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·*Original Article*·

Adriamycin induces H2AX phosphorylation in human spermatozoa

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Abstract

Aim: To investigate whether adriamycin induces DNA damage and the formation of γH2AX (the phosphorylated form of histone H2AX) foci in mature spermatozoa. **Methods:** Human spermatozoa were treated with adriamycin at different concentrations. γH2AX was analyzed by immunofluorescent staining and flow cytometry and double-strand breaks (DSB) were detected by the comet assay. **Results:** The neutral comet assay revealed that the treatment with adriamycin at 2 μg/mL for different times (0.5, 2, 8 and 24 h), or for 8 h at different concentrations (0.4, 2 and 10 μg/mL), induced significant DSB in spermatozoa. Immunofluorent staining and flow cytometry showed that the expression of γH2AX was increased in a dose-dependent and time-dependant manner after the treatment of adriamycin. Adriamycin also induced the concurrent appearance of DNA maintenance/repair proteins RAD50 and 53BP1 with γH2AX in spermatozoa. Wortmannin, an inhibitor of the phosphatidylinositol 3-kinase (PI3K) family, abolished the co-appearance of these two proteins with γH2AX. **Conclusion:** Human mature spermatozoa have the same response to DSB-induced H2AX phosphorylation and subsequent recruitment of DNA maintenance/repair proteins as somatic cells. (*Asian J Androl 2008 Sep; 10: 749–757*)

Keywords: adriamycin; human spermatozoa; DNA double strand-breaks; γH2AX

1 Introduction

Adriamycin, also named doxorubicin, is one of the most popular chemotherapeutic drugs used in the treatment of a variety of cancers. It elicits antitumour activity by inducing DNA damage through three major mechanisms: stabilizing the topoisomerase II cleavage complex, binding DNA and causing DNA damage via the production of free radicals. As a consequence, adriamycin produces single-strand and double-strand breaks (SSB and DSB) in DNA, which leads to the death of cancer cells [1].

Adriamycin has been reported to impair male fertility through the induction of DNA damage in germ cells [2].

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Male germ cells have different susceptibility to genotoxic agents in the course of their development [3]. The nature and extent of genotoxic effects of adriamycin on male germ cells depend on the developmental stage of germ cells when the exposure occurs. Most studies on the genotoxic effects of adriamycin have been conducted during the early stages of spermatogenic cells (e.g. during intermediate spermatogenia and when spermatocytes meiotically divide, rapidly undergoing proliferation and differentiation [2, 4]). In contrast, little information is available about adriamycin-induced genetic stress or DNA damage in post-meiotic germ cells, especially in mature spermatozoa, which are terminally differentiated, transcriptionally inactive and no longer dividing.

Recently, γH2AX (the phosphorylated form of histone H2AX) has been recognized as a sensitive indicator for DNA damage, particularly DSB [5, 6]. H2AX is phosphorylated by members of the phosphatidylinositol 3-kinase (PI3K) family and forms localized "foci" at the sites of DSB a few minutes after the exposure to ionizing radiation (IR) or other factors that induce DSB [7]. Our previous study indicated that γH2AX foci formation could be used to evaluate DNA damage induced by a variety of chemical or physical factors [8]. More importantly, such a localization of γH2AX is responsible for the recruitment of other repair or checkpoint proteins to the damaged sites, including the Mre11/RAD50/Nbs1 (MRN) complex, BRCA1 and 53BP1 [9, 10]. The complex formation of γH2AX with other repair/checkpoint proteins at the site of damage is believed to reflect the cellular stress response to DSB, which can lead to DNA repair, cell cycle arrest, and/or apoptosis. Therefore, the detection of these proteins may help to predict the final fate of cells when confronted with DNA damage [11]. H2AX, a highly conserved histone H2A variant, constitutes 10%–20% of total H2A proteins in mammalian somatic cells [5]. Moreover, H2AX is abundant in adult germ cells [12]. H2AX has been shown to be phosphorylated soon after the occurrence of DSB and subsequently recruit other DNA repair proteins in somatic cells; however, it remains largely unknown how H2AX functions in mature human spermatozoa in response to DSB. To date, the published literature on the relationship between γH2AX and DNA repair in male germ cells is limited and only spermatogenic cells undergoing proliferation and differentiation have been examined [3, 11]. How the mature spermatozoa respond to DNA damage is still unknown. In addition, whether γH2AX functions the same way in spermatozoa as in somatic cells, such as in the recruitment of other protein factors, is also unclear. In an effort to answer these questions, we examined H2AX phosphorylation and complex formation of γH2AX with RAD50 and 53BP1 in human spermatozoa after exposure to adriamycin. We found that DNA damage induced the phosphorylation of H2AX and subsequent recruitment of other repair proteins in human spermatozoa in the same manner as in somatic cells. However, unlike in somatic cells, there was no distinct "focus" observed in DNA-damaged spermatozoa.

