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Final Narrative Report
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Background: Following spinal cord injury (SCI), fertility of men is generally impaired, attributed to abnormal semen qualities characterized by decreases in sperm count and progressive motility, and an increase in sperm with abnormal morphology. These observations suggest that multiple factors may contribute to the deterioration of semen quality after SCI. In order to develop remedies capable of preserving normal spermatogenesis and sperm functions after SCI, understanding the mechanisms leading to reduction of sperm production and deterioration of sperm quality and function is essential.

Our previous studies using spinal cord-transected (SCX) rat as a model (supported by the Department of Veterans Affair Rehabilitation Research & Development [VARR&D] Service) have characterized changes in spermatogenesis during different phases of the injury. However, because complete cord transection occurs only in approximately 10-15% of SCI men, changes in spermatogenesis and sperm function observed after SCX may not reflect that occur in all SCI men.

Spinal cord injuries induced by cord contusion (SCC) are the function of sheer force generated by a weight drop. By using this method, the spinal cord of the rat can be injured to various extents mimicking those that occur in SCI men. The initial thrust of this project was to use SCC rat as a model to determine the relationship between the extent of cord injury and normalcy of spermatogenesis and Sertoli cell function.

Animal model: Spinal cord contusion (SCC) was induced in adult male Sprague-Dawley rats (275-300 gm) at the level of the 9th-10th vertebra. The rats were anesthetized with 45 mg/kg sodium pentobarbital, and the spinal cord will be exposed at the level of T9-T10 by laminectomy. Different extents of cord contusion were induced by weight drop delivered by the **NYU *IMPACTOR*** with different height settings (12.5, 25, 50 and 75 mm). The muscle layer will be sutured and the wound will be closed with surgical clips. The post-operative care of these SCI rats was undertaken using the protocol designed for the care of cord-transected rats.

Project Success

Results of multiple experiments have demonstrated that spermatogenesis persisted in >95% of SCC rats regardless the duration and/or the extent of cord injury. This was different from that in SCX rats in which the seminiferous epithelium regressed to various extents during early phase of the injury and totally regressed in >50% of the animals during the chronic phase of the injury. Nevertheless, abnormalities in spermatogenesis, including abnormal expression and cellular distribution of cAMP responsive element modulator (CREM, a master switch of post-meiotic germ cell differentiation), expression of Sertoli and germ cell specific transcripts, and defect in spermiogenesis (spermatid differentiation), in SCC rats resembled that seen in SCX rats. The extent of these changes varied according to the extent of the injury and time post injury. These results confirmed our postulate that the effects of SCI on spermatogenesis were related to the extent of the injury, and suggested that altered cAMP signaling events might mediate some of the effects of cord injury on spermatogenesis.

Furthermore, we found that sperm motility was impaired to various degrees, depending on the extent of the injury, in all SCC rats. These effects persisted in those rats with more severe injury but recovered in those suffered less severe injury. These results demonstrate a correlation between abnormal sperm motility and the extent and duration of the injury. The SCC rat thus provides an appropriate model to study the mechanisms leading to deterioration in sperm function after cord injury, and to test the effectiveness of various methods to improve sperm functions. As discussed and proposed in the Progress Report submitted at the end of the 1st year, we undertook additional experiments to characterize changes in sperm functions in SCC rats, and compared these effects to that in SCX rats. Highlights of some of these experiments are presented as the following

(A) Decreases in sperm motility after SCI were associated with altered cAMP signaling events

Cyclic AMP signaling is essential for various sperm functions and involves protein phosphorylation in various organelles and substructures. We first examined the effects of SCC and SCX on sperm motilty and its relationship with sperm cAMP contents. As shown in Figures 1-2, sperm cAMP contents were significantly elelvated ($p < 0.05$, 0.01) in SCC rats while their sperm motility was lowered compared to that in sham control rats ($p < 0.05$, 0.01). Similar increase in sperm cAMP content was also observed in SCX rats (see Figure 4). These results demonstrated that a normal relationship between sperm motility and endogenous cAMP production was disrupted after SCX and SCC.

To determine if increases in sperm cAMP affects sperm protein phosphorylation, Western blot was used to compared sperm protein phosphorylation between SCX rats and sham control rats. As

presented in Figure 3, the overall protein tyrosine phosphorylation was lower in the sperm of SCX rats, compared to that of sham control rats. Similar results were also observed in SCC rats. Tgether, these results demonstrated that impaired sperm motility after SCI (SCC or SCX) was associated with abnormal sperm cAMP status and its downstream signaling events. Since cAMP signaling events are involved in various sperm functions leading to fertilization, abnormalities in cAMP signaling events might compromise these sperm functions, and contribute to poor fertility rate commonly seen in SCI men.

