Spinal Cord Contusion Impairs Sperm Motility in the Rat Without Disrupting Spermatogenesis

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ABSTRACT: Our previous studies demonstrated various abnormalities in spermatogenesis after spinal cord injury (SCI) in cord-transected rats. In this study, we examined whether abnormalities in spermatogenesis in SCI rats were related to the degree of SCI. We used spinal cord-contused (SCC) rats as a model. Adult male Sprague-Dawley rats were subjected to various degrees of cord contusion caused by the weight of a rod dropped from different heights (12.5, 25, 50, and 75 mm) using a New York University IMPACCTOR. Testicular histology revealed persistent complete spermatogenesis in all SCC rats 4, 8, or 14 weeks after cord contusion regardless of the extent of SCI. Northern blot complementary DNA (cDNA) hybridization revealed transient but significant decreases in the levels of Sertoli cell-specific transcripts in SCC rats. In addition, levels of messenger RNA (mRNA) transcripts for germ cell-specific transition protein-2 and protamine-1 were consistently decreased in these rats. Such effects were related to the height of the weight drop and were associated with reduced levels of mRNA for cyclic adenosine monophosphate (cAMP) responsive element modulator (CREM). These results demonstrated specific effects of SCI on spermiogenesis and were consistent with altered cAMP signaling in testicular cells after SCI. Sperm motility was also significantly decreased in SCC rats and was related to the height of weight drop. Normal sperm motility recovered only in those rats injured by weight drop from 12.5- and 25-mm heights. In summary, current results demonstrate persistent abnormalities in spermiogenesis and sperm motility in rats that suffered spinal cord contusion by weight drop. Such effects were related to the height of the weight drop and thus to the extent of SCI.

Key words: Spinal cord injury, cAMP, sperm.

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More than 80% of the estimated 200,000 individuals who survive spinal cord injury (SCI) from all causes each year are men in the prime of their reproductive years (ages 15–29). These men are now expected to live longer and with more functions than in the past. Since most men become infertile after SCI, fertility may become a major concern for these men. SCI-related male infertiltiy is associated with abnormal semen quality attributable to abnormal spermatogenesis (Hirsch et al, 1991; Linsenmeyer and Perkaf, 1991; Brackett et al, 1994), impaired epididymal function (Linsenmeyer et al, 1999), or dysfunction in male accessory glands (Brackett et al, 2000). Our previous studies, using cord-transected SCI rats as a model, suggest that hormone deprivation accounts for the acute effects of SCI on spermatogenesis (Huang et al, 1995), whereas neurogenic mechanisms underlie abnormal spermatogenesis and or regression of the seminiferous epithelium during the chronic phase of SCI (Huang et al, 1998; Chow et al, 2000). However, because only 10%–15% of SCI men suffered complete cord transection, knowledge of changes in spermatogenesis and sperm function occurring after cord transection in rats may not equate to the same changes in SCI men.

The extent of SCI resulting from contusion is a function of the sheer force generated by the weight of a rod that drops from different heights (Kwo et al, 1989). By using this method, various degrees of SCI resembling those in SCI men can be induced in the rat. Spinal cord-contused (SCC) rats provide a unique animal model for evaluating the relationship between the extent of SCI and the integrity of peripheral functions. In this study, we examined the normalcy of spermatogenesis and sperm function in SCC rats with various degrees of SCI induced by weight drop from different heights.

Materials and Methods

Animals

Mature Sprague-Dawley rats (275–300 g, Taconic Farm, Taconic, NY) were caged individually in an air-conditioned, light-controlled animal room for 1 week prior to the experiment. They were fed Purina rat chow and water ad libitum. For induction of cord contusion, the rats were anesthetized with 45 mg/kg of so-

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dium pentobarbital, and the spinal cord was exposed at the level of T9–T10 by laminectomy. The spinal cord of each rat was then covered by a rod from different heights using a New York University IMPACTOR (Kwo et al., 1989). Sham control animals received laminectomy but without weight drop. After injury, the muscle layer was sutured, and the wound was closed with surgical clips. Details of the postoperative care of SCC rats were identical to those reported previously for cord-transected rats (Linsenmeyer et al., 1994). The use of the New York University IMPACTOR to induce spinal cord contusion in the rats was approved by Institutional Animal Care and Use Committees at both the East Orange VA Medical Center and the University of Medicine and Dentistry-New Jersey Medical School.

