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The expression of Bax protein in the early stages of spinal cord injury in the sperm cells of rats

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ABSTRACT

Introduction: Apoptosis is one of the most important biological processes, which occurs through the activation of intracellular cell death pathway.

Aim: The aim of this study was to determine the pattern of cell death within the early stages of spinal cord injury (SCI) and the evaluation of the changes in Bax protein expression of progressive apoptosis.

Material and methods: 48 adult male Sprague Dawley rats were randomly divided into two control and experimental groups. Animals were anesthetized and then the laminectomy procedure was performed in the area of T6–8. On days 1, 7, 14 and 28 after the surgery, one-third of the middle part of their testis tissue were taken for histological and immunohistochemical analysis.

Results: The immunohistochemical analysis indicated the presence of TUNEL-positive cells and cells containing the pro-apoptotic protein Bax in testicular sperms in the 1st day after SCI, as well as increased on 28 days.

Discussion: 1 day after SCI, the apoptosis occurred in testicular sperm lines as well as the apoptosis can be related to Bax protein expression. Therefore inhibition of caspase activity via caspase cascades-mediated apoptosis may have a protective role in testicular injury during the acute phase of SCI.

Conclusions: Our finding suggested that the damage and destruction after SCI can be controlled by therapeutic interventions at the appropriate time before the destructive changes of SCI.

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1. INTRODUCTION

The incidence rate of spinal cord injury (SCI) in most countries is estimated to be between 20 and 40 cases per 1 000 000 annually that it often occurs in young people under the age of 30 years.[1] Generally two cellular responses occur to the spinal cord after injury including: primary mechanical injury and pathologically-physiologically secondary injury which appears to have two main forms: necrotic and apoptotic.[2] The first response includes necrotic cell death induced by external mechanical damage the most important landmark of which is acute hemorrhage and ischemia, and the secondary response is the activation of the apoptotic death cascade which is mediated by multiple injury processes including inflammatory cytokines and formation of free radicals.[2] The apoptosis is distinguished from necrosis cells by biochemical and morphological characteristics.[3] The cellular destructions during apoptosis is characterized by cell shrinkage, immune cell transmigration, myelin degradation and chromatin condensation along with fragmentation of the nucleus.[4] In the necrosis process, cell swelling and destruction of mitochondria occurs.[5] Recently, apoptosis has been suggested as damage to the nervous system caused by ischemia, neuronal degeneration conditions, inflammatory diseases and traumatic damage.[6] B cell leukemia/lymphoma 2 (Bcl-2) is a gene that play a crucial role in the regulation of cell death as well as degree of apoptotic degeneration of neurons so that neuronal cell death is prevented when overexpressed in neurons.[7] After SCI, the expression of Bcl-2 can suppress apoptosis in the neurons of the spinal cord by preventing the release of cytochrome c from mitochondria, scavenging the free radicals, and rising the growth factor.[7] The Bcl-2 proteins were identified in vertebrate cells using a variety of techniques.[8] This family consists of many proteins causing increased cell survival, or cell death, such as Bax protein. The Bax protein acts as a reinforcing factor of the cell death in the general apoptosis process that has the opposite role to Bcl-2 to work to stabilize the mitochondrial membrane because its destabilization leads to the release of mitochondrial cytochrome c into the cytoplasm and inducing the uncoupling of oxidative phosphorylation.[9,10] Furthermore, Bax is critically involved in both necrosis and apoptosis, and these two morphologically distinct forms of cell death could be prevented simultaneously by increasing the quantitative ratio of Bcl-xL and Bax in mitochondria.[11] Moreover, activation of caspase-3 has been reported to cause apoptotic cell death, and caspase-3 activation is markedly increased following SCI.[12]

2. AIM

This study was conducted to investigate the testicular sperms' changes during the early stages of SCI. Therefore, the main objective of the present study was to investigate the apoptosis and Bax protein expression in testicular sperm during the early stages of SCI.

3. MATERIAL AND METHODS

3.1. Animals and ethics

In this study, 48 adult male Sprague Dawley rats aged 90 days, weighing 250–300 g and pathogen free were used (purchased from the Pasteur Institute, Tehran, Iran). The experiments were carried out in accordance with the principles of Helsinki protocol. All procedures were approved by the ethical committee of Kurdistan University of Medical Sciences, Sanandaj, Iran (IR.MUK.REC.1395.402).

