacrificed after 1day, 3days, 7days and 56 days post SCI, respectively. One group remained intact as control (Co). CASA analysis of sperm parameters and qRT-PCR (ASC and Caspase-1) were made in all cases.

Results: Our data showed a severe reduction in sperm count and motility, especially on day 3 and 7. ASC gene expression had a non-significant increase on day 1 and 56 after surgery compared to control group. Caspase-1 expression increased significantly on day 3 post injury versus the control group (P = .009). Moreover, Caspase-1 overexpression, had significant correlations with sperm count (r = -0.555, P = .01) and sperm progressive motility (r = -0.524, P = .02).

Conclusion: Inflammasome complex expression increase following SCI induction. This overexpression correlates to low sperm parameters in SCI rats.

Keywords: spinal cord injury; infertility; testis, inflammasome; ASC; Caspase-1.

INTRODUCTION

Spinal cord injury (SCI) is a devastating clinical issue affecting 40 to 80 new cases per million population each year throughout the world and up to 90% of these cases are due to traumatic causes[(1)]. According to a study conducted by a Specialized Spinal cord injury center in Tehran, Iran, the incidence of SCI is up to 2.36 persons per 10000 population with an average age of 29.1 years. SCI patients are at higher risk of morbidity and mortality because of complications related to the injury. Iran has younger SCI cases more than other developing countries and about 80% of whom are male. SCI people are usually at reproductive age, so fatherhood is a grave issue for this population[(2)]. About 85% to 97% of SCI men suffer from impaired fertility caused by erectile dysfunction and ejaculatory problems[(3)]. There are some assisted methods such as penile vibration (PVS) and electroejaculation (EEJ) to obtain the semen from these patients. However, most SCI men have a low semen quality[(4-6)].

Although testicular tissue becomes involved post-SCI, a limited number of studies have addressed this issue. Impaired spermatogenesis, vast germ cell apoptosis, inflammatory cytokines elevation, blood-testis barrier disruption and leukocytes influx have been demonstrated as abnormal changes in testis after SCI inducing an inflammatory environment and unstable niche in this tissue[(7-9)].

The inflammasome is a multi-protein complex that is a part of the innate immunity. The main component of this complex is called nucleotide oligomerization domain–like receptor (NLR), an inter-cellular receptor for pathogenic and non-pathogenic signals. The other parts of complex consist of an adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and caspase-1 (Casp-1). The Inflammasome activation induces auto-cleavage of pro-Caspase-1 into the it's active form that leads to converting pro-Interleukin-1β (Pro-IL-1β) and pro-Interleukin-18 (Pro-IL-18) to the biological active forms (IL-1β and IL-18). These pro-inflammatory cytokines trigger other inflammatory cascades playing

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Table 1. Standard terminology for variables measured by computer-assisted sperm analyzer (CASA) systems.

Parameters	Unit	Description
Curvilinear velocity (VCL)	µm/seconds	Time-averaged velocity of a sperm head along its actual curvilinear path.
Straight-line velocity (VSL)	µm/seconds	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last
Average path velocity (VAP)	µm/seconds	Time-averaged velocity of a sperm head along its average path
Linearity (LIN)	%	The linearity of a curvilinear path, VSL/VCL
Straightness (STR)	%	Linearity of the average path, VSL/VAP.
Wobble (WOB)	%	A measure of oscillation of the actual path about the average path, VAP/VCL.

an important role in the innate immunity[10,11]. However, higher activity of the inflammasome complex and resulted inflammation can induce damages in the involved tissues and cause a rapid pro-inflammatory form of cell death (Pyroptosis)[12].

In 2011, Dulin et al. revealed disruption in Blood-Testis-Barrier (BTB) and immune cell infiltration into the rat testis tissue following SCI. Accordingly, they found the elevation of IL-1β 72h post-injury[9]. In a recent study, Fortune et al. assessed gene expression pattern and metabolomics in acute (24h) and chronic (3 months) phases of SCI in rat testis. They detected many transcripts and metabolites related to inflammatory, oxidative stress and apoptotic pathways. Therefore, they concluded that an unstable niche have been established in testis after SCI causing SCI-dependent male infertility[13].

