

ORIGINAL ARTICLE

Assessment of nuclear DNA integrity of epididymal spermatozoa following experimental chronic spinal cord injury in the rat

Ali Reza Talebi,* Mohammad Ali Khalili* and Ahmad Hossaini†

*Research & Clinical Center for Infertility, Yazd University of Medical Sciences, Yazd, and †Department of Anatomy, Shahed Beheshti University of Medical Sciences, Tehran, Iran

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Correspondence:

Mohammad Ali Khalili, Research & Clinical Center for Infertility, Yazd University of Medical Sciences, Yazd, Iran.
E-mail: khalili59@hotmail.com

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Summary

Infertility is considered as one of the major problems associated with spinal cord injury (SCI). However, the exact underlying mechanism is still unknown. Therefore, the main objective of this experimental study was to evaluate the effect of chronic SCI on sperm parameters as well as chromatin integrity and DNA of spermatozoa aspirated from cauda epididymis of rats. Forty-five adult Wistar rats were divided into three groups – SCI, sham, and control. Following laminectomy, SCI was induced onto exposed dura matter (T10). The sham group underwent laminectomy of T10 only, while the control rats were not exposed to any type of injury or medication. The cauda epididymal sperms were aspirated after 8 weeks for analysis of sperm parameters and sperm chromatin integrity with aniline blue (AB), chromomycin A3 (CMA3), sodium dodecyl sulphate (SDS), and acridine orange (AO) tests. The sperm progressive motility and normal morphology of SCI rats were significantly changed when compared with other groups ($p < 0.05$). In addition, AB as well as CMA3 tests were insignificantly increased in the SCI group when compared with the sham and control groups. However, SDS and AO tests were significantly changed in SCI samples when compared with the sham and control groups ($p < 0.001$). The results showed that chronic SCI in rat disturbs sperm parameters as well as nuclear maturity and DNA integrity of sperms. Therefore, sperm chromatin structure is compromised in SCI animals as revealed by chromatin structural probes. These alterations may reduce the fertility potential of the male gamete following SCI.

Introduction

Multiple sperm defects including decreased viability, reduced progressive motility, and increased abnormal morphology have been observed in patients with spinal cord injury (SCI) (DeForge *et al.*, 2005; Utida *et al.*, 2005; Brown *et al.*, 2006). In addition, high levels of abnormal chromatin condensation and nuclear DNA fragmentation have been found in semen samples of SCI men (DeForge *et al.*, 2005; Utida *et al.*, 2005). Therefore, one speculates that alterations in sperm chromatin integrity may disturb the fertility potential of men with SCI (DeForge *et al.*, 2005). However, further research activities are needed to pinpoint the exact mechanism involved in the aforementioned fertility disturbances in SCI individuals.

In mammals, sperm nuclear DNA is recognized around protamine molecules during testicular spermiogenesis. In this phase, sperm-specific protamine, which is rich in cysteine and other basic amino acids, replaces nuclear histones. During epididymal transit, the cysteine-thiol groups of protamine molecules are oxidized to disulphide bonds (S-S), which are necessary for stability of sperm chromatin (Poccia, 1986; Said *et al.*, 1999). If the number of disulphide bonds between protamine molecules decreases, the chromatin becomes more susceptible to denaturation. In rat, spermatozoa that are collected from caput contain approximately 84% of total SH+S-S groups in the head region as thiols. This is reduced to 14% in spermatozoa obtained from the cauda epididymis (Said *et al.*, 1999). This difference indicates that during transit

between the two regions of epididymis, about 1.5 billion disulphide bonds are formed per spermatozoan. Therefore, spermatozoa become highly resistant to a variety of agents such as acids, proteases, DNase, and detergents (e.g., sodium dodecyl sulphate, SDS) after nuclear chromatin condensation (Mahi & Yanagimachi, 1975). Evenson *et al.* (1999) reported that sperm chromatin condensation is a complex process which is directly related to the capacity of sperm to fertilize an oocyte. Moreover, patients with defective sperm chromatin have low fertilizing ability *in vitro* (Hoshi *et al.*, 1996).

