

Beneficial Effects of Vitamin E in Sperm Functions in the Rat After Spinal Cord Injury

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ABSTRACT: Male infertility as a result of spinal cord injury (SCI) is associated with abnormal semen qualities including low sperm counts and poor sperm motility and morphology. Clinical studies suggest that reactive oxygen species (ROS)-related events might contribute to abnormal sperm functions after SCI. The current study examined whether impaired sperm functions after SCI can be ameliorated by an antioxidant, vitamin E. Vitamin E feeding of spinal cord transected (SCX) rats during the acute (maintenance) and chronic (restoration) phases of the injury partially preserved sperm viability and mitochondrial potential; similar effects were only seen in spinal cord contused (SCC) rats during the chronic phase. A beneficial effect of vitamin E on sperm motility, however, was only observed in SCX rats during the chronic phase of the injury. These results suggest that ROS-related events might account for some of the effects of cord injury on sperm functions, depending on the extent of injury and time postinjury. Furthermore, we found that sperm

heads from SCC and SCX rats were less condensed compared to those from sham control rats. Such effects were attenuated by vitamin E, suggesting that ROS-related events may also contribute to abnormal sperm morphology after SCI. Partial restoration of male accessory gland weights in those rats fed vitamin E further suggests its beneficial effects on the functions of these glands. Conclusion: Vitamin E feeding attenuated some of the effects of spinal cord injury on sperm functions and male accessory glands in the rat. These results support a role of ROS-related events in deterioration of semen quality after cord injury. Further understanding of the underlying mechanisms for effects of vitamin E on sperm functions and male accessory glands will provide scientific rationale for the use of vitamin E or other antioxidant as therapeutic means to preserve sperm functions and semen quality in SCI men.

Key words: Sperm motility, viability, mitochondrial potential.

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Infertility is one of the consequences that affect quality of life in men with spinal cord injury (SCI), attributable to deterioration of semen qualities characterized by poor sperm motility and morphology and frequently low sperm count (Hirsch et al, 1991; Linsenmeyer and Perlash, 1991). While decreases in sperm count and increases in sperm with abnormal morphology are likely to result from abnormalities in spermiogenesis (Huang et al, 1998, 2004), poor sperm motility could result from structural or biochemical defects in the sperm as a result of impaired spermiogenesis or abnormal maturation during epididymal transit (Ricker et al, 1996; Linsenmeyer et al, 1999), or changes in milieu bathing the sperm (Brackett et al, 1996, 2000). In spinal cord transected (SCX) rats decreases in sperm motility occurred in the presence of

elevated sperm cAMP content and an overall decrease in sperm protein phosphorylation (Wang et al, 2005), suggesting that abnormal cAMP-related cellular events may contribute to poor sperm functions after SCI.

Excess reactive oxygen species (ROS) in the sperm or seminal plasma is known to result in oxidative damages of sperm DNA and impair sperm functions (Aitken et al, 1998) and has been linked to male infertility (Alkan et al, 1997). While increases in ROS in the seminal plasma and sperm from SCI men (de Lamirande et al, 1995; Pardon et al, 1997) suggest a putative cause for impaired sperm functions and infertility after SCI, a link between changes in seminal ROS and specific sperm functions after SCI has not been defined. Antioxidants, including vitamin E, have been shown to improve sperm motility and fertility in men (Suleiman et al, 1996; Rolf et al, 1999).

In the rat, spermatogenesis undergoes extensive but reversible regression after SCX but is qualitatively maintained after spinal cord contusion (SCC). In both injury models, deterioration of sperm functions occurs depending on the time and extent of the injury (Huang et al, 2003a,b, 2004; Wang et al, 2005). To determine if the ROS-related events are indeed involved in impaired sperm functions after cord injury, the current study

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examined the efficacy of vitamin E on various sperm functions in SCC and SCX rats during the maintenance and restoration phases of the injury.

Materials and Methods

Animals

Mature Sprague Dawley rats 7–8 weeks of age (250–275 gm; Taconic Farm, Taconic, NY) were caged individually in an air-conditioned ($70 \pm 2^\circ\text{C}$), light-controlled (12L:12D) animal room for 2 weeks prior to the experiment. They were fed Purina rat chow and water ad libitum.

