

The impact of peritoneal fluid from healthy women and from women with endometriosis on sperm DNA and its relationship to the sperm deformity index

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Objective: To study the effect of peritoneal fluid (PF) from healthy women and from women with endometriosis on sperm DNA and its relationship to sperm morphology as assessed by the sperm deformity index (SDI).

Design: Experimental study.

Setting: Research laboratory at an academic hospital.

Patient(s): Healthy women undergoing laparoscopic tubal ligation and women with endometriosis.

Intervention(s): Aliquots of prepared sperm from 10 healthy donors were incubated with PF from healthy women undergoing laparoscopic tubal ligation (treatment 1, n = 10), with PF from patients with endometriosis (treatment 2, n = 10), and with human tubal fluid media with 10% bovine serum albumin (control, n = 10).

Main Outcome Measure(s): Sperm DNA fragmentation was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay and SDI.

Result(s): There was a significant increase in sperm DNA damage at 24 hours compared with at 1.5 and 3 hours. The SDI scores significantly correlated with sperm DNA damage after 1.5 and 24 hours of incubation in all aliquots. Percentage normal morphology did not correlate with DNA damage.

Conclusion(s): In vitro exposure of sperm to PF from patients with endometriosis is associated with significantly increased DNA damage. There is evidence of interdependence between the sperm morphology as assessed by SDI scores and DNA damage. The significant increase in sperm DNA damage observed after 24 hours of incubation may be clinically relevant. (Fertil Steril® 2009;92:61–7. ©2009 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, peritoneal fluid, infertility, sperm, DNA damage

Endometriosis is a common disease that affects up to 10% of women of reproductive age. In women with pelvic pain, infertility or both, its frequency is 35%–50% (1–3). Severe endometriosis decreases female factor fertility by many mechanisms such as distortion of pelvic anatomy, impairment of oocyte release in an inflammatory hostile peritoneal environment, difficulty in ovum pickup and transport through the tube, and impairment of implantation and embryo development (3, 4). Despite the vast amount of research in the last decade, the relationship between endometriosis and infertil-

ity is not completely understood. Oxidative stress (OS) has been proposed as a possible mechanism that is involved in the pathophysiology of endometriosis (5, 6) by promoting growth and adhesion of endometrial cells in the peritoneal cavity. Elevated levels of reactive oxygen species (ROS) have been found to increase in the serum and peritoneal fluid (PF) of patients with endometriosis (7, 8).

OS has been shown to exert toxic effects on sperm, damaging the sperm cell membrane, inducing DNA damage, and mediating sperm apoptosis (9, 10). Membrane lipid peroxidation is believed to be an important mechanism that occurs in the spermatozoa as a result of exposure to excessive ROS (11). As a consequence of lipid peroxidation, the plasma membrane loses the fluidity and integrity it requires for participation in the membrane fusion events associated with fertilization (11, 12). Furthermore, OS-mediated DNA damage interferes with the sperm DNA integrity required for achieving and sustaining normal embryo development (13).

Abnormally high levels of tumor necrosis factor- α (TNF- α) have been reported in the PF of females with endometriosis. TNF- α has been proposed as a sensitive marker for the nonsurgical diagnosis of endometriosis (7, 8, 14). In vitro incubation of motile sperm with TNF- α has produced sperm

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chromatin and DNA damage in a concentration- and time-dependent manner (15).

The routine evaluation of male factor fertility continues to rely on the assessment of perm count, motility, and morphology in ejaculated semen. The sperm deformity index (SDI) score is a novel expression of the quality of sperm morphology, which has been shown to be a more powerful predictor of male factor fertility and of IVF outcome than assessment of the proportion of sperm with normal morphology (16). High SDI scores have been shown to be associated with sperm OS (17, 18) and sperm cell apoptosis (19).

The goal of this study was to assess the impact of sperm incubation with PF from healthy women and from patients diagnosed with endometriosis on the integrity of sperm chromatin as assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The DNA damage was correlated with the percentage of normal sperm morphology and the SDI scores as indicators of sperm functional integrity.