(B) SCI resulted in decreases in sperm viability and mitochondrial potential

To determine if cell death and/or impaired sperm metabolism also contributed to decreases in sperm motility after SCI, we also examined the effects of SCX and SCC on sperm viability and mitochondrial potential using sperm uptake of fluorescent dyes, SYBR-14 and JC-1, specifically for viability and mitochondrial potential. As presented in Figure 2C, sperm viability and mitochondrial potential were significantly decreased in SCC rats that received weight drop from both the 25 and 75 mm heights. These initial results demonstrated that decreases in viable sperm and energy metabolism may contribute to lowered sperm motility after spinal cord injury. These effects could be due to intrinsic defects in the sperm as the result of abnormal spermiogenesis, or abnormal sperm maturation or aging resulting from dysfunction in epididymis.

(C) Exogenous testosterone (T) maintained sperm viability but not motility in SCX rats

Our previous studies demonstrated that spermatogenesis in SCX rats was partially maintained by exogenous T in dose-dependent manner. To determine if exogenous T also maintain sperm functions, we examined various sperm parameters in the sperm of sham and SCX rats given T as silastic capsules (TC) implanted immediately after the injury for 8 weeks. As presented in Figure 4A, sperm motility was suppressed by exogenous T in sham control and SCX rats, and the effects on SCX rats were more pronounced than that in sham control rats. Of note, treatment of SCX rats with low dose of exogenous T (1 or 2 cm TC) increased sperm cAMP contents whereas identical treatment of sham control rats did not (Figure 4B). Administration higher doses of T (5 or 10 cm TC) normalized sperm cAMP contents in SCX toward that of sham control rats. Failure to maintain sperm motility in these SCX rats (given 5 or 10 cm TC) despite normalization of cAMP contents was consistent with the notion that impaired sperm motility after SCX was not related to endogenous sperm cAMP contents. Lack of normal sperm motility, while sperm cAMP contents were normalized, in SCX rats given 0.1 IU of porcine FSH for 14 days prior to sacrifice also consistent with the above notion.

A follow-up experiment was performed to determine if the effects of exogenous T was due to changes in sperm viability or mitochodrial function. As shown in Figure 4C-E, sperm viability and mitochondrial potential in the sperm of sham control rats were not affected, whereas that in SCX rats were improved by high dose of exogenous T (10 cm TC). These results demonstrated that T-induced impairment of sperm motility after SCX was unrelated to sperm viability. Similar results were observed when exogenous T was administered during the chronic phase of SCI (Figure 5).

(D) Beneficial effect of vitamin E on sperm function in SCC and SCX rats

Recent clinical studies suggested that the reactive oxygen species (ROS) related cellular events might contribute to abnormal sperm functions in SCI men. We have performed preliminary experiments to determine the efficacy of an antioxidant, vitamin E, in the maintenance and restoration of sperm functions in SCC and SCX rats. In thess experiments, vitamin E (2 or 10 mg/kg) was administered to SCC (weight dropped from 75 mm height) and SCX rats immediately (maintenance) or 8-10 weeks post surgery (restoration) for 8 weeks.

Figure 6 shows that sperm motility, viability (uptake of SYBR-14) and mitochondrial potential (uptake of JC-1) were reduced significantly ($p < 0.05$, 0.01) in SCC and SCX rats in the maintenance experiment. While sperm viability and mitochondrial function in SCC and SCX rats were partially maintained by high dose of vitamin E, sperm motility in these rats was not maintained. In the restoration experiment sperm motility and viability (uptake of SYBR-14) were reduced significantly ($p < 0.05$, 0.01) in chronic SCC and SCX rats. While mitochondrial potential (uptake of JC-1) in the sperm of SCC rats was normal, that in SCX rats was also significantly reduced ($p < 0.01$). High does (10 mg/kg) vitamin E significantly improved sperm motility, viability and mitochondrial potential in SCX rats, but did not affect the same parameters in SCC rats.

Together, these results indicate that multiple mechanisms might contribute to poor sperm motility, and perhaps other sperm functions, during difference phases of the injury, and these effects were dictated by the extent of the injury. Maintenance and restoration of sperm functions in SCX rats with vitamin E supports the notion that ROS-related mechanisms might mediate some of the effects of SCX on sperm motility. Further investigation of the effects of vitamin E or other antioxidants on various sperm functions will provide further insight into the causes for abnormal sperm functions after cord injury, and will facilitate the development of therapeutic regimens to improve sperm function after SCI.