Spinal Cord Contusion and Transection—Details of the procedures employed to induce spinal cord injury in the rat by contusion (SCC rat) (Kwo et al, 1989) or transection (SCX rat), and post-op care of these rats, have been previously described (Linsenmeyer et al, 1994). These procedures were reviewed annually by the Institutional Animal Care and Use Committees at both the East Orange VA Medical Center and UMD-New Jersey Medical School.

The rats were given oral dose of vitamin E (2 or 10 mg/kg; Sigma Chemical Co, St Louis, Mo) or 0.1 mL vehicle (soybean oil) daily beginning immediately after the injury (maintenance) or 8–10 weeks postinjury (restoration) for 8 weeks. Timings for vitamin E feeding were selected because spermatogenesis is rapidly regressing within a week after cord injury, and significant recovery has occurred 8–10 weeks postinjury (Huang et al, 1995, 1998). At the end of treatment period, the rats were killed by decapitation. The epididymides were excised immediately, and spermatozoa were recovered from the caput and caudal epididymides as described (Wang et al, 2005).

Sperm Motility—Spermatozoa were recovered from the caudal epididymides in 37°C sperm buffer (Ringer solution containing 1 mmol sodium pyruvate, 5.5 mmol D-glucose, 10 mmol sodium bicarbonate, 25 mmol N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), and 2% bovine serum albumin (BSA)). Sperm motility was measured by the procedures described in previous publications (Huang et al, 2004; Wang et al, 2005).

Sperm Viability and Mitochondrial Potential—Sperm uptake of fluorescent dyes SYBR-14 and JC-1 (Molecular Probes, Eugene, Ore), specific for viability and mitochondrial potential, respectively (Graham et al, 1990; Gravance et al, 2001), was determined using a Becton-Dickinson FACScan flow cytometer (Franklin Lakes, NJ). Details of the procedure have been reported previously (Wang et al, 2005).

Sperm cAMP—The procedures of Wu et al (1995) were adapted to measure sperm cAMP. Details of these procedures have been described previously (Wang et al, 2005).

Sperm Protein Phosphorylation—To measure sperm protein phosphorylation, caudal epididymal sperm were processed by standard procedures as previously described (Wang et al, 2005). The proteins were electrophoresed on a standard 10% SDS-poly acrylamide gel, immunoblotted with antiphosphotyrosine monoclonal antibody (1:1000; Santa Cruz Biotech,

Santa Cruz, Calif), and visualized with Chemiluminescence Reagent (Perkin Elmer Life Sciences, Boston, Mass).

Sperm Head Decondensation—The procedures described by Calvin and Bedford (1971) were adapted for our experiments. Briefly, caudal epididymal sperm were washed with PBS, pelleted by centrifugation ($800 \times g$, 2 min), and resuspended in 0.1 mL 0.5 mol sodium borate (pH 9.0). Following brief sonication, spermatozoa were incubated in the presence of 0.1% SDS and 1 mmol dithiothreitol (DTT) for various times. Sperm head decondensation was recorded at various times using a Nikon phase contrast microscope. The conditions for such experiment were validated by pilot experiments.

Statistics—All data were analyzed with analyses of variance using vitamin E dose as the independent variable. When the treatment effects were significant ($P < .05$), Dunn's tests were used to determine statistical significance among treatment groups.

Results

Motility—In both the maintenance and restoration experiments sperm motility in sham control rats was not affected by vitamin E ($P > .1$), and sperm motility in untreated SCC and SCX rats was significantly lower compared to that in untreated sham control rats ($P < .01$, Figure 1A). While sperm motility in SCC and SCX rats in the maintenance experiment was not affected by vitamin E feeding, that in SCX rats that received high-dose vitamin E in the restoration experiment was significantly higher than that of untreated SCX rats ($P < .05$).