MATERIALS AND METHODS

The present study was approved by the Institutional Review Board of the Cleveland Clinic. Semen samples were collected from 10 healthy donors with ages ranging between 25 and 39 years after a period of 3–5 days of sexual abstinence. The mean sperm count was $66 \pm 30 \times 10^6/\text{mL}$, and the mean percentage progressive motility was $68\% \pm 13\%$. After liquefaction, semen analysis was performed according to World Health Organization standards (20). Only samples with $\geq 20 \times 10^6$ spermatozoa/mL and at least 50% progressive sperm motility were selected for the study to ensure the presence of sufficient spermatozoa for all of our planned evaluations.

Collection of PF

PF was collected from 10 women with pelvic endometriosis confirmed during a diagnostic and/or therapeutic laparoscopy procedure. Disease stage ranged from I to IV according to the revised American Fertility Society scoring system (21). Fluid was collected from the posterior cul-de-sac and transported on ice to the laboratory where it was centrifuged at 600 g for 5 minutes. The supernatant was then frozen and stored at -20°C . PF from 10 healthy women with no macroscopic evidence of endometriosis was collected during laparoscopic tubal sterilization. Samples were prepared as above. Individual peritoneal samples were used for incubation with one semen sample.

Sperm Preparation

Semen samples were prepared by double density gradient centrifugation (PureCeption; SAGE BioPharma, Bedminster, NJ). Samples were loaded onto a 40% and 80% discontinuous gradient and centrifuged at 300 g for 20 minutes at room temperature (25°C). The resulting 80% pellet was washed by centrifugation for an additional 7 minutes at 300 g and resus-

pended in 4.5 mL of human tubal fluid media (HTF; Irvine Scientific, Santa Ana, CA) enriched with bovine serum albumin (BSA 10%).

The sample was then divided into nine aliquots of 500 μL each. Three aliquots were for incubation with PF from healthy women undergoing tubal ligation (treatment 1), and three were for incubation with PF from women with endometriosis (treatment 2). The final three aliquots were incubated with HTF (with 10% BSA) alone and served as controls. For each treatment and the control, the incubation in 5% CO_2 at 37° continued for 1.5 hours for the first aliquot, 3 hours for the second aliquot, and 24 hours for the third aliquot. These intervals were selected arbitrarily to reflect the length of time sperm may remain in the fallopian tubes before fertilizing an egg. At the end of each time interval, sperm DNA fragmentation was assessed as described below.

Evaluation of DNA Fragmentation

DNA fragmentation of the sperm was assessed with terminal TUNEL using an Apo-Direct kit (BD Bioscience, San Jose, CA) in all nine aliquots at the end of the incubation period. Briefly, 1×10^6 spermatozoa were washed in PBS, resuspended in 1% paraformaldehyde, and placed on ice for 30–60 minutes. Subsequently, spermatozoa were washed again and resuspended in 70% ice-cold ethanol.

After a second wash in PBS to remove the ethanol, sperm pellet samples and the positive and negative controls provided with the assay kit were resuspended in 50 μL of the staining solution for 60 minutes at 37°C . The staining solution contained terminal deoxytransferase (TdT) enzyme, TdT reaction buffer, fluorescein-tagged deoxyuridine triphosphate nucleotides, and distilled water. All cells were further washed in rinse buffer, resuspended in 0.5 mL of propidium iodide/RNase solution, and incubated for 30 minutes in the dark at room temperature, followed by flow cytometric analysis.

Flow Cytometric Measurements

All fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan (BD Biosciences, San Jose, CA). Approximately 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/second. The excitation wavelength was 488 nm, supplied by an argon laser at 15 mW. Green fluorescence (480–530 nm) was measured in the FL-1 channel, and red fluorescence (580–630 nm) was measured in the FL-2 channel. Gating was done to exclude debris and aggregates using 90° and forward-angle light scatter. The percentage of positive cells and the mean fluorescence were calculated on a 1023-channel scale using the flow cytometer software FlowJo version 6.4.2 (Tree Star, Inc., Ashland, OR).

