

# Sperm DNA damage and seminal oxidative status after shock-wave lithotripsy for distal ureteral stones

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**Objective:** To investigate the relationship between level of sperm DNA damage, seminal oxidative status, and shock-wave lithotripsy (SWL) for distal ureteral stones.

**Design:** Prospective study with control.

**Setting:** Academic research institute.

**Patient(s):** Men who had undergone SWL for distal and upper ureter stones.

**Intervention(s):** Level of sperm DNA damage and seminal oxidative status assessed through the examination of semen on the day before and 3 days, and 3 months after SWL.

**Main Outcome Measure(s):** DNA damage score and oxidative stress in semen parameters.

**Result(s):** Sperm DNA damage score and semen total oxidant status (TOS) levels increased, but semen total antioxidant status (TAS) levels, sperm concentration, and motility decreased immediately after SWL. However, there were no statistically significant correlations between the DNA damage scores and the increased TAS and TOS levels in the study group. All of the changes returned completely to initial level during three months after SWL.

**Conclusion(s):** SWL may affect fertility in men. Therefore, we suggest other treatment modalities, such as ureteroscopy, for young men with distal ureteral stones to prevent the development of male infertility. (Fertil Steril® 2011;96:1087–90. ©2011 by American Society for Reproductive Medicine.)

**Key Words:** SWL, male infertility, DNA damage, seminal plasma oxidative status

The treatment of patients with ureteral calculi aims to remove the whole stone with minimum rates of morbidity (1). There is disagreement regarding the optimum treatment for patients who need elimination of distal ureteral calculi (2). Shock-wave lithotripsy (SWL) and ureteroscopy techniques are associated with high success rates and are considered to be effective therapies for distal ureteral stones (1). However, SWL and ureteroscopy are not without treatment-related side effects and complications. The mechanism of SWL-induced cellular injury is still controversial. Formation of free radicals during SWL is one of the mechanisms discussed for tissue destruction (3). During oxidative stress, free radicals, which are produced in large amounts, have a role in degeneration of cellular and subcellular membrane structures (4). In addition, it was reported that oxidative stress affects the integrity of the sperm genome by causing high frequencies of single- and double-strand DNA breaks, which are often observed in the semen of infertile patients (5). At present, DNA destruction in spermatozoa is considered to be a significant cause of male infertility (6).

The distal section of ureter lies anatomically close to the seminal vesicle. The epididymis is also close to the affected area by SWL. Therefore, it is possible that SWL can theoretically affect these organs in cases with distal ureteral stones. The oxidative status and DNA damage score in semen of these patients have not been reported. Therefore, in the present pilot study, we investigated the effect of SWL on the levels of sperm DNA damage score, seminal

plasma oxidative status, and semen parameters for patients who underwent SWL for distal ureteral stones.

## MATERIALS AND METHODS

In all, 12 patients who underwent SWL for distal ureteric stones from March 2010 to February 2011 were enrolled in this prospective study. We excluded patients with scrotal surgery, those with inguinal surgery, those with a history of smoking, and those with abnormal sperm and urine examination. Each of the patients gave written informed consent before they were enrolled in the study, which had been approved by the Institutional Review Board. Two patients with abnormal sperm examination were excluded.

In the study group, ten patients with stones in the distal ureter underwent one SWL session with 2,500 shock waves at an energy level of 18 kV. Diclofenac sodium (75 mg) was given intramuscularly 45 minutes before the procedure for analgesia. The SWL sessions were performed using the Piezolith 3000 (Richard Wolf) lithotripter. The delivery rate of the Piezolith 3000 was 120/min.

The semen from these patients was examined according to World Health Organization (WHO) criteria (7) on the day before and 3 days after SWL, and these data were used to assess the effect of SWL on sperm DNA damage score and plasma semen total antioxidant status (TAS) and total oxidant status (TOS). As a control group, semen was obtained from ten patients with stones in the upper ureter who underwent one SWL session with 2,500 shock waves at an energy level of 18 kV. The upper ureteral stones were fragmented under continued ultrasound monitoring, applying fluoroscopy to confine the stone and confirm the location if the stone was not detectable or moved out of the focal region on ultrasonography. Distal ureteral stones were managed with fluoroscopic guidance only. The mean fluoroscopy time was 57 seconds for the study group and 33 seconds for the control group.