Viability and mitochondrial potential—Sperm uptakes of SYBR-14 and JC-1 were significantly lowered in untreated SCC and SCX rats in the maintenance experiment ($P < .01$) (Figure 1); such effects were partially prevented by high dose of vitamin E ($P < .01$). In the restoration experiment, while SYBR-14 uptake was significantly lower in SCX and SCC rats ($P < .01$), uptake of JC-1 was only reduced in SCX rats ($P < .01$). Feeding of vitamin E partially restored sperm SYBR-14 and JC-1 uptake in SCX rats ($P < .01$) but did not affect that in SCC rats.

Cyclic AMP Content—Figure 2 shows that cAMP contents in caput sperm from untreated SCC and SCX rats were marginally elevated, but these in caudal sperm were significantly elevated ($P < .05$) when compared to that of untreated sham control rats. While vitamin E feeding resulted in dose-dependent increases of caudal sperm cAMP content in sham control rats ($P < .01$), such effects were not seen in SCC and SCX rats.

Sperm Protein Phosphorylation—Figure 3 shows that vitamin E feeding resulted in dose-dependent increases in the major phosphorylated protein band in sham control rats; such effect was not seen in SCX rats. Similar results were seen in at least 3 independent blots.

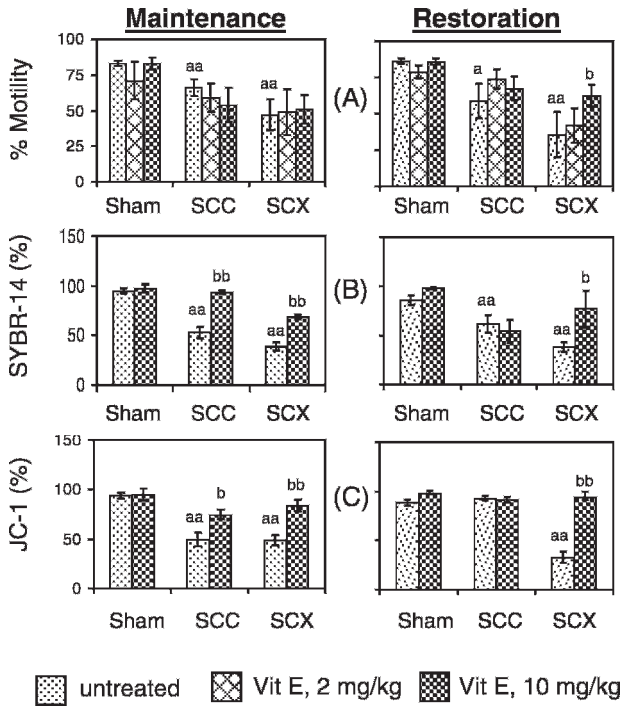


Figure 1. The effects of vitamin E feeding on sperm motility (A), sperm uptake of SYBR-14 (B), and JC-1 (C) in spinal cord contused (SCC) and transected (SCX) rats. Results are expressed as mean ± SEM. n = 6–8 rats per group. a: P < .05, aa: P < .01 vs untreated sham control; b: P < .05, bb: P < .01 vs untreated rats.

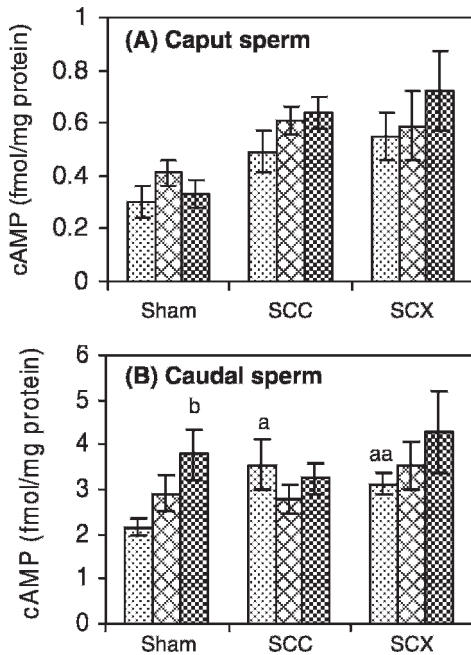


Figure 2. The effects of vitamin E feeding on cAMP content in (A) caput sperm and (B) caudal sperm from SCC and SCX rats of the restoration experiment. Results are expressed as mean ± SEM. n = 6–8 rats per group. a: P < .05, aa: P < .01 vs untreated sham control; b: P < .05. See legend to Figure 1 for key of treatment groups.

