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Final Narrative Report
Submitted to the New Jersey Commission for Spinal Cord Research

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A. Specific aims, summarized from the original proposal

Over 80% of individuals who suffer spinal cord injury (SCI) from all causes are men in the prime of their reproductive years (ages 15-29). Consequently, infertility due to deterioration of semen quality after the injury has become a major concern for these men. Our long term goal was to develop in vitro and in vivo approaches to improve sperm functions of SCI men. During the past 12 years, we have used spinal cord transected (SCX) and spinal cord contused (SCC) rats as models to study their effects on spermatogenesis and sperm functions. Results of our experiments suggest that altered cAMP signaling events might contribute to abnormal sperm functions after SCI. In addition, we found that vitamin E feeding improved sperm motility, viability and sperm head condensation in chronic SCX rats, consistent with reactive oxygen species (ROS) -related mechanisms in abnormal sperm functions after SCI.

Hypothesis: (I) Alteration of the cAMP signaling events in the sperm underlies abnormal sperm functions after spinal cord injury. (II) Reactive oxygen species (ROS)-related mechanisms also contribute to abnormal sperm function after SCI.

Specific Aims: (I) To identify the cAMP dependent sperm functions which are impaired after cord injury. (II) Determine beneficial effects of antioxidants on sperm functions in SCX/SCC rats.

B. Project success

With the support of the NJCSCR, we were able to investigate the feasibility to use antioxidant to preserve sperm functions after SCI. While some of the functions were preserved by vitamin E feeding, many of the essential functions of the sperm were not well preserved.

(1) The effect of vitamin E feeding during the acute phase of the injury

Our preliminary results demonstrated improvement of various sperm motility, viability (SYBR-14 uptake) and mitochondrial potential (JC-1 uptake) in SCC and SCX rats fed vitamin E during the chronic phase of the injury. We therefore also examine the effect of vitamin E on sperm functions in SCC and SCX rats during the acute phase of the injury. As shown in Figure 1, 10 mg/kg vitamin E attenuated the effects of SCC/SCX on sperm viability and mitochondrial potential. Impaired sperm motility in these rats, on the other hand, was not improved by vitamin E feeding. These results suggest that the ROS- related events may mediate the effect of SCI on sperm viability and mitochondrial potential, but not for impairment of sperm motility, during the chronic phase of the injury. This is different from that in chronic phase of the injury (see Figure 7 of the original proposal), thus suggests the involvement of multiple mechanisms in the effects of SCI on sperm functions during different phases of the injury.

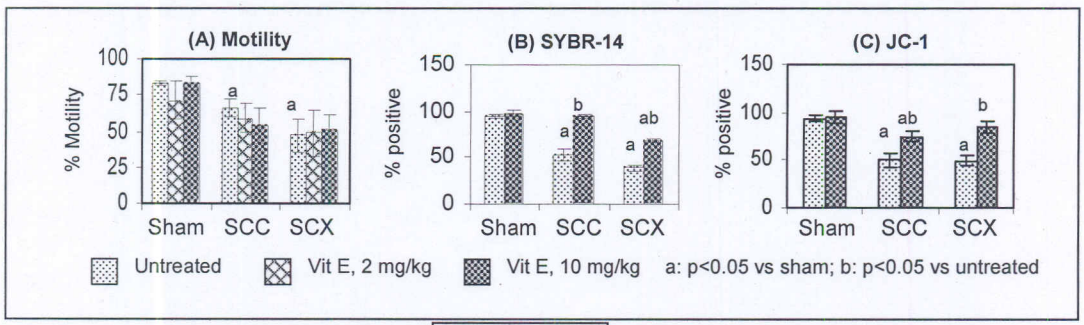


Figure 1

(2) Vitamin effects on sperm protein phosphorylation in chronic SCX rats

We have demonstrated that vitamin E feeding during the chronic phase of the injury improved sperm motility, viability and mitochondrial potential in SCX rats, but not in SCC rats (Figure 7, original proposal). To determine if the effect of vitamin E is mediated by cAMP/protein phosphorylation pathway, we further compared sperm cAMP content and protein phosphorylation in the sperm of these rats. As presented in Figure 2, sperm cAMP contents were elevated in caput and caudal sperm from SCC and SCX rats ($p<0.05$). Vitamin E feeding resulted in dose-dependent increases in cAMP in caudal sperm from sham control rats ($p<0.05$), but did not affect that from SCC and SCX rats (n=6-8 per group). Figure 3 shows a representative Western blot showing vitamin E feeding resulted in dose-dependent increases in protein phosphorylation in the sperm from sham control rats. Such effects were not seen in sperm from SCX rats. Together, these results indicate that improvement of sperm motility by vitamin E seen earlier was not related to sperm cAMP production and sperm protein phosphorylation. Stimulation of caudal sperm cAMP and protein phosphorylation in vitamin E fed sham control suggests a putative mechanism for the action of vitamin E. Failure to induce increases in sperm cAMP and protein phosphorylation in spermatozoa from SCX rats fed vitamin E was consistent with altered sperm cAMP signaling in these rats.

