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Oxidative stress induces H2AX phosphorylation in human spermatozoa

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Abstract **H2AX phosphorylation occurs following the induction of DNA double strand breaks (DSBs), thus collaborating with many other proteins to mediate important biological functions in somatic cells. In human spermatozoa, the present study showed that H_2O_2 induced H2AX phosphorylation in a time- and dose-dependent manner. Moreover, such effect could be abolished by the phosphatidylinositol 3-kinase inhibitor wortmannin. Meanwhile, the neutral comet assay also revealed DSBs production in correlation with H2AX phosphorylation assessed by flow cytometry. Besides H2AX phosphorylation, two other collaborating proteins, Rad50 and 53BP1, were also generated in spermatozoa after H_2O_2 exposure. However, unlike in somatic FL cells, there were no distinctive focuses, but rather a whole nuclei staining pattern of these three proteins in spermatozoa. Additionally, γH2AX (the phosphorylated form of H2AX) staining in spermatozoa persisted despite the fact of a decrease in the number of γH2AX foci in FL cells after H_2O_2 removal. Collectively, these results demonstrate that oxidative stress can induce H2AX phosphorylation in human spermatozoa through DSB induction, and that γH2AX may be used as a sensitive, novel marker for such DSBs. Moreover, the surveillance system involving γH2AX, Rad50, and 53BP1 in human spermatozoa cannot function effectively in DNA repair, but this system may possess other biological functions in response to DSBs.**

Keywords: Oxidative stress; Human spermatozoa; DNA double strand-breaks; γH2AX

1. Introduction

Reactive oxygen species (ROS) have been widely reported as having physiological and pathological significance in male germ cells. ROS play a vital role in regulating the normal functions of mature spermatozoa when present at physiological levels [1]. However, oxidative stress occurs if excessive ROS exposure overwhelms spermatozoa's antioxidant defenses. Under this abnormal condition, oxidative stress leads to a wide range of pathological changes that could impair the genomic integrity and fertilizing ability of spermatozoa [2].

Oxidative stress has been recognized as one of the main causes of male gamete DNA damage (for detailed reviews about this topic, please see Refs. [3–5]). Experimental studies revealed that in vitro ROS exposure results in high frequencies of DNA single-strand breaks (SSBs) and double-strand breaks (DSBs) [6–9]. Clinical evidence also suggests that high levels of ROS produce DNA fragmentation, a condition that is commonly observed in the spermatozoa of infertile men [10–12]. Moreover, sperm DNA damage also occurs frequently under other medical conditions that can stimulate free radical generation, such as chemotherapy and ionizing radiation treatments [13,14]. Since oxidative DNA damage in human spermatozoa has been proven to be associated with poor semen parameters, decreased fertilizing capacity, and even adverse pregnancy outcomes, it is critical in an attempt to understand how spermatozoa respond to and identify such threats for clinical purposes [5,15,16].

In somatic cells, H2AX phosphorylation occurs shortly after DSB induction, thus γH2AX (denoted the phosphorylated form of histone H2AX) has been identified as an early sensitive indicator of DSBs induced by ionizing radiation and chemical agents [17,18]. Our recent study also indicated that γH2AX formation can be used for evaluating DNA damage produced by a variety of chemicals/stress factors [19]. Besides functioning as a biomarker for DSBs, γH2AX also helps recruit many other 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 [20–24]. Thus, γH2AX and its cofactors are regarded as specific mediators of cellular response to DSBs in somatic cells. In addition, the kinases responsible for the phosphorylation of H2AX have also been investigated, and it is now clear that H2AX phosphorylation depends on the activation of members of the phosphatidylinositol 3-kinase family (PI-3K), including ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), and DNA-PK (DNA-dependent protein kinase) [25,26].

As for male germ cells, similar to somatic cells, there is also evidence suggesting the vital roles of H2AX phosphorylation during spermatogenesis. γH2AX staining has been observed in the nuclei of all intermediate and B spermatogonia, along with spermatocytes at all stages of meiotic prophase and round spermatids, but this staining is absent from Sertoli cells, Type A spermatogonia and elongated spermatids [27–29]. In response to genotoxic stress such as ionizing radiation and chemotherapy agents that cause DSBs, γH2AX foci were

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Abbreviations: ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; DSBs, double strand breaks; γH2AX, the phosphorylated form of H2AX

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induced in spermatogonia, spermatocytes, and round spermatids [29,30]. Therefore, it is generally agreed that γ H2AX plays an important role in sex chromosome condensation, synapsis and transcriptional inactivation, as well as in DSB sensing and repair during spermatogenesis [29–32]. However, as for the ejected spermatozoa, which are mature terminal cells, transcriptionally inactive and generally considered having lost their DNA repair ability, the response to such DSBs in terms of the H2AX phosphorylation and foci formation is still unknown.

