Research article

Sperm chromatin condensation, DNA integrity, and apoptosis in men with spinal cord injury

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Objectives: To evaluate the effect of cord injury on (1) sperm parameters and (2) DNA chromatin status.
Design: Case–control study.
Setting: Data were collected from men referred to Research and Clinical Center for Infertility, Yazd, Iran.
Participants: Thirty infertile men with the presence of any level of spinal cord injury (SCI) were compared with 30 healthy donors with definite fertility and normal sperm parameters.
Interventions: Not applicable.
Outcome measures: Sperm chromatin integrity was assessed using aniline blue (AB), chromomycin A3 (CMA3), toluidine blue (TB), and acridine orange (AO) assays. The rate of apoptotic spermatozoa was evaluated with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining.
Results: Sperm concentration, motility, and morphology in men with SCI were significantly decreased compared with control group ($P < 0.05$). In addition, with regard to cytochemical staining and TUNEL test, the rate of reacted spermatozoa was increased significantly in SCI group when compared with the controls ($P < 0.05$). The majority of AB, TB, AO, and CMA3-reacted spermatozoa were higher than the "cut-off" value in men with SCI, as were the number of apoptotic spermatozoa stained with TUNEL.
Conclusion: Results showed that SCI disturbs sperm parameters, nuclear maturity, and DNA integrity of spermatozoa. Therefore, the production of spermatozoa with less condensed chromatin and more apoptotic rate increases after cord injury and this may be one possible cause of infertility following SCI.

Keywords: Infertility, Male, Chromatin condensation, Apoptosis, Spermatozoa, Erectile dysfunction, Ejaculatory dysfunction, Semen quality, Spinal cord injuries, Cytochemical tests

Introduction

Spinal cord injury (SCI) affects approximately 300 000 people with 10 000 new cases occurring each year.[1] The predominant age range at the time of injury is 15–25 years with a male-to-female ratio of 4:1.[2] However, the average age increased to 37.6 years between 2000 and 2003, and is now 41%.[1,3] Despite the rise in average age at injury, 80% of the affected people are still men, with a preponderance of young men of reproductive age.[4] Male fertility is compromised after SCI because of impaired erection, ejaculatory dysfunction, and poor semen quality.[5–8] Only 10% of men with SCI will father a child without medical contribution.[9] In addition to abnormal semen analysis, a high level of increased sperm nuclear DNA fragmentation and abnormal chromatin has been found in semen samples of men with SCI[10] as well as in experimental animals.[11]

In mammals, throughout the process of spermiogenesis, the nuclear histones are replaced at first by transition proteins and then by the basic sperm-specific proteins which are named protamines. Furthermore, during sperm epididymal transit, the cysteine-thiol groups of protamine molecules are oxidized to disulfide bonds (S-S), which are necessary for sperm chromatin stabilization.[12,13] Therefore, spermatozoa become highly resistant to a variety of agents such as acids, proteases, DNAse, and detergents (e.g. sodium dodecyl sulfate) after nuclear chromatin remodeling.[14] However, sperm DNA integrity can be influenced harmfully by oxidative stress through loss of sperm mitochondrial membrane stability, selective permeability, and absence of antioxidant capacity.[15]

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With regard to SCI, high concentration of oxidative stress[16] and the increased level of dead sperm cells and leukocytes[6] may be considered as the main causes of increased DNA damage, but to date, little is known about the impact of SCI on human sperm chromatin condensation and apoptosis. It is obvious that in the cases of SCI, the sperm epididymal transit takes place for long period of time due to alterations in autonomic innervation of this organ.[17] So, it is obvious that these spermatozoa are overwhelmed by more reactive oxygen species (ROS) and as a result have more sperm nuclear maturation abnormalities.

On the other hand, a strong correlation has been shown between damaged sperm nuclear DNA and male infertility.[4,18] It has been reported that chromatin condensation was directly related to sperm capacity for fertilizing an oocyte in a natural cycle.[18] Similarly, damaged sperm chromatin is a well-documented cause of low fertilizing ability *in vitro*.[19] Evenson and Wixon[20] reported that pregnancy rate significantly decreased with semen samples containing more than 30% spermatozoa with fragmented DNA.

