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Involvement of Asc and Nlrp3 inflammasomes in the testes following spinal cord injury

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Objective. The exact mechanism, by which spinal cord injury (SCI) leads to a male subfertility is not well-known. Present study was conducted to determine the mechanisms that lead to the elevated end-product cytokines and inflammasomes in the testes of an SCI rat model. Moreover, we evaluated the inflammasome components following SCI in testis over a defined time periods.

Methods. Weight drop technique was used to induce SCI at the level of the T10 vertebra in male Wistar rats. The animals were sacrificed at specific time intervals (3, 7, 14, 21, and 28 day's post-SCI). mRNA levels of inflammasomes and cytokines were measured by real-time PCR, germ cells apoptosis was evaluated by TUNEL staining, and the epithelium of seminiferous tubules by Miller's and Johnsen's scores.

Results. The results showed activation of *Nlrp3* in the testes of SCI animals at different time points. Expression of *Nlrp3* and *IL-1β* sharply increased 14 days after the SCI. Upregulation of *IL-1β* and *IL-18* at days 14 and 21 post-SCI might disintegrate the epithelium of seminiferous tubules at day 14 and induce germ cells apoptosis, increase abnormal sperm cells, and attenuate motility and viability at 21 days post-SCI.

Conclusion. This study provided further evidence of innate immunity activation in testes that could lead to more disruption of spermatogenesis in SCI patients at specific times.

Key words: inflammasomes, spinal cord injury, testis

The majority of men with spinal cord injuries (SCI) have evidence of impaired infertility due to erectile dysfunction, ejaculation disruption, and poor sperm quality. In the United States, the average age for SCI patients is 33.9 years, 80.7% of which are male. Although erectile dysfunction and ejaculation discrimination can be treated, no specific treatment exists to improve sperm quality in SCI patients (Zhang et al. 2013).

SCI consists of an acute phase associated with an inflammatory response, which persists in the chronic phase and leads to tissue destruction and axonal loss. The inflammatory processes after SCI lead to germ cells apoptosis (Bao et al. 2004). Pro-inflammatory cytokines disrupt the blood-testis-barrier (BTB) integrity and permeability (Sarkar et al. 2008). Therefore, abnormalities in semen quality arise from inflammation that follows the SCI and result in disruption to the BTB. These occurrences cause infiltration of immune cells to the damaged sites (Hultling et al. 1994).

Pro-inflammatory cytokines, such as interleukins IL-1β and IL-18, are structured similarly. These cytokines are produced in inactive precursor forms that activate by a multi-protein complex known as an inflammasome. They play crucial role in the innate immunity and induction of the inflammatory response that contributes to the death of cells

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(Zhang et al. 2013). Inflammasomes are comprised of three main components: apoptosis-associated speck-like protein (Asc) that contains caspase activation and recruitment domain, caspase-1, and nucleotide-binding oligomerization domain-like receptor (NLR) family. There is a significantly higher rate of the mentioned components in the semen of SCI patients (Zhang et al. 2013). Following activation of *Nlrps* (*Nlrp1* and *Nlrp3*), Asc as an adaptor protein is employed and subsequently interacts with the CARD domain of pro-caspase-1. This interaction leads to caspase-1 activation and alters the pro-inflammatory forms of IL-1β and IL-18 into their biologically active forms (Zendedel et al. 2016). To the best of our knowledge, no study has shown that the inflammasome components in testes tissues at different time periods are differentially regulated after SCI. Increasing pro-inflammatory cytokines such as IL-1β and IL-18 in human semen following SCI leads to increased Asc and caspase after inducing abnormality of semen (Patki et al. 2008). However, neutralization of Asc leads to reductions in these cytokines with subsequent amelioration of sperm motility (Ibrahim et al. 2014).

Since, the role of Asc and Nlrp3 inflammasomes and IL-1β and IL-18 cytokines in BTB destruction and disturbance of spermatogenesis following SCI has been demonstrated, this study was conducted to find a specific time course, in which inflammasome genes overexpressed in the testicles after SCI as well as elucidating their relationships with spermatogenic cells apoptosis, so that future therapeutic interventions can be performed before chronic and permanent consequences affect spermatogenesis.

Materials and methods

Animals. Sixty-six adult male Wistar rats were obtained (200–250 g) from the Animal House at Zanjan University of Medical Sciences (ZUMS; Zanjan, Iran). The Animals were maintained in a pathogen-free and climate-controlled environment with access to clean drinking water and standard laboratory diet (Pars Dam Co., Zanjan, Iran). They were kept at a temperature of 24±1 °C, relative humidity of 45–55%, and a 12:12 h dark/light cycle. They were allowed to adapt for at least one week in the animal room before surgery. The ZUMS Ethics Committee approved the current research and animal care procedures.

Animal subjects and surgeries. The 66 rats were divided into 11 groups (n=6 per group) as follows: a control group, and 5 sham and 5 SCI groups from days 3, 7, 14, 21, and 28 after surgery. Animals in the control group were left intact with uninjured spinal cords. The sham-operated groups underwent the same surgical procedures and laminectomy as the SCI groups at the above-mentioned time points, but without any spinal cord contusion. The exposed spinal cords were injured in the SCI groups. The rats were sacrificed after 3, 7, 14, 21, or 28 days. Based on similar results from the behavioral test in the sham (laminectomy) group, it was considered as calibrator in the qPCR analysis.

All SCI surgeries were performed based on the Kearney et al. (1988) protocol. Briefly, the rats were anesthetized with intraperitoneal injections of 80 mg/kg ketamine and 10 mg/kg xylazine. The back region of each rat was shaved and the spinal cord exposed at the T8–T10 vertebra level. A complete single level (T10) laminectomy was performed. A severe contusion injury was delivered by dropping a 10 g rod from a height of 25 mm. Immediately following the injury, the overlying muscles and skin layers were sutured. The animals received subcutaneous injections of 2 ml lactated Ringer's solution for 3 days and daily intramuscular injections of 50 mg/kg cefazolin also for 3 days to prevent infection. The animals' bladders were manually emptied twice every day for 2 weeks.

Behavioral test. The Basso, Beattie, and Bresnahan (BBB) (Basso et al. 1995) locomotor rating scale were used to assess movement from zero (complete paralysis) to 21 (normal gait). Each animal was placed individually in an open field that had a diameter of 110 cm and a height of 50 cm. The animal was allowed to wander freely. Five BBB tests were performed collectively on days 1 and 3 as well as 2, 3 and 4 weeks post-injury. The mean scores of the bilateral hind limbs at day 3 and weeks 1–4 were recorded for each of the rats in the experimental groups.