3.2. SCI model and tissue preparation

All animals were randomly divided into experimental and control groups. Animal spinal cord injury (SCI) model was performed under general anesthesia (80 mg/kg ketamine; 10 mg/kg xylazine, i.p.). Briefly, rats were anesthetized under sterile conditions and a laminectomy was performed at T7 to expose the spinal dura mater. The T8 of spinal cord contusion was performed using the modified spinal cord impactor device which was developed so that a 25 g metal rod (2.5 mm in diameter) length of 12 mm falls from a 10 cm height.[13,14] In the control group, the surgery was performed without spinal cord manipulation/contusion. During the postoperative period, all animals were monitored three times daily, were given an analgesic (ketoprofen 2,5 mg/kg; sc twice daily) for 2 days and gentamycin (5 mg/kg; sc) for 4 days to prevent of the bladder infection.[15] The Basso, Beattie, Bresnahan (BBB) scoring is used to evaluate hind-limb locomotor function by two observers who were blinded to the any intervention. They were killed at 1, 7, 14 and 28 days after surgery. Surgical and animal care procedures were conducted strictly in accordance with the guidelines published in the *NIH Guide for the Care and Use of Laboratory Animals*: National Institutes of Health Publications.[16] The testis tissues were fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) for 4 h, incubated overnight at 4°C in 100 mM sodium phosphate buffer (pH 7.4).[17]

3.3. Histological examination and immunohistochemical staining

At the end of the survival period, animals intraperitoneally with a mixture of ketamine (3.75 mg/kg), and xylazine (1.9 mg/kg), were anesthetized and perfused intraventricular with 0.1 mL of heparin (5000 IU/mL), and subsequently transcardially with 4% paraformaldehyde in 0.1 M PBS, pH 7.[17] Briefly, on the 1st, 7nd, 14th and 28th days after SCI, the peritoneal cavity of animals were dissected for weighting of testes with digital scale (Sartorius; model-BL210S). The testis tissues were prepared in the same fixative at room temperature for 4 h, sectioned and processed for histochemistry and immunohistochemistry analysis. To determine the histochemistry of tissues, the hematoxylin (Merck KGaA, Darmstadt, Germany) and eosin (Sigma, St. Louis, MO, USA) were used. In total, 100 seminiferous tubules of each sample were conducted in accordance with the Johnsen method (1970).[18] For immunohistochemistry and visualization of Bax, endogenous peroxidase activity in the sectioned tissues was blocked with 3% H_2O_2, and incubated at 4° overnight in PBS, pH 7.4, con-

Table 1. Rating seminiferous tubules in both control and experimental groups according to Johnsen classification (results are presented as a percentages).

Groups	Johnsen scores									
	1	2	3	4	5	6	7	8	9	10
Control group									1.4	98.6
Experimental group										
One week after SCI									25.5	74.5
One week after SCI								20.3	37.4	53.3
Two weeks after SCI							18.4	46.6	23.8	11.2
Four weeks after SCI				20.5	23.5	34.4	13.9	6.6	2.1	

taining 0.01% mouse anti-Bax antibody (sc-7480, Santa Cruz, USA) in blocking buffer (3% goat serum in 1× PBS with 0.2% Tween 20). After rinsing, sections were incubated for 2 h at room temperature in blocking buffer containing anti-goat antibody conjugated to peroxidase (diluted as per the recommendations of the supplier; sc-516102, Santa Cruz, USA). Sections were rinsed again and were incubated with the chromogen diaminobenzidine (DAB; Sigma, St. Louis, MO) for 5 minutes to yield a permanent deposit. Sections were rinsed in distilled water, then mounted on gelatin-coated slides, air-dried overnight, and cover slipped.