All semen was separated into sections immediately after ejaculation. One sample was examined for semen characteristic directly, and one was used in DNA damage determination and measurement of oxidative status. All patients initially showed no abnormality of sperm characteristic before SWL. Sperm DNA damage, semen oxidative status, and semen quality were examined once again 3 months after SWL.

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## Isolation of Mature Sperm Population for Assessing Sperm DNA Damage

One milliliter of liquefied semen samples was prepared to use two-step discontinuous Percoll gradient (95.0–47.5%; Pharmacia Biotech), and liquefied semen was layered on top of the gradient and centrifuged at 450g for 12 minutes. The resulting sperm pellet was concentrated by centrifugation at 200g for 6 minutes. The final sperm preparation was suspended in a suitable volume of BWB medium (without adding any exogenous substrates) and centrifuged at 500g for 7 minutes and finally diluted with a suitable volume of BWB medium to achieve a concentration of  $1 \times 10^5$  sperm cells in 10  $\mu$ L BWB (8). Sperm membrane integrity was assessed by means of the trypan blue exclusion method.

The remaining semen samples were centrifuged at 1,500g for 10 minutes to obtain the semen plasma. The separated semen plasma was then stored at  $-80^\circ\text{C}$  until further analysis of TAS and TOS content.

## Determination of Sperm DNA Damage Using Comet Assay

Sperm DNA damage was investigated using a single-cell gel electrophoresis (comet) assay that was generally performed under highly alkaline conditions. Embedding of sperm in agarose gel:  $1 \times 10^5$  cells in 10  $\mu$ L BWB medium was mixed with 80  $\mu$ L 0.7% low-melting-point agarose (Sigma) and added to microscope slides (with frosted ends) that had been covered with a bottom layer of 1% normal-melting-point agarose (Sigma) in phosphate-buffered saline solution and covered with a coverslip. Slides were allowed to solidify for 5 minutes at  $4^\circ\text{C}$  in a moist box. The coverslips were removed and the slides were immersed in a freshly prepared cold lysis buffer containing 2.5 mol/L NaCl, 100 mmol/L  $\text{Na}_2\text{-EDTA}$ , 10 mmol/L Tris, 1% Triton X-100, and 10% dimethyl sulfoxide, pH 10, for 1 hour. Then the slides were incubated for 4 hours at  $37^\circ\text{C}$  in 100  $\mu$ M proteinase K (Roche Diagnostics) and added to the lysis buffer. The slides were removed from the lysis buffer, drained, and placed in a horizontal electrophoresis unit filled with a fresh alkaline electrophoresis solution, containing 300 mmol/L NaOH and 1 mmol/L EDTA, pH 12.5, for 20 minutes to allow the DNA to unwind. Electrophoresis was performed for 10 minutes at room temperature at 12 V (0.714 V/cm) and was adjusted to 300 mA by raising or lowering the buffer level. Then the slides were washed with a neutralized solution of 0.4 mol/L Tris, pH 7.5, to remove alkali and detergents. After neutralization, the slides were stained with 50  $\mu$ L 2  $\mu$ g/mL ethidium bromide (Sigma) and covered with a coverslip. All steps were performed under dim light to prevent further DNA damage.

## Image Analysis

One hundred cells from each slide were selected randomly (50 cells from each of the two replicate slides) and analyzed visually. Observations were made at magnification  $\times 400$  using an epifluorescence microscope (Olympus

BX51) Each image was classified according to the intensity of the fluorescence in the comet tail and given a value of 0, 1, 2, 3, or 4 (from undamaged [class 0] to maximally damaged [class 4]), so that the total score of the slide could be between 0 and 400 arbitrary units (AU). The interslide variation between the means of the three control slides showed coefficients of variation ranging from 0.6% to 5.0%.

## Measurement of Total Antioxidant and Total Oxidant Status in Semen Plasma

The TAS of semen samples was determined using a novel automated measurement method developed by Erel (9). The TOS of semen samples was determined using a new automated colorimetric measurement method (9).

## Statistical Evaluation

All data were entered into statistical software for analysis (SPSS v11.5). Continuous variables were expressed as mean  $\pm$  SD. Distributions of data were tested with one-sample Kolmogorov-Smirnov test and not found to be normally distributed. For multiple comparisons, repeated measurement of variance analysis was used for comparisons between groups and the Bonferroni test used if any statistical significance was found. Spearman correlation ( $\rho$ ) test was used for group comparison. All statistical tests were two sided. A  $P$  value of  $<.05$  was considered to be significant.