As having children is of great importance among men with SCI, the current study was conducted to evaluate the effect of SCI on chromatin condensation and apoptosis of spermatozoa. According to our knowledge, this study is a unique report on evaluation of sperm chromatin condensation using different cytochemical tests in patients with SCI compared with fertile men.

Methods

Patients

Semen samples from 60 men referred to our research and clinical center for infertility were divided into two groups for analysis. Case group consisted of 30 infertile men with any level of SCI who were injured at least 1 year before the start of the study. The majority of patients had been injured on the battlefield or in vehicular accidents. Cases with azoospermia and semen samples contaminated with blood were excluded from the study. The control group consisted of 30 healthy donors with proven fertility and normal spermograms and whose partners had successful pregnancies within the last 24 months. Written informed consent was obtained from the patients in both groups. This prospective study was approved by the institutional review board of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Semen analysis

Samples from all patients in case group were taken using penile vibratory stimulation or using electroejaculator (EEJ) (Seager model 14 electroejaculator system, Dalzell, VA, USA) by the same urologist. Stimulation was performed by using 2.5 mm amplitude and a frequency of 100 Hz in 2–3 minutes period of time until ejaculation occurred. Regarding EEJ, 12–15 V stimulation rate was applied in short time intervals until ejaculation. All specimens in control group were collected by masturbation following 2–4 days of abstinence. All raw semen samples were analyzed according to World Health Organization criteria.[21] After semen liquefaction, to evaluate morphological abnormalities, Diff quick staining was applied. To determine sperm motility we used Makler chamber (Sefi Medical Co., Haifa, Israel) and phase-contrast microscopy (Olympus Co., Tokyo, Japan) at ×200. All analyses were performed by one experienced laboratory technician blinded to the study.

Sperm chromatin and DNA study

For the evaluation of sperm DNA integrity and chromatin condensation, four different cytochemical tests including aniline blue (AB), chromomycin A3 (CMA3), toluidine blue (TB), and acridine orange (AO) test were used.

AB staining

Regarding AB staining, fresh sperm smear of each case was air dried and then fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 minutes at room temperature. Each smear was treated with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 5 minutes. At least 200 spermatozoa were counted in each slide by light microscopy (Olympus Co., Tokyo, Japan).

CMA3 staining

At first, smears were dried and then fixed in Carnoys solution (methanol/glacial acetic acid, 3:1) at 4°C for 10 minutes. Each slide was stained with 100 µl of CMA3 (Sigma, St Louis, MO, USA) (0.25 mg/ml in McIlvaine buffer; 7 ml citric acid, 0.1 M + 32.9 ml Na_2HPO_4 $7H_2O$ 0.2 M, pH 7.0 containing 10 mM $MgCl_2$) for 20 minutes. Finally, the slides were washed in buffer and mounted with buffered glycerol (1:1).

TB staining

To do this staining, after air drying of smears, they were fixed in fresh 96% ethanol-acetone (1:1) at 4°C for 30 minutes and then hydrolyzed in 0.1 NHCl at 4°C for 5 minutes. The slides were rinsed thrice in distilled water for 2 minutes and finally stained with 0.05% TB in 50% McIlvaine buffer (pH 3.5) for 10 minutes at room temperature.[22,23] The chromatin quality of

spermatozoa was determined according to metachromatic staining of sperm heads with the aid of light microscopy at ×100 magnification.

AO test

In this staining, smears were fixed overnight in Carnoy's solution after primary air dried. Each sample was stained for 10 minutes in freshly prepared AO (0.19 mg/ml) in McIlvaine phosphate citrate buffer (pH 4) for 5 minutes. Smears were assessed on the same day using fluorescent microscopy (460 nm filter). The duration of illumination was restricted to 40 seconds per field.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

To determine the percentage of apoptotic spermatozoa in each sample, we used the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL) staining by In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). At first, air-dried smears were washed in phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde for 15 minutes at room temperature. Then the slides were permeabilized with 0.5% Triton X-100 in 0.1% sodium citrate for 5 minutes on ice. The permeabilized spermatozoa were washed in PBS, and then covered with TUNEL reaction mixture in a dark room at 37°C for 1 hour. Smears were treated with enhanced by peroxidase (POD) convertor at 37°C for 1 hour. We did the second washing with PBS and then enhanced in 50–60 µl diaminobenzidine for 15 minutes in room temperature. Final washing with PBS was done before staining with hematoxylin for 30 seconds and dehydration by serial alcohols (70, 90, and 100°) for 3 minutes. To clear the slides, the xylens I and II were used before mounting. At least 200 sperm cells in each smear were counted using light microscopy at ×100 magnifications.