3.4. TUNEL assay

As described previously, the sections were also tested with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. They were placed in terminal deoxynucleotidyl transferase (TdT) buffer (containing 50 μM dUTP-biotin, 100 μM dATP, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 7.6; Invitrogen, Carlsbad, CA) for 30 minutes followed by reaction with a TdT reaction mixture (consisting in equilibration buffer, biotinylated nucleotide mix and TdT enzyme) and incubated in a humidified chamber at 37°C for 90 minutes. The sections were rinsed two times in buffer solution (150 mM sodium chloride, 15 mM sodium citrate, pH 7.4) for 10 minutes followed by washing in PBS (pH 7.4) two times for 10 minutes. The avidin–biotin technique was applied, and stained with chromogen diaminobenzidine (DAB) solution. After counterstaining with hematoxylin (0.5%), slides were dehydrated and mounted. TUNEL-positive ($TUNEL^+$) cells (%) were quantified as apoptotic index and it was characterized by the core of $TUNEL^+$ cells was brown in appearance.[19]

3.5. Statistical analysis

All statistical analyses were performed using the SPSS software (v.19, SPSS Inc., Chicago, IL, USA) using *t*-test. Differences were considered as significant at $P < 0.05$.

4. RESULTS

4.1. Johnsen scoring

In this study the rate of spermatogenesis in the control and experimental groups were evaluated. All cases were ranked according to the Johnsen scoring system categories as shown in Table 1. The quantitative results showed that in the control group the average total score (as score 10) spermatogenesis was 98.6%. Following the experimental groups as 'one day after SCI' group, 'one week after SCI' group, 'two weeks after SCI' group and 'four weeks after SCI' group were 74.5%, 53.3%, 11.2% and 0%, respectively. Therefore the rate of spermatogenesis in four weeks after SCI group was significantly decreased ($P = 0.0027$).

4.2. Histological examination

In the histological sections of control group, testicular parenchyma consisted of seminiferous tubules which appeared rounded or oval in shape with regular contour as well as spermatogenesis was observed in all seminiferous tubules. Therefore, there was no evidence of irregularities in spermatogenesis or degenerative changes in seminiferous tubules and the interstitial connective tissue. Finally, histological evaluations reveal normal architecture of the spermatogenic cells at various stages of development (Figure 1). One day after SCI in the experimental group, the shape of seminiferous tubules was regular and round with external border. Also, the spermatogonia cells, primary spermatocytes and round spermatid, often exhibited quite condensed nuclei. Furthermore, the spermatogenesis in most of the seminiferous tubules were more 90% and a few evidence of seminiferous tubule degeneration were observed. But, vasculature and increased number of inflammatory cells was observed in the interstitial connective tissue (Figure 2). One week after SCI, the irregular outline of the seminiferous tubules was observed as well as thickened and irregular basement membrane. Also a sign of degeneration, vacuolated cytoplasm and pyknotic nuclei was observed in the spermatogenic cells in some seminiferous tubules. On histological examination of spermatogonial cells, the primary spermatocyte, round spermatid and sperm were observed (Figure 3). Two weeks after SCI, in the experimental group, the seminiferous tubules basement membrane of tubules was slightly corrugated. In the histological study, a degeneration of epithelial lineage, thick corrugated basement membrane and absence of well-organized elongated spermatids was observed. Furthermore, results showed evidence of spermatogenesis in 83% of the seminiferous tubules and the other tubules. Illustrations show evidence of degenerative changes including disorganization of seminiferous tubule and heterochromatin of nucleus in spermatogonia, primary spermatocyte and round spermatocyte germ cells.