Experiments

Experiment 1—Animals were subjected to a weight drop from a 12.5-, 25-, 50-, or 75-mm height. Animals were killed at 5-7 days or at 14 weeks after injury by decapitation to collect trunk blood for hormone measurement.

Experiment 2—Animals were subjected to a weight drop 1 (X) or 3 times (3X) from 25- or 75-mm heights. Animals were hormonally castrated 4 weeks after the injury and killed by decapitation 4 weeks later.

In both experiments, a portion of 1 testis was fixed in Bouin solution and processed for histology. The remaining tissues were stored at -80°C for further analysis.

Sperm Motility

The epididymides were dissected immediately from the testes after killing and immersed in 4-5 mL of 37°C Kreb Ringer solution supplemented with sodium pyruvate (1 mM), D-glucose (5.57 mM), sodium bicarbonate (10 mM), HEPES (25 mM), and bovine serum albumin (2%). The distal end of the caudal epididymis was punctured in 20-30 locations with a 19-G needle, and the sperm were flushed out with a gentle stream of buffer. The sperm suspension was kept at 37°C for 10-15 minutes. A drop of sperm suspension was then placed on a hemacytometer and examined. Sperm in 10-20 microscopic fields were videotaped, and sperm motility was later determined. A sperm was considered "motile" if it did not remain at the same location during the 5- to 10-second taping time.

Northern Blot Complementary DNA Hybridization

Detailed procedures for isolation of tissue RNA by the single-step method (Chomczynski and Sacchi, 1987), enrichment of poly-A+ RNA by oligothymine dinucleotide cellulose chromatography (Aviv and Leder, 1972), and Northern blot complementary DNA (cDNA) hybridization (Sambrook et al., 1989) of Sertoli and germ cell-specific transcripts have been previously reported (Huang et al., 1999, Ottenweller et al., 2000).

Hormone Measurement

Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone concentrations were determined by radioimmunoassay as previously described (Huang et al., 1991).

Immunostaining of Cyclic Adenosine Monophosphate Responsive Element Modulator

Five-micron-thick sections of Bouin solution fixed testicular tissue were deparaffinized in xylene, rehydrated in graded ethanol, and boiled in 0.01 M citrate buffer (pH 6.0) for 10-15 minutes. After a phosphate-buffered saline (PBS) wash (twice for 5 minutes each), the sections were incubated with 1.5% hydrogen peroxide for 10 minutes and washed twice in distilled water, washed in 2 changes of PBS (5 minutes each), and blocked in 4% normal goat serum in PBS containing 0.01% Tween 20 for 20 minutes. After 2 washes in PBS, the sections were then incubated with polyclonal anti–cyclic adenosine monophosphate (cAMP) responsive element modulator (CREM) antibody (1:300 dilution in blocking solution, Santa Cruz Biotechnology Inc, Santa Cruz, Calif) at 4°C overnight. For the negative control, the CREM antibody was incubated with a fivefold excess of purified antigen at 4°C overnight and diluted to 1:300 in blocking solution before use. The sections were subsequently washed in 2 changes of PBS, incubated with biotin-labeled anti-rabbit immunoglobulin G (IgG; 1:2000, Sigma Chemical Co, St Louis, Mo) for 30 minutes, and washed with PBS. The sections were then incubated with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, Calif) for 30 minutes, washed in 2 changes of PBS, and visualized after adding stable diaminobenzidine solution (Research Genetics, Huntsville, Ala). The slides were dehydrated in graded ethanol solution, cleared in xylene, and covered.

Statistics

All data were analyzed to determine whether they were normally distributed. The data then were evaluated using analyses of variance of treatment groups (sham and SCC from different heights). When there was a significant treatment effect (P < .05), Dunn tests were used to determine the significance of the differences among groups and to correct for multiple comparisons. In addition, regression analysis was used to determine whether changes in parameters were related to the height of weight drop.