Assessment of Sperm Morphology

Thin smears of the well-mixed ejaculated semen were prepared in duplicate by placing 2–5 μL on clean poly-L-lysine

coated slides. Thin semen smears facilitated sperm morphology assessment by avoiding sperm cell overlap and ensuring that the sperm were scattered at the same focal depth. After drying in air, the slides were stained with Papanicolaou. Slides were coded and randomly evaluated by one of the investigators (N.A., Liverpool Women's Hospital, Liverpool, UK).

The method for assessing sperm morphology for the purpose of calculating the sperm morphology is described elsewhere (16). In brief, a total of 200 spermatozoa were scored per slide using bright-field illumination and an oil immersion objective with a total magnification of $\times 2000$. At least 10 high-power fields selected at random from different areas of the slide were examined. A calibrated micrometer on the eyepiece of the light microscope was used to measure sperm dimensions when there was doubt over sperm classification. All slides were assessed using a morphological classification based on a modification of the method of Eliasson (22) and applying the strict criteria for normal sperm morphology (23). A multiple-entry scoring technique was adopted in which an abnormal sperm was classified more than once if more than one deformity was observed (16). The SDI was calculated by dividing the total number of deformities observed by the number of sperm randomly selected and evaluated irrespective of their morphological normality.

Statistical Analysis

Data were analyzed using built-in functions within the Statistical Package for Social Science (SPSS UK Ltd., Chertsey, Surrey, UK). Summary statistics are presented as mean \pm SD. The analysis of variance (ANOVA) was used to compare mean sperm DNA damage for different treatments. Simultaneous multiple pairwise comparisons of different treatments and control were performed with the Tukey test. Spearman's rank correlation was used to provide a test of independence between sperm DNA damage and sperm morphological fea-

tures. All hypothesis testing was two-tailed, and $P < .05$ was considered statistically significant.

RESULTS

Sperm DNA Damage after Incubation

The mean (\pm SD) of sperm DNA damage for treatment 1, treatment 2, and controls at the end of each incubation period is given in Table 1. The incidence of DNA damage at different incubation periods for the two treatments and controls is shown in Figure 1. When the three aliquots for treatment 1 (1.5, 3, and 24 hours) were compared, evidence of a significant difference in the mean sperm DNA damage was found (ANOVA variance ratio between treatments [F] = 35.6; $P = .0003$). Simultaneous pairwise comparison of the three aliquots revealed that the mean sperm DNA damage after 24 hours of incubation was significantly higher compared with the mean after incubation at 1.5 and 3 hours. However, the mean sperm DNA damage after 1.5 and 3 hours was comparable. Similar results were demonstrated when the three aliquots of treatment 2 ($F = 16.7$; $P < .0001$) and the three control aliquots were compared ($F = 6.6$; $P = .007$).

When the three aliquots incubated for 11.5 hours (two treatments and control) and were compared, evidence of a significant difference in the mean sperm DNA damage was found ($F = 21.2$; $P < .0001$). Simultaneous pairwise comparison of the three aliquots revealed significantly higher mean sperm DNA damage with treatment 2 (PF from endometriosis patients) compared with treatment 1 (PF from healthy women) and controls. On the other hand, there was no evidence of a statistical difference in mean sperm DNA damage when treatment 1 and control aliquots were compared. Similar findings were demonstrated when treatments and controls were compared after incubation for 3 hours ($F = 13.5$; $P = .0003$) and 24 hours ($F = 17.8$; $P < .0001$). Similarly, simultaneous pairwise comparison of the three aliquots revealed significantly higher mean sperm DNA damage

TABLE 1

Sperm DNA damage (mean \pm SD) after incubation with PF from healthy women undergoing tubal ligation (treatment 1), PF from patients with endometriosis (treatment 2), and HTF only (controls) for 1.5, 3, and 24 hours.