Laboratory analyses, body and reproductive organ weights. Weight gain was considered as the difference between the initial and final body weights for each group of rats. The animals were anesthetized after obtaining their weights. Subsequently, we removed and weighed one testis from each of the rats. The testes were maintained at –70 °C until real-time RT-PCR analysis. Each rat was perfused transcardially with saline, glutaraldehyde, and 4% paraformaldehyde (mass/vol) (Merck, Germany) in PBS (Sigma, USA). The other testis was fixed in a 10% neutral buffered formaldehyde solution and kept for histopathological analyses. The selected testes sections were stained with hematoxylin and eosin (H & E), and assessed according to the TUNEL assay. The accessory sex organs (seminal vesicles, prostate, and epididymides) were dissected, cleaned of fat,

and weighed. The weight of the testicles was calculated as percentage in comparison with body weight. The lesion area in the spinal cord was removed and paraffin-embedded for H & E staining. The mean cavity percentage in a 3000 µm length of injured spinal cord was evaluated by Image J 1-44 software (Abdanipour et al. 2012).

Sperm characteristics and histopathological evaluation of spermatogenesis. In order to assess sperm characteristics, one epididymis was randomly selected. The caudal section was dissected, minced in 5 ml pre-warmed Ham's F10 (Sigma, USA) solution, and incubated for 15 min at 37 °C. A total of 10 µl of the pipetted sperm suspension was placed on a slide and cover slipped for evaluation of sperm motility with an optical microscope (Olympus BX51, Tokyo, Japan). A total of 10 microscopic fields were observed at 400x magnification by counting more than 200 spermatozoa. Progressive and in situ motility were expressed as percentages of the total motility of sperm according to Shokri et al. (2010). A prepared slide was subsequently examined for the presence of abnormal spermatozoa in at least 10 fields at 400× magnification. Abnormal spermatozoa were defined as those with abnormalities in the head, neck, and tail sections. The suspension was diluted with saline, placed on an erythrocytometer, and examined under a light microscope (Olympus, Japan) to determine the sperm count (Shokri et al. 2010). In order to categorize spermatogenesis, the numbers of germinal epithelial layers were counted in 10 seminiferous tubules as described by Miller et al. (1990). Johnsen's method assigned a score of 1–10 for each tubule cross-section according to the presence or absence of the main cell types arranged in the order of maturity (Johnsen 1970).

Germ cells apoptosis. The TUNEL assay kit (Roche, Germany) was used to evaluate germ cells apoptosis. Briefly, 6 µm thick paraffin-embedded sections were deparaffinized and rehydrated in a graded alcohol series. The slides were microwave-pretreated in 10 mM citrate buffer (pH 6.0) for 10 min and incubated with blocking solution that consisted of 3% H_2O_2 in methanol (Merck, Germany) for 10 min. After washing in PBS, the specimens were incubated with TUNEL reaction mixture comprised of terminal deoxynucleotidyl transferase and nucleotide mixtures in a reaction buffer at 37 °C for 60 min. After washing, the slides were stained with Converter-POD anti-fluorescein antibody, and conjugated with horse-radish peroxidase-POD at 37 °C for 30 min. DAB substrate (Roche, Germany) was applied for color development. Positive cells that contained fragmented nuclear chromatin characteristic of apoptosis exhibit a brown nuclear stain. For each group, we have counted the numbers of stained cells from 10 tubules in non-necrotic areas. The apoptotic index-1 (AI-1) was defined as the number of tubules that contained apoptotic cells per 100 tubules. The apoptotic index-2 (AI-2) was the number of apoptotic TUNEL-positive cells per 100 tubules. A pathologist blinded to the source of testicular tissue performed all measurements (Shokri et al. 2010).

Table 1
List of primers.

Primer	Sequence (sense/antisense)	Base pair	Annealing temp.
GAPDH	AACCCATCACCATCTTCCAG GTGGTTCACACCCATCACAA	143	62
IL-1β	TGGCAACTGTCCCTGAACTC GTCGAGATGCTGCTGTGAGA	170	62
IL-18	GGACTGGCTGTGACCCTATC TGTCCTGGCACACGTTTCTG	152	61
NLRP3	TCTGTTCATTGGCTGCGGAT GCCTTTTTCGAACTTGCCGT	314	65
ASC	GCTGCAGATGGACCCCATAG ACATTGTGAGCTCCAAGCCA	80	64

Real-time PCR. The amount of total RNA extracted from testes in all groups and purity of the extracted RNA by a spectrophotometer (Nanodrop 1000, PeqLab, Germany) was established. A total of 1 µg purified RNA (DNA-free) was applied to synthesize 20 µl of cDNA according to a Revert Aid™ First Strand cDNA Synthesis kit (Fermentas, Germany). cDNA (0.5 µg) was used to quantify *NLRP3*, *IL-18*, *IL-1β*, and *ASC* mRNA levels. As an internal control, we used primers for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Table 1 lists the applied primers and analyzed genes. The PCR reaction was synthesized in a 13 µl volume (sense and anti-sense primers, cDNA, Sybr green) and carried out for 40 cycles in an Applied Biosystems cycler (USA). The Pfaffl method was used to analyze relative changes in mRNA levels according to the following equation (Yuan et al. 2006):

$$\Delta Ct_{target} = Ct_{control} - Ct_{treatment} \text{ and } \Delta Ct_{reference} = Ct_{control} - Ct_{treatment}$$

All mRNAs were normalized for *GAPDH* mRNA.

Statistical analysis. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, SPSS, Inc., USA) version 16. All data are presented as mean ± standard error of mean (S.E.M.). One-way ANOVA followed by Tukey's post

hoc comparison test was used to compare multiple means within groups for all experiments. The statistical significance level was set at p<0.05.

Results

Behavioral assessment. Figure 1 shows a comparison of mean BBB scores from all SCI and sham groups. The mean BBB scores in the SCI groups gradually increased at day 28 (3.37±0.31) post-SCI. The results of the mean BBB scores in the SCI groups for days 3 (1.26±0.19), 7 (2.35±.0.31), 14 (1.96±0.32), and 21 (2.85±0.31) did not significantly differ. The mean BBB scores in the sham groups were approximately 20 with no significant differences.

Histological assessments of spinal cord tissue cavities. Histological assessments were performed 4 weeks post-SC. Image J software analysis of the percent of cavity present in 3000 μm length of the injured spinal cord showed significant differences between days 3, 7, 14, 21, and 28 (Figure 2A). The mean cavity percentage at days 14 (16.51±1.2) and 21 (16.78±0.67), and 28 (16.74±0.68) significantly (p=0.05) increased in comparison to days 3 (6.75±0.47) and 7 (10.79±1.08) (Figure 2B). No alterations were observed in the spinal cords of the sham groups. All groups were compared with the sham (laminectomy) group.

Body weight gain and sex organ weights. Mean body weight gain drastically decreased at the different time points post-injury compared to the control-operated animals (p<0.0001). Decreases were observed at days 3 (–13.75±5.9), 7 (–27±4.63), 14 (–27±4.73), 21 (–16.66 ±6.66), and 28 (–15±2.88) compared to the control group (48.33±6) (Figure 3).