2 Materials and methods

2.1 Chemicals and antibodies

Adriamycin, wortmannin and 4',6-diamidino-2-phenylindole (DAPI) was purchased from Sigma (St.Louis, MO, USA). Rabbit antibodies against 53BP1 and RAD50 were from Gibco (Carlsbad, CA, USA); mouse monoclonal antibody against γH2AX was purchased from Upstate Technology (Lake Placid, NY, USA); FITC-conjugated goat anti-mouse IgG, TRIC-conjugated goat anti-rabbit IgG and goat blocking serum were obtained from Beijing Zhongshan Biotechnology (Beijing, China). Adriamycin was dissolved in double distilled water at a concentration of 2 mg/mL as a stock solution, and wortmannin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mmol/L as stock solution. Both solutions were diluted with culture medium prior to use.

2.2 Sperm preparation

Semen samples were obtained from donors attending the Fertility Clinic, the Centre of Reproductive Medicine, Women's Hospital, Zhejiang University School of Medicine, China, to receive assisted reproduction technology (ART) owing to female factors. All subjects provided their informed consent to participate in the research. Ethical permission to use semen samples in this study was obtained from the Institutional Review Board of Zhejiang University School of Medicine. To exclude sperm abnormalities, a physical examination, together with an ultrasonography of scrotal contents, a basic semen analysis and a male endocrine test, were performed in all volunteers before enrollment. The mean sperm concentration was (117.06 ± 35.02) × 10^6/mL (range: [80–171] × 10^6/mL) and the sperm count was (339.86 ± 131.70) × 10^6/ejaculate (range: [170–600] × 10^6/ejaculate). Progressive forward motility was 49.3% ± 7.3%

(range: 37.0%–50.4%) and morphological abnormalities reached 24.8% ± 8.0% (range: 11.0%–38.0%).

Semen samples were obtained by masturbation after 3–5 days of abstinence. After liquefaction for 30 min at 37ºC, a sperm fraction of high quality was isolated by discontinuous Percoll gradient separation (95% and 50% layers). Briefly, 2 mL of semen was carefully placed on the Percoll layers and centrifuged at 500 × *g* for 20 min. Following centrifugation, spermatozoa at the base of the high density Percoll fraction were collected (designated 95% Percoll fraction). The pellet was washed with Biggers Whitten and Whittingham buffer containing 0.3% (w/v) human serum albumin (HSA) and centrifuged again at 500 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in human tubal fluid medium supplemented with 0.5% (w/v) HSA.

2.3 Cell culture and experiment design

For each experiment, the sperm pellets from two or three donors were pooled and resuspended in medium at a concentration of 5×10^6/mL. Aliquots of sperm suspension in 24-well culture plates were incubated at 37ºC with 5% (v/v) CO_2 with different concentrations of adriamycin (0.4, 2 and 10 μg/mL) for 8 h, or with 2 μg/mL of adriamycin for different times (0.5, 2, 8 and 24 h). To clarify the role of PI3K signaling pathway in adriamycin-induced DSB, spermatozoa were pretreated for 30 min with wortmannin (50 μmol/L) followed by an adriamycin treatment (2 μg/mL) for 2 h and afterwards washed three times with phosphate buffered saline (PBS). Aliquots from the same donors without adriamycin treatment were used as mock controls at 0, 0.5, 2, 8 and 24 h and DMSO control was set when spermatozoa were treated with wortmannin. Incubation was stopped by washing the spermatozoa with PBS.