In an effort to determine if H2AX phosporylation could occur in response to oxidative stress-induced DSBs in human spermatozoa, and γH2AX could be used as a biomarker for such DSBs, H2AX phosphorylation and DSB formation were examined in parallel after H_2O_2 exposure. Moreover, the response of other two cofactors 53BP1 and Rad50 were also evaluated after DSB induction in human spermatozoa in order to explore the possible biological functions of the γH2AX-involved surveillance system. In parallel with human spermatozoa, somatic FL (Human amnion cell line) cells were also employed as the comparison group to our experimental model.

2. Materials and methods

2.1. Materials

Hydrogen peroxide (H_2O_2, 30% w/w), 4′,6-diamidino-2-phenylindole (DAPI), and wortmannin were purchased from Sigma (St. Louis, MO). Rabbit antibody against 53BP1 and Rad50 were purchased from Gibco (Carlsbad, CA); mouse monoclonal antibody against γH2AX was purchased from Upstate Technology (Lake Placid, NY); FITC-conjugated goat anti-mouse IgG, TRIC-conjugated goat anti-rabbit IgG, and goat blocking serum were obtained from Beijing Zhongshan Biotechnology Co., China. Stock solutions of DAPI and wortmannin were prepared in dimethyl sulfoxide (DMSO), protected from light, and stored at 4 °C.

2.2. Sperm sample preparation

Semen samples were obtained from male donors attending the fertility clinic (Center for Reproductive Medicine, Woman's Hospital, Zhejiang University School of Medicine) to receive Assisted Reproduction Technology (ART) treatment for female factors. Informed consent was obtained from all the donors. Ethical permission to use semen samples in this study was also obtained from the Institutional Review Board (Zhejiang University School of Medicine). To exclude sperm abnormalities, a physical examination together with ultrasonography of scrotal content, basic semen analysis, and male endocrine test, was performed in all donors before enrollment.

Semen samples were obtained by masturbation after 3–5 days of abstinence. After liquefaction for 30 min at 37 °C, the sperm fraction with high quality was isolated by discontinuous Percoll gradient separation (95% and 50% layers). Briefly, two milliliters of semen was carefully placed on the Percoll layers and centrifuged at $500 \times g$ for 20 min. Following centrifugation, spermatozoa at the base of the high density Percoll fraction were collected (designated 95% Percoll fraction). The pellet of the 95% layer was washed with Biggers Whitten and Whittingham buffer (BWW) containing 0.3% human serum albumin (HSA), and centrifuged at $500 \times g$ for 10 min. The supernatant was discarded, and the pellet was resuspended in human tubal fluid (HTF) medium supplemented with 0.5% HSA.

The presence of white blood cells (WBCs) in all specimens was assessed using myeloperoxidase staining. Specimens with WBCs greater than 1×10^6/ml were excluded from the study, as leukocytospermia may lead to abnormal ROS production.

2.3. Cells culture and treatment

The sperm pellets were finally resuspended in HTF medium at a concentration of 5×10^6/ml, and aliquots of sperm suspension were incubated at 37 °C with 5% CO_2 with different concentrations of H_2O_2 (0.1, 0.5, and 1.0 mM, respectively) for 8 h, or with 0.5 mM H_2O_2 for different times (0.5, 2, 8, and 24 h, respectively). In order to examine whether DNA repair occurs in human sperm after H_2O_2 treatment, sperm samples were first treated with 1 mM H_2O_2 for 2 h. After H_2O_2 removal, sperm samples were further incubated for another 2 h or 4 h. Aliquots from the same donor without H_2O_2 treatment served as controls. Incubation was stopped by washing the samples with PBS immediately at the end time-point.

Human amnion cells (FL) were routinely subcultured in Eagle's minimum essential medium (EMEM) (Invitrogen, Carlsbad, CA), containing 10% newborn calf serum, 100 U/ml penicillin, 125 μg/ml streptomycin, and 0.03% glutamine. About 1×10^5 cells were seeded into 6-well culture plate containing a glass cover slip in each well.

2.4. Immunofluorescence staining

Human spermatozoa were harvested after various treatments and washed three times in PBS for the evaluation of H2AX phosphorylation. About 5×10^4 sperm cells in a 50 μl volume were dropped onto polylysine-coated slides and fixed in 4% (w/v) paraformaldehyde for 15 min at 4 °C, followed by permeabilization in 0.2% (v/v) Triton X-100 for 15 min. Afterwards, slides were blocked with goat blocking serum for 60 min prior to incubation with mouse monoclonal anti-γ H2AX antibody (1:1000) overnight at 4 °C, and then washed in PBS. The slides were further incubated with FITC-conjugated goat-anti-mouse secondary antibody (1:500) for 60 min at 37 °C, followed by PBS washes. Nuclei were counterstained with DAPI (1 μg/ml in PBS) for 15 min. After another wash in PBS, slides were mounted with coverslips and viewed using an Olympus AX70 fluorescent microscope (Olympus, Tokyo, Japan).

For human FL cells, the immunofluorescent staining procedure and counting of the number of foci was the same as described before [19,33].