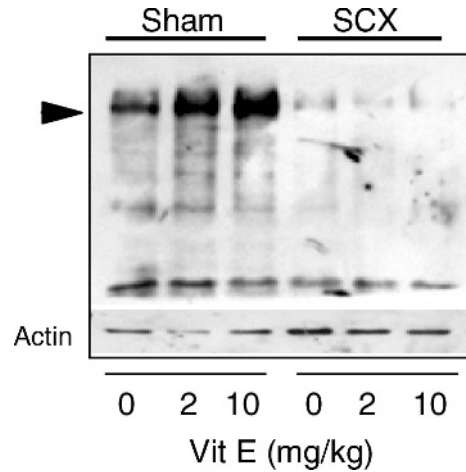


Figure 3. Effects of vitamin E feeding on sperm protein phosphorylation. Sperm from randomly selected sham or SCX rats received 0, 2, or 10 mg/kg vitamin E of the restoration experiment were compared. Vitamin E feeding resulted in dose-dependent increases in the major phosphorylated sperm protein band (arrowhead) in sham control rats; similar effect was not seen in sperm from SCX rats. Similar results were seen in 3 different blots.

To determine if cord injuries affect sperm capacitation, preliminary experiments were undertaken using BSA as a capacitation agent and sperm protein phosphorylation as an indicator for capacitation. Incubation of caudal sperm from sham control rats with BSA (0.2–20 mg/mL) in sperm buffer for 60 minutes resulted in dose-dependent increases in sperm protein phosphorylation (data not shown). Subsequently, caudal sperm from sham control and SCX rats were incubated with 2 mg/mL BSA for various times. Figure 4A is a representative blot showing time-dependent increases in the major phosphorylated protein in caudal sperm from a sham and an SCX rat; that of SCX rat were consistently lower than that of sham control rat at each time. Further comparison of the BSA-induced sperm phosphorylation in randomly selected rats from each group revealed a consistently lower abundance of the major phosphorylated protein band in spermatozoa from SCC and SCX rats (Figure 4B).

Sperm Head Decondensation—To determine if decreases in the expression of spermatid nuclear proteins in SCC and SCX rats (Huang et al, 2003a,b) might compromise condensation of the sperm heads, we compared the SDS-DTT induced swelling (decondensation) of the sperm head among treatment groups. Pilot experiments revealed time- and DTT concentration-dependent increases in sperm head swelling (Figure 5). Subsequently, caudal sperm from various groups were incubated with 0.1% SDS and 1 mmol DTT for 30 minutes. Results of these experiments show that sperm heads from chronic SCC and SCX rats were