Results

Serum Hormone Concentrations

Fourteen weeks after cord contusion, serum testosterone levels of SCC rats in the 12.5- or 25-mm height groups were not different from sham control levels (P > .1), but serum testosterone levels of those rats in the 50- and 75-mm height groups were significantly lower than sham control levels (P < .05, P < .01, Table). Serum testosterone levels were also marginally lowered in those SCC rats 4 weeks after they were injured by 3X weight drops from the 75-mm height (P < .058). In both experiments, serum FSH and LH levels of SCC rats were not different from those of sham control rats.

Organ Weights

The testis weight of SCC rats remained normal during the first week after injury (experiment 1, data not shown).
was a significant increase in CREM in young spermatocytes, including the preleptotene spermatocytes and sometimes the spermatogonia in SCC rats (Figure 1F through H). This phenomenon was least pronounced in those rats injured by a weight drop from the 12.5-mm height and most pronounced in those rats injured by a weight drop from the 75-mm height. Such changes were no longer apparent 8 or 14 weeks after the injury (data not shown).

**Northern Blot**

Fourteen weeks after the cord contusion, the levels of messenger RNA (mRNA) for Sertoli cell–specific androgen binding protein (ABP) and the inhibin subunit (α1: 2.4 kilobase [kb], α2: 1.3 kb) of SCC rats were not different from those of sham control rats. Transferrin (Trf) transcript levels, however, were significantly lower in SCC rats in the 25- and 50-mm height groups (P <.05 and P <.01, Figure 2A). On the other hand, several germ cell–specific mRNAs exhibited various patterns of changes among the SCC rats. The mRNA levels of nuclear transition protein-2 (TP-2) and lactate dehydrogenase C (LDHC) were only significantly reduced in the 50-mm height group (P <.05). In contrast, the levels of protamine-1 (Pm-1) mRNA were significantly lowered in SCC rats injured by weight drops from the 25-, 50-, and 75-mm heights (P <.01, Figure 2B), and this effect was negatively correlated with the height of weight drop (r = -0.65, P <.005). Hemiferrin (Hemif) mRNA levels in SCC rats were comparable to those in sham controls. Linear regression analysis also revealed a negative correlation between levels of CREM mRNAs and the height of the weight drop (CREM1, 3.8 kb: r = -0.52, P = .018; CREM2, 2.3/1.6 kb: r = -0.70, P <.01). However, a significant decrease in mRNA level was observed only in CREM2 mRNA of the 50- and 75-mm height groups (P <.05, Figure 2C).

In the second experiment, while levels of mRNA for ABP were only marginally reduced in SCC rats injured by 1X weight drop from both the 25- and 75-mm heights (P <.06), Trf mRNA levels were significantly lower in most groups of SCC rats 4 weeks after injury (P <.05, Figure 3A). At 4 weeks postinjury, the levels of inhibin α1 mRNA were not affected in those rats injured by a weight drop from the 25-mm height, but they were significantly decreased in the rats in the 75-mm height group (P <.01). The level of inhibin α2 mRNA remained unaffected. As in the first experiment, there were general decreases in the levels of mRNA for TP-2 and Pm-1 in most SCC rats (P <.05 and P <.01, Figure 3B); such effects were not related to the height of weight drop. On the other hand, levels of mRNA for hemif and LDHC were not significantly affected (Figure 3B). While there was a general decrease in the level of CREM mRNA transcripts, it was marginally significant (P <.06).
Sperm Motility
Fourteen weeks after the sham operation (experiment 1), more than 65% of caudal epididymis sperm were motile in the control rats. Sperm motility of SCC rats in the 12.5- and 25-mm weight-drop groups was not different from that of sham control rats but was significantly lower in the SCC rats that were injured by weight drops from the 50- and 75-mm heights ($P < .05$, Figure 4A). In the second experiment, sperm motility was reduced more than 50% in all SCC rats 4 weeks after they were injured by 1× or 3× weight drops from both the 25- and 75-mm heights ($P < .01$ and $P < .05$, Figure 4B); however, this decrease was not related to the height of weight drop. By the eighth week, the sperm motility of rats injured by a 1× weight drop from the 25-mm height had returned to approximately 60% of normal but remained below 50% in all other groups ($P < .01$).