To further explore the cellular response to DSBs, double immunofluorescence staining of γH2AX and Rad50 or 53BP1 was conducted. The procedure is the same as described above except that both anti-γH2AX and anti-Rad50(or anti-53BP1) antibodies were used together as first antibodies, followed by FITC-conjugated goat-anti-mouse and TRIC-conjugated goat-anti-rabbit antibody (1:500) as secondary antibodies.

2.5. Flow cytometry analysis for γH2AX

Human sperm cells were first fixed in 2% paraformaldehyde for 15 min and then kept in 70% ethanol at −20 °C for up to 2 weeks before analysis. All samples were analyzed on the same day. Staining for γH2AX was conducted as described above. Briefly, fixed cells were rehydrated for 10 min, then centrifuged and resuspended in 200 μl of monoclonal mouse anti-γH2AX antibody (1:1000 dilution) for 2 h at 37 °C. Samples were then rinsed and resuspended in 200 μl of secondary antibody (1:500 dilutions) for 1 h at 37 °C. Finally, the sperm cells were rinsed and resuspended in PBS before analysis with a Flow Cytometer (Coulter, USA). The average γH2AX antibody staining relative to the untreated control was calculated based on mean fluorescence, and the population of γH2AX-labeled cells were gated according to control histograms to determine the percentage of cells with γH2AX antibody labeling.

2.6. Comet assay

To detect DSBs in sperm, the neutral Comet assay was performed as previously described with slight modifications [34,35]. The fully frosted microscope slides were covered with 100 μl of 0.7% normal melting-point agarose in PBS and then dried at room temperature. Approximately 10^5 sperm cells were mixed with 0.7% low melting point agarose (80 μl) to form a cell suspension. This suspension was pipetted onto the first agarose layer, spread, and solidified on ice. After removal of the cover slip, a third layer of 0.65% low melting-point agarose was added, spread and again allowed to solidify on ice for 5 min. The slides were then immersed in the neutral lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH adjusted to 10 before adding 1% Triton X-100 and 10 mM DDT) at 4 °C for 1 h, then incubated at 37 °C in 10 μg/ml of proteinase K in lysis buffer for 2 h. After 20 min of incubation to unwind the DNA, electrophoresis was conducted at 25 V, 100 mA for 15 min in electrophoresis buffer (TBE neutral buffer containing 10 mM Tris, 80 mM Boric acid, 0.5 M EDTA, pH 8.2).

Following electrophoresis, slides were immersed in Tris (pH 7.5, 0.4 M) for 15 min of neutralization and then in DAPI solution (1 μg/ml) for 15 min, rinsed in PBS, and coverslips were placed on the gels.

The slides were then examined using an Olympus AX70 fluorescent microscope (Olympus, Tokyo, Japan). Images were saved as BTM files and tail moments were determined using CometScore™ Freeware from TriTek Corp. (Sumerduck, VA). The tail moment is the integrated value of density multiplied by migration distance and proportional to the levels of damaged DNA; moreover it is considered the most sensitive parameter of comet assay for DNA damage. Twenty-five cells were scored from each replicate slide (50 cells total) and subsequently pooled.

2.7. Statistical analysis

Statistical analysis was carried out with the SPSS version11.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using ANOVA and presented as means ± S.D. A *P*-value smaller than 0.05 was deemed statistically significant. Each experiment performed in duplicate was repeated at triplicates.

3. Results

3.1. H_2O_2 induces DSBs in human spermatozoa

The comet assay, which is widely used for assessing DNA damage in somatic cells, has been adapted for measuring DNA strand breaks in human sperm [34,35]. In contrast to alkaline comet assay, neutral comet assay is specific for detecting DSBs. Therefore, the neutral comet assay was conducted to detect DSBs in human sperm cells in response to H_2O_2 exposure. As shown in Fig. 1, it was found that H_2O_2 had a clear time- and dose-dependent effect on DNA fragmentation as indicated by the tail moment of neutral comet assay. Although incubation with 0.5 mM H_2O_2 for 30 min did not induce significant DSBs, DNA fragmentation became evident at 2 h, and lasted to 24 h (Fig. 1A). Similarly, while the effect on DNA fragmentation was not obvious after exposure to 0.1 mM H_2O_2 for 8 h, exposure to 0.5 or 1.0 mM H_2O_2 for 8 h induced significant DNA fragmentation ($P < 0.05$, Fig. 1B).