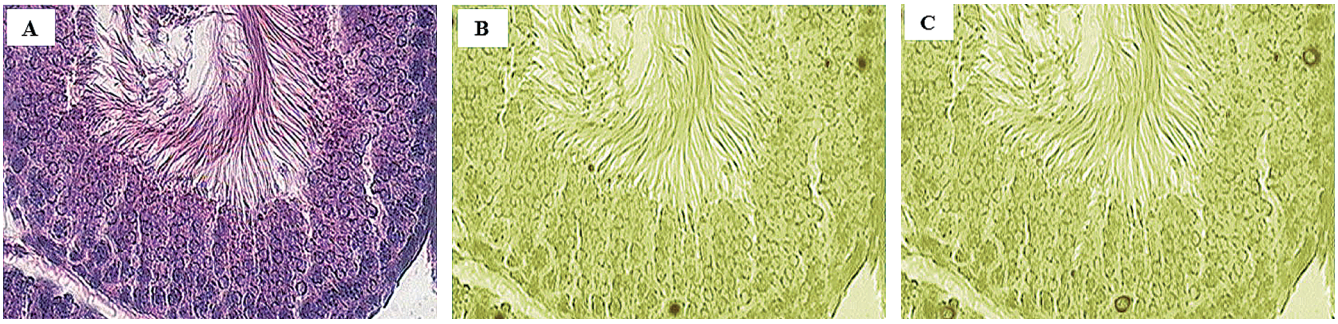

Figure 1. Control group: A) spermatogenesis in all of the seminiferous tubules without any degeneration (HE staining); B) TUNEL assay (Bax protein expression) and C) TUNEL$^+$ and Bax$^+$ cells in the entire spermatogenic cell lines in the seminiferous tubules (Bax protein expression).

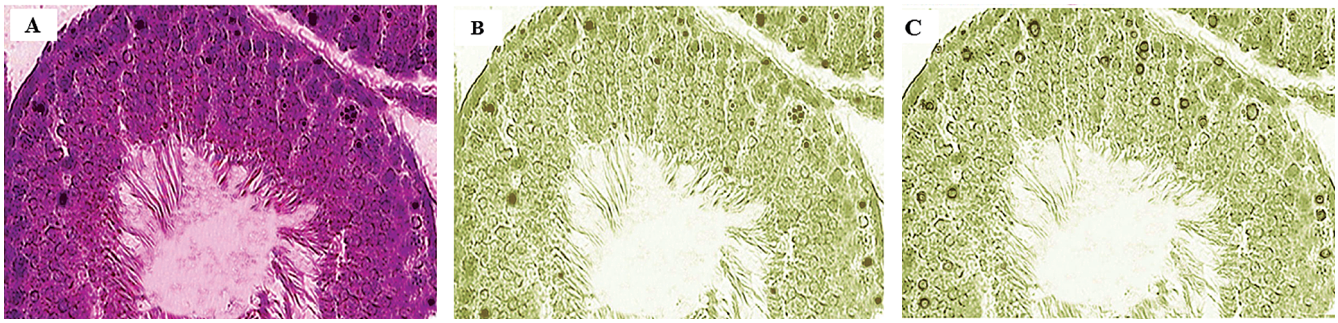

Figure 2. Experimental group one day after SCI: A) regular and round seminiferous tubules, dense core of primary spermatocyte and round spermatid (HE staining); B) TUNEL assay (Bax protein expression) and C) the testis of experimental group stained with TUNEL (Bax protein expression). Some of the TUNEL$^+$ and Bax$^+$ sperm cells were observed with brown core. Especially, most of these cells were in the lines of spermatocytes and round spermatids.

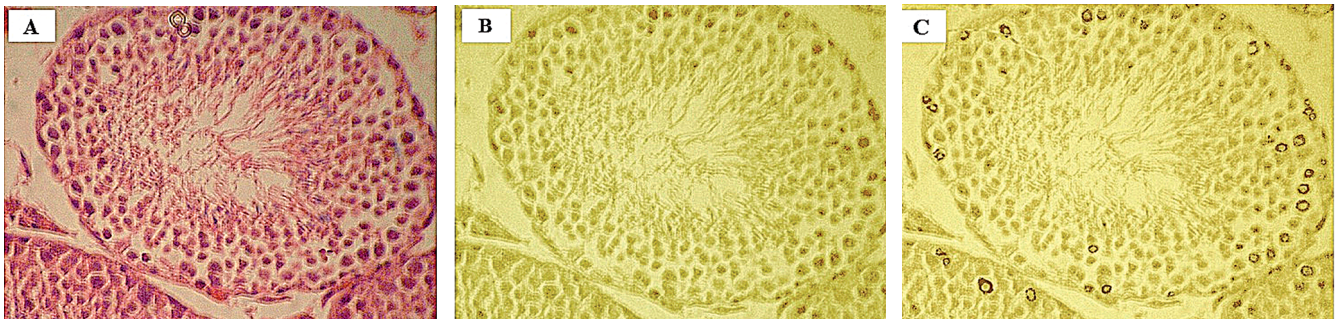

Figure 3. Experimental group one week after SCI: A) seminiferous tubules with irregular basement membrane (HE staining); B) TUNEL assay (Bax protein expression) and C) some of the TUNEL$^+$ and Bax$^+$ cells in the lines of spermatocytes, round, and long spermatids (Bax protein expression).

