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Evaluating γH2AX in spermatozoa from male infertility patients

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Objective: To investigate whether γH2AX levels were different in the spermatozoa of healthy men compared with infertility patients, and to assess the possible correlations between γH2AX and conventional semen parameters and double-stranded breaks (DSBs) identified with the use of comet assay.
Design: Prospective study.
Setting: Clinical laboratory.
Patient(s): Semen from 100 male infertile patients and 100 healthy sperm donors.
Intervention(s): Human sperm samples were analyzed in terms of World Health Organization parameters. The γH2AX levels were detected by means of flow cytometry. DSBs of sperm were detected by means of comet assay. Morphology slides were made and the sperm morphology assessed according to strict criteria.
Main Outcome Measure(s): Conventional semen analyses, γH2AX levels in sperm, DNA DSBs in sperm, and correlations among γH2AX, conventional semen analyses, and DSBs.
Result(s): Concentration, viability, motility, and normal sperm morphology were significantly lower in male infertility patients compared with healthy men. Also, γH2AX levels and the number of DSBs were significantly higher in the sperm of infertile subjects compared with healthy men. γH2AX levels correlated negatively with conventional semen parameters and positively with DSBs. A threshold γH2AX level of 18.55% was identified as a cutoff value to discriminate infertile subjects from fertile control subjects with a specificity of 86.0% and a sensitivity of 83.0%. The positive and negative predictive values of the 18.55% γH2AX threshold were high: 87.7% and 85.5%, respectively.
Conclusion(s): γH2AX levels were higher in the sperm of male infertility patients than in healthy men. γH2AX levels in sperm, as evaluated with the use of flow cytometry, might be a useful biomarker for evaluating DSBs in human spermatozoa. (Fertil Steril® 2015;■: ■–■. ©2015 by American Society for Reproductive Medicine.)
Key Words: Male infertility, H2AX phosphorylation, DNA double-stranded breaks

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Sperm quality is one of the factors that determines the success rate of fertilization. Currently, sperm quality is evaluated by means of conventional semen analysis, which includes assessing the sperm concentration, motility, and percentage of morphologically normal forms. Although conventional semen analysis provides a considerable amount of information, such as the correlation between normal sperm morphology and fertilization potential (1), its limitations for predicting the ability to achieve pregnancy are also well acknowledged (2), and the application of additional more reliable technologies is still needed.

Over the past two decades, sperm DNA damage has been a useful indicator for evaluating the quality of sperm. Several studies have demonstrated that infertile men had higher levels of damaged spermatozoa DNA than healthy men, even when sperm morphology was normal (3). In addition, the integrity of DNA in sperm is essential for the accurate transmission of genetic information to offspring, which is one of the crucial factors that affects reproductive outcome (4–10). As a matter of "best practice," the DNA quality of male gametes should be tested before the sperm are used clinically. Although the American Society for Reproductive Medicine does not recommend sperm

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DNA damage evaluation as a diagnostic tool for the management of male infertility, a growing number of studies report that sperm DNA damage is thought to impair fertilization and disrupt embryo development, which result in miscarriage and even birth defects in the offspring (11–15). In addition, the high level of sperm DNA damage is thought to influence the rate of IVF treatment (13, 16, 17).

Currently, there are several ways to assay sperm DNA damage: sperm chromatin structure assays (SCSAs), single-cell gel electrophoresis (comet) assays, and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assays. However, all of these methods have their limitations. And the use of these methods to explore DNA damage and evaluate male-factor infertility and their application toward understanding the relationship between DNA damage and adverse pregnancy outcomes have not yet reached a consensus (18–20). Several studies have demonstrated an inability to achieve IVF/ICSI and natural conception when there is 27%–30% sperm DNA damage. However, other studies have reported opposing conclusions (5, 21). The reasons for these differences are not clear; however, Perreault et al. (22) thought that it is difficult to use a single method to evaluate sperm DNA damage comprehensively and predict the clinical treatment outcome of IVF/ICSI. Therefore, exploring new DNA damage markers for the clinical detection of sperm DNA damage and predicting male infertility is still an important research topic.