Following SCI which may alter the epididymal autonomic innervation, the sperms accumulate in the cauda epididymis for a long period of time, resulting in ageing (Billups *et al.*, 1991). The normal activity of the epididymis, where spermatozoa undergo final maturation, is altered with SCI, which in turn may disturb sperm nuclear maturation as well as disulphide bonds in protamine molecules (Hirsch *et al.*, 1999). As a result, with instability of chromatin, the sperm DNA becomes more susceptible to denaturing agents. It has been suggested that altered nuclear chromatin/DNA structure in spermatozoa is implicated as a possible cause of increased infertility in SCI men (Hoshi *et al.*, 1996; Utida *et al.*, 2005).

Several assays are currently employed for evaluating the nuclear chromatin integrity/maturity of sperms. These include: (i) aniline blue (AB) staining, which selectively stains lysine-rich histones, and has been used for distinguishing chromatin condensation anomalies (Auger *et al.*, 1990), (ii) chromomycin A3 (CMA3), a fluorochrome specific for GC-rich sequence which is used for evaluation of the degree of protamination of mature spermatozoa (Nasr-Esfahani *et al.*, 2001), (iii) acridine orange (AO) that is used for measurement of the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* by quantifying the metachrome shift of AO fluorescence from green (undenatured DNA) to orange-red (denatured DNA) (Nasr-Esfahani *et al.*, 2001; Khalili *et al.*, 2006), and (iv) SDS analysis with use of toluidine blue (TB) staining which measures the rate of nuclear chromatin condensation of spermatozoa (Rosenborg *et al.*, 1990; Barrera *et al.*, 1993). The aforementioned techniques are considered as independent measures of sperm quality, which can detect the subtle defects in chromatin/DNA integrity, thereby assisting in quality assessment of spermatozoa (Evenson *et al.*, 1999; Erenpreiss *et al.*, 2001).

The main objectives of the current investigation were to: (i) evaluate the effect of experimental SCI on motility and morphology of spermatozoa aspirated from the cauda epididymis of rats, and (ii) assess the nuclear DNA and chromatin integrity of spermatozoa retrieved from the

cauda epididymis of rats after chronic SCI (one spermatogenesis cycle is about 45 days in rat) with the application of four different standard assays. The results can highlight the mechanism behind decreased fertility in patients with SCI.

Materials and methods

Animals

A total of 45 adult male Wistar rats (300–325 g) were assigned to one of the three groups – control, sham, and SCI. Animals were caged individually in an air-conditioned, light-controlled room for 2 weeks prior to the experiment. They were fed rat chow and had access to water *ad libitum*. The experimental proposal was approved by our university ethics committee.

Control group

A total of 15 adult rats were randomly assigned to non-surgical, non-injected controls.

Sham group

Rats ($n = 15$) were laminectomized as described by Hirsch *et al.* (1999). Briefly, animals were anesthetized with *i.m.* injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) before a laminectomy was performed at the 10th thoracic vertebrae (T10), leaving the dura mater intact. The exposed vertebral column was stabilized by clamping the rostral T9 and caudal T11 vertebral bodies with appropriate surgical forceps. After surgery, the muscles and skin were immediately sutured together in layers. Each rat then received injections of 10 cm³ sterile saline (*s.c.*) and 33.3 mg/kg cefazoline antibiotic (*s.c.*) before being placed on a heating pad during recovery. The rats were housed in individual cages (one animal per cage) and their daily activities were monitored by a technician.

Spinal cord injury group

Fifteen rats were first laminectomized as described for the aforementioned sham group. Immediately after laminectomy, a contusion injury was performed with a 10-g brass rod with a tip diameter of 2.5 mm dropped from a height of 5 cm onto the exposed, intact dura overlying the dorsal spinal cord as described by Hirsch *et al.* (1999). The rod was immediately removed, the wound irrigated with saline and observed under a dissecting microscope for the presence of bruised cord. The muscles and skin were then sutured together in layers. Following surgery, each rat was handled as described above. The postoperative care included sterilizing of the wound. Manual expression of the bladder was performed twice daily until bladder function returned. Antibiotics (cefazoline) were administered twice a day for up to 1 week.

Chemicals

All chemicals used in this study were purchased from Merck chemical company (Darmstadt, Germany), unless otherwise stated.

Epididymal sperm preparation

Animals surviving for 50 days (one cycle of spermatogenesis in rat is approximately 45 days) were anaesthetized, as described above. A small piece of the cauda epididymis was dissected and placed in 1 mL of pre-warm T6 culture medium (37 °C, 5% CO₂). Gentle agitation along with tearing of the tissue was applied to make spermatozoa to swim out into the culture medium placed in a Falcon culture dish (Kempinas *et al.*, 1998). Each sample was briefly incubated at 37 °C, 5% CO₂ for further analysis.