Human amnion FL cells were cultured in Eagle's Minimum Essential Medium (Invitrogen, Carlsbad, CA, USA), containing 10% (w/v) fetal calf serum, 100 U/mL penicillin, 125 μg/mL streptomycin, and 0.03% (w/v) glutamine. Approximately 1×10^5 cells were transferred to 6-well culture plates containing a glass cover slip in each well and treated with indicated drugs.

2.4 Immunofluorescent staining

To evaluate H2AX phosphorylation, spermatozoa were treated with adriamycin, and were then harvested and washed three times with PBS. Approximately 5×10^4 cells in a volume of 50 μL were dropped onto polylysine-coated slides and fixed in 4% (w/v) paraformaldehyde for 15 min at 4ºC, followed by permeabilization in 0.2% (v/v) Triton X-100 for 15 min. After permeabilization, slides were blocked with goat blocking serum for 60 min prior to incubation with mouse monoclonal anti-γH2AX antibody (1:1 000) overnight at 4ºC. After washing in PBS, the slides were incubated with FITC-conjugated goat-anti-mouse secondary antibody (1:500) for 60 min at 37ºC, followed by further PBS washes. Sperm nuclei were counterstained with DAPI (1 μg/mL in PBS) for 15 min. After another wash, slides were mounted with coverslips and viewed using an Olympus AX70 fluorescent microscope (Olympus, Tokyo, Japan). For each slide, the spermatozoa were first viewed for DAPI staining and then assessed for γH2AX staining. Five representative images of spermatozoa were captured on each slide. In parallel with spermatozoa, human FL cells were also treated and stained for γH2AX using the same method. A negative control for γH2AX staining was performed by replacing anti-γH2AX antibody with PBS.

Double immunofluorescent staining of γH2AX and RAD50 or 53BP1 was performed in human spermatozoa and FL cells. The procedure is the same as above except that when the antibody was first applied, γH2AX with RAD50 or 53BP1 was used to substitute γH2AX alone, and, subsequently, both FITC-conjugated goat-anti-mouse and TRITC-conjugated goat-anti-rabbit secondary antibody (1:500) were added and incubated for 60 min at 37ºC.

2.5 Comet assay

To detect DSB in human spermatozoa, the neutral comet assay was performed as previously described with slight modifications [13]. Briefly, 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 100 000 sperm cells (10 μL) were mixed with 0.7 % (w/v) low melting agarose (80 μL) to form a cell suspension, and this suspension was pipetted onto the first agarose layer, spread and solidified on ice. After removal of the coverslip, a third layer of 0.65% (w/v) 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 mol/L NaCl, 100 mmol/L ethylenediaminetetraaceticacid [EDTA], 10 mmol/L Tris [adjusting pH to 10 and adding 1% {v/v} Triton X-100], 10 mmol/l DL-Dithiothreitol before using) at 4ºC for 1 h. The slides were then incubated at 37ºC in lysis buffer

with 10 μg/mL of proteinase K for 2 h. Electrophoresis was conducted at 25 V, 100 mA for 15 min in electrophoresis buffer (Tris-Borate-EDTA [TBE] neutral buffer: Tris 10 mmol/L, Boric acid 80 mmol/L, EDTA 0.5 mol/L, pH 8.2) after a first 20-min incubation for unwinding of the DNA. Following electrophoresis, the slides were first immersed in Tris buffer (pH 7.5, 0.4 mol/L) for 15 min for neutralization, and then in DAPI solution (1 μg/mL) for 15 min. After washing with PBS, coverslips were placed on the gels.

The slides were examined using an Olympus AX70 fluorescent microscope (Olympus). Images were obtained and saved as BTM files and tail moments were determined using CometScore Freeware from TriTek (Sumerduck, VA, USA). The tail moment is the integrated value of fluorescence intensity multiplied by migration distance and is proportional to the levels of damaged DNA, which is considered the most sensitive parameter of the Comet assay for DNA damage. For each experiment, 25 cells were scored from replicate slides (50 cells total) and subsequently pooled, and each experiment was repeated three times.