## RESULTS

The mean ages for the study group and control group were  $28.3 \pm 4.80$  (23–35) years and  $28.8 \pm 5.30$  (21–34) years, respectively. There was no statistically significant difference between the groups regarding mean age ( $P>.05$ ). Semen parameters of the study and control groups are detailed in Table 1. Sperm concentration and motility characteristics were highly significantly different between the day before and 3 days after SWL in the study group ( $P<.001$ ). However, repeated examinations 3 months after SWL were not significantly different. Comparison of semen oxidative stress indices and sperm DNA damage score in patients with the study and control groups are presented in Table 2. In the levels of TAS and TOS, there were significant differences between the levels initially and 3 days after SWL in the study group. DNA damage scores also increased to compare with initial levels 3 days after SWL in the study group. However, in evaluations made 3 months after SWL, all changes were observed to return to initial levels.

Correlations between semen TAS, TOS, and sperm parameters and DNA damage index in the study group are presented in Table 3. In the

**TABLE 1**

Semen characteristics of the study and control group, mean  $\pm$  SD.

	Study group (n = 10)				Control group (n = 10)			
	Initial	3 d after SWL	3 mo after SWL	P value	Initial	3 d after SWL	3 mo after SWL	P value
Concentration ( $\times 10^6/\text{mL}$ )	53.32 $\pm$ 8.70	38.00 $\pm$ 6.10	51.23 $\pm$ 7.80	<.05	52.00 $\pm$ 9.60	51.00 $\pm$ 10.30	53.00 $\pm$ 8.80	>.05
Total sperm count ( $\times 10^6$ )	173 $\pm$ 23	133 $\pm$ 19	176 $\pm$ 26	<.05	163 $\pm$ 22	164 $\pm$ 24	169 $\pm$ 27	>.05
Motility (% motile sperm)	61.67 $\pm$ 12.44	41.22 $\pm$ 8.7	58.56 $\pm$ 9.7	<.05	58.66 $\pm$ 12.4	56.90 $\pm$ 11.6	60.80 $\pm$ 12.4	>.05
Microscopic hemospermia (n)	0	5	0	NS	0	0	0	NS

Note: SWL = shock-wave lithotripsy.

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**TABLE 2****Comparative analysis of DNA damage score and oxidative status of the groups, mean  $\pm$  SD.**

	Study group (n = 10)				Control group (n = 10)			
	Initial	3 d	3 mo	Sig.	Initial	3 d	3 mo	Sig.
TAS (mmol Trolox equiv/L)	17.7 $\pm$ 3.3	8.9 $\pm$ 1.8	15.9 $\pm$ 2.6	<i>P</i> < .05	14.8 $\pm$ 2.7	13.9 $\pm$ 2.5	15.1 $\pm$ 3.4	<i>P</i> > .05
TOS ( $\mu$ mol H <sub>2</sub> O <sub>2</sub> /L)	13.9 $\pm$ 8.7	24 $\pm$ 9.6	15 $\pm$ 8.1	<i>P</i> < .05	16.4 $\pm$ 9.8	15.8 $\pm$ 7.7	14.6 $\pm$ 8.2	<i>P</i> > .05
DNA damage score (AU)	11.5 $\pm$ 5.9	29 $\pm$ 11.7	13 $\pm$ 6.3	<i>P</i> < .05	10.3 $\pm$ 5.5	11.5 $\pm$ 6.7	9.5 $\pm$ 5.8	<i>P</i> > .05

Note: AU = arbitrary units; TAS = total antioxidant status; TOS = total oxidant status.

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study group, although there was a positive correlation between TOS with DNA damage and TAS with sperm parameters and a negative correlation between TAS with DNA damage and TOS with sperm parameters, these correlations were not statistically significant (*P* > .05 for all of them). In the control group, in the levels of TAS, TOS, and DNA damage score, there were no significant differences between the day before and 3 days and 3 months after SWL. No correlations between DNA damage score and TAS and TOS levels were found in the control group.

## DISCUSSION

In the past 20 years, the biologic effects of SWL have been intensively investigated (10–13). In some of these studies, it was reported that shock waves applied at a therapeutic level of energy may cause double-strand DNA damage (12, 13). There are various mechanisms that potentially affect DNA damage during shock wave exposure, including the system of generating the shock wave, the presence or lack of cavitation impact, and generation of sonochemicals, such as oxygen superoxide. Although clinical and experimental investigations have suggested the safety of SWL, its possible effects on the male reproductive system are still not known. Furthermore, the literature contains a limited body of research with contradictory results on the possible effects of SWL on semen characteristic and gonads (10, 11, 14–19). Although Puppo et al. (16) reported no adverse effect on male fertility, Andreessen et al. (10) noted macroscopic and microscopic hemospermia and decreased sperm motility and sperm density.