Based on this background, SCI promotes an inflammatory microenvironment in the testis tissue leading to a large germ cells apoptosis. Inflammasome activation has not been reported in testis post-SCI in the literature. Therefore, we launched a time course study (Acute and chronic phases of SCI) regarding the role of essential genes (ASC and Casp-1) responsible for inflammasome complex in rat testis.

MATERIALS AND METHODS

Experimental animals

In this study, 25 male Wistar rats (weight 200-250 g, age 8–10 weeks) were used in a random sampling design. All animals were kept and maintained at 20–24 °C, 55 ± 10 % humidity and on a 12-hour light/dark cycle at the animal Laboratory Core Facility of Royan Institute, Tehran, Iran. They were fed by standard diet ad libitum, with access to tap water. All animal handlings, surgeries and cares were managed in compliance with the Tehran University of Medical Sciences ethics committee. The animals acclimatized to the laboratory at least one week before surgery.

Study design

The rats were randomly divided into 5 groups and SCI induction and sacrifices were performed based on the study time-line (**Figure 1**). Contusion injury model at T10-T11 levels was chosen because there is no direct innervation from these levels to testes. Also, this model has the most similarity to traumatic injuries of the SCI patients in the clinics. Surgeries for contusion injury induction were done at four groups: SCI 1, SCI 3, SCI 7 and SCI 56. Rats in each group were killed and analyzed at a specific time point that is to say one day, three days, seven days and 56 days after surgery. One group remained intact as control (Co).

SCI surgery

The rats were anesthetized with an intraperitoneal administration of ketamine (80 mg/ kg) and xylazine (10 mg/kg) mixture. A dissection along the midline of the cord was performed on the 10th to 11th thoracic (T10–T11) vertebrae to create a 4mm longitudinal cut. After cutting the muscles and tissues, the laminectomy was performed. The vertebrae around T10 were stabilized and the contusion injury model was induced by NYU MASCIS (New York University Multicenter Animal Spinal Cord Injury Study) impactor [14,15]. A 10-gram rod was dropped from a height of 25 mm. Complete contusion injury was obtained on spinal cord after 1-3 minutes (**Figure 2**). After surgery for recovery time, all rats were placed on a warm plate (38°C) for an hour. Manual bladder emptying was daily done from the day after surgery to remove residual urine, blood or any infection until the return of reflexive control of bladder function.

Behavioral test

From day one, all SCI rat models were functionally examined. Open field locomotor test was performed by the non-invasive, Basso, Beattie, Bresnahan (BBB) locomotor rating scale[16]. All animals with BBB scoring less than 2 were kept and others were removed from our study because of insufficient damage of spinal cord.

Epididymis sampling

The cauda epididymis was dissected before perfusion to avoid negative effects on sperm motility. This procedure was done with care without any damage to vessels in that area. Epididymis, after one or two incision, minced in 1 mL pre-warmed Ham's F-10 medium (St. Louis, MO, USA) fortified with bovine serum albumin (BSA, Sigma, Louis, USA) for 30 to 45 min in a 37°C incubator.

Semen analysis

After 30-45 min incubation, epididymal sperm released from the tissue and swim up into the medium. Sperm parameters were analyzed with a Computer-assisted Sperm Analyzer (CASA) as pointed out by Krause[17]. The CASA system consisted of a phase contrast microscope (Eclipse E-200, Nikon Co., Japan) with a heat plate equipped with Sperm Class Analyzer® software

Table 2. The sequences of rat specific primers for Caspase-1 and ASC cDNA. All primers were designed by Perlprimer v1.1.21.