Statistical analysis

According to the results of a pilot investigation which was done on a few patients, we considered 30 cases in each group to find a difference by 80% power with a 5% significance ($a = 0.05$, $b = 0.2$). The Statistical Package for the Social Sciences (SPSS) 15.0 software was used to analyze the data. Data were expressed in mean ± SD. The baseline characteristics of the two groups of patients were compared using the Student *t*-test for normal distributed variables and Mann–Whitney *U* test for abnormal distributed variables. A *P* value of <0.05 was considered statistically significant.

Results

The mean age was similar between case and control groups (35.62 ± 5.65 and 36.43 ± 5.44, respectively). The mean post injury years in case group was 13.37 ± 7.03 ranging from 3 to 28 years and the most frequent level of injury was at lumbar region with frequency of 66.6%. The other injury levels were high thoracic (20.8%), low thoracic (8.3%), and cervical (4.2%). The results of sperm parameters analysis of both groups are listed in Table 1. It should be noted that the duration and level of injury have no effect on sperm parameters and DNA chromatin status.

There was a significant difference between case and control groups regarding sperm concentration and morphology ($P < 0.05$). In addition, the total motility (grades "a" and "b" and "c"), the progressive (grades "a" and "b") and non-progressive motility (grade "c") were significantly higher in control men than patients with SCI ($P < 0.05$). In addition, 75% of samples of SCI patients have contaminated with bacteria and white blood cells ($\geq 10^6$) that specified the leukocytospermia.

The sperm nuclear integrity of patients and control group was shown in Table 2.

In sperm chromatin study, the rates of spermatozoa that reacted with AB (AB+) were significantly different in both groups ($P < 0.05$). The spermatozoa with unstained or pale-blue nuclei were recorded as normal and those with dark-blue nuclei were regarded as abnormal ones.[11,22] Furthermore, 87.5% of patients with SCI

Table 1 Mean and standard deviation of sperm parameters of two groups under investigation

Sperm variables	SCI ($n = 30$)	Control ($n = 30$)	*P* value*
Concentration ($\times 10^6$/ml)	70.16 ± 46.32	105.70 ± 59.24	0.020
Total motility (%) (grade "a" + "b" + "c")	19.58 ± 18.30	71.36 ± 8.38	0.000
Progressive motility (%) (grade "a" + "b")	11.58 ± 13.94	58.30 ± 10.93	0.000
Non-progressive motility (%) (grade "c")	8 ± 5.92	13.06 ± 4.63	0.001
Normal morphology (%)	13.04 ± 7.05	37.43 ± 15.66	0.000

SCI, spinal cord injury.
Data are presented as mean ± SD.
*SCI vs. control.

Table 2 Characteristics of sperm nuclear integrity of two groups under investigation

Variables	SCI ($n = 30$)	Control ($n = 30$)	*P* value
AB+ (%)	52.45 ± 19.16	29.10 ± 12.17	0.000*
CMA3+ (%)	49.95 ± 14.39	22.80 ± 8.88	0.000*
AO+ (%)	62.66 ± 18.14	30.06 ± 10.78	0.000*
TB+ (%)	63.54 ± 22.09	30.20 ± 11.07	0.000*
TUNEL (%)	32.91 ± 12.55	9.03 ± 2.94	0.000**

SCI, spinal cord injury.
Data are presented as mean ± SD.
*SCI vs. control using Student's *t*-test.
**SCI vs. control using Mann–Whitney *U* test.

showed higher AB-reacted spermatozoa than "cut-off" value (30%) (Fig. 1).[24]

Regarding CMA3 staining, there was a statistically difference between the rates of CMA3-reacted spermatozoa in both groups. In other words, the percentage of men with SCI who had higher CMA3+ sperm cells than the "cut-off" value (30%)[25] was 91.7% (Fig. 1). The CMA3-reacted spermatozoa (CMA3+) showed bright yellow stain, and non-reacted ones (CMA3−) were yellowish green when viewed under axiplane fluorescent microscope with a 460 nm filter (Zeiss Co., Jena, Germany) at ×100 magnification.