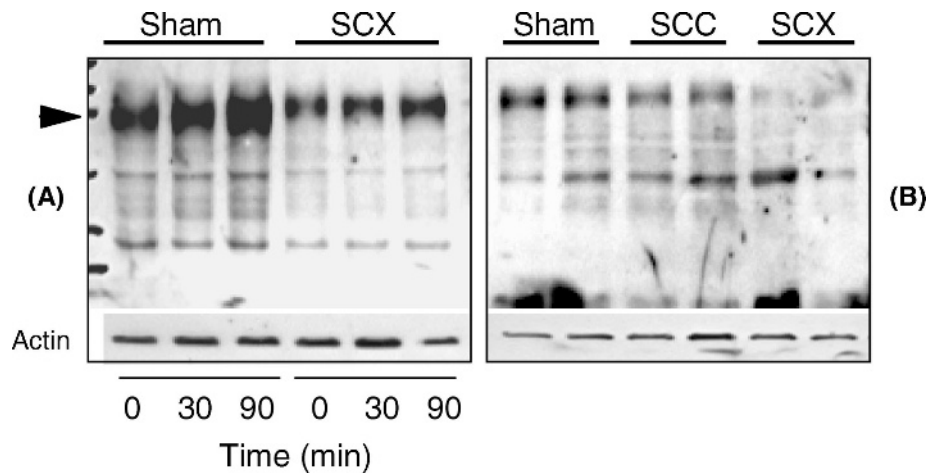


Figure 4. Representative Western blots showing the effect of bovine serum albumin (BSA) on sperm protein phosphorylation. **(A)** Caudal sperm from a sham control and a SCX rat of the restoration experiment were incubated in sperm buffer containing 2% BSA for various times. Spermatozoa from both sham control and SCX rats exhibited time-dependent increases in the major phosphorylated protein band. However, that of the SCX rat was significantly lower in each time point. Similar results were seen in 3 different blots. **(B)** Caudal sperm from pairs of sham control, SCC and SCX rats were incubated with 2% BSA for 90 minutes, showing that the BSA-induced protein phosphorylation was lower in sperm from SCC and SCX rats compared to that from sham control rats.

decondensed to greater extents when compared to sperm heads from sham control rat (Figure 5); such effects were less pronounced in those rats that received vitamin E feeding (10 mg/kg).

Effects of Vitamin E on Male Accessory Glands—At the end of the maintenance experiment, ventral prostate (VP), dorsal prostate (DP), and seminal vesicle (SV) weights were slightly lower in SCC and SCX rats than in sham control rats; only that of the VP was significant ($P < .05$, Figure 6). Vitamin E feeding during the maintenance phase did not affect the weights of these organs ($P > .1$). At the end of the restoration experiment, weights of the VP, DP, and SV of SCC and SCX rats were all significantly lower than those of sham control rats ($P < .01$, Figure 6). Vitamin E feeding during the chronic phase of the injury significantly increased the weight of the VP, DP, and SV in SCX rats ($P < .05$, $.01$, Figure 6), but only increased SV weight in SCC rat ($P < .05$).

Discussion

In SCC and SCX rats, the lowered sperm motility concurs with decreases in sperm viability and mitochondrial potential (Wang et al, 2005), suggesting a causal relationship between these changes. Such a relationship is, however, only observed in SCX rats fed high-dose vitamin E during the restoration phase. These results suggest that factors other than mitochondrial function and viability might also contribute to impaired sperm motility after cord injury.

Improvement of various sperm functions in SCC and SCX rats fed vitamin E is indicative of a role of ROS-related events in impaired sperm functions after cord injury. ROS has been shown to affect sperm motility through tyrosine phosphorylation of the fibrous sheath protein (Luconi et al, 2005), and epididymal sperm are protected from ROS by both antioxidant enzymes in epididymal fluid and endogenous vitamin E (Zini and Schlegel, 1997; Tramer et al, 1998). In the Brown Norway rat, manipulation of vitamin E nutrition status in vivo has been shown to alter the expression of oxidative-related gene in the epididymis, with the most profound effects in the corpus epididymis (Jervis and Robaire, 2004). The observed beneficial effects of vitamin E on sperm functions in SCC and SCX rats could thus be accounted for by changes in epididymal environment, perhaps due to the suppression of oxidative-related genes.

Previously we reported that impaired sperm motility in SCX rats is associated with altered cAMP-protein phosphorylation signaling events (Wang et al, 2005). Since vitamin E has been shown to modulate adenylate cyclase and cAMP-related signaling events, as well as protein kinases (Sahu and Prasad, 1988; Sahu et al, 1988; Ottino and Duncan, 1996; Keaney et al, 1999; Kempná et al, 2004), we further examined if effects of vitamin E were associated with normalization of cAMP-protein phosphorylation status of the sperm. The dose-dependent and parallel increases in sperm cAMP and protein phosphorylation in caudal sperm from sham control rats fed vitamin E suggest a link between vitamin E and sperm cAMP-protein phosphorylation