Target gene	Primer sequence (5′–3′)	Annealing temperature(°C)	Gene bank code	Product size(bp)
Caspase-1 Forward	CTTTCTGCTCTTCAACACCAG	59.61	NM_012762.2	122
Caspase-1 Reverse	AATGTCCTCCAAGTCACAAGA	59.64		
ASC Forward	CCCATAGACCTCACTGATAAACT	59.55	NM_172322.1	127
ASC Reverse	GCTCCAGACTCTTCCATAATCTT	60.13		

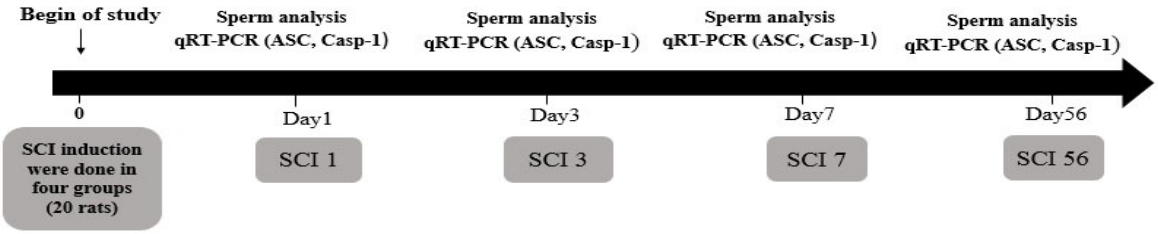

Figure 1. The time line of the study. There were four groups that have undergone SCI surgery. Rats in each group were killed at a specific time point (Day1, Day3, Day7 and Day56) post injury and epididymis (for sperm analysis) and testis (for real-time PCR) were dissected.

(SCA, full research version 5.1, Microptic Co., Barcelona, Spain). In order to make sperm analysis, 4 µl sperm samples were placed in a standard count analysis chamber (Leja, Nieuw Vennep Co., Netherlands). Specimens were observed with a Nikon microscope 10x/0.25 negative phase contrast field Ph1 BM, with an intermediate magnification of 0.7 and a green filter. At least 400 spermatozoa were counted for each sample. (**Table1**)

Testis sampling and RNA extraction

At each of the time points of study, the tissue reperfusion was done with normal saline to eliminate blood from all tissues especially testes. After 45-60 min, testes were dissected and cut into the three parts. Each part was snap frozen in liquid nitrogen and stored individually in -80□C, for further investigation. RNA extraction procedures were done under an RNase-free condition. Total RNA was isolated from testis samples using TRIZOL reagent (Sigma, St. Louis, MO, USA), based on the manufacturer's protocol. Briefly, samples were warmed at lab temperature. Then, they were placed in 1.5 mL RNase free tubes and homogenized thoroughly with a needle. Afterward, 800 µl (1 ml per 50 to 100 mg tissue) TRIzol® Reagent was added to each tube and homogenized by hand with a tissue-homogenizer tip until tissue was completely dissociated. Then, 200 µl chloroform was added to each tube, capped tightly and shaken firmly. After 3 min incubation in lab temperature, the tubes were centrifuged at 12000 rpm, 15min, and 4□C. The aqueous (top) phase (containing RNA) was decanted in another RNase-free tube and the same volume of 100% isopropanol was added to the tube for RNA precipitation. Tubes are placed in -20°C for an hour and centrifuged (12000 rpm, 15min, and 4°C). The pellet was washed with 70% ethanol, air dried and dissolved in diethyl pyrocarbonate (DEPC) treated water. The extracted RNA was quantitated at 260 nm (NanoDrop 2000 spectrophotometer, Thermo Scientific, Wilmington, DE).

Quantitative Real-time PCR

The isolated RNA was reversely transcripted to complementary DNA (cDNA) using Primescript RT reagent kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer guidelines. Primers were designed by PerlPrimer software version 1.1.21 (Marshall, 2004) and the sequences were listed in **Table 2**. The mRNA expression levels of the genes (ASC and Casp-1) were quantified using ABI/StepOnePlus Real-Time PCR System (Applied Biosystems). All real-time PCR assays were run in a total reaction volume of 20 µL. The result was shown as relative gene expression by the comparative Ct method ($2^{-\Delta\Delta Ct}$)[18]. All Ct values were determined and normalized in comparison to a housekeeping gene (b-actin). Relative quantification was calculated by StepOneTM Real-Time PCR Software version 2.2 (Thermo Fisher Scientific, Waltham, MA).