(E) Beneficial effect of vitamin E on sperm head condensation

Replacement of somatic histone in the spermatid by spermatid specific nuclear proteins during the second half of spermiogenesis results in the condensation of spermatid nuclei, a step that is essential for the morphogenesis of sperm head. Our previous experiments demonstrated that the expression of these spermatid nuclear proteins was altered after SCI. We postulated that these changes may affect the condensation of spermatid nuclei and thereby, morphogenesis of sperm head. This could result in the production of sperm with abnormal morphology, a common finding in SCI men. We performed preliminary experiments to evaluate the response of sperm heads to a reducing agent, dithiothreotol (2 mM) in the presence of 1% SDS using sperm recovered from frozen epididymis of chronic SCC and SCX rats (16 weeks post surgery), and the effect of vitamin E on sperm head condensation.

As presented in Figure 7, sperm heads of untreated sham control rat (A, without vitamin E feeding) was less de-condensed compared to that of SCC (B) and SCX (C) rats. Vitamin E feeding resulted in less sperm head de-condensation in all groups (D-F). These results demonstrate that vitamin E also benefit sperm head condensation and justify additional experiments to verify beneficial effects of vitamin E or other antioxidants on sperm morphology after SCI, as well as the mechanisms involved.

In addition to various sperm functions, we found vitamin E also attenuated the effects of SCX and SCC on male accessory glands, as indicated by the weights of these organs (Figure 8). Since secretion of these glands are the major component of seminal fluid that contains antioxidant enzymes, vitamin E or other antioxidants may also benefit sperm functions through their effects on these accessory glands.

(F) SCI altered the expression of spermatid nuclear transition proteins

The results described above strongly suggest that impaired spermatid nuclear condensation or maturation may account for abnormal sperm morphology commonly reported after SCI. Since our previous results demonstrated that the expression of mRNA transcripts for spermatid nuclear proteins (protamin and TP-1) was reduced after SCI, and these proteins provide biochemical forces for spermatid nuclear condensation and maturation, we postulated that abnormal expression of these proteins may contribute to the less-condensed sperm heads in SCC and SCX rats (figure 7). For this reason, we performed preliminary immunohistochemistry experiments, using antibodies specific for transition protein (TP)-1 and TP-2 to localize these proteins in spermatogenic cells. As presented in Figure 9, we observed reduced TP-1 and TP-2 in elongated spermatids of SCI rats (n=3) compared to that in sham control rats (n=2). In addition, we noted the TP-1 was not present in the step 17 spermatids in sham control rats, but in those of SCI rats, indicating alteration of the timing of TP-1 translation. These results indicated that the expression of TP-1 and TP-2 were affected after SCI, and could contribute to abnormal spermiogenesis and/or sperm morphology.

In summary, with the support of the NJCSCR, we were able to perform multiple experiments to validate and use SCC and SCX rats as models to investigate the effects of SCI on sperm functions. Results of these experiments provide significant new insights into possible mechanisms leading to impairment of sperm functions after SCI. These new informations fill many of the gaps in our knowledge regarding the cause for male infertility after SCI, and enalbe us to postulate new working hypothesis and design new experiments to test the feasibility of using in vitro or in vivo approaches to improve sperm functions after SCI.

Project challenges

Male infertility is a secondary effect of SCI and affects the quality of life of SCI men. Understanding the immediate causes that underlie the effect of SCI on sperm functions is essential for the development of therapeutic regimens. However, due to logistical difficulties, these informations cannot be obtained from clinical studies. Results obtained from this project demonstrated that changes in sperm

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functions in the rat after SCC and SCX in many ways mimic those that occured in SCI men, and therefore qualify SCC and SCX rats as models to study the mechanisms leading to these changes. Thus, our challenges are to utilize the results obtained from this project and SCC/SCX rats as models to test the feasibility of using various therapeutic remedies to prevent or restore abnormal sperm functions during different phases of the injury. A success in these attempts will provide scientific rationale to test the efficacy of effective remedies in clinical trials, and will lead to the development of therapies capable of preserving sperm function and thereby, fertiltiy in SCI men.