According to Table 2, the relative weights of the left testes increased on days 3 (0.61±0.01), 7 (0.57±0.04), 14 (0.61±0.02), 21 (0.57±0.02), and 28 (0.59±0.03) compared to the control animals (0.61±0.01). However, these findings held no statistical significance. The relative weights of the right testes increased on days 3 (0.57±0.005), 7 (0.57±0.03), 14 (0.55±0.02), and 28 (0.59±0.04), but not on day 21 (0.54±0.02), compared to the control animals (0.56±0.02). These findings were not statistically significant.

The relative weights of the right epididymis increased at days 3 (0.18±0.007), 7 (0.18±0.01), 14 (0.18±0.01), 21 (0.17±0.002), and 28 (0.17±0.008) compared to the control animals (0.16±0.01), although it was not statistically significant. The relative weights of the left epididymis increased at days 3 (0.19±0.01), 14 (0.18±0.009), and 28 (0.18±0.01) compared to the control animals (0.17±0.006). Weights of the left epididymis did not increase on days 7 (0.16±0.01) and 21 (0.16±0.01) compared to the control group. These findings were not statistically significant.

The relative weights of the right seminal vesicles increased at days 3 (0.12±0.01), 7 (0.13±0.005), 14 (0.12±0.01), 21 (0.13±0.01), and 28 (0.12±0.01) compared to the control animals (0.11±0.003). These increases were not statistically significant. The relative weights of the left seminal vesicles increased at days

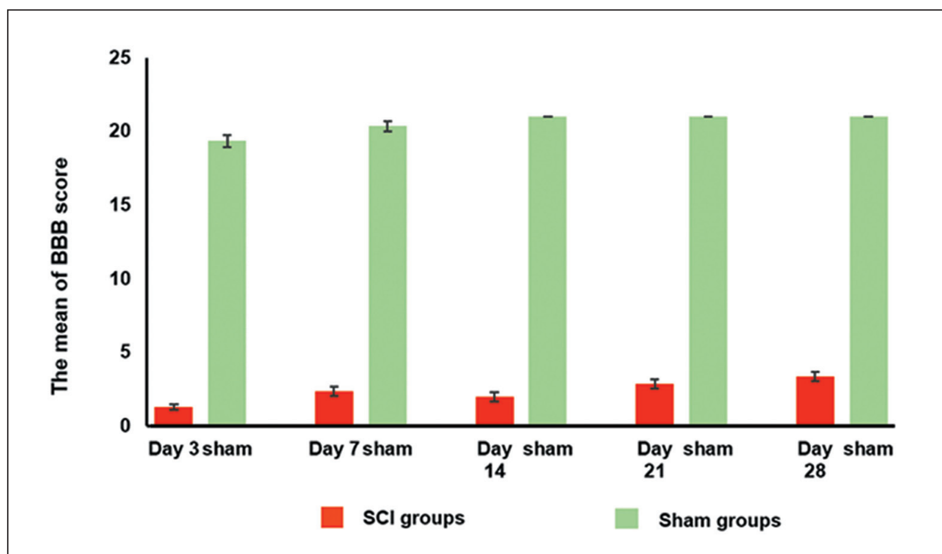

Figure 1. The Basso, Beattie, and Bresnahan (BBB) scores were obtained from days 3, 7, 14, 21, and 28. This is exhibited numerical difference (delta number) of BBB scores between day 3 and others time points.

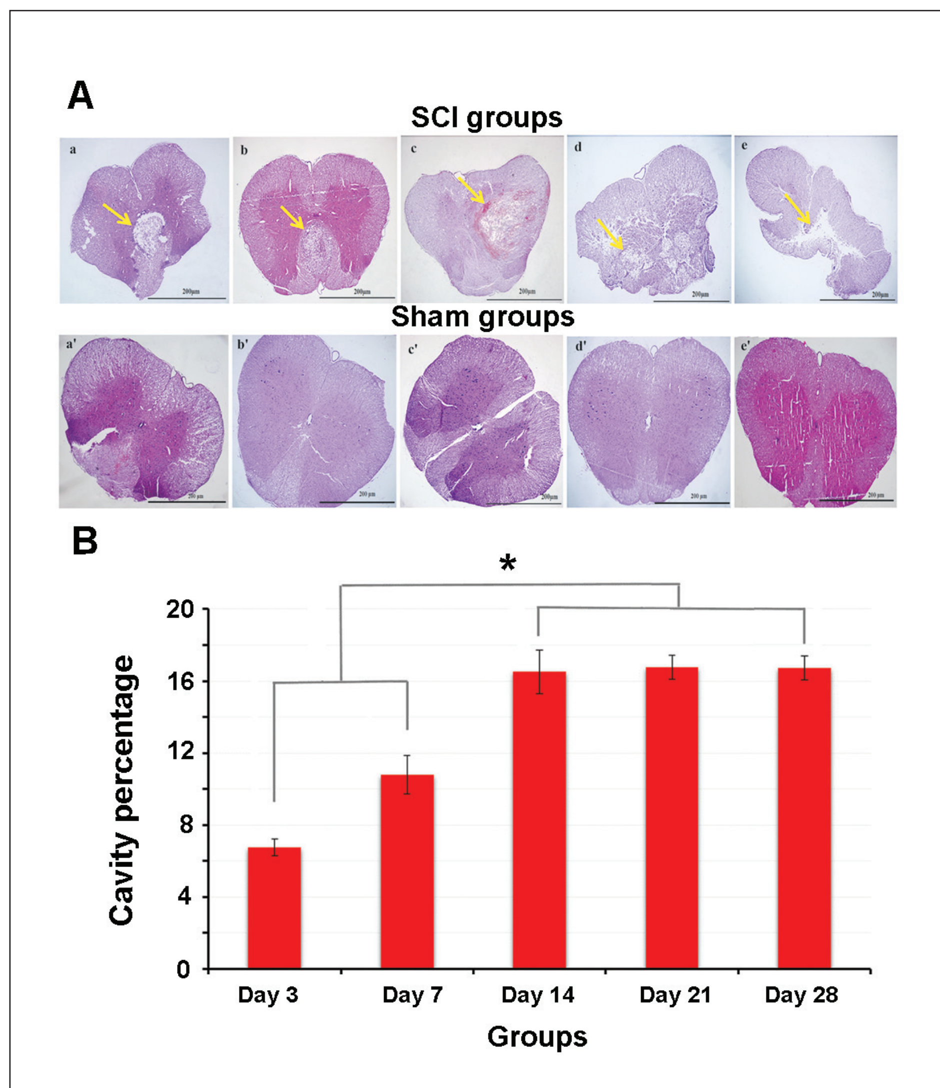

Figure 2. Evaluation of cavity percentage in the spinal cord in different time points after injury. **(A)** This illustration exhibits the lesion extending from the different regions of spinal cord. The extensive destruction of tissue loss in the SCI and sham groups with magnification ×40. The arrows indicate legion sites. **(B)** This chart indicates mean cavity of spinal cord injury as mean ± S.E.M. (n=6); *p<0.05 days 3 and 7 vs. days 14, 21, and 28. a, a' – day 3; b, b – day 7; c, c' – day 14; d, d' – day 21; e, e' – day 28.