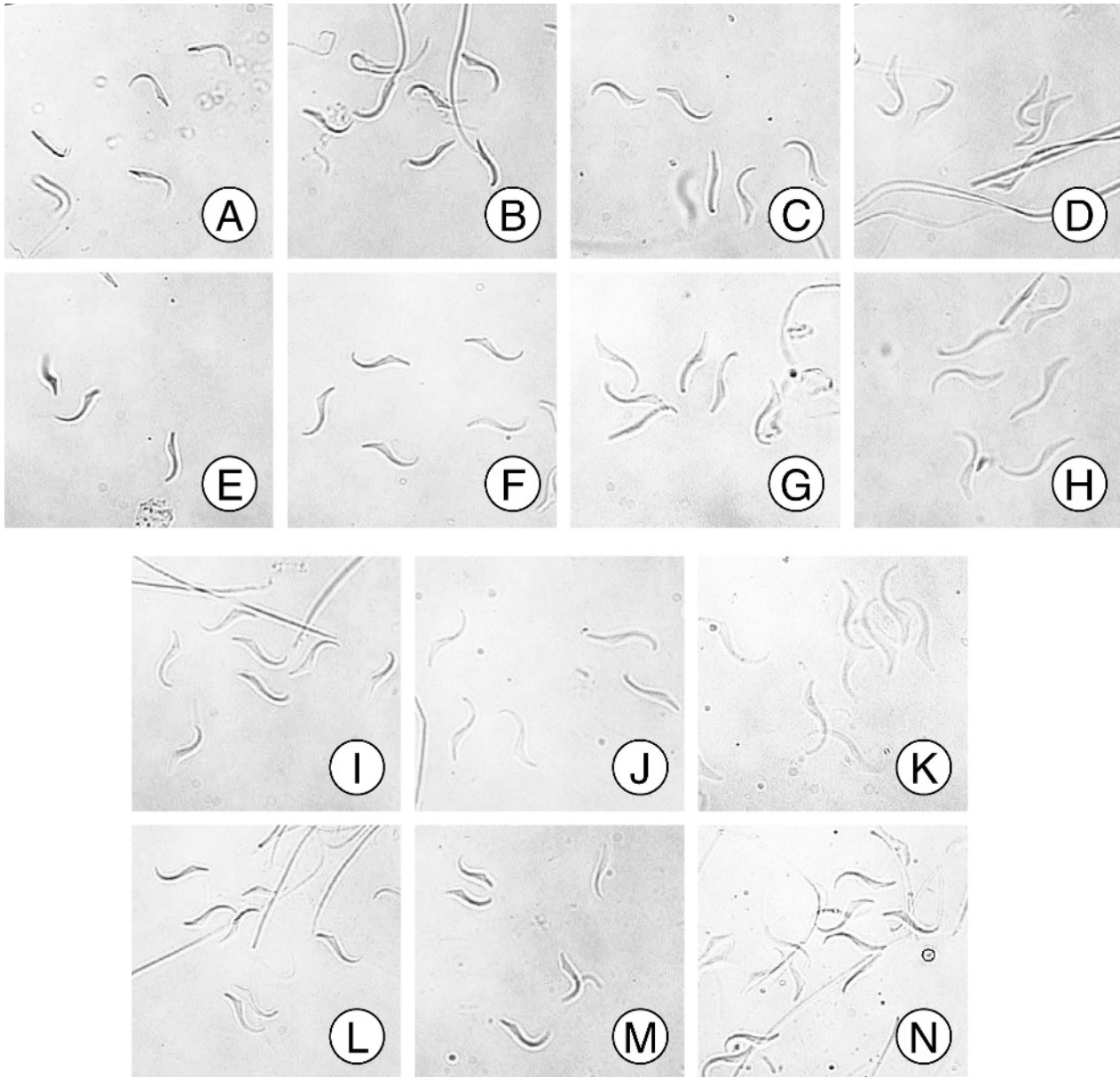


Figure 5. Photomicrographs of decondensed rat sperm head. **(A–D)** Dithiothreitol (DTT) concentration-dependent decondensation of sperm heads. Caudal epididymal sperm from a sham control rat were incubated in 0.5 mol sodium borate (pH 9) in the presence of 0.1% SDS and **(A)** 0, **(B)** 0.5, **(C)** 1, and **(D)** 2 mmol DTT for 20 minutes. **(E–H)** Time dependent decondensation of sperm heads. Spermatozoa were incubated in the same buffer in the presence of 1 mmol DTT for 5 **(E)**, 10 **(F)**, 20 **(G)**, and 40 **(H)** minutes. **(I–N)** The effects of cord injury and vitamin E feeding on sperm head decondensation. Spermatozoa from untreated sham control **(I)**, SCC **(J)**, and SCX **(K)** rats, and high-dose vitamin E fed sham control **(L)**, SCC **(M)**, and SCX **(N)** rats killed at the end of restoration experiment were incubated in 0.5 mol sodium borate in the presence of 0.1% SDS and 1 mmol DTT for 30 minutes.

cascade. However, since sperm motility, viability, and mitochondrial potential in sham control rats were not affected by vitamin E, the purported links between vitamin E and sperm cAMP and protein phosphorylation cascade probably have no direct impact on these functions. Since cAMP content in caput sperm from sham control rats was not affected by vitamin E feeding, vitamin E is likely to affect sperm cAMP and protein

phosphorylation posterior to the caput epididymis. Lack of similar increases in cAMP and protein phosphorylation in caudal sperm from SCX rats fed vitamin E is consistent with altered sperm cAMP signaling after cord injury. Thus, the beneficial effects of vitamin E on sperm motility, viability, and mitochondrial functions seen in SCC and SCX rats probably are not mediated by cAMP-protein phosphorylation cascade. Nevertheless,