Parameter	Percentage sperm with DNA damage at different incubation periods (mean \pm SD)			P^b
	1.5 hours	3 hours	24 hours	
Control	8 \pm 4.7	10.3 \pm 6.5	18 \pm 13	.0074
Treatment 1	8 \pm 3.9	10.5 \pm 4.3	19 \pm 6	.0003
Treatment 2	18 \pm 8	20.5 \pm 10	36 \pm 17	.0004
P^a	< .0001	= .0003	< .0001	

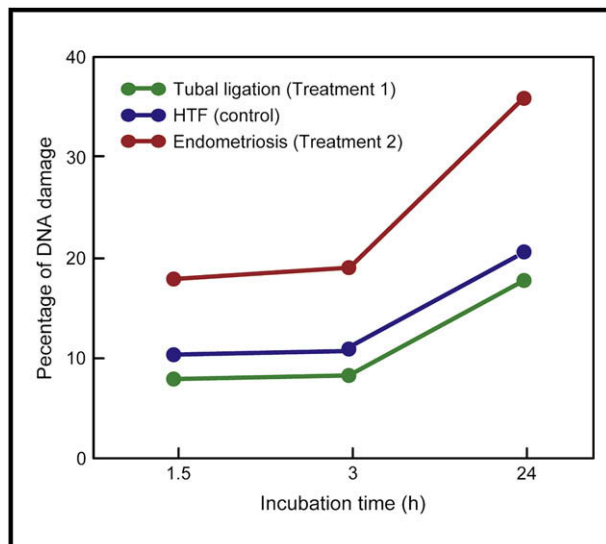
^a $P < .05$ was considered significant for sperm DNA damage in different groups at each incubation period using ANOVA.

^b $P < .05$ was considered significant for sperm DNA damage for each group at different incubation times using ANOVA.

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FIGURE 1

Sperm DNA damage (mean \pm SD) after incubation human tubal fluid (HTF) only (control), peritoneal fluid (PF) from healthy women undergoing tubal ligation (Treatment 1), and PF from patients with endometriosis (Treatment 2) for 1.5, 3 and 24 h.



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with treatment 2 compared with treatment 1 and with controls. On the other hand, there was no evidence of a statistical difference in mean sperm DNA damage when treatment 1 and control aliquots were compared.

Sperm Morphology

Normal sperm morphology and sperm DNA damage The mean percentage normal morphology as assessed applying the strict criteria was $13\% \pm 6\%$. There was no evidence of interdependence between the percentage normal morphology and sperm DNA damage as assessed with the TUNEL assay at any incubation period from either treatments or control.

SDI scores and sperm DNA damage The mean SDI score was 1.74 ± 0.19 . There was evidence of significant positive interdependence between the SDI scores in ejaculated semen and sperm DNA damage in all aliquots incubated for 1.5 and 24 hours (Table 2). Significant positive interdependence between the SDI scores in ejaculated semen and sperm DNA damage after 3 hours of incubation was observed only in samples incubated with PF from patients with endometriosis.

DISCUSSION

To the best of our knowledge, this is the first study that compares the impact of PF from healthy women and from women with endometriosis on sperm DNA and its relationship to the SDI. Our study demonstrated that under standardized in vitro incubation conditions, progressive sperm DNA fragmenta-

tion occurs over time irrespective of the incubating media used (HTF, PF from healthy women, or PF from patients with endometriosis). However, samples incubated with PF from patients with endometriosis showed evidence of significantly higher sperm DNA damage compared with samples incubated with PF from healthy women and controls at the three incubation periods studied. The study demonstrated significant positive interdependence between the SDI scores and the extent of sperm DNA damage both in controls and in samples incubated in PF. Percentage normal sperm morphology as assessed applying the strict criteria did not correlate with sperm DNA damage at any stage of our study.