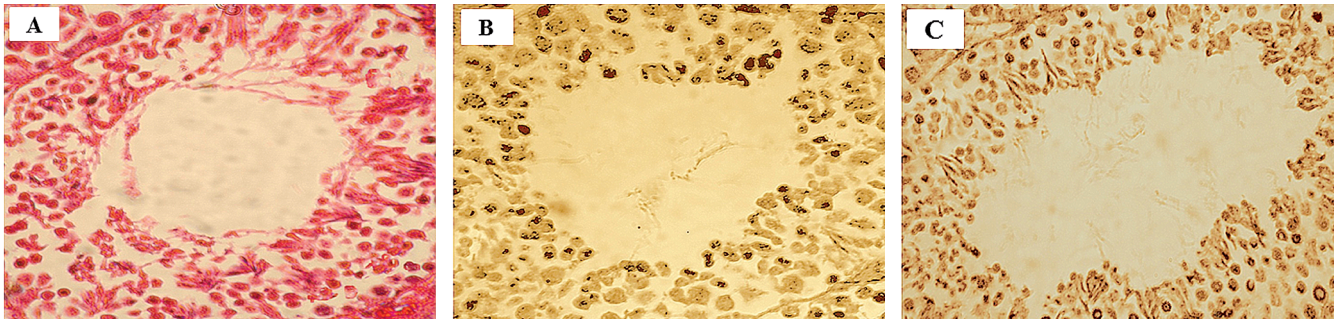

Figure 4. Experimental group two weeks after SCI: A) seminiferous tubules with regular shaped, but irregular basement membrane (HE staining). Also, stopping spermatogenesis trend and unstructured sperm cells was observed in some seminiferous tubules; B) TUNEL assay (Bax protein expression) and C) lines of spermatocytes, round and long spermatids with brown cytoplasm (Bax protein expression).

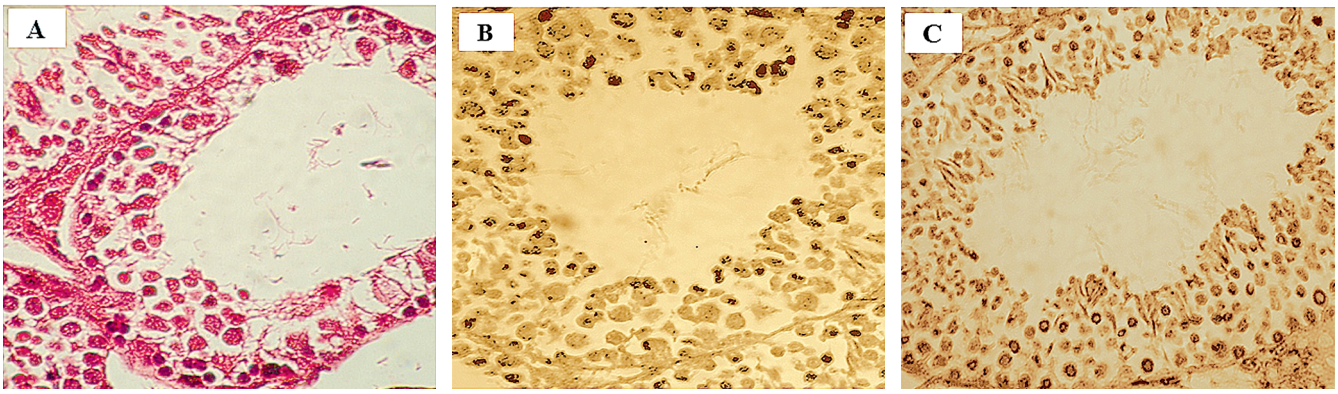

Figure 5. Experimental group four weeks after the SCI: A) the testis tissues that epithelium atrophy in some seminiferous tubules (HE staining); B) TUNEL assay (Bax protein expression) and C) the testis tissue which TUNEL$^+$ and Bax$^+$ in the line of spermatogonia cells with brown cytoplasm (Bax protein expression).

Table 2. Number of Bax+ cells and TUNEL$^+$ cells in both experimental and control groups.