Statistical analysis

All data were analyzed using SPSS statistical software version 22.0 (SPSS Inc., Chicago, IL). Results were expressed as mean ± standard error of the mean (S.E.M). Analyses of the parametric data were done by one-way analysis of variance (ANOVA) with Turkey's post hoc statistical tests. Non-parametric data were analyzed statistically using Kruskal-Wallis Test (nonparametric ANOVA) and Dunn's Multiple Comparisons for post-test. In all analyses, $P < .05$ was set as a significant level.

RESULTS

Semen analysis

Sperm parameters were evaluated by recruiting CASA system and statistically analyzed compared to those of the control group and $P < 0.05$ was considered statistically significant (**Table 3**). Sperm concentration was significantly lower in comparison to the control group, on day 1, 3 and 7 after SCI ($P = .034$, $P = .002$ and P

Table 3. Effects of SCI on sperm parameters in rats, on day 1, 3, 7 and 56 after injury. (Mean ± Standard error)

	Groups				
	Co	**SCI 1**	**SCI 3**	**SCI 7**	**SCI 56**
Count (106 /ml)	27.64 ± 2.33	15.38 ± 1.41*	9.92 ± 0.77*	14.99 ± 1.47*	24.13 ± 4.95
Total Motility (%)	83.22 ± 3.52	52.09 ± 9.39	44.28 ± 4.58*	57.66 ± 0.93	52.19 ± 15.67
Progressive Motility (%)	64.11 ± 7.68	31.66 ± 7.44	19.11 ± 2.89*	28.02 ± 1.36*	42.24 ± 14.26
Non-progressive Motility (%)	19.10 ± 4.59	20.42 ± 4.82	25.16 ± 1.99	29.62 ± 0.70	9.72 ± 1.66
Immotile sperms (%)	16.79 ± 3.52	47.91 ± 4.70	55.71 ± 4.58*	42.34 ± 0.93	47.78 ± 15.64
VCL (%)	107.40 ± 12.61	53.71 ± 6.57*	35.65 ± 9.07*	41.03 ± 9.85*	76.60 ± 20.20
VSL (%)	28.62 ± 1.61	13.02 ± 3.69*	5.87 ± 1.20*	8.56 ± 2.74*	15.78 ± 5.30
VAP (%)	47.41 ± 3.96	26.37 ± 4.84	14.80 ± 4.24	16.99 ± 4.76	37.53 ± 10.78
LIN (%)	27.76 ± 3.60	23.30 ± 3.79	17.89 ± 1.98	18.72 ± 3.09	18.95 ± 2.84