After semen is deposited in the vagina, only a few thousand sperm reach the fallopian tubes, where they are maintained in a fertile state, for hours and possibly days, by interacting with endosalpingeal epithelium. As the time of ovulation approaches, sperm become capacitated and hyperactivated, which enables them to proceed toward the tubal ampulla (24). At this site sperm may be exposed to PF coming through the patent fallopian tubes. Our study has demonstrated that incubating sperm with PF from patients with endometriosis for periods as short as 1.5 hours was associated with significantly higher sperm DNA damage compared with the level of damage that occurred when sperm were incubated with PF of healthy women and controls. Based on this, an assumption could be made that sperm DNA damage is induced relatively quickly in endometriosis cases and in an exaggerated manner. Throughout the study's incubation period, the proportion of sperm with DNA damage remained significantly higher in sperm exposed to PF from patients with endometriosis compared with controls and with sperm exposed to the PF from healthy women.

Increased generation of ROS by activated macrophages has been reported in the PF of patients with endometriosis (8, 25). OS due to exogenous excessive free radicals has been shown to have a negative impact on sperm DNA (26), leading to the formation of oxidative products such as 8-oxo-7,8-dihydroxyguanosine (27). This compound causes fragmentation of sperm DNA. OS was shown to induce genomic and mitochondrial DNA damage (28). Spermatozoa have limited antioxidant defenses because their cytoplasm contains low concentrations of scavenging enzymes (29, 30). Moreover, they are very susceptible to OS-induced damage because of the high content of polyunsaturated fatty acids in their membranes (31). Nevertheless, the sperm midpiece contains superoxide dismutase, and glutathione peroxidase and glutamyl transpeptidase both regulate the glutathione content of the oocyte, thus providing protection against OS (11). Peroxidation of DNA can lead to chromatin cross-linking, base changes, and DNA strand breaks.

Spermatozoa do not possess functional repair enzymes and so are unable to repair DNA damage. The biological impact of sperm DNA damage may depend on the combined effects of the level of sperm DNA damage and the competence of the oocyte or early embryo to repair the DNA damage. However, it has been reported that if the damage occurs beyond a certain

TABLE 2

Correlation between the SDI scores and sperm DNA damage after incubation with PF from healthy women undergoing tubal ligation (treatment 1), PF from patients with endometriosis (treatment 2), and HTF only (controls) for 1.5, 3, and 24 hours.

Parameter	Spearman's rank correlation coefficient <i>r</i> (two-tailed <i>P</i>)		
	1.5 hours	3 hours	24 hours
Control	0.72 (<i>P</i> =.02)	0.58 (<i>P</i> =.08)	0.71 (<i>P</i> =.02)
Treatment 1	0.74 (.018)	0.57 (<i>P</i> =.08)	0.73 (<i>P</i> =.02)
Treatment 2	0.87 (<i>P</i> =.002)	0.81 (<i>P</i> =.0068)	0.85 (<i>P</i> =.003)

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level, it results in a low rate of embryonic development and early pregnancy loss due to implantation failure (32). To complicate matters further, strong evidence exists that oocyte quality is altered in endometriosis (33–35), which may affect the oocyte's ability to repair sperm DNA damage. As such, sperm DNA damage induced as a result of contact with PF may be considered one of the underlying mechanisms of endometriosis-related infertility and poor pregnancy outcome. Equally, successful IVF treatment in these cases may be attributable to sparing sperm the exposure to PF.

In a case-control study, increased percentages of B lymphocytes, natural killer cells, and monocyte-macrophages in the PF of patients with endometriosis compared with those with other causes of infertility have been noted (36). TNF- α , a potent cytokine that exerts inflammatory, cytotoxic, angiogenic, and growth modulatory effects on different target cells (26, 37), is mainly produced by neutrophils, activated lymphocytes, natural killer cells, and activated macrophages (38). Its presence within the male and female reproductive tracts suggests its involvement in infertility modulation. In the female it is involved in the pathogenesis of endometriosis and is associated with infertility (39, 40). The toxic effects that TNF- α exerts on the spermatozoa could be mediated by inducing the generation of free radicals and promoting apoptosis (41, 42). Elevated levels of the marker of lipid peroxidation lysophosphatidyl choline, a potent chemotactic factor for monocytes/T lymphocytes, have been reported in the PF of women with endometriosis (43).