Discussion
Our previous experiments using cord-transected SCI rats as a model demonstrated significant suppression of the pituitary-testis hormonal axis that was believed to account for abnormalities in spermatogenesis during the acute phase of the injury (Huang et al, 1995). The transient or permanent regression of the seminiferous epithelium in cord-transected rats during the chronic phase of the injury occurred in the presence of normal serum hormone levels, suggesting that nonhormonal factors are involved in the chronic effects of SCI on spermatogenesis (Huang et al, 1998). The presence of relative normal serum FSH and LH levels in SCC rats was consistent with that observed in chronic SCI rats (Huang et al, 1995, 1998). The lowered serum testosterone in those SCC rats injured by weight drops from a 50- or 75-mm height (perhaps stress related) was insufficient to result in higher serum LH levels. To our surprise, complete spermatogenesis was maintained in all SCC rats. Nevertheless, apparent abnormalities in the nuclei of pachytene spermatocytes and total regression of the seminiferous epithelium in some tubular cross sections from rats that were injured by a weight drop from the 75-mm height were consistent with abnormal spermatogenesis after SCI (Huang et al, 1998). Since damages to the spinal cord were manifested by paralysis of the hind limbs and loss of spontaneous voiding capability in cord-contused animals, the absence of more pronounced spermatogenic effects in SCC rats perhaps was because injuries induced by contusion were not severe enough to impair spermatogenesis. To test this postulate, rats were subjected to repeat cord contusion in the follow-up experiment (experiment 2). The persistence of relatively normal testis weights and complete spermatogenesis in those SCC rats injured by 3× weight drops from the 75-mm height argued against our postulate. Thus, the lack of severe spermatogenic effects in SCC rats was most likely due to the presence of a neuronal connection between the central nervous system and the testis.

Previously, we reported a significant decrease in Trf mRNA levels (Huang et al, 1995) and changes in the response of Sertoli cell-specific transcripts to testosterone and/or FSH (Huang et al, 1999, 2003; Ottenweller et al, 2000) after spinal cord transection, which illustrated an alteration in Sertoli cell functions after SCI. While decreases in Sertoli cell transcript levels 4 weeks after cord contusion were consistent with abnormal Sertoli cell func-