The rate of orange-red fluorescence spermatozoa was significantly higher in patients with SCI in comparison with control group (62.66 ± 18.14 vs. 30.06 ± 10.78; $P < 0.05$). The percentage of green fluorescence spermatozoa (normal double-stranded DNA) and orange/red fluorescence spermatozoa (abnormally denatured DNA) per sample were calculated.[11,22,26] Only 29.2% of SCI men showed AO-reacted sperms below 50% which considered as "cut-off" value for this test.[27]

In TB staining, we also found a significant difference between control and case groups ($P < 0.05$). The percentage of men with SCI who had samples with more than 45% TB-reacted spermatozoa,[28] was notably higher than controls ($P < 0.05$) (Fig. 1). The scores were as followed: score 0 = light blue (good chromatin), score 1 = dark blue (mild abnormal chromatin), and score 2 = violet and purple (severe chromatin abnormality).[29] So, the sum of spermatozoa with scores 1 and 2 (TB+) were assumed as abnormal chromatin, whereas score 0 (TB−) as sperm cells with normal chromatin.

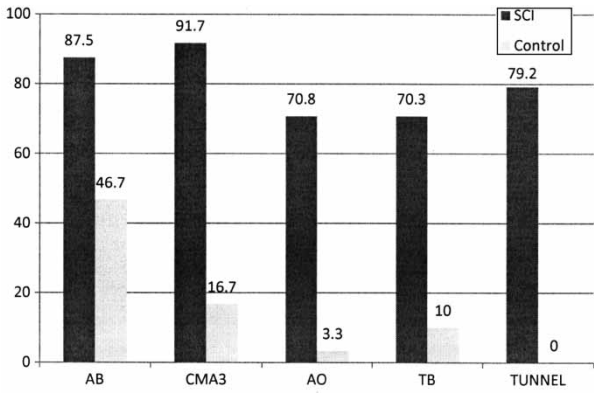

Figure 1 The percentages of samples that were above the "cut-off" value in case and control groups regarding cytochemical tests and TUNEL assay.

In TUNEL assay, the results showed that the rate of sperm apoptosis was higher in patients with SCI when compared with controls (32.91 ± 12.55 and 9.03 ± 2.94), respectively, ($P < 0.05$). The 79.2% of the samples of patients with SCI had above 20% apoptotic sperm cells (Fig. 1).[30] Spermatozoa with light-blue-stained nuclei were considered as alive and those with dark brown pigments in their heads were counted as apoptotic ones.

Discussion

SCI may affect male fertility due to sexual dysfunction, abnormal semen parameters, and damage to sperm nuclear DNA. In this study, we evaluated semen parameters as well as sperm nuclear chromatin/DNA status and sperm apoptosis in SCI cases using cytochemical tests and TUNEL assay. The results showed that all semen parameters dramatically decreased in SCI group when compared with control men. It has been reported in some studies that SCI accompany with poor semen quality, specially decreased sperm motility, morphology, and viability.[31–34] With regard to sperm concentration, our result was in contrast with Salsabili *et al.*[33] (2009) study in which a significant elevation was found in the SCI group compared with fertile men. However, in another studies, sperm count had been remained in normal range after SCI.[4,34,35] These differences may be seen because using different kinds of sperm retrieval methods in SCI patients. Many factors may be responsible for abnormal semen parameters, and the most important one could be high amount of ROS. It is believed that semen samples from men with SCI have higher levels of ROS than fertile men.[16,36] A well-known potential source of ROS production in semen is activated leukocytes[28,37] which are significantly increased among patients with SCI.[33,34,38] It is shown that high levels of oxidative stress in the semen of patients with SCI are negatively related to both semen parameters and sperm DNA integrity.[37] We should consider that each 25% increase of seminal ROS induces 10% increase in sperm DNA fragmentation.[39]

This study is unique because we used four different cytochemical tests at the same time to evaluate the

sperm chromatin/DNA status in men with SCI. According to our results, significant differences were found between patients with SCI and the control group in all of the tests. It should be noted that in our previous experimental study, high levels of sperm chromatin/DNA abnormalities were found in SCI animals.[11]