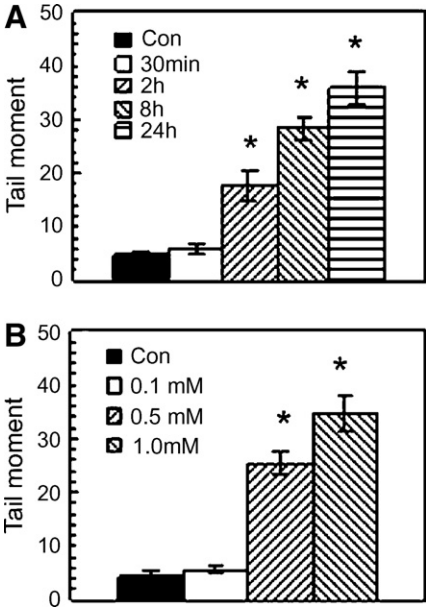

Fig. 1. H_2O_2 induces DSBs in human spermatozoa. After exposure to various concentrations of H_2O_2 (0.1, 0.5, 1.0 mM) for 8 h, or to 0.5 mM H_2O_2 for different times (30 min, 2, 8, and 24 h), human sperm cells were subjected to the neutral comet analysis to evaluate the formation of DSBs. (A) Time response of DSB formation as indicated by tail moment. (B) Dose response of DSB formation as indicated by tail moment. $^*P < 0.05$.

3.2. H_2O_2 exposure induces H2AX phosphorylation in human spermatozoa

Since H_2O_2 exposure induced DSBs, we further tested if H_2O_2 can induce H2AX phosphorylation in human spermatozoa using immunofluorescent microscopy. It was found that H_2O_2 exposure induced H2AX phosphorylation also in a time- and dose-dependent manner. As shown in Fig. 2A, γH2AX staining became visible as early as 30 min after exposure to 0.5 mM H_2O_2, and the fluorescent intensity became stronger as exposure time lengthened; Exposure to 0.1, 0.5, and 1.0 mM H_2O_2 for 8 h also induced the formation of γH2AX, with higher concentrations inducing stronger fluorescent intensity. This observation was further confirmed by quantitative analyses of flow cytometry data (Fig. 2C and D). However, unlike in somatic FL cells (Fig. 2B), H_2O_2 exposure did not induce the formation of distinct γH2AX foci but rather a whole nucleus staining pattern in human spermatozoa.

3.3. Wortmannin inhibits γH2AX formation induced by H_2O_2

Wortmannin is an inhibitor of PI3K family members, which has been shown to inhibit the phosphorylation of H2AX induced by IR or other agents that cause DSBs [25,26]. Therefore, the effect of wortmannin on H_2O_2-induced γH2AX formation was also examined. In order to prevent the early decomposition of wortmannin (19,33), sperm cells were first pre-incubated with wortmannin (200 μM) for 30 min before H_2O_2 treatment, and then with a second dose added 2 h after H_2O_2 (0.5 mM) treatment. Eight hours later, the phosphorylation of H2AX was determined by both immunofluorescent microscopy and flow cytometry. Microscopy observation revealed that wortmannin addition could lead to a nearly complete abrogation of the H_2O_2-induced phosphorylation (data not shown). Such effect was further confirmed by flow cytometry analysis, which showed that wortmannin reduced the H_2O_2-induced phosphorylation to the basic level in terms of both the mean intensity of γH2AX staining and the percentage of γH2AX-positive sperms (wortmannin and H_2O_2 combination treatment compared with mock control: mean intensity 0.267 ± 0.038 vs 0.235 ± 0.031 and positive percentage 5.13 ± 0.57% vs 4.62 ± 0.73% respectively, $P > 0.05$). Therefore, the phosphorylation of H2AX in response to H_2O_2-induced DSBs in human spermatozoa is also dependent on PI3K family kinases.

3.4. H_2O_2 exposure induces the co-localization of γH2AX with 53BP1 and Rad50 in human spermatozoa

It has been reported that γH2AX is responsible for the recruitment of many DNA maintenance and repair proteins, including 53BP1 and MRN complex, to the damaged sites and it co-localizes with these proteins in somatic cells [20–24]. Thus, the co-localization of γH2AX with 53BP1 and Rad50 was also evaluated in human sperm after H_2O_2 exposure. Shown in Fig. 3B are representative images for the co-appearance of γH2AX with Rad50 and 53BP1 in FL cells after 0.5 mM H_2O_2 exposure for 8 h. Similarly, H_2O_2 also induced the co-appearance of γH2AX with Rad50 and 53BP1 in human