Sperm analysis

In this study, only sperm motility (%) and normal morphology (%) were evaluated for 100 sperms from each of the 45 animals. Sperm motion analysis was performed using Makler chamber (Sefi Medical Co., Haifa, Israel) and phase-contrast microscopy (Olympus Co., Tokyo, Japan) at ×200. Motility was expressed as percentage of progressive (fast and slow) and non-progressive sperms. Moreover, the percentage of normal morphology of 100 spermatozoa per rat was assessed using Giemsa staining and light microscopy at ×400 (Seed *et al.*, 1996). All analyses were performed by one experienced technician blinded to the study.

Evaluation of sperm nuclear chromatin

For evaluation of sperm nuclear chromatin, four different techniques were applied.

Aniline blue staining

Smears from cauda epididymal samples were first air-dried and then fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 5 min (Auger *et al.*, 1990). Normal (unstained or pale blue stained) and abnormal (dark blue stained) spermatozoa were evaluated with light microscopy (Olympus).

Chromomycin A3 staining

Air-dried smears were fixed in Carnoy's solution (methanol/glacial acetic acid, 3 : 1) at 4 °C for 10 min. Each slide was treated for 20 min with 100 µl of CMA3 (Sigma, St Louis, MO, USA) solution (0.25 mg/mL in

McIlvain buffer; 7 mL citric acid, 0.1 M + 32.9 mL Na₂HPO₄·7H₂O 0.2 M, pH 7.0 containing 10 mM MgCl₂). The slides were finally rinsed in buffer, mounted with buffered glycerol (1 : 1) and viewed with the aid of a fluorescent microscope with a 460-nm filter (Zeiss Co., Hamburg, Germany) (Nasr-Esfahani *et al.*, 2001). Bright yellow stained spermatozoa were considered CMA3+; while, yellowish green stained sperms were considered CMA3-.

SDS treatment with toluidine blue stain

Fifty microlitres of the epididymal sperm suspension in T6 medium was mixed with 350 µl of 1% SDS in 0.05 M borate buffer (88 mL NaOH 0.1 M + 4.2 mL Na-tetraborate 0.25 M + 400 mL DW, pH 9.0). Following 120 min of incubation at 37 °C, the reaction was stopped by adding an equal volume of 2.5% glutaraldehyde in 0.05 M borate buffer (Rosenborg *et al.*, 1990). Sperm nuclear swelling was assessed after TB staining in different scores. For TB staining, the air-dried smears of SDS-treated spermatozoa were fixed in fresh 96% ethanol-acetone (1 : 1) at 4 °C/30 min, and finally hydrolysed in 0.1 N HCl at 4 °C/5 min. The slides were then rinsed thrice in distilled H₂O for 2 min, and finally stained with 0.05% TB for 10 min. The staining buffer was composed of 50% citrate phosphate (McIlvain buffer, pH 3.5) (Erenpreiss *et al.*, 1997). Four different scores (0–3) were applied: score 0 = good chromatin (light blue), lacking head swelling; score 1 = mild abnormal chromatin (dark blue) with sperm head swelling; score 2 = abnormal chromatin (violet) with head swelling; and score 3 = severe chromatin abnormality (purple) with highly swelling of sperm head. The following formula was applied for assessing the 'total score': total score = (S₀ × 0) + (S₁ × 1) + (S₂ × 2) + (S₃ × 3) (Rosenborg *et al.*, 1990).

Acridine orange staining

For detection of sperm DNA integrity, the smears were air-dried for 1 h and then fixed overnight in Carnoy's solution. Each sample was stained for 10 min in freshly prepared AO (0.19 mg/mL) in McIlvain phosphate-citrate buffer (pH 4) for 5 min, as explained by Hirsch *et al.* (1999). Smears were evaluated on the same day using a fluorescent microscope with a 460-nm filter. The duration of illumination was limited to 40 sec/field. The percentage of 100 green (normal double-stranded DNA), orange/red (abnormally denatured) fluorescence sperms per sample was calculated (Khalili *et al.*, 2006).

Statistical analysis

Data are presented as mean ± standard deviation. Statistical significance was set at *p* < 0.05 for sperm parameters

and $p < 0.01$ for nuclear chromatin evaluations. Two-way ANOVA was considered for comparison of the results for all tests, except the CMA3 test. Both nonparametric Kruskal–Wallis and Mann–Whitney U -tests were applied for the data generated from the CMA3 test.