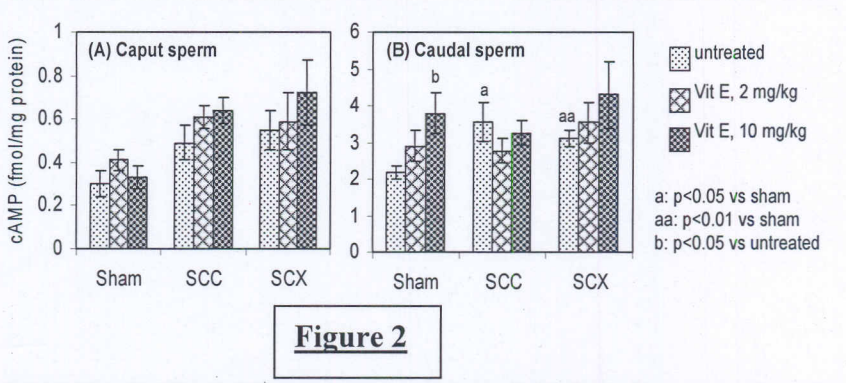


Figure 2

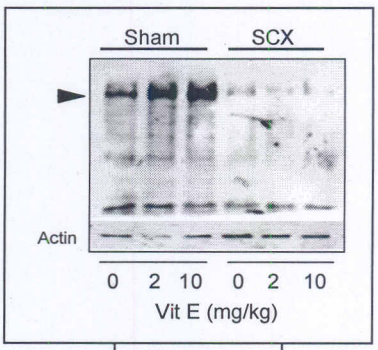


Figure 3

(3) Sperm capacitation is compromised after SCI

The sperm undergoes capacitation in female tract to acquire its final fertilization capability. Capacitation is cAMP dependent and involves protein phosphorylation, and can be induced in vitro by known capacitation agents (BSA, Ca++ and bicarbonate). Figure 4 shows that when incubated with 2% BSA, spermatozoa from a sham rat exhibited a time –dependent increases in protein phosphorylation. Spermatozoa from a SCX rat also exhibited similar increases in protein phosphorylation, but to less extent. These results suggest that the potential of spermatozoa to undergo capacitation probably is not totally abolished, but compromised, after cord injury.

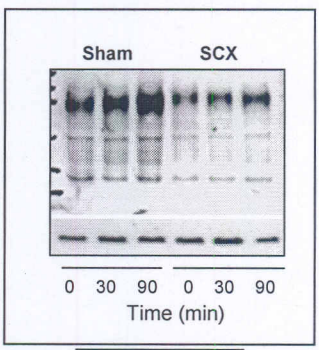


Figure 4

To verify this possibility, we examined hyperactive sperm motility, an indicator for capacitation, in spermatozoa from sham control, chronic SCC and SCX rats (n=6/group) after they were incubated in 2% BSA in PBS for 6 hrs. Figure 5 shows that more than 80% of spermatozoa from shame control rats exhibited hyperactive motility despite that only less than 60 % of the un-stimulated spermatozoa remained motile. On the other hand, less than 20% of untreated spermatozoa from SCC and SCX rats were motile after 6 hrs. While 30-35% of spermatozoa from SCC rats exhibited hyperactive motility when exposed to 2% BSA, only approximately 20% of the BSA treated spermatozoa from SCX rats exhibited hyperactive motility. These results demonstrated that motility of spermatozoa from SCC and SCX rats cannot be maintained under physiological condition for prolonged period, and only a small fraction of these spermatozoa were able to respond to capacitation agent (2% BSA) and perhaps were able to undergo capacitation. These results may thus provide some mechanistic explanation for poor sperm fertility in men after suffering SCI. While vitamin E feeding improves sperm initial motility in SCC and SCX rats to some extent, it failed to improve sperm capacitation potential in these rats.