Implications for future research and/or clinical treatment

Cyclic AMP-related signaling events are essential for various sperm functions. Further identification of cAMP-dependent sperm functions which are altered after SCI, and mechanisms underlying these effects, will facilitate the use of cAMP analogs or related agents to improve sperm functions in vitro. If successful, these approaches could be used to improve sperm function of SCI men in vitro without the use of pharmacological agents in vivo, and could have immediate clinical applicability.

The beneficial effects of vitamin E on various sperm functions in SCC and SCX rats were consistent with the involvement of ROS-related events in abnormal sperm functions after SCI. Since beneficial effects of various antioxidants on sperm functions and fertiltiy have been demonstrated in animal experiments and clinical trials, antioxidants are likely to be beneficial for sperm functions after SCI. Thus, antioxidant therapy offers a simple and inexpensive therapeutic option to restore or preserve sperm functions after SCI. If successful, this approach will provide a low cost alternative to costly in vitro fertility technologies to restore reproductive capability of SCI men.

Plans for future research

Based on the results described above, we have hypothesized that (I) alteration of the cAMP signaling events and/or (II) ROS-related mechanisms are responsible for abnormal sperm function after SCI. To test these hypotheses, we have submitted a proposal entitled "Preservation of sperm functions after spinal cord injury" to the NJCSCR to test various ways to improve sperm functions in SCC and SCX rats. In addition, we have also submitted a complimentary proposal entitled "Mechanisms underlying the effect of spinal cord injury on sperm functions" to the VARR&D to investigate mechanisms leading to abnormal sperm functions after SCI.

Decreases in sperm motility after spinal cord injury were associated with higher sperm cAMP content

The effects of cord injury on sperm motility were related to the extent of cord injury. We examine sperm motility and its relationship with cAMP content in SCC rats (their spinal cord was contused by the weight of a rod (10 mg) dropped from various heights). Figure 1 shows that percent motility (**mean±sem**) of caudal epididymal sperm was decreased in SCC rats 8 weeks after the surgery (Figure 1A); such decreases were inversely related to the height of weight drop (i.e. the extent of injury, *p<0.05; **p<0.01). These effects were associated with a significant increase in cAMP level in sperm recovered from the caudal epididymides (p<0.05, Figure 1B). The cAMP level of caput epididymal sperm of these SCC rats, however, was not different from that of sham control rats.
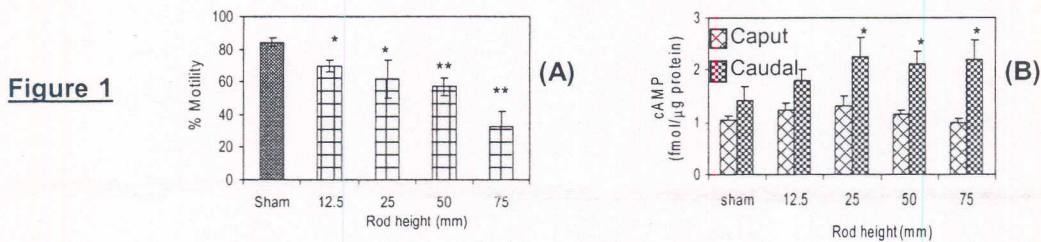
Figure 1

A follow-up experiment was undertaken to determine the effects of cord contusion on sperm functions at an earlier time (4 weeks post injury). As shown in Figure 2A motility of caudal epididymal sperm was significantly reduced in SCC rats which received a weight drop from the 25 or 75 mm height (**p<0.01). Significant increase in sperm cAMP levels was noted in sperm recovered from both the caput and caudal epididymides of these SCC rats (**p<0.01, Figure 2B). Such effects were associated with a significant decrease in sperm uptake of SYBR-14 and JC-1 fluorescent dyes (**p<0.01, Figure 2C). The latter results denoted decreases in viable sperm and sperm mitochondrial function in SCC rats. **All results were presented as mean±sem**.

Figure 2
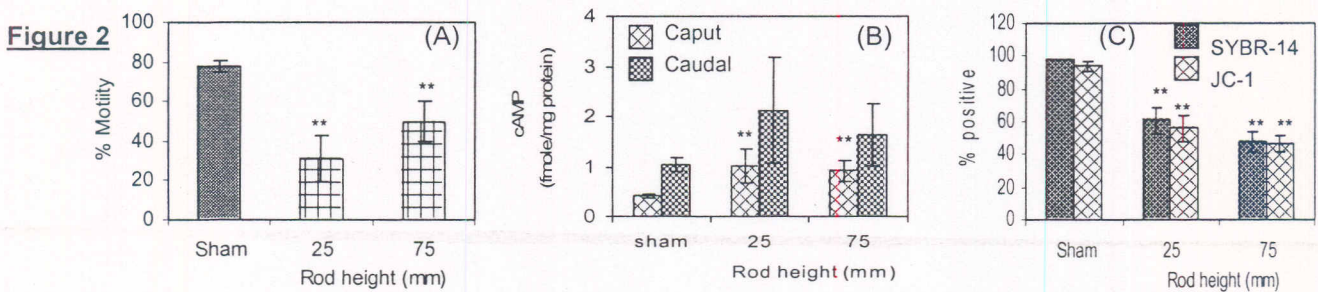
SCI resulted in decreases in sperm protein phosphorylation