Table 2

Effect of SCI on the testis, epididymis, seminal vesicle and prostate weight in male rats.

Relative Weight (%)	Control	Day 3	Day 7	Day 14	Day 21	Day 28
Left testis	0.61±0.01	0.61±0.01	0.57±0.04	0.61±0.02	0.57±0.02	0.59±0.03
Right testis	0.56±0.02	0.57±0.005	0.57±0.03	0.55±0.02	0.54±0.02	0.59±0.04
Left epididymis	0.17±0.006	0.19±0.01	0.16±0.01	0.18±0.009	0.16±0.01	0.18±0.01
Right epididymis	0.16±0.01	0.18±0.007	0.18±0.01	0.18±0.01	0.17±0.002	0.17±0.008
Left seminal vesicle	0.11±0.003	0.11±0.01	0.13±0.01	0.14±0.006	0.13±0.01	0.12±0.01
Right seminal vesicle	0.11±0.003	0.12±0.01	0.13±0.005	0.12±0.01	0.13±0.01	0.12±0.01
Ventral prostate	0.17±0.006	0.18±0.03	0.17±0.01	0.18±0.02	0.18±0.02	0.14±0.03

All values were scaled to set the control as mean ± S.E.M.

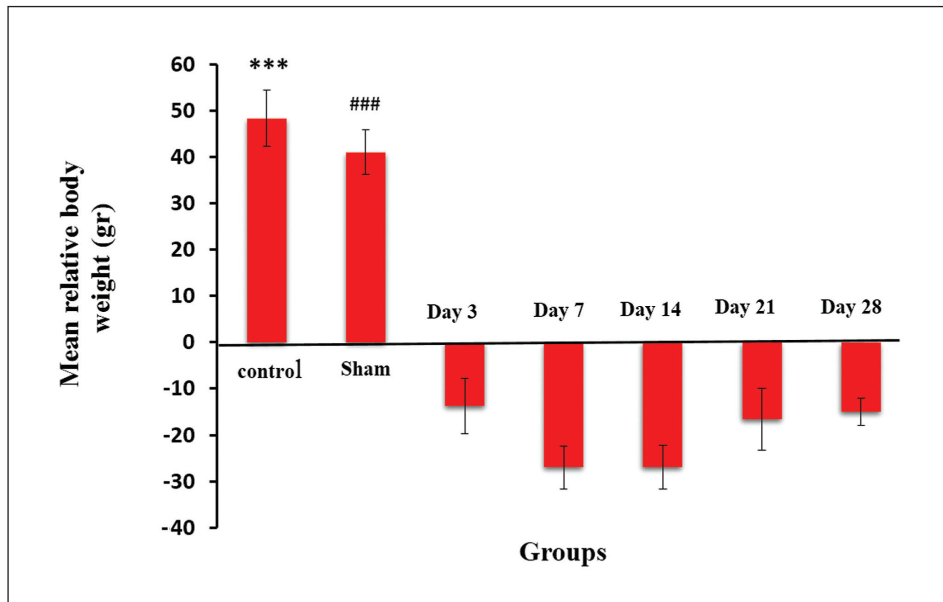

Figure 3. Effects of SCI on the body weight changes in different time courses in male rats. Values are presented as mean ± S.E.M. (n=6). ***p<0.0001 vs. all groups and ###p<0.0001 vs. all groups.

Table 3
The sperm parameters following SCI.

Sperm parameters	Control	Day 3	Day 7	Day 14	Day 21	Day 28
Count (×107)	10.32±2.17	5.63±2.0	4.98±1.17[2]	3.27±1.09[1]	5.51±1.51	1.81±1.44[1]
Total motility (%)	63.33±1.81	28.61±2.30[3,13]	39.71±4.62[4]	46.99±2.895	33.31±2.98[3,15]	47.11±3.65[14]
Progressive motility (%)	20.44±1.91	6.35±1.62[7]	10.45±2.62[9]	14.83±2.86	3.6±1.98[6,16]	7.93±1.51[8]
Viability (%)	63.38±3.56	48.28±9.04	52.91±8.37	54.29±8.41[11]	36.94±4.50	33.30±5.90[10]
Total abnormal (%)	7.29±2.4	16.66±4.30	7.36±3.11	16.44±4.25	21.65±7.30[12,17]	8.95±1.72

All values were scaled to set the control as mean ± S.E.M. Compared with control, after spinal cord injury (SCI) including sperm count: [1]days 14 and 28 (p=0.006); [2]day 7 (p=0.04). Sperm motility: [3]days 3 and 21 (p<0.0001); [4]day 7 (p=0.002,) and 514 (p=0.02). Progressive motility: [6]day 21 (p<0.001); [7]day 3 (p=0.001); [8]day 28 (p=0.007); and [9]day7 (p=0.05). Sperm viability: [10]day 28 (p=0.004) and [11]day 21 (p=0.008). Total abnormal sperm: [12]day 21 (p=0.026); between [15]days 21 and 14 (p=0.03). Progressive motility compared between [16]days 21 and 14 (p=0.009). Sperm viability compared between [17]days 21 and 14 (p=0.03).

7 (0.13±0.007), 14 (0.14±0.006), 21 (0.13±0.01), and 28 (0.12±0.01), except for day 3 (0.11±0.01), compared to the control animals (0.11±0.003) although it was not statistically significant.

The relative prostate weight decreased at day 28 (0.14±0.03) compared to the control animals (0.17±0.006). The prostate weights increased at days 3 (0.18±0.03), 14 (0.18±0.02), and 21 (0.18±0.02), but did not change at day 7 (0.17±0.01). These findings were not statistically significant.

Sperm count. Table 3 shows decreased sperm count in all groups compared with the control group. There was a significant decrease at days 7 (4.98±1.17; p=0.04), 14 (3.27±1.09), and 28 (1.81±1.44) compared with the control group (10.32±2.17; p=0.006).

Total motility. Total motility compared between day 3 and day 14 (p=0.001) and day 28 (p=0.005), also total sperm motility decreased in all groups compared to the control group. The percentages of sperm motility on days 3 (28.61±2.30) and 21 (33.31±2.98) were significantly lower compared to the control group (63.33±1.81; p<0.0001). We observed decreased sperm motility on days 7 (39.71±4.62) and 14 (46.99±2.86) compared to the control group (p=0.028). Among the experimental groups, there was a greater decrease on days 3 and 21 than days 3 and 14 when compared with day 28 (47.11±3.65; p=0.005) (Table 3).