*$P < 0.05$ compared to Co

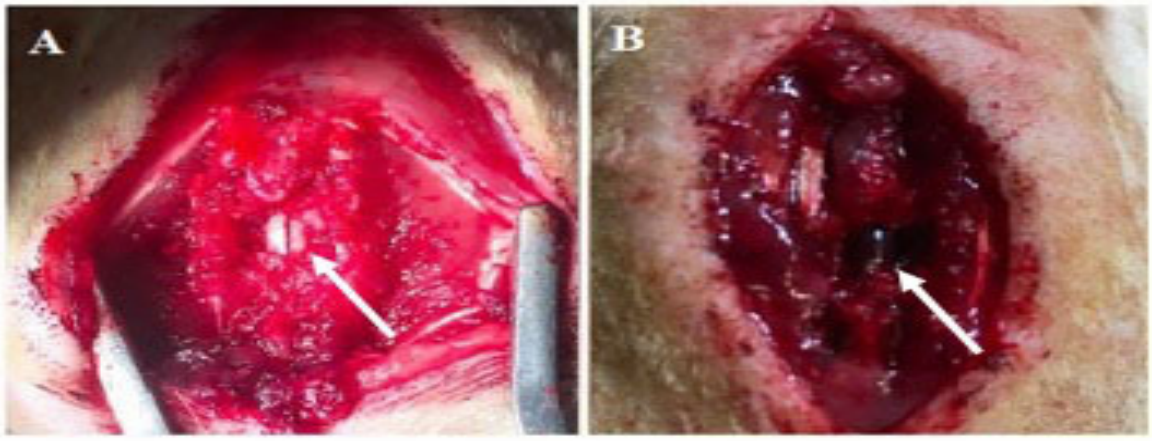

Figure 2. Rat model of spinal cord injury (T10-T11): A. Exposed spinal cord following a dorsal laminectomy procedure. B. bruising of the spinal cord after contusion induction

= .028, respectively). After 56 days post injury, sperm count had returned almost to amount of control group. Total motility (%) had a decline in all groups, but it was significant just on 3 day group, compared to control group (P = .02). Sperm progressive motility was reduced significantly on day 3 and 7 after SCI (P = .01 and P=.034, respectively). Non-progressive motility had no significant differences at any time points, compared to that of control. The most immotile sperm number was observed on day 3, although there was a non-significant growth in immotile sperm percent at four-time points. VCL and VSL (%) significantly reduced on day 1, 3 and 7, respectively. But the most reduction was seen on day 3. There was a decrease in VAP and LIN levels at every mentioned time points post-injury, but the changes were not significant.

ASC and Casp-1 mRNA Gene expression

ASC and Casp-1 mRNA expression in rat testes of the control group, without any intervention, was at a low basic level (**Figure 3**, Co). In one day group the amount of mRNA expression level for ASC increased, but it was not significant. The ASC had a non-significant expression peak again, 56 days after SCI (**Figure 3**). Casp-1 also had an increased expression only one day after SCI. The peak level of Casp-1 was on day 3 (P = .009). After that, the expression level became lower, even on 56 days post-SCI.

The correlation of gene expression and sperm parameters

The correlations were assessed between sperm parameters and gene expression (**Figure 4**). Sperm count correlated negatively to Casp-1 expression (r = -0.555, P = .01). Moreover, there is a significant negative correlation between sperm progressive motility and Caspase-1 expression (r = -0.524, P = .02)

DISCUSSION

Impaired fertility is a common feature of men with SCI that is attributed to erectile and ejaculation dysfunctions. In most cases, these problems are solvable with some methods like PVS and EEJ. However, semen quality of these patients is often poor. The majority of the studies have emphasized that low sperm motility and viability, leukocytospermia, and high sperm DNA fragmentation are common among SCI men. Many reports indicate that sperm count remained unchanged following SCI[6,19]. In the present study, we examined sperm parameters of SCI rats at the acute (1,3,7 days after injury) and the chronic (56 days after injury) phases. Interestingly, sperm count fell by half just after one

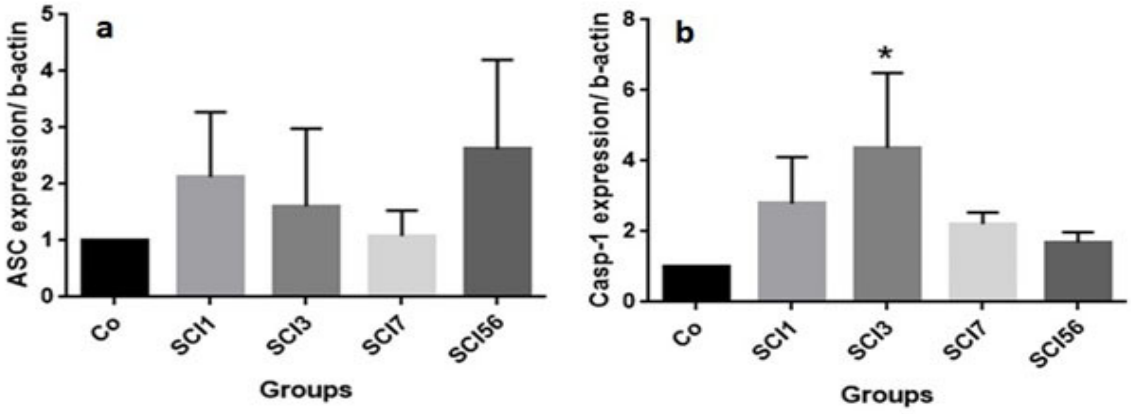

Figure 3. Effects of SCI on gene expression of ASC and Caspase-1 in rat testis, on day, 1, 3, 7 and 56 post injury (*P < .05 compared to Co).