Results

Sperm parameters

The results showed that more than 70% of spermatozoa retrieved from the caudal epididymis were motile in control animals. This rate was slightly increased in sham-operated rats (73.44%). However, 50 days after the weight-drop contusion, the total motility of spermatozoa was significantly reduced to 51.77% in the SCI group (Table 1). The non-progressive motility was slightly increased from 17.08% in controls to 22.72% in SCI rats ($p > 0.05$). With regard to the sperm normal morphology, a significant reduction was noticed in spinal cord-lesioned animals (control: 88.40%; sham: 84.00%; SCI: 64.88%). In general, laminectomized rats (sham) did not show any significant alteration in sperm progressive motility or normal morphology profiles (Table 1).

Assessment of sperm DNA and chromatin

Aniline blue test

The majority of epididymal spermatozoa in three groups of control, sham and SCI rats were shown to be mature with intact morphology (Table 2). The rates of AB-reacted sperms (AB+) were similar in all groups of animals under investigation.

Chromomycin A3 test

There was an insignificant difference between control/sham and SCI group with regard to CMA3 results. Table 2 shows that the rate of reacted spermatozoa to CMA3 was 0.90% and 1.0% in control and sham animals respectively. This rate was slightly increased to 1.4% in animals with SCI.

Sodium dodecyl sulphate analysis

The total score (%) was found to be significantly altered in the SCI group when compared with the other two

Table 2 Characteristics of nuclear integrity of spermatozoa retrieved from the cauda epididymis of three different groups of rats

Variable (%)	Control	Sham	SCI	p -value ^a
AB+	1.00 ± 1.05	1.00 ± 0.94	1.10 ± 0.99	>0.05
CMA3+	0.90 ± 1.59	1.00 ± 1.41	1.40 ± 1.50	>0.05
Total score	27.16 ± 8.51	26.41 ± 9.80	72.06 ± 22.75	<0.001
AO+	13.60 ± 8.75	16.10 ± 2.84	18.50 ± 13.48	<0.05

AB+: aniline blue-reacted sperm heads showing immature sperms with increased histone; CMA3+: chromomycin A3-reacted sperms deficient in protamine; total score: SDS test for evaluation of sperm head swelling; AO+: acridine orange-reacted sperms that are immature with single-stranded DNA; SCI, spinal cord injury. ^aSCI vs. control/sham.

groups. Table 2 shows that the rate of SDS was increased from 27.16% in spermatozoa of the control group to 72.06% in animals with SCI.

Acridine orange test

Sperm nuclei from SCI rats showed a significant evidence of DNA damage (increase in orange-red fluorescence) when compared with those of control and sham-operated animals. Over 18% of spermatozoa retrieved from the epididymal cauda of SCI rats were abnormally denatured.

Discussion

In rats and humans, spermatozoa are capable of fertilizing an oocyte by the time they reach the cauda epididymis. The biochemical changes that sperm undergo during their transit to the cauda epididymis will increase the motility of sperm and their ability to fertilize oocytes (Shalgi *et al.*, 1989; Kempinas *et al.*, 1998; Brown *et al.*, 2006). Previous investigations confirm that autonomic innervation plays a profound role in normal epididymal activity and sperm transit. Following SCI, however, the epididymal innervations are altered, which would decrease contractility of the duct, causing epididymal dysfunction, delay in sperm transition and ageing of spermatozoa (Kempinas *et al.*, 1998). Linsenmeyer *et al.* (1999) induced SCI at T9 of rats to study its effect on sperm motility. Their results showed that progressive motility of epididymal sperms were significantly reduced after SCI. They believed that delaying sperm transit from the head to the cauda region

Variables (%)	Control	Sham	SCI	p -value ^a
Progressive motility	54.75 ± 10.95	50.25 ± 8.22	29.61 ± 2.72	<0.05
Non-progressive motility	17.08 ± 8.07	22.83 ± 9.95	22.72 ± 9.04	>0.05
Total motility	70.91 ± 5.72	73.44 ± 7.02	51.77 ± 8.85	<0.05
Normal morphology	88.40 ± 4.40	84.00 ± 8.40	64.88 ± 10.16	<0.05

SCI, spinal cord injury. ^aSCI vs. control/sham.