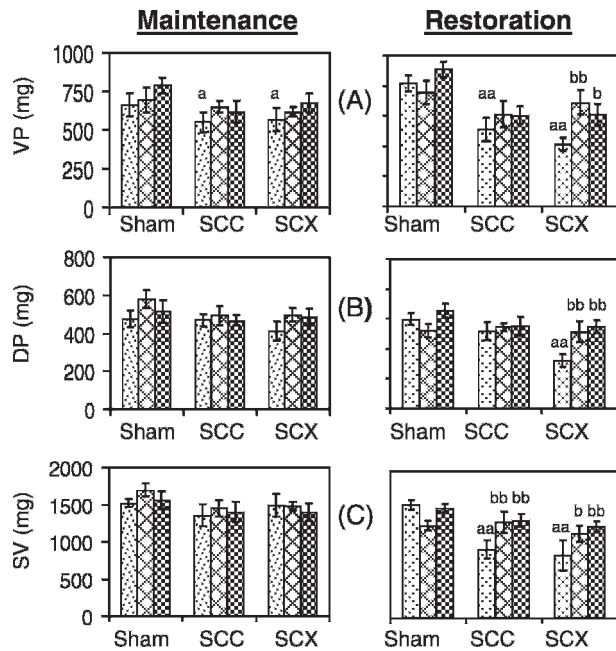


Figure 6. The effects of vitamin E feeding on weights of the ventral prostate, VP (A), dorsal prostate, DP (B), and seminal vesicle (SV) (C) from SCC and SCX rats. Results are expressed as mean \pm SEM. $n = 6-8$ rats per group. a: $P < .05$, aa: $P < .01$ vs untreated sham control; b: $P < .05$, bb: $P < .01$ vs untreated rats. See legend to Figure 1 for key to the treatment groups.

involvement of proteins phosphorylated by other kinase system in the effects of vitamin E on sperm functions cannot be ruled out.

Sperm undergo capacitation to acquire final competence to interact and fertilize ova. This process involves the activation of multiple forms of adenylate cyclases (Kopf and Vacquier, 1984; Rojas et al, 1993; Jaiswal and Conti, 2003; Esposito et al, 2004) and phosphodiesterases (Fisch et al, 1998), resulting in increases in cAMP and sperm protein phosphorylation (Galatino-Homer et al, 1997; Visconti and Kopf, 1998; Adeoya-Osiguwara and Fraser, 2002; Baker et al, 2003). Since we have demonstrated altered cAMP-protein phosphorylation cascades in the sperm from SCC and SCX rats, we sought to examine if sperm capacitation is impeded after cord injury. Sperm capacitation is initiated by cholesterol efflux, a process that can be triggered in vitro by serum proteins, including BSA (Visconti et al, 1999). We first examined the BSA-induced sperm protein phosphorylation. An overall decrease in the BSA-induced protein phosphorylation in spermatozoa from SCC and SCX rats suggests that these spermatozoa might not be able to initiate capacitation cascade normally. Additional experiments using capacitation-specific end points such as acrosome reaction and hyperactive motility would be required to confirm our postulate.