Progressive motility. Mean progressive motility at day 21 (3.6±1.98) sharply decreased compared to the

control (20.44±1.91; p<0.0001) group. We observed decreases on days 3 (6.35±1.62), 7 (10.45±2.62), and 28 (7.93±1.51) compared to the control animals. A comparison between the experimental groups showed that progressive s perm motility sharply decreased on day 21 compared to day 14 (14.83 ± 2.86; p=0.009) (Table 3).

Sperm viability. According to Table 3, the percentage of live sperms at days 21 (36.94±4.50; p=0.008) and 28 (33.30±5.90; p=0.004) significantly decreased compared to the control group (63.38±3.56; p=0.02). Among the experimental groups, day 21 showed a greater decrease compared to day 14 (54.29±8.41; p=0.04).

Sperm morphology. The mean total abnormal sperm (head, neck, and tail) increased in the experimental groups compared to the control group. The percentage of abnormal sperm at day 21 (21.65±7.30) increased greatly compared with the control group (7.29±2.40; p=0.02). Among the experimental groups, there was more decrease on day 7 (7.36±3.11; p=0.02) compared to day 21. An increase was observed on day 3 (16.66±4.30) (Table 3) although it was not statistically significant. According to Table 4, there were more abnormal sperm head on day 21 (19.37±7.45) compared to the control group (4.02±1.04; p=0.01). Day 21 increased compared to days 3 (5.84±3.13; p=0.02), 7 (4.92±2.04; p=0.01), and 28 (7.44±1.02; p=0.05). Abnormal sperm in the neck area increased on day 3 (2.16±1.49) compared to the control group (0.2±0.2; p=0.05). Among the experimental groups, day 3 significantly increased compared to day 28 (0.0; p=0.04). The amount of abnormal sperm tails significantly increased on day 3 (8.6 ±2.62; p=0.001) compared to the control (1.26±0.81), days 7 (2.03±0.96; p=0.003), 14 (3.7±1.55; p=0.01), 21 (1.1±0.55; p<0.0001), and 28 (1.51±1.04; p=0.002) groups.

Architecture of testes tissue according to Miller's and Johnsen's scores. Based on Figure 4 and Table 5,

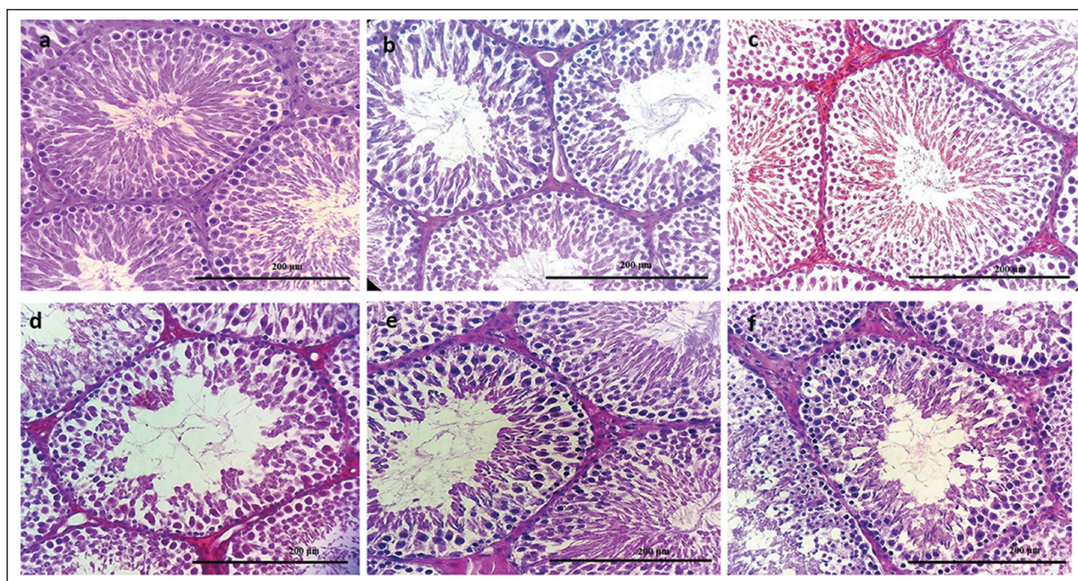

Figure 4. The effect of SCI on the number of epithelial cell layers and quality of seminiferous tubules in the testes in different time points (magnification ×400). a - control; b – day 3; c – day 7; d – day 14; e – day 21; f – day 28.

Table 4
Abnormal sperms (head, neck, and tail) following spinal cord injury.

Sperm parameters	Control	Day 3	Day 7	Day 14	Day 21	Day 28
Head abnormal (%)	4.02±1.47[1]	5.84±3.13	4.92±2.04	11.80±3.58	19.37±7.45[1,3,4]	7.44±1.02
Neck abnormal (%)	0.2±0.2	2.16±1.49	0.38±0.23	0.96±0.59	1.16±0.43	0.0[5]
Tail abnormal (%)	1.26±0.81[2]	8.6±2.62	2.03±0.96[6]	3.70±1.55[7]	1.1±0.55[8]	1.51±1.04[9]

All values were scaled to set the sham and control as mean ± S.E.M. Compared with control, after spinal cord injury (SCI): abnormal sperm head [1]day 21 (p=0.01) and abnormal sperm tail [2]day 3 (p=0.001). Abnormal sperm head in day 21 compared with: [3]day 7 (p=0.01) and [4]day 3 (p=0.02). Abnormal sperm neck in day 28 in compare with [5]day 3 (p=0.04). Abnormal sperm tail in day 3 compared with in all groups respectively [6, 7, 8, 9](d7: p=0.003, d14: p=0.01, d21: p<0.0001, and d28: p=0.002).

Miller's scale had the lowest percentage at day 28 (4.91±0.05) compared to the control (5.0±0.0). This finding was not statistically significant. Johnsen's score indicated that all groups on days 3 (8.44±0.06), 7 (8.39±0.13), 14 (8.24±0.05), 21 (8.51±0.1), and 28 (8.18±0.13) significantly decreased compared to the control group (9.46±0.008) (Table 5).

Germ cells apoptosis. According to Figure 5A, AI-1 significantly increased on days 3 (35±4.04; p=0.004) and 21 (31.66±4.8; p=0.02) compared to the control group (9.33±4.37). AI-2 significantly increased on day 3 (82.33±1.28) compared with the control (13.66±6.69; p=0.002). AI-2 increased on day 21 (64.0±1.36) compared to the control (p=0.02) (Figure 5B).

Table 5
Effect of spinal cord injury on the number of epithelial cell layers and quality of seminiferous tubules.