The AB staining specifies sperm residual histones and indicates anomalies in sperm chromatin condensation.[40,41] Based on the significant difference regarding AB staining between two groups, the results showed that SCI increased the rate of spermatozoa with residual histones. In agreement with our results, Salsabili *et al.*[35] (2006) used AB test and found that men with SCI had a lower sperm chromatin condensation and chromatin stability when compared with patients with idiopathic infertility. In contrast, Hou *et al.*[42] did not observe any significant difference in unstained spermatozoa with AB between men with SCI and healthy men, which may be due to difference in sample sizes.

The CMA3 is a polymerase inhibitor fluorochrome-specific for guanosine-cystosine-rich sequence, which shows the degree of sperm protamination.[43] According to our results, the percentage of CMA3-positive sperm cells was remarkably higher in the SCI group than in control subjects. It may be concluded that cord injury has a negative effect on sperm protamination. Although, the authors could not find any reports on CMA3 staining of spermatozoa in men with SCI, in our previous study, we did not find any significant difference between SCI rats and control ones.[11] This lack of significance may be due to fully condensed chromatin in spermatozoa of rats in comparison with human sperm cells that have lower levels of chromatin condensation.[44]

For evaluation of both sperm DNA fragmentation rate and chromatin condensation, the TB staining was applied. TB is a structural probe for assess DNA arrangement and chromatin packaging.[29] In current study, the rate of spermatozoa that had dark-blue, violet, and purple heads was significantly lower in healthy fertile men than in patients with SCI. This indicated that SCI has increased sperm DNA fragmentation and abnormal chromatin condensation as well. To confirm our results, we also did not find any study on using TB for sperm assessment in men with SCI.

AO staining detects DNA susceptibility to *in situ* acid-induced DNA denaturation via shifting AO fluorescence from green to red. The present data showed a significant increase in spermatozoa with red fluorescence among SCI group when compared with controls. This means that there is more DNA damage in sperm cells from men with SCI. The findings also confirmed increase rates of sperm apoptosis through DNA denaturation in patients with SCI. Although, there is no research on using AO staining in men with SCI, it had been found that these tests are positive in SCI rats.[11]

TUNEL test was applied for direct detection of sperm DNA fragmentation that is a specific sign of apoptosis. In the final part of our study, spermatozoa of SCI patients presented significantly higher apoptosis-specific DNA fragmentation than the control group. In accordance with our findings, Restelli *et al.*[45] indicated that men with SCI have higher percentages of DNA fragmentation than fertile men. In another study, the DNA fragmentation index was higher in semen samples from men with SCI in comparison with controls.[46] Our previous study on SCI rats demonstrated that chronic cord injury alters the stability of sperm chromatin by influencing the epididymal phase of chromatin condensation and disulfide bond formation, but histone-protamine replacement phase may remain intact.[11] Alteration of normal epididymal activity following SCI disturbs sperm nuclear maturation. The duration of the presence of spermatozoa in the cauda epididymis increases following abnormal epididymal autonomic innervations in men with SCI. The higher the sperm accumulation, the greater the exposure to oxidative stress and sperm DNA damage.[47] As explained before, ROS with high concentration in the semen of 96% of men with SCI[48] plays a key role in abnormalities of sperm nuclear DNA.

Another explanation for DNA fragmentation is apoptosis, which has a strong cause–effect relationship with oxidative stresses.[49] A high incidence of sperm apoptosis was observed in men with SCI in our study using TUNEL test. In agreement to our study, Restelli *et al.*[45] has also reported an increased sperm apoptotic rate among patients with SCI. The proposed mechanism is occurrence of "abortive apoptosis" in spermatozoa of subfertile men and therefore the normal clearance of apoptotic marked spermatozoa does not occur properly.

Conclusions

In conclusion, our results indicated a statistically significant increase of chromatin/DNA anomalies among men with SCI, which may explain the low fertility potential of these patients. On the other hand, due to the high frequency of abnormal spermatozoa in men with SCI, their semen samples should be checked for sperm chromatin status especially protamine deficiency, before applying assisted reproductive technologies.

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