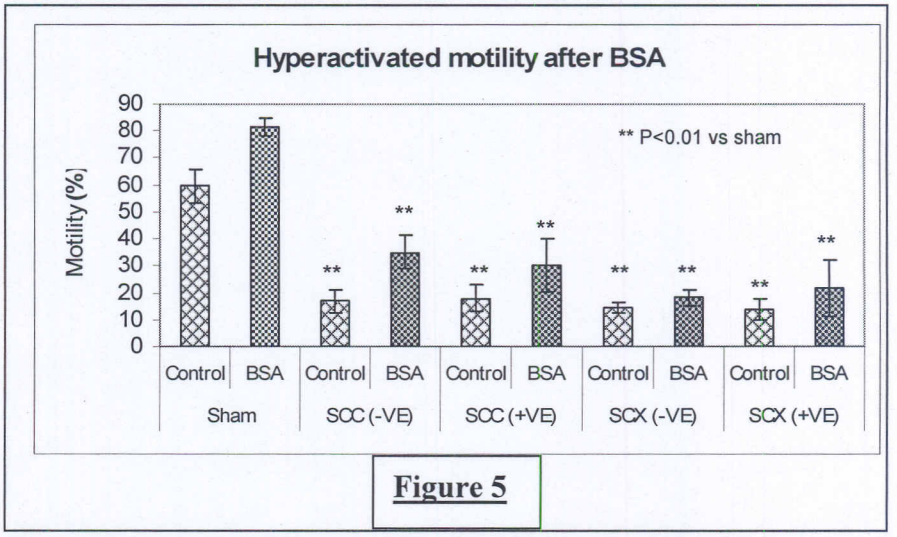
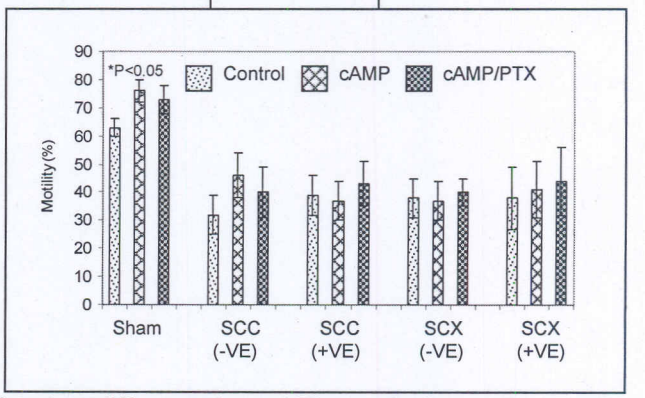


Figure 5

(4) Response of spermatozoa to AMP

Our preliminary results suggested that the cAMP-signaling events in rat sperm might be altered after SCI. Because cAMP signaling is vitally important for sperm functions including motility and capacitation, we further examine if spermatozoa can respond to cAMP after cord injury, spermatozoa from chronic sham, SCC and SCX rats (n=6-8 per group) were incubated with dibutyryl (db)cAMP (10 uM) in the presence or absence of a phosphodiesterase inhibitor pentoxyfylline (PTX, 10 uM). Sperm motility and protein phosphorylation were examined at different times. Figure 6 shows that exposure of spermatozoa from sham control rats to cAMP for 30 min resulted in a significant increase in sperm motility even though this effect was not enhanced by PTX. On the other hand, motility of spermatozoa from SCC and SCX rats did not respond to cAMP, and vitamin E feeding did not improve the responses of sperm motility to cAMP and PTX in these rats. As expected, cAMP treatment enhanced protein phosphorylation in spermatozoa from sham and SCX rats in time dependent manner. However, vitamin E feeding did not improve the cAMP-

Figure 6



induced sperm protein phosphorylation (data not shown). These results are consistent with impaired cAMP signaling the sperm after SCI.

(5) Failure to confirm changes in CREM switch in spermatogenic cells after SCI

Our preliminary RT-PCR experiments revealed abnormal pattern of cDNA of the cAMP responsive element modulator (CREM) in isolated spermatids from the rat after spinal cord injury (SCI). These results, and abnormal distribution of CREM and spermatid transition proteins in spermatogenic cells from SCX rats, suggest that the switch of CREM from its inhibitory form to the activating form during spermatogenesis might be altered. Such changes could thus responsible for abnormal expression of spermatid nuclear proteins essential for sperm head morphology. We sought to confirm this finding by repeating the same experiment using additional animals. Unfortunately, our experiment failed to confirm the previous findings in at least 6 SCC and SCX rats. These results indicate that the abnormal CREM expression and regulation are not the only causes that lead to abnormal spermiogenesis after SCI.

C. Project challenges

Due to the gap of funding, the original technicians were no longer available. While new technicians were hired, the project has not progressed as anticipated. In addition, we were encountering unexpected among-animal variability in different sperm functions. Worst of all, due to the fact that I was unable to renew my VARR&D project, the newly hired technicians were let go, and we were unable to undertake many of the experiments planned in the proposal.