Group	Miller criteria (%)	Johnsen criteria (%)
Control	5.0±0.0	9.46±0.008[1,2]
Day 3	4.99±0.001	8.44±0.06
Day 7	4.99±0.006	8.39±0.13
Day 14	4.99±0.008	8.24±0.05
Day 21	4.99±0.001	8.51±0.1
Day 28	4.91±0.05	8.18±0.13

Values are represented as mean ± S.E.M. The Johnsen's criteria compared with between control group and [1]days 3, 7, 14, 28 (p<0.0001) and [2]day 21 (p=0.001).

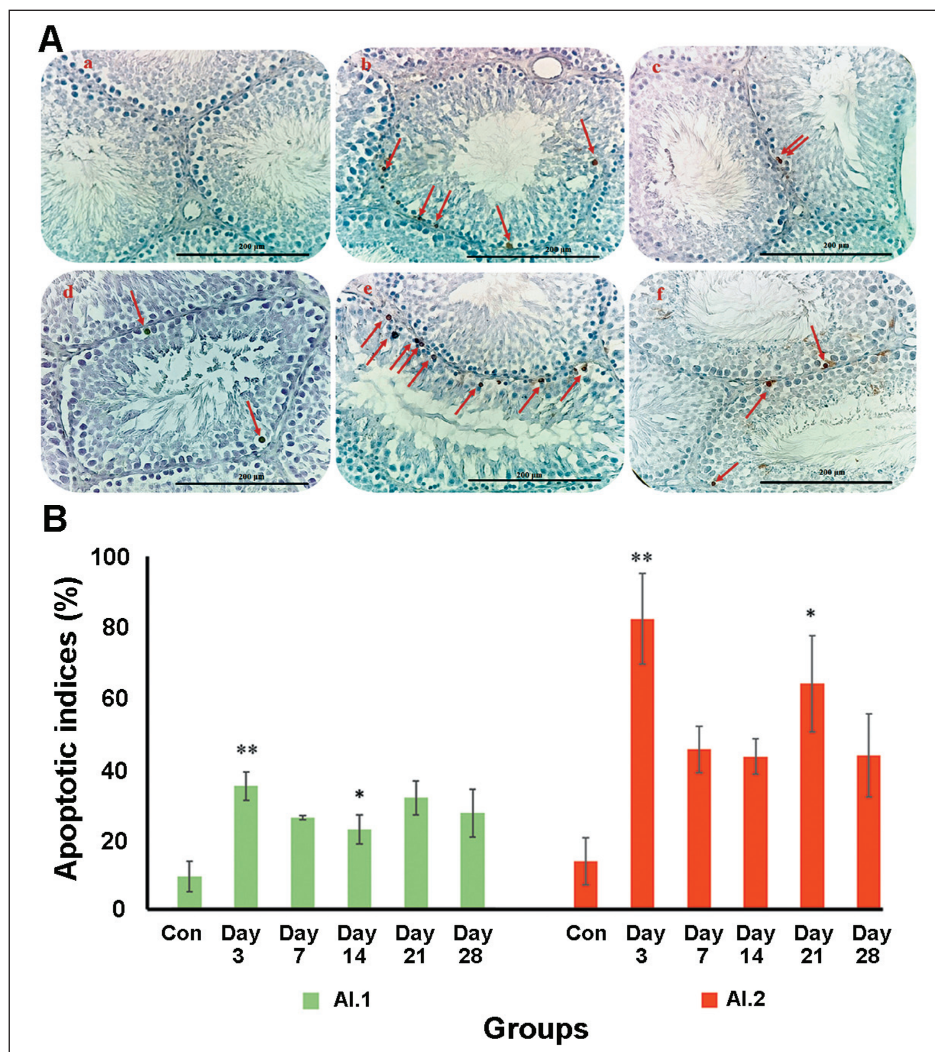

Figure 5. (A) The effects of SCI on the apoptotic indices in seminiferous tubules in different time points by TUNEL staining. The arrows indicated apoptotic cells (magnification ×400). **(B)** Values are presented as means ± S.E.M. (n=6). AI-1 – control group vs. **day 3 (p=0.009) and *day 21 (p=0.02). AI-2 – control group vs. **days 3 (p=0.002) and *21 (p=0.02).

Cytokine and inflammasomes expression levels. The mRNA expression profiles of the main inflammatory markers were analyzed 3–28 days after the SCI. A decrease of *IL-1β* mRNA expression was noted on days 3 (0.09±0.02) and 7 (0.18±0.01). However, expression of *IL-1β* dramatically increased on day 14 (1.67±0.14; p<0.0001) compared with the other experimental groups. The *IL-1β* expression rapidly declined by day 21 (0.01±0.002) and significantly increased on day 28 (0.74±0.12) compared with days 3, 14, 21 (p<0.0001), and 7 (p=0.001) (Figure 6A). The *IL-18* mRNA expression decreased on days 3 (0.17±0.02), 7 (0.18±0.04), and 14 (0.05±0.007). However, this expression dramatically increased on day 21 (0.91±0.07) and remained elevated until day 28 (0.85±0.1) when compared to the days 3, 7, and 14. (p<0.0001) (Figure 6B).

The *Nlrp3* mRNA levels increased on day 3 (8.97±0.56) compared to days 7 (2.46±0.31), 14 (6.2±0.54), 21 (3.71±0.32), and 28 (1.52±0.12) (p-value of <0.0001), followed by a decline on day 7. There was a significant increase on day 14 compared to days 7 and 28 (p<0.0001), as well as day 21 (p=0.005). We observed a correlated, stepwise decrease on days 21 and 28 (p=0.009) (Figure 7A). The *Asc* mRNA expression had a stepwise increase from days 3 (0.88±0.09) to 7 (1.27±0.18) and a decrease on day 14 (0.45±0.05) compared to day 7 (p<0.0001), followed by a dramatic increase on day 21 (1.69±0.14) where it was at its highest level compared to days 3 and 14 (p<0.0001), and 28 (0.76±0.09; p<0.0001). We observed decreased expression on day 28 (Figure 7B).