Days	Groups	Parameters			
		TUNEL$^+$		Bax$^+$	
One day	Control	2.85 ± 0.04	$P = 0.073$	2.97 ± 0.41	$P = 0.067$
	SCI	11.95 ± 0.53		14.81 ± 0.09	
One week	Control	2.91 ± 0.28	$P = 0.014^a$	3.28 ± 0.61	$P = 0.019$
	SCI	16.32 ± 1.04		19.32 ± 1.35	
Two weeks	Control	2.01 ± 0.02	$P = 0.0055^{b,c}$	3.7 ± 0.16	$P = 0.0072$
	SCI	22.28 ± 0.73		24.3 ± 0.62	
Four weeks	Control	2.85 ± 0.07	$P = 0.072^d$	3.63 ± 0.13	$P = 0.081$
	SCI	8.95 ± 0.15		10.58 ± 0.3	

Comments: [a] $P \leq 0.022$ vs. one day group; [b] $P \leq 0.009$ vs. one day group; [c] $P \leq 0.014$ vs. one week group; [d] $P \leq 0.006$ vs. one week group.

There were gaps between spermatogonia, primary spermatocytes and round spermatid. The spermatogenic cells are less closely arrayed, and some of the spermatogenic cells desquamate. Moreover, spermatogonial cells, primary spermatocyte, and round spermatid were detected, but a small number of sperm was found (Figure 4). Four weeks after SCI in the experimental group, the seminiferous tubules were quite irregular and there was a shrunken basement membrane with relatively thick as well as abnormal cellular debris in the tubules. Also a failure and disruption was observed in the trend of spermatogenesis in all seminiferous tubules as spermatogenesis in 55% of the seminiferous tubules were found. In addition, the other tubules often exhibited degenerative changes such as partial regression of the seminiferous epithelium due to the absence of proliferating spermatogonia and other spermatogenic cell (Figure 5).

4.3. The TUNEL technique and expression of the Bax protein

In control group TUNEL-positive (TUNEL$^+$) and Bax-positive (Bax$^+$) sperm cells were rarely observed (Figure 1 and Table 2). The immunohistochemical results one day after SCI in the experimental group showed that most of TUNEL$^+$ and Bax$^+$ cells were found in the spermatogonial and primary spermatocyte series, as these cells were especially in the line of round spermatid and sperm. There was no statistically significant difference in the TUNEL$^+$ and Bax$^+$ cells at one day after SCI when compared to control group ($P = 0.073$ and $P = 0.067$, respectively) (Table 2 and Figure 2). In contrast the differences at 7 days after SCI were statistically significant ($P = 0.014$ and $P = 0.019$, respectively) and the TUNEL$^+$ and Bax$^+$ cells has significantly increased. Most of these cells were spermatogenic cell lines including round spermatid and spermatozoa. Results of 1 week after SCI showed more TUNEL$^+$ and Bax$^+$ cells compared to 1 day SCI group. Most of TUNEL$^+$ and Bax$^+$ cell were in the spermatogonial and primary spermatocyte series. TUNEL$^+$ and Bax$^+$ cells for this group are represented in Table 2 and Figure 3. In 14 days after SCI in experimental group, the results show that the TUNEL$^+$ and Bax$^+$ cells has significantly increased when compared to control group ($P = 0.0055$ and $P = 0.0072$, respectively). Furthermore, a significant increase was observed when we compared this group with days 1 and 7 after SCI (Table 2). The TUNEL$^+$ and Bax$^+$ cells were significantly elevated in all of the spermatogenic series cells in the seminiferous tubules including spermatogonia primary spermatocyte, round spermatid and spermatozoa (Figure 4 and Table 2). There was no significant increase in the TUNEL$^+$ and Bax$^+$ cells at 28 days after SCI group compared to the control group. In contrast, a sig-

nificant decrease in TUNEL$^+$ and Bax$^+$ cells was seen when compared with the 14 days after SCI group ($P = 0.006$) (Table 2), the TUNEL$^+$ and Bax$^+$ cells were observed in line of spermatogonial cells, and primary spermatocyte (Figure 5).