An increase in cellular cAMP including that in the sperm usually results in phosphorylation of proteins that initiate changes in cellular functions. Immunoblotting of phosphorylated sperm protein however revealed an overall decrease in phosphorylated protein in sperm of SCX rats (Figure 3), despite the elevated sperm cAMP content. These results indicate that the relationship between sperm cAMP content and protein phosphorylation was interrupted after SCI.

Figure 3
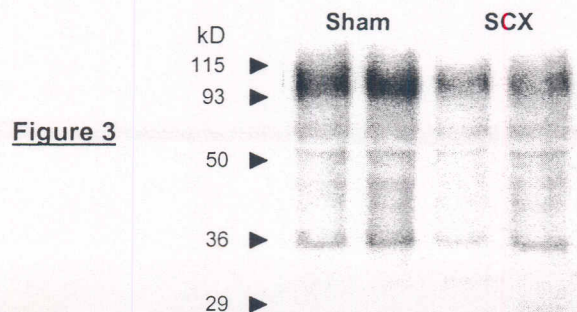
Exogenous testosterone (T) maintained sperm viability but not motility in SCX rats

Our earlier results demonstrated that sperm motility in SCX rats was maintained by low doses of testosterone (T), but suppressed by higher doses. We further examined the relationship between such effects and sperm cAMP contents in cord-transected SCX rats that were given exogenous T immediately after the surgery for 8 weeks. Sperm motility was significantly lower in untreated SCX rats compared to untreated sham controls (Figure 4A, $p < 0.05$). Implantation of 1-10 cm TC resulted in slight but dose-dependent decreases in sperm motility in sham control rats (Figure 4A, $**p < 0.01$). Similarly, sperm motility of SCX rats was not affected by 1 cm TC ($p > 0.1$), but was suppressed by higher doses of T in biphasic manners ($p < 0.01$). Sperm motility in SCX rats received 5 or 10 cm TC implants rebounded, but remained lower than that of untreated SCX rats and their sham control counterparts ($*p < 0.05$). Daily injection of FSH for 2 weeks prior to sacrifice also reduced sperm motility in sham control rats. None of the sperm of FSH-treated SCX rats were motile ($**p < 0.01$). **All results were presented as mean±sem**.

cAMP level in caudal epididymal sperm of SCX rats was also increased significantly compared to sham controls ($*p < 0.05$, Figure 4B). Administration of exogenous T resulted in a dose-dependent increase in sperm cAMP level in sham control rats, reaching a maximum in those that received 5 cm TC implants ($**p < 0.01$). Implantation of 1 or 2 cm TC further increased sperm cAMP level in SCX rats. However, variability among animals precluded statistical significance. On the other hand, implantation of 5 or 10 cm TC lowered sperm cAMP level in SCX rats to the level of their sham control counterparts. FSH injections also elevated sperm cAMP slightly in sham control rats ($*p < 0.05$) but suppressed that in SCX rats.