2.6 Flow cytometric analysis of γH2AX

Human spermatozoa were first fixed in 2% paraformaldehyde for 15 min and then kept in 70 % (v/v) ethanol at –20ºC until analysis. Staining for γH2AX was conducted as described. Briefly, fixed cells were re-hydrated for 10 min, then centrifuged and re-suspended in 200 μL of monoclonal mouse anti-γH2AX antibody (1:1 000 dilution) for 2 h at 37ºC. Spermatozoa were then rinsed and re-suspended in 200 μL of secondary antibody (1:500 dilution) for 1 h at 37ºC. Finally, the sperm cells were rinsed and re-suspended in PBS before analysis with a flow cytometer (Coulter, Fullerton, CA, USA). The average γH2AX antibody staining relative to the untreated control was calculated from the mean fluorescence intensity and the percentage of γH2AX-positive cells.

2.7 Statistics

Statistical analysis was carried out with using SPSS version 11.0 (SPSS, Chicago, IL, USA). Data are presented as mean ± SD and compared with analysis of variance. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Adriamycin induces DSB in human spermatozoa

A previous study has shown that adriamycin could induce DNA strand breaks in human spermatozoa that could be detected with the alkaline comet assay [14]. Because the alkaline comet assay can detect both SSB and DSB or even base modifications, and the neutral comet assay is specific for detecting DSB [15], we performed the neutral comet assay in the present study to examine the integrity of chromosomal DNA after adriamycin exposure. As shown in Figure 1, adriamycin induced DSB in a time-dependent and dose-dependent manner in human spermatozoa. Treatment with adriamycin at 2 μg/mL for different times (0.5, 2, 8 and 24 h), or for 8 h at different concentrations (0.4, 2 and 10 μg/mL), induced significant DSB in human spermato-

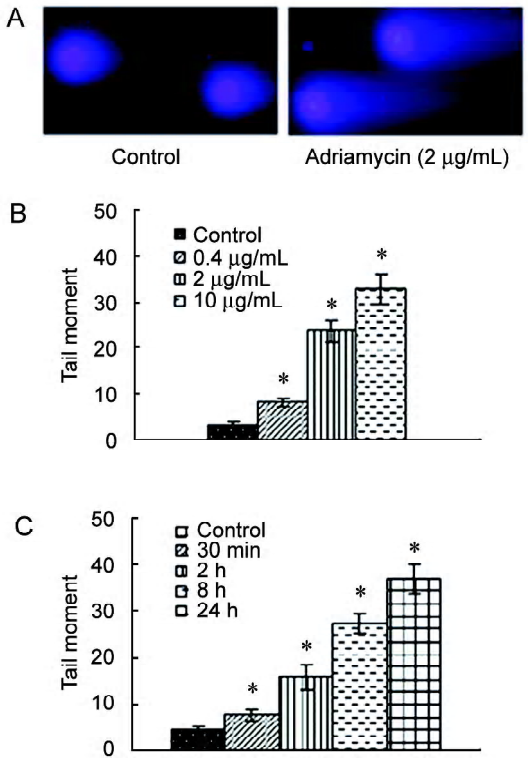

Figure 1. Adriamycin causes double strand breaks (DSB) in human spermatozoa. After exposure to various concentrations of adriamycin (0.4, 2 and 10 μg/mL) for 8 h, or to 2 μg/mL adriamycin for different times (0.5, 2, 8 and 24 h), human spermatozoa were subjected to neutral comet analysis to evaluate the formation of DSB. (A): Representative images from neutral comet assay. (B): Dose response of DSB formation as indicated by tail moment. (C): Time response of DSB formation as indicated by tail moment. $^{*}P < 0.05$, compared with the control.

zoa, which was indicated by the tail moment of the neutral comet assay.

3.2 Adriamycin induces H2AX phosphorylation

To explore whether adriamycin could induce H2AX phosphorylation in human spermatozoa, immunofluorescent staining specific for γH2AX was performed. We found that γH2AX was stained in the nucleus of spermatozoa after adriamycin exposure for 8 h at different concentrations (0.4, 2 and 10 μg/mL) (Figure 2A), or at 2 μg/mL for different times (0.5, 2, 8 and 24 h) (Figure 2B). The immunofluorescent intensity, which reflects H2AX phosphorylation levels in the spermatozoa treated with adriamycin increased in a dose-dependent and time-de-