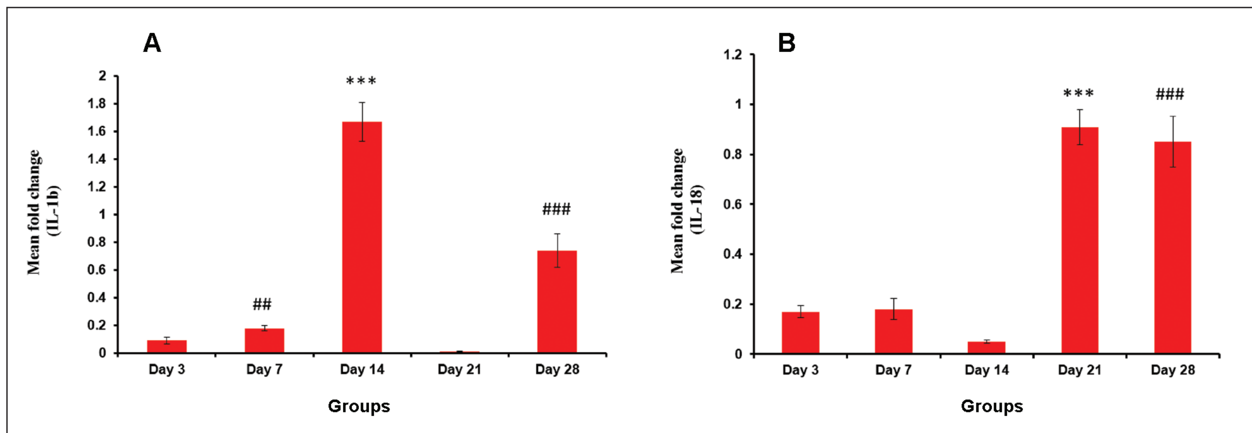

Figure 6. mRNA levels of *IL-1β* **(A)** and *IL-18* **(B)** in the testes following SCI in different time courses. Values are presented as means ± S.E M. (n=6). **(A)** ***day 14 vs. all groups (p<0.0001); $^{###}$day 28 vs. days 3, 14, 21 (p<0.0001), and $^{##}$day 7 (p=0.001). **(B)** ***days 3, 7, and 14 vs. days 21 (p<0.0001) and $^{###}$28 (p<0.0001).

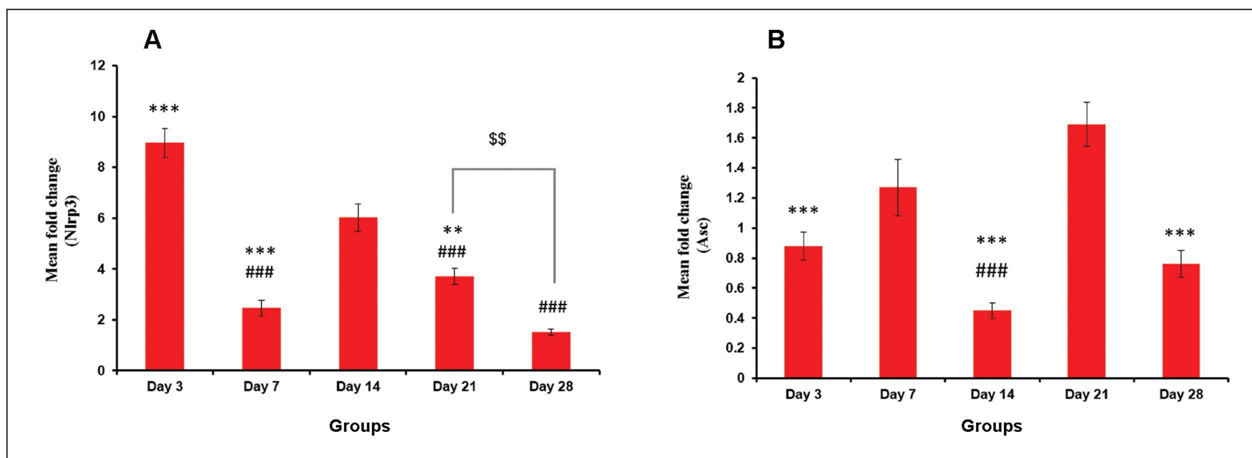

Figure 7. The mRNA levels of NLRP3 **(A)** and ASC **(B)** in the testes following SCI in different time courses. Values are presented as means ± S.E.M. (n=6). **(A)** ***day 14 vs. day 3, 7, 28 (p<0.0001), and **21 (p=0.005); $^{###}$day 3 vs. days 7, 21, and 28 (p<0.0001); $^{##}$day 21 vs. day 28 (p=0.009). **(B)** ***day 21 vs. days 3, 14 and 28 (p<0.0001); $^{###}$day 7 vs. day 14 (p<0.0001).

Discussion

Male SCI patients have severely disrupted fertility with normal sperm counts and poor sperm motility and morphology. However, there are increased inflammatory cytokines in this abnormal semen profile. Whether exact trend of these cytokines and the inflammasome components signaling pathway that are active in the testes of SCI men is yet to be determined. The present study has indicated, for the first time, expression of inflammasome components genes in the testicles of an SCI rat model at multiple time points and demonstrated their relationships to spermatogenic cells apoptosis and sperm quality.

The current study revealed that the expression of inflammatory cytokines and inflammasome components *IL-1β* and *Nlrp3* increased on day 14. Mallidis et al. (1994) and Anderson et al. (2018) indicated that semen sample is normal within acute phase, whereas two weeks after SCI, it dropped to poor quality. These results supported the decreased sperm count and quality of seminiferous epithelium at the same time point. Current studies have revealed that *Nlrp3*, was detected whereas the discrete expression profiles in the testis proposed a site-specific role in the inflammatory response. High levels *Nlrp3* expressed specifically in Sertoli cells (Lech et al. 2010). These studies have proposed that *Nlrp3* plays a vital role in the first line of defense against pathogens. Linsenmeyer et al. (1996) have indicated that testicular blood flow decreased to 77±8% at 14 days after the SCI in a rat model. In addition, other studies reported that chronic testicular ischemia in non-SCI animals disintegrated the spermatogenesis (Altavilla et al. 2012; Cvetkovic et al. 2015). Minutoli et al. (2015) have stated that increased reactive oxygen species (ROS) generation after testes ischemic-reperfusion (I/R) and over-production of nitric oxide (NO) were the most likely reasons for damage to the testes. They confirmed the important role of *Nlrp3* in I/R through *Nlrp3* knockout mice and activation blocking. These researchers have suggested that the *Nlrp3* inflammasome components signaled inflammatory triggers during low-flow situations and resulted in an apoptotic response in the testis. Following these pathological events, the numbers of germ cells decreased due to increased numbers of apoptotic cells and vacuolization of the seminiferous epithelium. Eventually, testicular atrophy and impaired spermatogenesis would occur (Minutoli et al. 2016). This finding closely resembled the current study results where increased *Nlrp3* expression in SCI animals have occurred 3 and 14 days post-SCI. Since *Nlrp3* specifically expressed in Sertoli cells and this cell is the orchestra of the seminiferous epithelium, it is likely that upregulation of *Nlrp3* in Sertoli cells might induce apoptosis in germ cells and eventually disrupt the architecture of the seminiferous epithelium, which closely resembles our data. Any changes in relation to immune cells in testis in order to prevent autoimmunity against the antigens present in the sperm must first be overcome by the inhibitors of Sertoli cells (Kaur et al. 2014). In addition, according to the study of Minutoli et al. (2015), NLRP3 is a specific target for innovative drugs directed at treating altered spermatogenesis and male infertility.