Figure 4C-E shows that exogenous T did not affect sperm uptake of SYBR-14 and JC-1, but increased that in SCX rats ($\#p < 0.5$). These results indicate that impairment of sperm motility after exogenous T treatment was not due to a decrease in sperm viability or mitochondrial function. (C) and (D) Representative dot plot of sperm uptake of SYBR-14 and JC-1, respectively. (E) Quantitative comparison of sperm uptake of SYBR-14 and JC-1 among groups. $**$ $p < 0.01$ vs sham control, $\#$ $p < 0.05$ vs untreated SCX. Similar results were observed in SCX rats received 10 cm TC during the chronic phase of the injury (Figure 5)
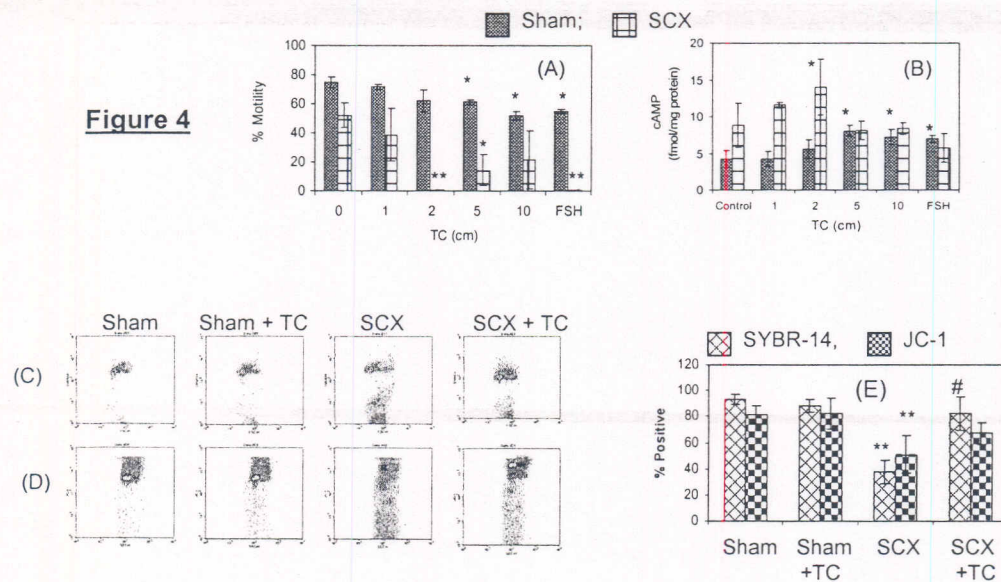

Figure 4

Figure 5. Effects of exogenous T on sperm motility (A) and SYBR-14 and JC-1 uptake in chronic SCX rats.
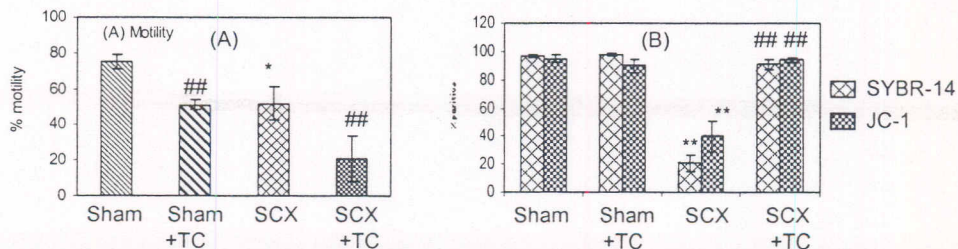
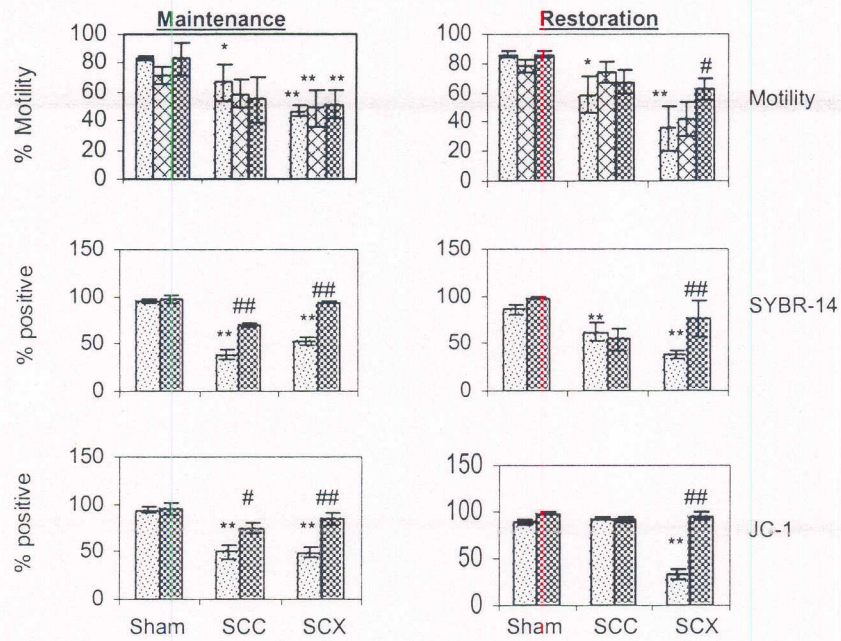# Figure 6. Beneficial effect of vitamin E on sperm function in SCC and SCX rats