In the present study, there was no sign of microscopic hemospermia before shock wave use, but it was detected in five of ten patients with lower ureteral calculi after SWL. Macroscopic hemospermia

was not detected in either group. Hemospermia could have been due to the close anatomic locality of the distal ureter to the seminal vesicles and epididymis.

Although the mean sperm concentration and motility of the study group were within the normal range, as judged by WHO criteria, we found a significant decrease in sperm concentration and motility after SWL. However, sperm concentration and sperm motility returned to initial levels 3 months after SWL in the study group. A direct effect on the sperm in the seminal vesicle and epididymal tail may also affect sperm motility. But the mechanism responsible for the decrease in sperm concentration is not clear. Andreessen et al. explained this mechanism as a breakdown of spermatozoa in the seminal vesicle (10). Although the extensive agreement in the literature is that SWL temporarily affects male fertility parameters in patients with lower ureteral stones, there are also some studies that report different results (17). Some studies have investigated the effects of interruption of the organogenesis of germ cells caused by the teratogenic potential of high-energy shock waves in a number of systems, such as in vitro effects of human spermatozoa (18). Ohmori and Matsuda (15) and Bedir et al. (17) noted that spermatozoa and testis were irreversibly injured with an increasing number of shock waves. However, Deng et al. (19) and Basar et al. (11) showed only temporary histologic changes in testicular tissue in vivo.

Nuclear and mitochondrial DNA damage are serious outcomes caused by oxidative stress. Therefore, the decrease in reactive oxygen species might reduce DNA damage (20). To our knowledge, no trial has yet determined the relationship between sperm DNA damage and semen oxidative status after SWL for distal ureteral stones; therefore, in the present pilot study we investigated the effect of SWL on sperm DNA and semen oxidative parameters 3 days and 3 months after SWL.

**TABLE 3****Correlations between semen TAS, TOS, and sperm parameters and DNA damage index in the study group.**

	Semen concentration ( $\times 10^6$ /mL)	Semen motility (% motile sperm)	DNA damage index (AU)
TAS (mmol trolox equivalent/L)	$\rho = 0.160$ <i>P</i> = .406	$\rho = 0.370$ <i>P</i> = .130	$\rho = -0.430$ <i>P</i> = .095
TOS ( $\mu$ mol H <sub>2</sub> O <sub>2</sub> equivalent/L)	$\rho = -0.042$ <i>P</i> = .828	$\rho = -0.170$ <i>P</i> = .320	$\rho = 0.390$ <i>P</i> = .125

Note: Abbreviations as in Table 2.

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We found that sperm DNA damage score and semen TOS levels increased but that semen TAS levels decreased immediately after SWL (statistically significant). However, there were no statistically significant correlations between the DNA damage scores and the increased TAS or TOS levels in the study group. All of the changes had returned completely to initial levels 3 months after SWL.

Oxidative stress refers to a disproportion between the production of reactive oxygen species and antioxidant protections buffering the oxidative harm (21). The decreased antioxidative agents may be associated with buffering the increased oxidative agents because of maintaining the balance (22). Therefore, inadequate antioxidant capacity and excessive oxidative agents might be associated with increased DNA damage in patients who have undergone SWL for distal ureteric stones.

Another controversial side effect of SWL on infertility is fluoroscopic exposure. Patients suffering from renal or vesical stones are

treated preferably under ultrasound imaging, whereas fluoroscopic imaging is favored for treating ureteral stones (23). Vesicals and gonads receive the highest dosage when the stone is located in the distal ureter (24). Therefore, fluoroscopic exposure may also be a cause of oxidative stress and DNA damage in patients who undergo SWL for distal ureteral stones.

SWL has been used successfully to treat urinary tract stones for the past 3 decades. However, we found that SWL for distal ureteral stones caused increase in DNA damage score and oxidative stress in semen parameters. There is a correlation between sperm DNA damage and lower normal, intrauterine insemination, and in vitro pregnancy rates. (25). Therefore, alternative techniques, such as ureteroscopy, should be taken into account when treating young male patients who have borderline sperm parameters. Considering the limitations of the present study group, prospective studies with larger populations should be conducted to further elucidate these results.

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