H2AX is a member of the H2A histone family. The phosphorylation of Ser-139 of the histone variant H2AX, forming γH2AX, is an early cellular response to the induction of DNA double-strand breaks (DSBs) (23). In somatic cells, γH2AX is a key step in DNA damage repair (24). When cells undergo ionizing radiation (IR), H2AX phosphorylation occurs shortly after the induction of DSBs (25), and each DSB corresponds to one IR-induced γH2AX focus (24). Therefore, recent studies have used γH2AX to evaluate the activity of chemotherapy drugs that kill cancer cells and the sensitivity of tumor cells to radiation therapy (26, 27). Besides functioning as a biomarker for DSBs, γH2AX also plays a role in signaling and initiating the repair of DSBs (24). When γH2AX focus occurs shortly after DSB induction, it creates an epigenetic signal that can help recruit DNA damage response proteins involved in DNA damage sensing and repair, such as the Mre11/Rad50/Nbs1 (MRN) complex, BRCA1, 53BP1, etc., to form multiple protein complexes (termed foci) at the damaged site (28–32). Then, γH2AX facilitates DSB rejoining by anchoring broken ends together through nucleosome repositioning at damaged sites and a reduction in chromatin density (33–35). Moreover, cohesins, which help maintain chromatid cohesion, are recruited by γH2AX to keep ends in close proximity during repair, preventing the loss of large chromosomal regions (36, 37).

Regarding male germ cells, several studies have demonstrated that germ cells, similarly to somatic cells, could also induce γH2AX when injured by genotoxic stresses that cause DSBs, such as ionizing radiation and chemotherapy agents (38–40). Mature spermatozoa lose their effective DNA repair capacity, although γH2AX exists in mature spermatozoa (40). However, there is a lack of information regarding the clinical value of the γH2AX assay for predicting outcome regarding pregnancy. In addition, it remains unknown whether the presence of γH2AX can be detected in the sperm of healthy men and male infertility patients. A definite answer as to whether sperm γH2AX levels are higher (as expected), lower, or the same in male infertility patients compared with healthy men could have useful implications for the pathogenesis of the infertility and, potentially, its prognosis.

The present study was conducted in an effort to determine whether γH2AX could be detected in healthy men and male infertility patients, and to assess whether sperm γH2AX levels differ between these populations. We also investigated the possible correlations between γH2AX and conventional semen parameters and DSBs identified with the use of comet assays, as well as the area under the receiver operating characteristic (ROC) curve (AUC) of γH2AX.

MATERIALS AND METHODS

Experimental Design

The study was performed after approval by the Institutional Review Board at the First Affiliated Hospital of Guangxi Medical University. A total of 200 semen samples were obtained from 100 male infertility patients (aged 25–40 years) and 100 healthy donors (aged 24–39 years). The diagnosis of infertility was assessed clinically by andrologists at the First Affiliated Hospital of Guangxi Medical University. All of the male infertility patients did not have a successful pregnancy after unprotected intercourse with their healthy female partner for $\geq$12 months. All of the healthy donors got children by natural conception with their female partners and had documented paternity. The criteria for selecting healthy fertile individuals included no serious chronic or contagious diseases and normal genital examinations. The inclusion criteria for the male infertility patients included normal sex hormone levels, no medications or surgery within the preceding 3 months, semen volume $>$2 mL, sperm concentration $>15 \times 10^6$/mL, sperm motility rate $>$20%, and negative results for antisperm antibodies. The patients provided semen by masturbating into sterile cups after 3–5 days of sexual abstinence. All semen samples were allowed to liquefy for 1 hour at room temperature and then analyzed according to the World Health Organization (WHO) criteria to detect sperm concentration and motility with the use of computer-aided sperm analysis. In addition, sperm morphology was evaluated manually.

Flow Cytometry to Detect γH2AX

Sperm cell concentrations were adjusted to $\sim 1 \times 10^7$/mL, and samples were then fixed in 4% paraformaldehyde for 15 minutes at room temperature. The samples were then mixed with phosphate-buffered saline solution (PBS), and permeabilized with the use of 0.5% Triton X-100 for 15 minutes. Subsequently the samples were centrifuged and resuspended in 20 μL fluorescein isothiocyanate (FITC)–conjugated