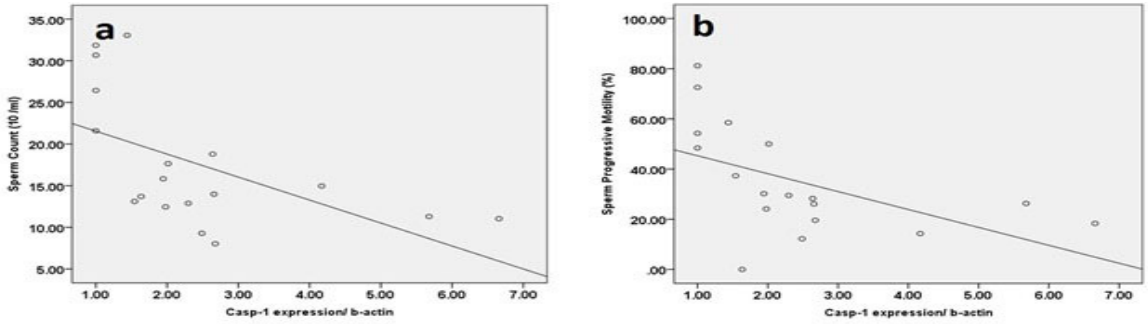

Figure 4. The correlation of Casp-1 expression and sperm parameters of rat model of spinal cord injury. a. Casp-1 overexpression was significantly correlated to decline in sperm count (r = -0.555, *P* = .01). b. Sperm progressive motility reduced with increase in Casp-1 expression (r = -0.524, *P* = .02).

day and a third after 3 days post-injury. This severe reduction was significant in the acute phase (1,3 and 7 days) but after 56 days (chronic phase) it had rebounded almost to the control group level. Sperm motility had a sharp decline after injury, as well. Total motility decreased in the acute (1,3 and 7 days after injury) and the chronic (56 days after injury) phases of SCI but it was statistically significant just on day 3. The most effect of SCI was on progressive motility with a significant reduction on day 3 and 7. SCI caused a large increase in the percent of immotile sperm, especially on day 3 with a threefold increase.

Most studies have analyzed sperm parameters post-SCI in human samples, and few researches have been conducted on the experimental models. In this field, the majority of studies indicated that sperm count after SCI was normal and most changes happen in sperm motility and viability[19]. Interestingly, our study showed a rapid decline in sperm count in the acute phase of SCI.

Low sperm motility in our research is in concordance with previous studies that they have been mentioned it as one of the most important causes of impaired fertility following SCI[19,20]. However, some investigations have claimed seminal plasma of SCI men is toxic to sperm, and cauda epididymis and vas deferens[21,22] have sperm with better quality[23,24]. In the present study, we analyzed sperm from caudal part of epididymis, to diminish toxic effects of ejaculated semen on sperm motility after SCI. intriguingly, total motility dropped rapidly just after one day, and it remained low after 56 days from surgery. It seems that the greatest effect of SCI is on progressive motility, especially on day 3 and 7 post-injury (Acute phase).