Figure 7. Beneficial effect of vitamin E feeding on sperm nuclear condensation. Caudal epididymal sperm of sham control (A,D), SCC (B, E) and SCX (C,F) rats were incubated with SDS/DTT.
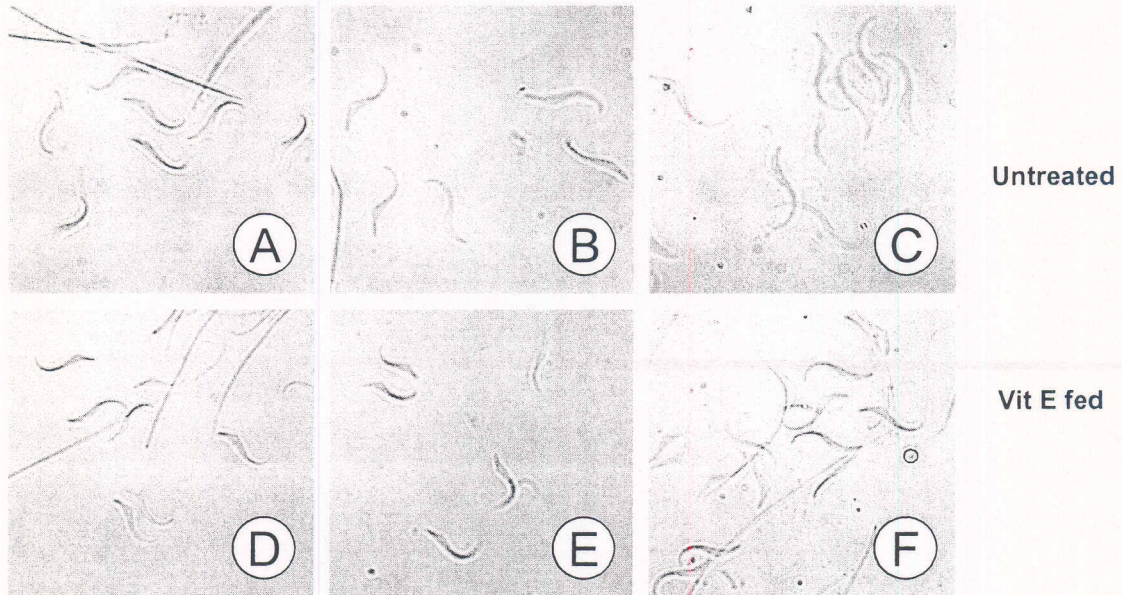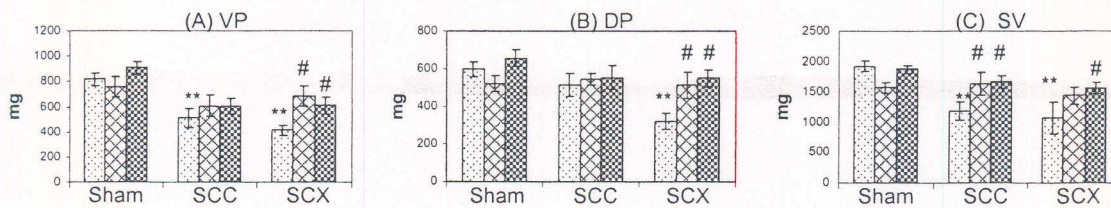

Figure 8   Vitamin E feeding attenuated the effects of SCC and SCX on male accessory glands.