5. DISCUSSION

The availability of suitable mouse models for the study of sperm cell apoptosis has a key role in the understanding of the process during the early stages of the SCI in the seminiferous tubules.[20] In this regard, cell apoptosis and Bax protein expression were studied after SCI in the early stages of testicular sperms. Results showed that one day after SCI in the testis tissues of experimental group the morphology of seminiferous tubules were regular and round with external border and regular basement membrane. Moreover, the results of the histological examination showed that the core of spermatogonial cells in the primary spermatocyte and round spermatid were dense. However, one day after SCI, the basement membrane was irregular. Also, the core of spermatogonial cells, primary spermatocyte and round spermatid were dense. These results could be attributed to the variation in the testicular temperature. This may be due to a disorder which may occur in the temperature of the testis after SCI, which is caused by denervation.[21] On the other hand, Chow et al. (2002) reported that the disorders of producing spermatogenesis after SCI in the longterm were not related to the etiology of endocrine chronic SCI. They showed that the neurological disorders, particularly in superior spermatic nerve (SSN) can cause alteration and arrest in the spermatogenesis after SCI.[22] In another study, Patki et al. (2008) reported that the quality of semen in men with SCI was poor, and these changes were observed in two weeks after SCI.[23] It should be noted that the initial variations in the acute period of the SCI may be due to hormonal imbalance.[21] According to the histological examination, an irregularity was observed in the trend of spermatogonial cells distribution one week after SCI. Moreover, after two weeks, cessation in the spermatogenesis trend and unstructured sperm cells were observed in some seminiferous tubules. In addition, cessation of spermatogenesis trend, unstructured sperm cells and epithelium atrophy were observed in some seminiferous tubules after four weeks of SCI. Moreover, the core of spermatogonial cells, primary spermatocyte, and round spermatid were dense. Also, eosinophilic masses were in different sperm categories. Therefore, the changes in the produced spermatogenesis increased over time. This result is consistent with the results of previous studies;[22,24,25] bearing in mind that the increased rate of aneuploidy for sperm with fragmented DNA was related to the occurrence of aneuploidy during sperm maturation, thereby TUNEL assay was widely used to identify apoptotic cells in the tissue sections. Chohan et al. (2006), it has been reported that the TUNEL assay is an accurate method for quantifying apoptosis in the germ cells.[26] In the present study the TUNEL assay was used for the study of sperm cells after SCI during the experiments. Results obtained indicated that one day after SCI, some TUNEL$^+$ sperm cells were observed with brown core. Especially, most of these cells were in the category of spermatocytes and round spermatids. This trend was also observed one week after SCI, and most of the observed cells were in the category of spermatocytes, round and long spermatids. Moreover, results showed that in those two weeks after SCI the cytoplasm of sperm cells were also brown. Four weeks after SCI, Bax$^+$ sperm cells were observed with brown cytoplasm, especially, the cells were in the category of spermatogonia. Therefore, the results concluded that the round cells increased over time after SCI, and after four weeks, long cells appeared as well. Previous studies have reported that the increase of round cells may be related to the response of body round cells to changes in sperm parameters after SCI, such as white blood cells.[27] Meanwhile, in a similar study Hirsch et al. (1999) reported that the morphology of sperm cells obtained from the epididymis changed between 2 to 12 weeks after SCI.[21]. Furthermore, caspase are a group of cysteine proteases and key effectors of apoptotic cell death. The caspase-3 is the principal effector whose activation has been implicated in the prevention of apoptotic cell death and its down-regulation in disease conditions leading to necrosis. Sperm count reduction in SCI rats observed in the present study appears to be due to the inhibitory action of SCI on spermatogenesis, including spermatogenic arrest and reduced spermatogenic cell number which is in accordance with previous studies. Also, the lesion surrounding the spinal cord cascades a series of inflammatory changes within the spinal cord tissue leading to diminutive effects on neuronal conduction homeostasis and integrity. This phenomenon follows a biphasic pattern. TNF-α could potentiate glutamate-mediated neuronal cell death in the rat spinal cord.

6. CONCLUSIONS

Our data revealed that degenerative changes occurs in the germinal cells in testicular seminiferous tubules during acute phase of SCI. Some of the degenerative cells exhibit morphological features of Bax-dependent apoptosis as well as the increase in Bax protein expression. The evaluation of Bax protein expression of SCI could be important for prognostic outcome. Therefore, suggest that the inhibition of caspase activity may play a protective role in the testicular atrophy through acute phase of SCI.

Conflict of interest

The authors declare no conflict of interest.

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