Table 1 Characteristics of sperm parameters retrieved from the cauda epididymis of three different groups of rats

of the epididymis was the main reason for reduced sperm motility. In addition, Huang & Li (2003) induced SCI at T10 in Sprague–Dawley rats, and noticed defects in sperm parameters. They observed a correlation between the severity of cord injury and alteration in sperm motility. Similar alterations were noticed in sperm parameters of 24 young patients with SCI at the level of the cervical or thoracic region (Padron *et al.*, 1997). Our findings are also in agreement with the aforementioned investigations that have shown epididymal sperm morphology and motility indexes were severely damaged after induction of injury at the T10 level, when compared with the sham-operated or control animal groups.

We assessed the susceptibility of nuclear integrity of epididymal spermatozoa to denaturation in order to determine whether changes in the chromatin stability occur after chronic SCI in rats. These changes may be associated with altered nucleoprotein content or disulphide bonding levels. It appears that SCI did alter the formation of disulphide bonds between protamine molecules in spermatozoa residing in the epididymis of the rat. This leads to the suggestion that in the epididymal phase of chromatin condensation, following disulphide bond disruption, the chromatin become unstable. Therefore, our results show that after SCI, the testicular phase of chromatin condensation may remain intact. This was shown with application of two assays of AB and CMA3 – a guanine–cytosine-specific fluorochrome. AB is a marker for detection of extra lysine-rich histone proteins, while CMA3 detects protamine defects during histone–protamine replacement in the testicular phase of sperm chromatin condensation (Romanini *et al.*, 1986; Auger *et al.*, 1990). Our results showed that both assays were unsuccessful in detecting chromatin defects in epididymal sperms retrieved post-SCI. In agreement with our findings, Hou *et al.* (1995) did not observe an alteration in the testicular phase of chromatin condensation in spinal cord-injured individuals after staining of spermatozoa with AB.

There was an evidence of increased red fluorescence with AO staining, which is a biomarker of increased susceptibility to acid denaturation that can result from DNA damage or altered chromatin structure in sperms from our experimental group. A possible mechanism for reduced fertility following SCI could involve changes in the molecular structure of the disulphide bond and DNA damage in the nucleus of the sperm (Romanini *et al.*, 1986; Said *et al.*, 1999). Disulphide bonding is primarily responsible for the stabilization of sperm chromatin in the epididymis (Rosenborg *et al.*, 1990). The disulphide levels may be critical during decondensation process following fertilization, at the time when sperm chromatin is decondensed within the

ooplasm (Poccia, 1986; Romanini *et al.*, 1986; Nasr-Esfahani *et al.*, 2001). This process is dependent upon the reduction of disulphide bonding. Furthermore, the timing of decondensation is dependent upon the number of disulphide bonds within the spermatozoal nucleus (Nasr-Esfahani *et al.*, 2001). The process of decondensation is necessary for both male and female genomes to combine and form the zygote (Sakkas *et al.*, 1996). Therefore, the alterations in disulphide bonding and DNA damage (increase in red fluorescence) which was noticed in sperm nuclei following chronic SCI in our study can affect the fertility potential of spermatozoa. Sperms characterized by increased nuclear DNA/chromatin anomalies are often associated with decreased fertilization rates and embryo cleavage after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Lopes *et al.*, 1998; Sakkas *et al.*, 1998). Contrary to these reports, others have suggested that infertile men with high level of sperm DNA damage can successfully undergo ICSI treatment (Bungum *et al.*, 2004; Payne *et al.*, 2005). It has been suggested that ICSI can result in higher pregnancy rates with semen samples with high rates of DNA damage when compared with conventional IVF (Bungum *et al.*, 2004). Infertile men generally have a higher fraction of sperms with sperm chromatin defects than the fertile population (Evenson *et al.*, 1999). All the above studies included infertile patients without spinal cord lesions. Therefore, different results may be obtained among infertiles with SCI. This needs further clarification in a controlled clinical setting.

Our findings provide evidence that sperm chromatin integrity is compromised in animals with chronic SCI. This was revealed by sensitive chromatin structural assays such as AO and SDS-TB (but not by means of less sensitive AB and CMA3) due to the abnormal maturation of sperm nuclei during the testicular and/or the epididymal phase. Thus, the low rate of fertility in men suffering from SCI may be the result of damage to the sperm nucleus.

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