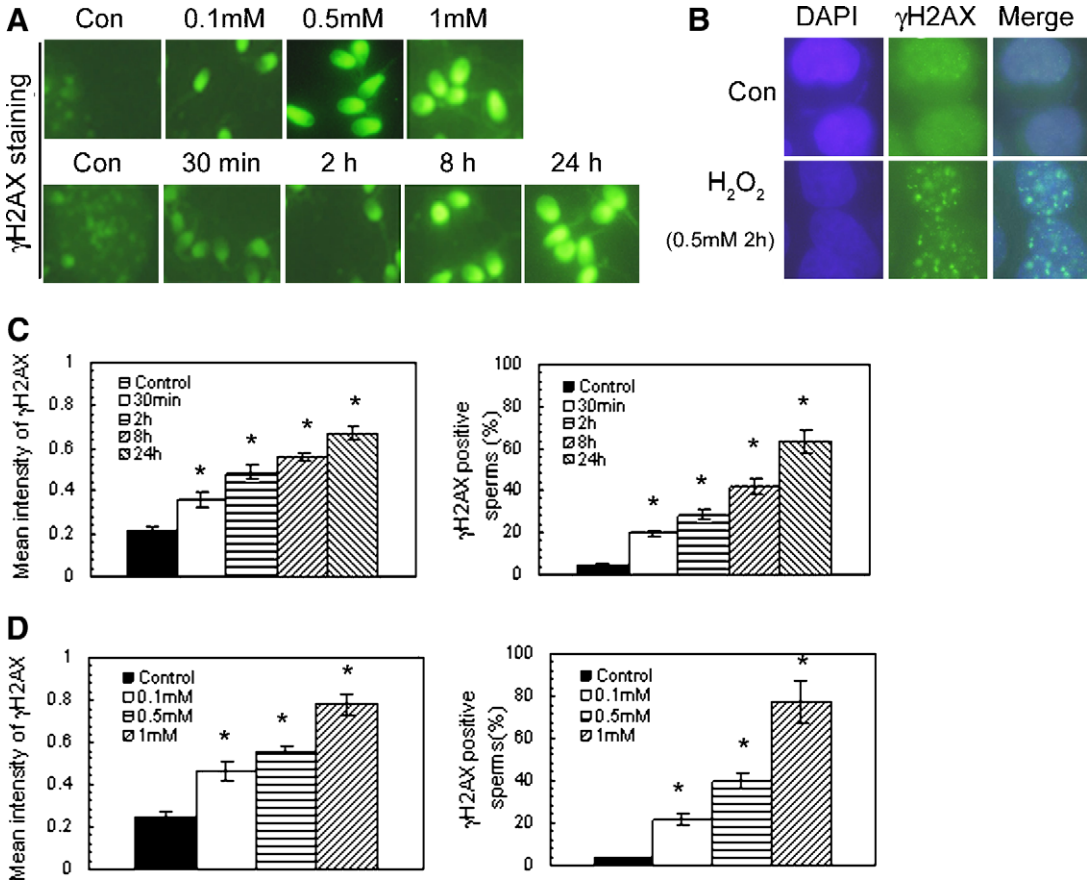

Fig. 2. H_2O_2 induces H2AX phosphorylation in human spermatozoa. Human spermatozoa were exposed to various concentrations of H_2O_2 (0.1, 0.5, and 1 mM) for 8 h, or to 0.5 mM H_2O_2 for different times (30 min, 2, 8, and 24 h). Cells were then fixed and stained with anti-γH2AX antibody, and subjected to immunofluorescent microscopy. Meanwhile, flow cytometry was also performed to determine the mean intensity of γH2AX immunofluorescence staining and the percentage of γH2AX-positive sperms. Human amnion FL cells were similarly treated and used as positive controls. (A) Shown are representative images for human spermatozoa, and (B) representative images for FL cells. (C) The time response of H2AX phosphorylation in sperm cells as indicated by the mean intensity of γH2AX immunofluorescence staining (left) or by the percentage of γH2AX-positive sperms (right). (D) The dose-response of H2AX phosphorylation in sperm cells as indicated by the mean intensity of γH2AX immunofluorescence staining (left) or by the percentage of γH2AX-positive sperms (right). $^*P < 0.05$.

spermatozoa (Fig. 3A). However, unlike in somatic FL cells, no distinct "foci" could be observed in spermatozoa.

3.5. Removal of H_2O_2 does not lead to the disappearance of γH2AX in human spermatozoa

In somatic cells, the γH2AX-involved repair system plays an important role in DNA repair. The disappearance of γH2AX foci is associated with the complication of DSB repair [17,24]. Because it has been widely accepted that human spermatozoa lose their ability of DNA repair, γH2AX would persist even after H_2O_2 removal. To demonstrate this assumption, we observed γH2AX variance in human spermatozoa, along with FL cells as a comparison. After 1 mM H_2O_2 incubation for 2 h, H_2O_2 was removed by changing to fresh media, and both types of cells were further incubated for 2 h and 4 h. The presence of γH2AX was evaluated by immunofluorescent microscopy and/or flow cytometry. As shown in Fig. 4A, after H_2O_2 removal, the number of γH2AX foci in FL cells gradually decreased with time. However, in human spermatozoa, there were no significant changes in γH2AX fluorescent intensity from both image analysis (data not shown) and flow cytometry data (Fig. 4B) at 2 h and 4 h after H_2O_2 removal. In parallel with γH2AX evaluation, the comet assay further confirmed that DNA repair functioned efficiently in FL cells, but did not in human spermatozoa (data not shown). These results show that γH2AX persistence in human spermatozoa indicated DNA repair defect, and that the γH2AX-involved repair system in human spermatozoa was not functional or might not function as efficiently as in somatic cells.