In this study, the expression level of both inflammasomes and inflammatory cytokines decreased on day 28 and at the same time the rate of apoptosis in testis decreased, although it was not significant. In addition, the rate of total motility, progressive motility improved, and total abnormal sperm increased on day 28 (41.11±3.65, 7.39±1.51, and 8.95±1.72, respectively) compared to day 21 (33.31±2.98, 3.6±1.98, and 21.65±7.30). The pro-inflammatory cytokines are protective agents of paracrine in male glands and the multifactorial interaction of cellular homeostasis and apoptosis in the testis has been attributed to IL-18, which is a positive effect of pro-inflammatory cytokines (Lotti et al. 2011; Carp et al. 2012). Sanocka et al. (2003) have suggested that measurement of IL-6, IL-8 and/or IL-18 levels in the plasma can be a permeable sign of early infection/inflammation in the male reproductive tract and a mark for rapid intervention with an inflammatory anti-inflammatory drug. The overexpression of some inflammatory cytokines in the male genital tract due to persistent infection/inflammation may enhance the peroxidation process and results sperm function with subsequent infertility (Matalliotakis et al. 2006). Generally, there is still no documented study of the cause of cytokine depletion. Therefore, before day 21 interventions may be suggested to suppress inflammation and prevent the lasting effects of inflammatory factors on male infertility, but it is need further studies after day 28.

The expression of the NLRP3, ASC and caspase-2 inflammasomes following SCI increases with increasing ER stress (Yanagisawa et al. 2019). However, in the study of Fortune et al. (2017) 1.5 years after SCI, there is an elevated level in T cells and chronic low-level immune response. They have suggested that pathological process is not confined to the acute stage of injury but are chronic and potentially life-long. Given this, a unique therapeutic intervention may not be successful.

In our study, the expression level of NLRP3 decreased on day 7 after SCI, which is conforming with the study by Jiang et al. (2017), which have shown that nlrp3 was elevated in the spinal cord tissue 6 h post injury and continued to rise until day 3, and then dropped at day 7. Although many hormonal, neurological, and immunological factors have been mentioned to increase the expression of these agents, unfortunately, there is still no accurate reason for the reduction of inflammatory genes and inflammasomes at a particular time.

Tumor-necrosis factor-α *(*TNF-α) might stimulate inflammasome components activation via reactive oxygen species (ROS). Another pathway is pro-*IL-1β* synthesis via NF-kB activation (Altavilla et al. 2012). The present study has shown higher expressions of *Nlrp3*, and *IL-1β* at 14 days after SCI. During the acute phase of SCI, pro-inflammatory cytokines such as *IL-1β* cause BTB discrete and increase its permeability (Sarkar et al. 2008). Dulin et al. (2011) have reported disintegration of the BTB, reduced tight junction proteins (occludin) in BTB, and the presence of immunoglobulin G within the seminiferous tubules.

The IL-1β were not found in blood serum after SCI, which indicated that the inflammatory response was limited to the urogenital system. According to Basu et al. (2004) there were elevated IL-1β level in the seminal plasma of men with SCI.

It is plausible that IL-1β has an association with germ cells apoptosis following SCI. The current study findings were closely related. We observed upregulation of *IL-1β* at 14 and 28 days post-SCI. This might lead to reductions in the quality of seminiferous epithelium according to Johnsen's score and sperm counts during the same time periods.

Huang et al. (1999) have reported significantly reduced testosterone levels 14 days post-SCI. However, they observed significant increases in FSH and LH levels. Reduction of spermatogenesis during SCI is pertinent to non-hormonal factors such as lack of nerve impulse and morphological changes in Sertoli cells (Huang et al. 1998). Disruption in nerve impulses due to SCI leads to an imbalance phospholipid membrane, membrane phospholipase hydrolysis, and production of biologically active eicosanoids which result in peroxidation of oxygen-free radicals. It has been reported that the concentration of lipid peroxidation immediately increased during the early acute phase of SCI, which could lead to the over-production of ROS (Harijith et al. 2014). A number of studies have reported that ROS activates inflammasome-activating signal transduction pathways through MAP kinases, ERK1/2, and PI3K. ERK1/2 results in both pro-and anti-apoptotic states during inflammation. Results from recent studies have indicated that cytokines such as TNF-α, IL-1β, and IL-18 expressions via a positive feedback cycle induce intracellular aggregation of ROS by uncoupling antioxidant enzymes. Decreased rate of ROS production results in decreased activation of ERK1/2 and *IL-1β* expression (Harijith et al. 2014). Studies have implicated increased ROS production following SCI. Therefore, in the present study, the increased expression of *Nlrp3* and inflammatory cytokine *IL-1β* in the testes tissue might result from induction of ROS production 14 days after the SCI. However, further studies should clarify the presence of ROS production in the testes tissue following SCI and determine the association between ROS and the inflammasome components at different time points.

Inflammasome components are present in the semen of SCI patients. Increased levels of these proteins are associated with increased pro-inflammatory cytokines IL-1β and IL-18 (Zhang et al. 2013). Ibrahim et al. (2014) and Brackett et al. (2007) have observed significant improvements in sperm motility via neutralizing Asc as one of the key components of inflammasomes and inflammatory cytokines IL-1β, TNF-α, and IL-6 into the semen of SCI men. They observed increased levels of Asc and IL-1β in the semen of SCI men where Asc localized in the acrosome, equatorial segment, and midpiece sperm cell sections of these patients. They have proven that Asc, as an adaptor of the inflammasome components, is required for the activation process of this complex in men with SCI. Asc activates caspase-1, which subsequently activates IL-1β and IL-18. The present study has shown upregulation of Asc in the testis tissue at 7 and 21 days after SCI. Most likely low sperm motility along with increased abnormal sperm and apoptosis indices at day 21 occurred via activation of Asc and IL-18 at the same time point.

In the present study, the NLR family members *Nlrp3* activated in the testes of SCI animals at different time points. However, there was over-expression of *Nlrp3* on days 3 (MFC: 8.97) and 14 (MFC: 6.02).

In conclusion, the results of this study provide the first evidence of involvement of Asc and Nlrp3 inflammasome components in testes tissue and their associations with sperm quality, spermatogenic cells apoptosis, and the epithelium of seminiferous tubules in an SCI rat model at different time points. Sharp increases in the expression of *Nlrp3* and inflammatory cytokines *IL-1β* and *TNF-α* in

the testes tissue might result from induction of ROS production following SCI 14 days after the injury. Finally, upregulation of end-product cytokines *IL-1β* and *IL-18* at days14 and 21 post-SCI might disintegrate the epithelium of seminiferous tubules on day 14 and induce germ cell apoptosis, increased number of abnormal sperm cells, and attenuate motility and viability at 21 days after SCI. This study provides an additional evidence to propose that the activation of innate immunity in the testes lead to abnormal sperm quality in SCI patients. Thus, SCI induces a low-grade inflammatory situation that is implicated by a bidirectional relationship between the nervous, endocrine, and immune systems.

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