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Figure 1. Immunohistochemical localization of cyclic adenosine monophosphate responsive element modulator (CREM) in testicular cells. (A) A sham control rat showing various intensities of CREM immunostaining in the nuclei of spermatocytes and spermatids at different stages of spermatogenesis. The roman number indicates the seminiferous stage of the tubular cross section when identifiable. (B) An overview of CREM distribution in spermatogenic cells of a spinal cord-contused (SCC) rat that was injured by a weight drop from a 50-mm height. While CREM was present in the nuclei of young spermatids in stage IV and VI epithelium, it was also present in the nuclei of young and pachytene spermatocytes in other tubules. (C) A SCC rat injured by a weight drop from a 75-mm height. In this testis, CREM in young spermatids was absent or greatly reduced compared to that in (A). In contrast, CREM intensity in the nuclei of spermatocytes was significantly increased in all tubular cross sections. (D) Portion of a stage VIII tubule of the sham control rat shown in (A), demonstrating the presence of moderate strength of CREM signal in the nuclei of pachytene spermatocytes (P) and young spermatids (Sd). The nuclei of preleptotene spermatocytes (red arrows) at the periphery of the stage VIII epithelium were not stained. In a neighboring stage I or II epithelium, a weak CREM signal was seen in the nuclei of young pachytene spermatocytes (P), whereas young spermatids nearby showed a relatively strong CREM signal. (E) Stages VI and XII epithelium of the same sham control rat. A CREM signal was present in the nuclei of pachytene spermatocytes (P) and young spermatids but was not detected in type B spermatagonia (arrows) at the periphery of the stage VI epithelium. A weak-to-moderate strength of CREM signal was detected in the nuclei of zygotene (arrowheads) and pachytene (P) spermatocytes as well as in elongated spermatids in the neighboring stage XII epithelium. (F) Adjacent stages IX and VI epithelium of the SCC rat shown in (B). Note the presence of a very strong CREM signal in the nuclei of preleptotene spermatocytes (red arrowheads) in the stage IX epithelium and that of type B spermatagonia (black arrowheads) in the stage VI epithelium. In both tubules, the intensity of CREM in pachytene spermatocyte (P) nuclei was much stronger than that in comparable stages in the sham control rats shown in (D) and (E). Note that the nuclei of these pachytene spermatocytes appeared to be less decondensed than those seen in stages VIII and VI in (D) and (E), respectively. (G) Neighboring stage IV and VI epithelium of the 50-mm SCC rat shown in (B). Note the presence of a relatively strong CREM signal in pachytene spermatocyte (P) nuclei that appeared to be less decondensed than that seen in sham control rats in (E). In addition, a very strong CREM signal was detected in the nuclei of intermediate spermatagonia (black arrows) and type B spermatagonia (red arrows) in stage IV and VI epithelium, respectively. (H) Neighboring stages VIII and XII of the 75-mm SCC rat shown in (C), showing the presence of strong CREM immunostaining in the nuclei of preleptotene (P) and pachytene (P) spermatocytes in a stage VIII epithelium and zygotene spermatocytes (arrowheads) in a neighboring stage XII epithelium.
Figure 2. Northern blot analysis of the steady-state level of messenger RNA (mRNA) transcripts for (A) Sertoli and (B) germ cell–specific proteins and (C) cyclic adenosine monophosphate responsive element modulator (CREM) 14 weeks after spinal cord contusion (experiment 1). Upper panel: representative radiographs of different mRNA levels. Each lane contained 15 μg poly(A)+-enriched RNA, and the radiographs were developed after 1–2 days of exposure. Lanes 1 and 2: sham control; lanes 3 and 4: 12.5-mm spinal cord-contused (SCC) rats; lanes 5 and 6: 25-mm SCC rats; lanes 7 and 8: 50-mm SCC rats; lanes 9 and 10: 75-mm SCC rats. Lower panel: quantitative analysis of the changes of different mRNA transcripts after they were normalized against the abundance of 18s RNA in each sample. * \( P < .05 \), ** \( P < .01 \).
Figure 3. Northern blot analysis of the steady-state level of messenger RNA (mRNA) transcripts for (A) Sertoli and (B) germ cell–specific proteins and (C) cyclic adenosine monophosphate responsive element modulator (CREM) in spinal cord-contused (SCC) rats 4 weeks after repeated cord contusion (experiment 2). Upper panel: representative radiographs of different mRNA levels. Lanes 1 and 2: sham control; lanes 3 and 4: 1x from 25-mm height SCC rats; lanes 5 and 6: 3x from 25-mm height SCC rats; lanes 7 and 8: 1x from 75-mm height SCC rats; lanes 9 and 10: 3x from 75-mm height SCC rats. Lower panel: quantitative analysis of the changes of different mRNA transcripts. See legend of Figure 2 for other details.
tion in SCC rats, the return of normal levels of these transcripts 14 weeks postinjury indicated recovery of Sertoli cell functions in these rats. On the other hand, reduced levels of mRNA transcripts for germ cell–specific TP-2 and Pm-1 in SCC rats in both experiments were indicative of abnormal spermiogenesis. TP-2 and Pm-1 are spermatid-specific nuclear proteins that are involved in the condensation and maturation of spermatid nuclei, the processes essential for the final morphogenesis of sperm head (Bower et al., 1987; Kleene and Flynn, 1987). Abnormal expression of these genes could impair such processes and contribute to abnormal morphogenesis of the sperm head. Abnormal sperm morphology has been found in some studies of men with SCI (Hirsch et al., 1991). Decreases in these transcripts were not correlated with levels of serum hormones or Sertoli cell mRNA transcripts, suggesting that they were specific effects of SCI on spermiogenesis.