D. Changes in research plan

Due to incomprehensible causes, I was unable to renew my VARR&D project which has supported most of the expenses of my lab including my salary during the last 10 years. As such, there is no plan to continue in pursuing this project. Attempts have been made to collaborate with other faculties (Drs Alan Siegel of Neuroscience and Pranela Rameshwar of Medicine) to study the feasibility of using mesenchymal stem cells (MSC) to facilitate spinal cord regeneration using the SCC rat as a model. We had carried out a number of preliminary experiments to characterize rat MSC.

Isolation and characterization of MSC

Figure 1 shows the micrograph of rat MSC after 3rd passage. To verify that these cells are indeed MSC, we used flowcytometry to detect presence of MSC specific markers, or lack of, in these cells. As present in Figure 2, presence of CD9, CD 106, CD44, and lack of CD 34, CD 14, CD 46, and MHC1 in these cells illustrate that these cells are indeed MSC.

Figure A

Figure B

Expression and stimulation of RAR α in MSC

Due to the importance of retinoic acid in trans-differentiation of stem cells to neuronal cells, we have examined the expression of retinoic acid receptors (RARs) in MSC. Rat MSC (3rd passage) were sub-cultured onto culture slides in DMEM supplemented with 10 % FCS for 48 hrs. To determine the effect of retinoic acid, MSC were cultured overnight in charcoal-stripped FCS, and treated with retinoic acid for 24 hrs. Figure C-A shows the presence of RAR α in untreated MSC; nuclear RAR α was increased in those treated with retinoic acid (B: 0.1 or C:1 □M).

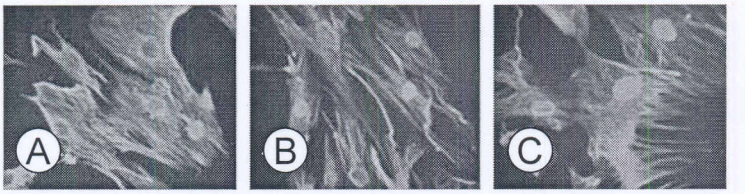


Figure C

In vitro trans-differentiation of MSC

To further determine differentiation potential of MSC, we also examined the effect of retinoic acid on trans-differentiation of MSC to neuronal cells. The MSC were treated with retinoic acid according to the published protocol (4-/4+), and cells were examined for neuronal markers. As presented in Figure D, the MSC were stained positive for stem cell marker, STRO-1. These cells express neuronal cell markers, SOX2, MAP2, Tau, Neu-N and b-tubulin. These results demonstrate the feasibility of these MSC to differentiate to neuronal cells.

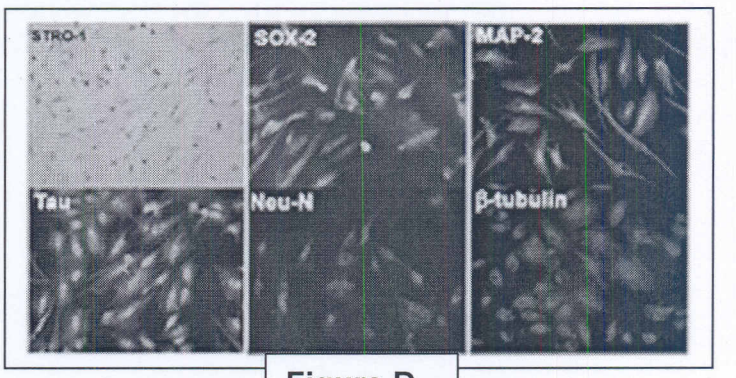


Figure D

With these preliminary data, a proposal had been submitted to the NJCSCR. Even though at least two of the reviewers were interested in our hypothesis and approaches, the proposal was not funded due to insufficient preliminary data, and **was not allowed to resubmit**. As such, unless we can obtain some funds and undertake additional preliminary experiments, our attempts to study the feasibility of using MSC in spinal cord regeneration are most likely unable to flourish, unfortunately.

E. List of publications emerging from this research, including those in preparation.

Wang S, Wang G, Barton BE, Murphy TF, Huang HFS. Impaired sperm function after spinal cord injury in the rat is associated with altered cyclic adenosine monophosphate signaling. J Androl 2005; 26: 592-600.

Wang S, Wang G, Barton BE, Murphy TF, Huang HFS. Beneficial effects of vitamin E in sperm functions in the rat after spinal cord injury. J Andrology 2007, 28: 334-341.

Haleem S, Shu P, Huang HFS. Failure of sperm capacitation in the rat after spinal cord injury. (tentative title, In preparation).