During the second half of spermiogenesis, replacement of somatic histones by sequential expressions of spermatid nuclear transition protein (TP)-1, TP-2, and protamine results in the condensation of spermatid nuclei and initiates morphogenesis of the sperm head (Grimes et al, 1977; Hecht, 1990). Heterogeneity of sperm chromatin structure and impaired DNA packaging have been linked to reduced expression of spermatid nuclear proteins (Steger et al, 1999; Yu et al, 2000; Steger et al, 2001; Zhao et al, 2001) and are implicated in abnormal sperm morphology (Evenson et al, 1980; Ballachey et al, 1987). We postulate that abnormal expression of these nuclear proteins in the rat after cord injury might compromise the condensation of spermatid nuclei. Increases in the SDS/DTT-induced decondensation of sperm heads from SCC and SCX rats are consistent with this postulate. Such an effect could hinder final steps of spermiogenesis and completion of spermatogenesis, leading to a decrease in sperm production. It could also impede morphogenesis of the sperm head, thus providing a mechanistic explanation for increases in sperm with abnormal morphology semen from SCI men. Of note, the SDS/DTT-induced decondensation of sperm heads from SCC and SCX rats was attenuated by vitamin E feeding. These effects could be attributed to changes in the expression of spermatid nuclear proteins, since vitamin E is known to affect spermiogenesis (Bensoussan et al, 1998), or to the direct effects of vitamin E on sperm maturation during epididymal transport (Tramer et al, 1998).

Significantly lower weights of the prostate and seminal vesicles in SCC and SCX rats are consistent with the need for an intact neural component for normal function of male accessory glands (Wang et al, 1991; Huang et al, 1997). Of note, in the restoration experiment both VP and DP weights of SCX rats were significantly lower than those at the end of the maintenance experiment. Since VP and DP weights of SCC rats were comparable between the maintenance and restoration experiments, SCC might have limited the growth of the prostate during the chronic phase, and that of SCX rats might have undergone further deteriorating changes during the same time. Similar changes were also observed in the SV in SCC and SCX rats during the chronic phase of the injury. These long-term effects of cord injuries could impair functions of these accessory glands and compromise normalcy of seminal fluid. In this regard, the seminal fluids from SCI men have been reported to have abnormal biochemical properties and to impair motility of spermatozoa from normal men (Brackett et al, 1996, 2000).

Partial restoration of prostate and SV weights in SCC and SCX rats by vitamin E in the restoration experiment implies a role of the ROS-related mechanisms in the

effect of cord injury on these glands. These effects probably are unrelated to infection of these organs due to daily manipulation of the bladder, since similar effects were not seen in the maintenance experiment. Vitamin E modulates prostate cancer cells through the expression of androgen receptor (AR) (Zhang et al, 2002). Our previous results suggest a putative role of androgen/AR signaling events in acute effects of SCI on prostate functions (Huang et al, 1997). However, since serum testosterone and prostate AR level are relatively normal in chronic SCX rats (Huang et al, 1997), variations in effects of cord contusion and transection, as well as that of vitamin E, on male accessory glands are likely to be independent of AR/androgen signaling.

In conclusion, current results demonstrate beneficial effects of vitamin E feeding on various sperm functions and properties, as well as male accessory glands, in SCC and SCX rats. Further understanding the underlying mechanisms responsible for these effects will provide scientific rationale for the use of vitamin E or other antioxidants to preserve semen quality and sperm functions in SCI men. Success in this endeavor will provide an easy and cost-effective therapeutic option to improve sperm functions, and ultimately fertility, in men with SCI.

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