Recently, we reported changes in the response of testicular cell cAMP responsive genes to FSH and/or testosterone treatments after SCI (Huang et al., 2003), which suggest that altered cAMP signaling mediates some of the effects of SCI on spermatogenesis. Abnormal cellular distribution of CREM in testicular cells shortly after cord contusion was consistent with this thesis. Similar changes in CREM localization in spermatogenic cells have been observed in cord-transected rats given 3- or 5-cm testosterone capsule implants but not in sham control rats given identical regimens (Huang et al., unpublished data). This suggests that the changes in CREM localization resulted from the interaction between disruption of normal neural input to the testis and reduced testicular testosterone production. A similar mechanism might also exist for abnormal CREM in testicular cells in acute SCC rats, since these effects were more pronounced in those rats injured by a weight drop from 50- and 75-mm heights and had a lower testosterone production.

CREM is a nuclear transcription factor that, upon phosphorylation, modulates the function of cAMP-responsive genes (Habener et al., 1995; Daniel and Habener, 1998). Because of differences in gene splicing and promoter usage, translation products of this gene could either promote or repress the expression of cAMP-dependent genes (Foulkes et al., 1992; Daniel and Habener, 1998). The CREMs (a and β) expressed in spermatogonia and early meiotic cells in prepubertal testes were of repressor isoforms (Foulkes et al., 1992). On the other hand, the CREMt transcript is transcribed in early spermatocytes and translated in maturing spermatocytes and young spermatids and is a functional switch for postmeiotic germ cell differentiation (Sassone-Corsi, 1998). The importance of CREM signaling in spermiogenesis and sperm production has been demonstrated in clinical studies (Peri et al., 1998; Steger et al., 1999). The precocious expression of CREM in early spermatocytes and the lack of it in young spermatids during the acute phase after cord contusion could indicate abnormal cAMP signaling. Such changes could disrupt the differentiation of spermatogenic cells and could result in the formation of sperm with abnormal morphology or function. In this regard, the promoter of Pm-1 and LDHC genes contains the putative cAMP response element (Ha et al., 1997; Bonny et al., 1998) and therefore could be affected by abnormal cAMP signaling. Premature translation of Pm-1 in the transgenic mouse has been found to result in precocious condensation of spermatid nuclei and arrest of spermatogenesis at the spermatid stages (Lee et al., 1995).

Persistently reduced sperm motility after a prolonged recovery period in cord-contused rats injured by weight drops from the 50- and 75-mm heights resembled that which occurred in SCI men after they had suffered various degrees of SCI (Perkash et al., 1985; Brackett et al., 1994). Normal sperm motility observed in rats 14 weeks after they were injured by a weight drop from the 25-mm height may reflect recovery of sperm function, since sperm motility was significantly decreased 4 weeks after the rats were injured by a 1X weight drop from the 25-mm height. Of note, the sperm motility in rats injured by
3X weight drops from the 25-mm height was comparable to that of rats injured by a 1X weight drop from the 75-mm height at both time points, and sperm motility did not recover 8 weeks after the injury. This observation illustrates that cord injured by repeated minor contusions could impair sperm motility (and perhaps other sperm functions) as much as does a single contusion with greater force. Together, the current results demonstrate, first, a relationship between impaired sperm function and the extent of SCI and, second, that impaired sperm motility resulting from minor SCI is reversible, whereas that from more severe SCI might be permanent. Impaired sperm maturation due to abnormal epididymal function (Billups et al, 1990) might contribute to reduced sperm motility after SCI. However, persistent decreases in the expression of spermatid-specific proteins suggest that abnormal spermiogenesis could also contribute to defects in sperm function after SCI. These findings offer alternative mechanisms, in addition to the abnormal seminal fluid property (Brackett et al, 2000), for reduced sperm motility in SCI men (Ohl et al, 1999).

In summary, current results demonstrate persistent spermiogenesis in SCI rats with varying degrees of cord injury. However, abnormal CREM distribution in spermatogenic cells and decreased levels of germ cell–specific transcript denote abnormalities in spermatogenic differentiation. These effects were associated with a decrease in sperm motility that correlated with the extent of SCI and the time postinjury. Further understanding of the causes and mechanisms underlying such effects will form the basis for therapeutic interventions to preserve sperm function after SCI and could be beneficial to the reproductive health of SCI men.

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