The time-dependant increase in DNA fragmentation observed in our study control aliquots is in agreement with a previous report (44). In that study, DNA fragmentation was investigated in spermatozoa that were selected by the swim-up procedure and incubated long term. It was found that in vitro sperm DNA fragmentation occurred after ejaculation under experimental conditions without the involvement of any external factor. There was evidence that endogenously produced ROS were the possible cause of in vitro sperm DNA fragmentation (44).

Another interesting finding in our study was that DNA damage in sperm exposed to PF from healthy women and in controls was comparable. This observation is reassuring

for both practitioners and consumers involved in assisted reproduction technologies. From a different perspective, we observed significant sperm DNA damage after 24 hours of incubation, which may have important clinical implications. Secondary insemination of unfertilized oocytes after 18 hours of incubation is a standard practice in many IVF laboratories. In light of our study results of increased sperm DNA damage with extended incubation, this practice may not be as safe as it is perceived. Similarly, for in vivo fertility, the longer the sperm is present in the fallopian tubes waiting for the egg, the higher the sperm DNA damage.

The importance of linking traditional sperm parameters with these newer aspects of sperm functional integrity arises from the fact that assessing semen for evidence of sperm OS, apoptosis, and DNA damage remains beyond routine clinical work. We reported previously on the relationship between high SDI scores and sperm chromatin damage (18, 45). In this study, we have demonstrated a positive interdependence between SDI score and the liability for DNA damage when incubated with PF as well as under standardized in vitro incubation. This indicates that in the presence of a higher frequency of morphological abnormalities, sperm nuclear chromatin is more susceptible to fragmentation even under the standard incubation conditions observed in assisted reproductive technologies. We have shown previously that this increased susceptibility is in part due to the fact that an increased SDI score is associated with increased proportions of sperm with cytoplasmic residues, which may result in increased ROS production (18, 46). The lack of a relationship between sperm DNA damage and percentage normal morphology is in agreement with the results of a previous report on OS-induced DNA damage in infertile men (18). This lack of relationship lends further support to the suggestion that in the presence of high SDI scores the integrity of morphologically normal sperm observed with light microscopy may be compromised on the subcellular level (18). In this study, we correlated the SDI scores in the unprepared semen to the outcome observed in the motile fraction for two reasons. First, in clinical situations it is the ejaculated semen that is subjected to assessment to evaluate the sample fertility potential. Second, we have demonstrated previously that the SDI score in ejaculated semen is a more powerful predictor of

IVF outcomes compared with the same score assessed in the prepared sperm (16).

In this study, each semen sample was exposed to PF from a different healthy woman and a different patient with endometriosis. We were interested in mapping sperm DNA damage over time in aliquots of the same semen sample when exposed to three incubation media. Thus, this study does not address questions such as the relationship between the clinical severities of endometriosis and the extent of induced sperm DNA damage, nor does it explore the link between the duration of endometriosis-related infertility and sperm DNA damage. We are currently undertaking work addressing these questions.

In summary, our study demonstrated that under standardized in vitro incubation conditions there is progressive sperm DNA fragmentation over time irrespective of the incubating media used (HTF, PF from healthy women, or PF from endometriosis patients). However, there was evidence of significantly higher sperm DNA damage in samples incubated with PF from patients with endometriosis compared with samples incubated with PF from healthy women and controls at the three incubation periods studied. The study demonstrated the significant positive interdependence between the SDI scores and the extent of sperm DNA damage. Percentage normal morphology as assessed applying the strict criteria did not correlate with sperm DNA damage. We are proposing that endometriosis-induced sperm DNA damage may be one of the underlying causes of reduced natural fertility in these cases.

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