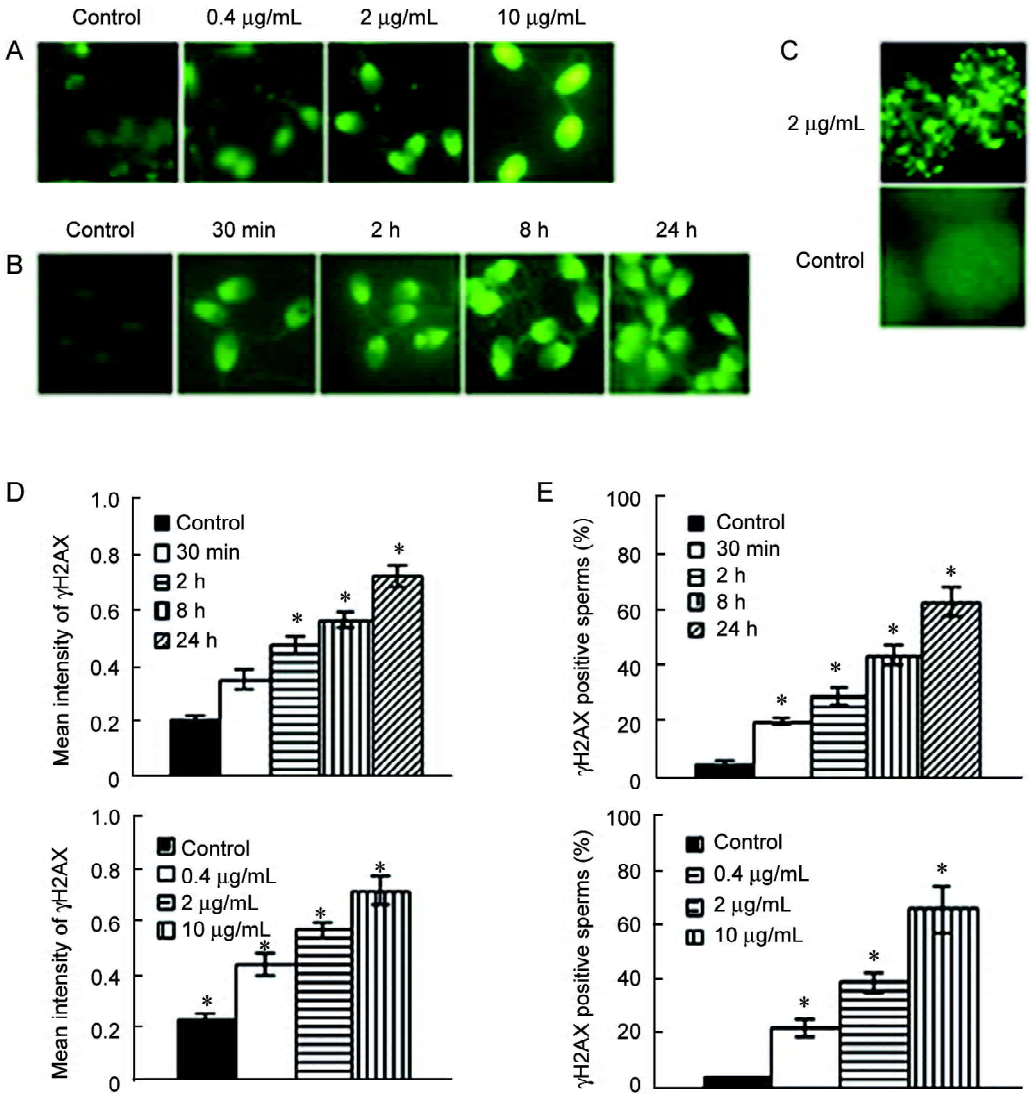

Figure 2. Adriamycin induces H2AX phosphorylation in human spermatozoa. Human spermatozoa were exposed to various concentrations of adriamycin (0.4, 2 and 10 μg/mL) for 8 h, or to 2 μg/mL adriamycin for different times (0.5, 2, 8 and 24 h). Cells were then fixed and stained with anti-γH2AX antibody, and subjected to immunofluorescence microscopy. Human amnion FL cells were similarly treated and used as positive controls. Flow cytometry was also performed to determine the mean intensity of γH2AX immunofluorescence staining and the percentage of γH2AX-positive spermatozoa. Shown are representative images for spermatozoa (A and B) and FL cells (C). The time-response and dose-response of H2AX phosphorylation (D and E, respectively) in sperm cells as indicated by the mean intensity of γH2AX immunofluorescence staining (left) or by the percentage of γH2AX-positive sperms (right).

pendent manner. This observation was further confirmed by quantitative analysis of flow cytometry (Figure 2D, E). Moreover, γH2AX intensity by flow cytometry analysis was well correlated with the tail moment of the neutral comet assay, indicating a significant relationship between DSB and γH2AX formation in human spermatozoa.