** p<0.01 vs sham control, # p<0.05 vs untreated.

Figure 9. SCI affected the expression of TP-1 and TP-2 in spermatids
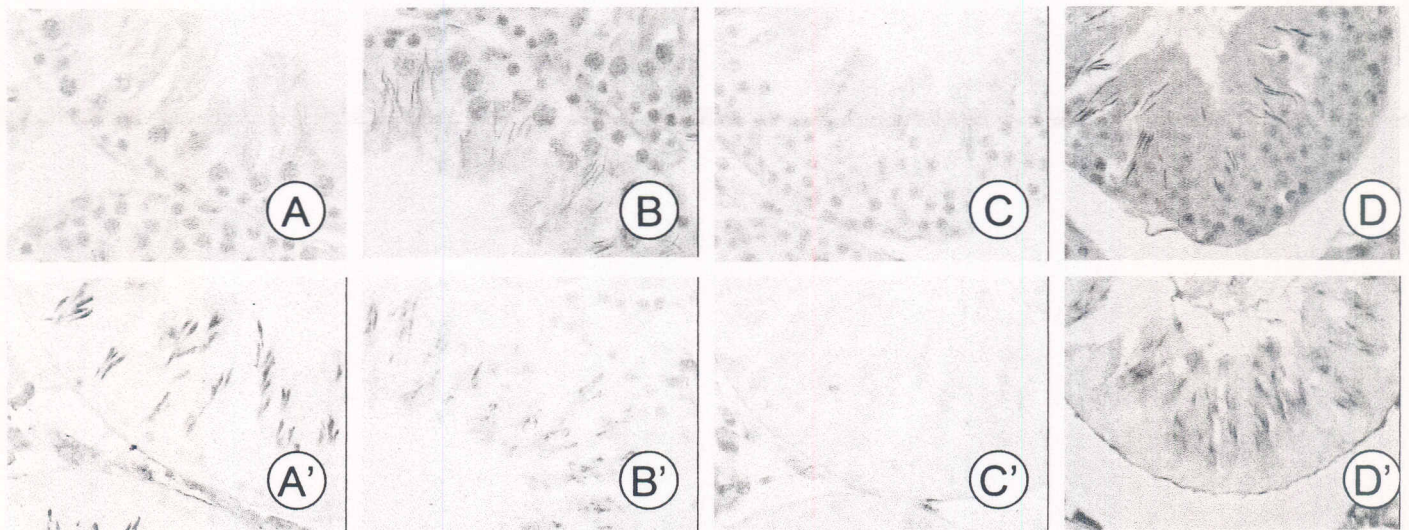

Figure 10A. Upper panel: Hematoxylin stained; lower panel: same areas immunostained with TP-1 in stage XII and IV epithelia of a sham control (A,C) and an SCI rat (B,D). TP-1 staining in elongated spermatids of SCI rat was lower in step 12 spermatids (B') but significant higher in step 17 spermatids (D'). The presence of TP-1 in step 4 spermatids of SCI rats indicates abnormal timing of the translation of this protein.
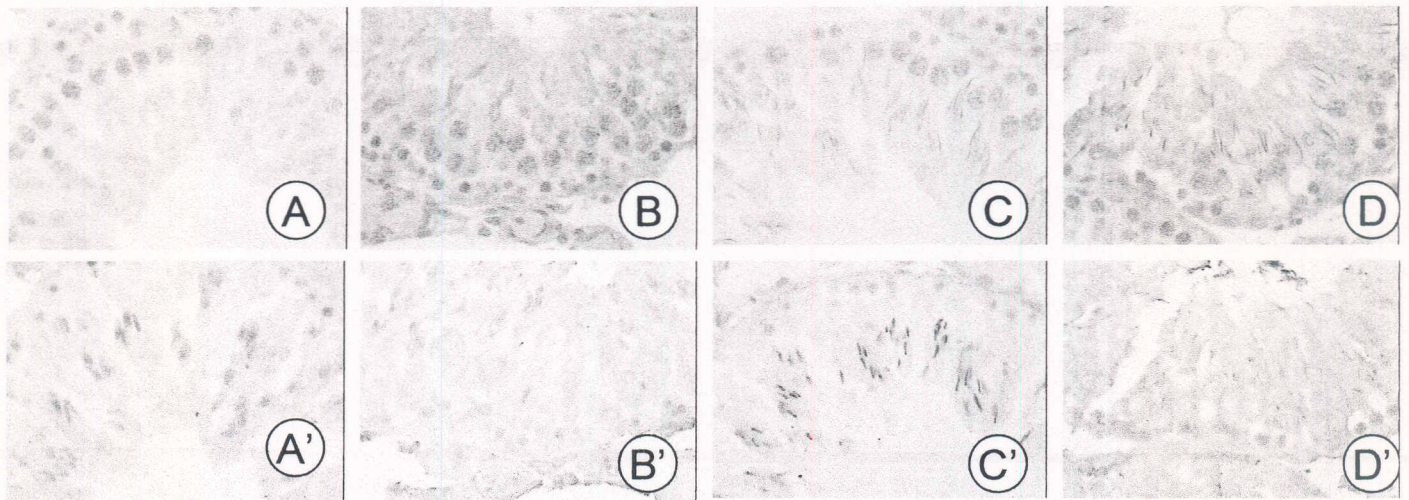

Figure 10 B. Upper panel: Hametoxylin stained; low panel: same areas immunostained with TP-2 in stage X and XII epithelia of a sham control rat (A,C) and a SCI rat (B,D). Note that TP-2 staining in steps 10 and 12 spermatids was significantly lower in SCI rats.