4. Discussion

Oxidative stress results in DSBs in human spermatozoa. In somatic cells, it has been confirmed that H2AX phosphorylation and complex formation with other proteins occur shortly after DSB induction, thereby mediating a series of cellular responses such as damage sensing and DNA repair, cell cycle arrest, and apoptosis. However, in human spermatozoa, little is known about how these proteins respond to the oxidative stress-induced DSBs. Since H_2O_2 has been generally used as an effective activator of oxidative stress in human spermatozoa, we therefore employed this agent to produce oxidative DNA damage in the present study.

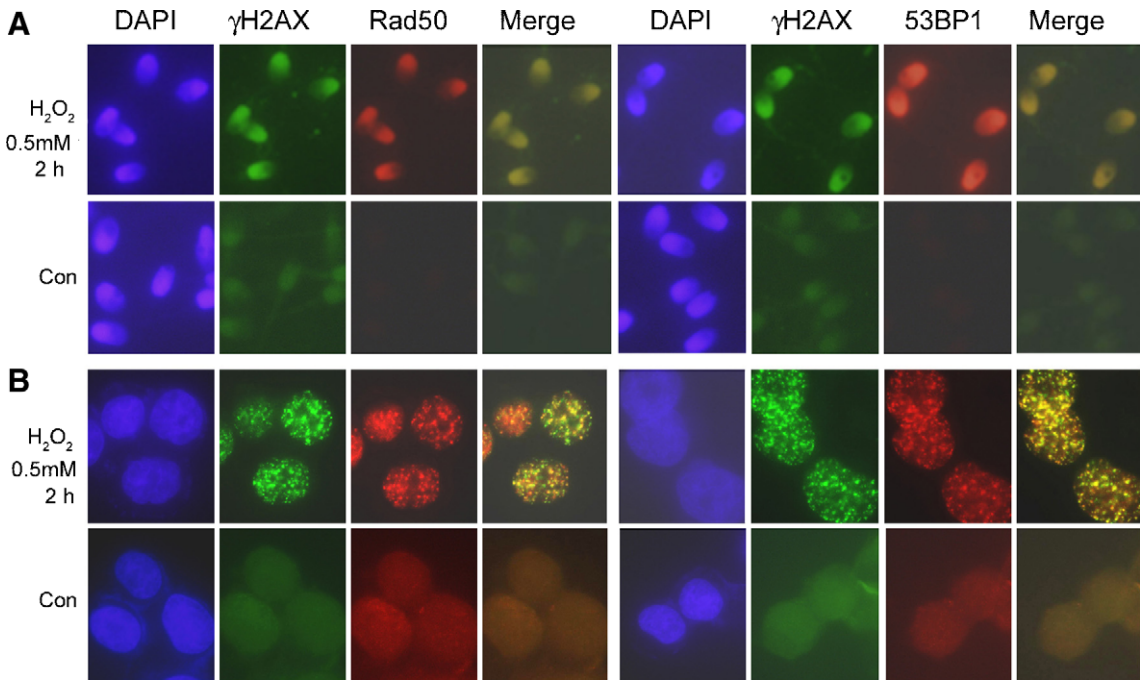

Fig. 3. γH2AX colocalizes with 53BP1 and Rad50. After exposure to 0.5 mM H_2O_2 for 2 h, human spermatozoa and FL cells were double stained with anti-γH2AX and anti-53BP1 or anti-Rad50 antibodies, and observed under a fluorescent microscope. Shown are representative images for human spermatozoa (A) and FL cells (B).

Our results demonstrated that H_2O_2 induced H2AX phosphorylation in human spermatozoa for different incubation times or at different doses (Fig. 2). Moreover, this effect could be abolished by wortmannin, an effective inhibitor of PI3K family members. Therefore, we propose for the first time that oxidative stress can induce H2AX phosphorylation in human spermatozoa via the kinase activities of PI3K pathway. It has been well established that oxidative stress can produce DNA damage in spermatozoa; our results by comet assay further supported such a notion (Fig. 1), and we can conclude that DSBs also generate along with H2AX phosphorylation in response to oxidative stress. Given the established fact that γH2AX is a specific indicator for DSBs in somatic cells, it can be suggested here that H2AX phosphorylation may be a specific response to H_2O_2-induced DSBs in spermatozoa as well. Moreover, other strong evidence supporting such conclusion comes from our observation that after H_2O_2 removal, γH2AX staining did not show any tendency of decreasing (Fig. 4). It is very clear that such discovery rules out the possibility of a direct induction of H2AX phosphorylation by H_2O_2, but in contrast, strongly implies an indirect effect of H_2O_2 on H2AX phosphorylation via DSB induction. Our results are consistent with others' demonstrating that γH2AX was induced by genotoxic stresses such as ionizing radiation and chemotherapy agents in the developing male germ cells [29,30].