Although many reasons have been raised for male subfertility following SCI, spermatogenesis defects, large germ cell apoptosis and inflammatory conditions in testicular tissue are not well-defined. In this regard, only a few investigations have been performed on experimental models. Huang et al. carried out a time course study (3, 7, and 14 days after the SCI induction) on spermatogenesis abnormalities following SCI on male rats. They showed delayed spermiation and vacuolization of the nucleus of spermatids just 3 days after SCI. Other spermatogenic abnormalities were observed on day 14 group. Also, they demonstrated that hormone alteration is not the only reason for the impaired spermatogenesis following SCI[25]. Choobineh et al. reported exogenous testosterone therapy after SCI in adult mice could not compensate sexual hormone insufficiency and it caused reduction in natural testosterone production of testes[26]. For the first time, Dulin et al. (2011) illustrated that Blood-testis barrier (BTB) integrity was disrupted after SCI on male Sprague-Dawley rats. In that study, BTB became permeable to immunoglobulin G at both 72 hours and 10 months post SCI. The results indicated immune cell infiltration into the testis tissue and high expression of the pro-inflammatory cytokine IL-1β. Moreover, widespread germ cell apoptosis was observed at 72 h after SCI[9]. In 2016, Fortune et al. showed many pathological events in testis in both acute and chronic phases of SCI. They revealed a pro-inflammatory environment established after SCI in rat testis. Afterwards, other inflammatory cascades are activated resulting cell cycle dysregulation and apoptosis within the seminiferous tubules[13].

Inflammasome is an inflammatory complex that is activated under pathogenic and non-pathogenic conditions. Association of this complex with many diseases was previously detected[23-25], but it is not clearly defined in male infertility. Ibrahim et al. (2013) showed higher concentrations of some inflammasome indicators including IL-1□, IL-18, Casp-1 and ASC in the semen of SCI affected men with chronic injury and even the inflammasome suppression treatments have been applied to improve the semen quality in SCI patients[27,28].

In this study, we evaluated the expression of ASC and Casp-1 in rat testis at the acute (1,3 and 7 days) and the chronic (56 days) phases of SCI. Interestingly, both of two genes were expressed higher than the control group, just after one day post-injury. ASC expression was high on day 1, dropped on day 3 and 7 and again raised on day 56 after injury but it was not significant at of the four time points of study. ASC is an adaptor protein that makes a connection between NLRs and Casp-1. However, recent investigations showed that some NLRs can be attached to Casp-1 directly without ASC (29). It means that ASC overexpression can indicate inflammasome activation but is not necessary.

Casp-1 is the best-known type of the inflammatory caspases and it is an indispensable component of inflammasome complex. There are some pathways to activate the inflammasome complex (canonical and non-canonical), but in all pathways, Casp-1 is necessary for inflammasome formation and activation[30,31]. In this study, Casp-1 expression increased more than two-fold on day 1 after injury and peaked on day 3 with a four-fold increase, compared to the control group. Following that, Casp-1 expression dropped on day 7 and remained

unchanged on day 56 after injury. Since the Casp-1 is a direct marker of the inflammasome, it seems the SCI could activate inflammasome gene expression.
Our data showed there are significant correlations between some sperm parameters and inflammasome gene expression. Casp-1 expression negatively correlated with sperm count and progressive motility. The expression of Casp-1 elevated after one day, and it was in the highest level on day 3 post-injury. As we know Casp-1 is the critical enzyme of the inflammasome complex (Interleukin-1□/18 converting enzyme or ICE) activating pro-inflammatory cytokines of IL-1□ and IL-18. These cytokines have been known as the negative factors on the sperm parameters, especially on motility (22). In the current study, a sharp decline in sperm progressive motility was detected on day 3 and 7 after injury. It seems that this reduction could happen secondary to the Casp-1 overexpression on day 1 and 3. Identification of gene expression pattern of inflammasome in testis during the acute and the chronic phases of SCI is essential to therapeutic purposes.

CONCLUSIONS

ASC and Casp-1 are two inflammasome specific genes and are not related to other signaling pathways. Therefore, the expression of those genes on rat testis following SCI can indicate an abnormal cell situation. With these data, it seems there is a pattern in inflammasome gene expression in both acute and chronic phases of SCI. Such a pattern has been specified in other tissues but not in testis. Moreover, SCI had negative effects on sperm progressive motility that correlated negatively with Casp-1 overexpression.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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