In somatic cells, γH2AX has been accepted and used as a specific, sensitive biomarker for DSBs induced by a variety of genetic insults such as ionizing radiation and chemotherapy treatment [36,37]. As discussed above, H2AX phosphorylation in human spermatozoa after H_2O_2 exposure may be also a specific response to oxidative stress-induced DSBs. Therefore, we intended to testify whether γH2AX can be used as a sensitive indicator of DSBs in spermatozoa. In this study, it was observed that in response to H_2O_2 exposure under most conditions, the levels of H2AX phosphorylation measured by flow cytometry displayed in a time- and dose-dependent manner, correlating well with those of DSBs assessed by comet assay. Interestingly, it was also found that the flow cytometry could reveal significant γH2AX staining in response to H_2O_2 incubation at 0.1 mM or for a period of 30 min, while the comet assay could not identify any significant DSBs formation under such conditions. In our previous study in somatic cells, it was also found that the γH2AX staining is more sensitive as an indicator of DNA damage induced by chemical reagent at low concentrations or for short incubation times than the comet assay [33]. Taken together, these data imply that γH2AX may be used as a sensitive marker for DSBs in human spermatozoa.

γH2AX foci formation at the DSB sites is essential for subsequent recruitment of other damage response proteins. For example, 53BP1 and Rad50, denoted "mediator proteins", are usually recruited to γH2AX foci and appear to facilitate phosphorylation of several ATM substrates and prompt DNA repair or apoptosis [38–40]. In addition, γH2AX disappears shortly after the complication of DNA repair, or on the contrary, it may persist for a long time and initiate apoptosis if DNA repair fails. In the present study, in somatic FL cells, the co-localization of γH2AX with 53BP1 and Rad50 was also observed (Fig. 3B). Furthermore, after H_2O_2 removal, the number of γH2AX foci decreased with time (Fig. 4A), suggested that DSB repair occurred in these damaged FL cells, which was later confirmed by our comet results (data not shown). On the contrary, in human spermatozoa, even though H_2O_2 exposure induced the concurrent presence of γH2AX with 53BP1 and Rad50 (Fig. 3A), no changes were observed in γH2AX fluorescent intensity after H_2O_2 removal (Fig. 4B), implying that DSB repair did not occur in these damaged germ cells. In fact, it was further confirmed by comet assay that no DNA repair occurred even after up to 4 h of H_2O_2 (data not

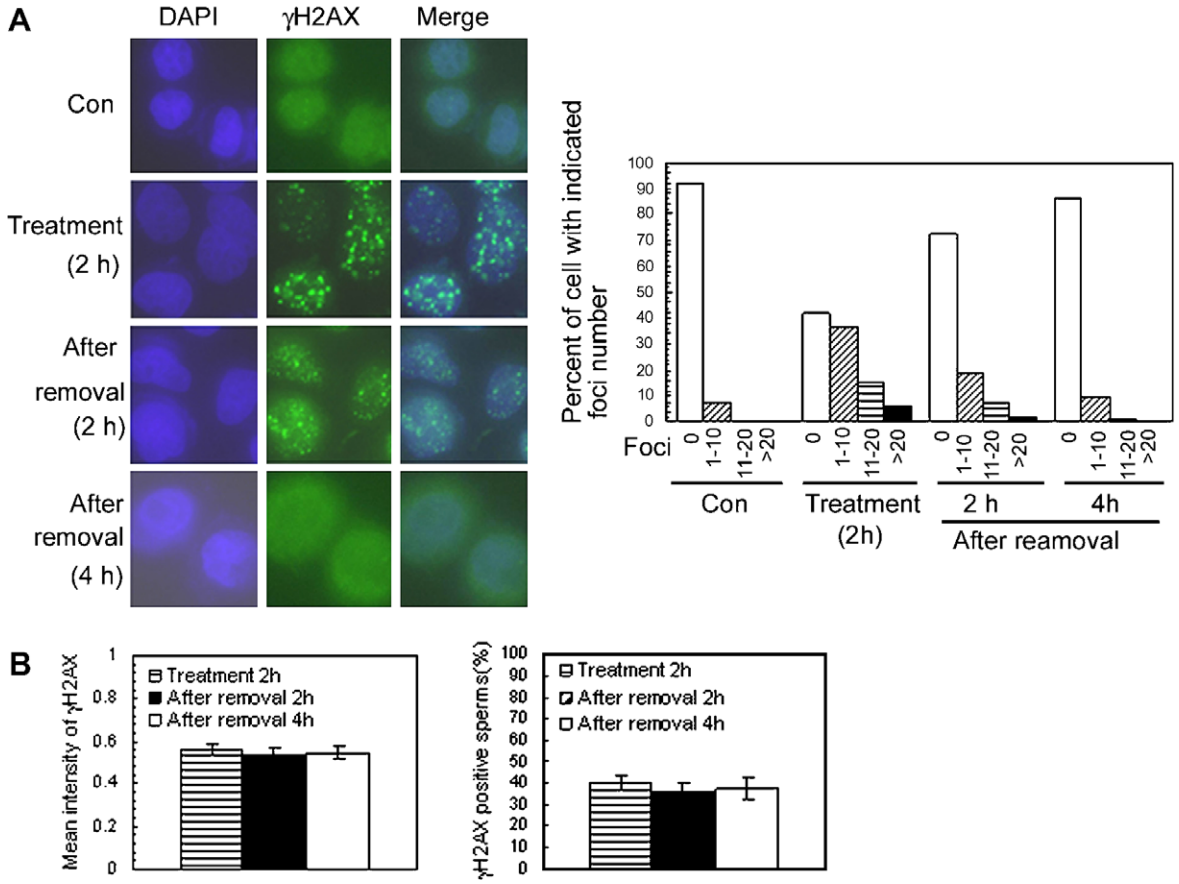

Fig. 4. Different responses of γH2AX staining in FL cells and human spermatozoa after H_2O_2 removal. FL cells or human spermatozoa were first exposed to 1 mM H_2O_2 for 2 h. After removing H_2O_2 by changing to fresh medium, cells were further incubated for 2 h and 4 h, then fixed and stained with anti-γH2AX antibody. FL cells were subjected to immunofluorescent microscopy, and the number of γH2AX foci was counted. Human spermatozoa were subjected to flow cytometry. (A) Left panel, representative images of immunofluorescent microscopy for FL cells. Right panel, quantification of H_2O_2-induced γH2AX foci in FL cells. (B) Flow cytometry analysis of H2AX phosphorylation in human spermatozoa as indicated by mean intensity of γH2AX immunofluorescence staining (left) or by percentage of γH2AX-positive cells (right).

shown). Moreover, it is also generally believed that DNA repair can only function efficiently in meiotic spermatocytes through early elongating spermatid stages in physiological conditions or after treatment with a variety of genotoxic chemicals, whereas elongated spermatids and mature spermatozoa are thought to lose their effective DNA-repair capacity [41–43]. In conclusion, our results suggest the probable existence of a specific surveillance system involving at least γH2AX, 53BP1 and Rad50 in human spermatozoa, which mediates cellular response to oxidative stress-induced DSBs. Although these proteins do not function effectively in DNA repair, they may play other biological roles, possibly, for example, activating apoptosis. A possible explanation for the inconsistence between somatic cells and spermatozoa may be that the later cells are terminally differentiated and transcriptionally inactive, thereby having different biological characters compared with the former.

Accumulating evidence has suggested that apoptosis can occur in ejaculated human sperm cells under various conditions [11,44,45]. For example, recent studies clearly demonstrated that carapace-dependent or other apoptotic pathways exist in the ejaculated sperm [10,45,46]. More importantly, it has been reported that apoptosis in human spermatozoa can be induced by ROS-mediated oxidative stress [47,48]. In our experimental system, sperm death was also observed after H_2O_2 exposure (data not shown). Unfortunately, it has not been further investigated in detail whether the cell death was due to apoptosis or necrosis, thus making it impossible to determine with confidence the possible role of γH2AX-53BP1/Rad50 pathway in these types of cell death. However, this project is currently underway in our laboratory.

It is worth noting that H_2O_2-induced γH2AX displayed in a homogeneous staining fashion in sperm nuclei, while it presented as typical foci in somatic FL cells. Meanwhile, Rad50 and 53BP1 were also observed in similar homogeneous patterns in human spermatozoa after H_2O_2 exposure. Such discrepancy between these two types of cells may be in part due to their different biological characters, i.e., the non-dividing mature spermatozoa versus the actively dividing somatic cells. In addition, the highly condensed histone in spermatozoa might prevent these proteins from moving to the damaged sites to form foci. In fact, we have previously found that such homogeneous staining also existed in human FL cells after a harsh treatment with other agent at high dose [33]. Halicka et al. have reported the presence of a HL-60 subpopulation with very bright H2AX phosphorylation in response to UVB radiation, and identified those as apoptotic cells [49]. Recently, Marti et al. also reported that UV radiation mainly induces

H2AX phosphorylation in somatic cell as a diffuse staining pattern, which depends on nucleotide excision repair, but not DNA DSBs [50]. However, while others have showed non-foci staining only in parts of cells under few specific conditions, our study revealed that such homogeneous staining occurred in most human spermatozoa under all experimental conditions. Therefore, such distribution of homogeneous staining possibly reflects some unique and important biological characteristics of human spermatozoa, which deserves further research.

In conclusion, our data suggest that H2AX phosphorylation in human spermatozoa is a specific response to oxidative stress-induced DSBs. However, the γH2AX-involved surveillance system does not function well in DNA repair, but may have other biological significance, such as initiating apoptosis, which will need to be confirmed in future studies. Moreover, γH2AX can be taken as a sensitive and early biomarker of DSBs in human spermatozoa, and γH2AX evaluation by flow cytometry may be a useful tool for "scanning" sperm DNA damage induced by oxidative stress. Further clinical studies are required to evaluate the reliability of γH2AX as a marker of DNA integrity in predicating the effects of sperm DSBs on fertility and pregnancy outcomes. Such information would be of great importance, in particular, to the infertile individuals seeking assisted reproduction therapy.

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