The distribution of γH2AX in the nucleus of human spermatozoa was presented in a diffuse pattern (Figure 2A, B). At the same time, we performed immunofluorescent staining in FL cells as control and found that the typical fashion of discrete nuclear foci were formed in FL cells after adriamycin (2 μg/mL for 2h) treatment (Figure 2C).

3.3 Colocalization of γH2AX with RAD50 or 53BP1 in human spermatozoa after adriamycin exposure

γH2AX has been shown to recruit and colocalize with other proteins such as RAD50 and 53BP1 in response to DSB in somatic cells. These proteins are thought to play important roles in cellular stress response such as DNA repair and/or apoptosis. To determine whether γH2AX functions in human sperm cells the same way as in somatic cells, double immunofluorescent staining was performed in parallel in both spermatozoa and FL cells.

In human FL cells, adriamycin (2 μg/mL for 2 h) induced the colocalization of γH2AX foci with RAD50 or 53BP1. In untreated FL cells, these proteins displayed a diffused nuclear distribution and the signals of the staining were comparatively weak (Figure 3B). Adriamycin treatment led to the formation of RAD50 and 53BP1 nuclear foci that, in part, colocalized with the induced γH2AX foci (Figure 3B). Adriamycin treatment (2 μg/mL for 2 h) also induced the concurrent appearance of RAD50 and 53BP1 with H2AX phosporylation in human spermatozoa, although no distinct foci were observed as in FL cells (Figure 3A).

3.4 Wortmannin inhibits adriamycin-induced H2AX phosphorylation and concurrently abolishes RAD50 and 53BP1 appearance in human spermatozoa

It was reported that the phosphorylation of H2AX and subsequent recruitment of RAD50 or 53BP1 depends on the PI3K signaling pathway. Wortmannin is an inhibitor for PI3K family members, including ataxia telangiectasia mutated (ATM) and ATM and Rad3-Related (ATR), which can inhibit the phosphorylation of H2AX induced by IR or other agents that cause DSB. To clarify the possible role of the PI3K signaling pathway in the phosphorylation of H2AX and subsequent complex formation of γH2AX with RAD50 or 53BP1 in human spermatozoa in response to adriamycin-induced DSB, human sperm cells were pretreated for 30 min with wortmannin (50 μmol/L) followed by an adriamycin treatment (2 μg/mL) for 2 h. Spermatozoa were then examined by immunofluorescence microscopy. Pretreatment of the cells with wortmannin significantly attenuated the phosphorylation of H2AX induced by adriamycin (Figure 4A). Furthermore, the accumulation of RAD50 or 53BP1 was also inhibited (Figure 4B). Therefore, the phosphorylation of H2AX and concurrent appearance of RAD50 or 53BP1 in human spermatozoa in response to the exposure of adriamycin were also dependent on PI3K family kinases.

4 Discussion

Adriamycin has been extensively studied for its ability to induce DNA damage in male germ cells during spermatogenesis. It has been shown that adriamycin-induced cytotoxicity is mainly concentrated in early spermatogenic cells, which undergo rapid proliferation and differentiation [2]. In addition, apoptosis is one of the earliest signs of genotoxic damage caused by adriamycin [16]. Observations on adriamycin-induced DNA damage in mature human spermatozoa have not been reported yet. However, it was implied that adriamycin may induce DNA damage in sperm cells at post-meiosis stage, possibly through the generation of reactive oxygen species (ROS)[17]. In the present study, we have provided evidence showing the genotoxic effects of adriamycin on mature human spermatozoa: adriamycin significantly increased DSB in human spermatozoa in a dose-dependent and time-dependent manner, as revealed by the neutral comet assay. Our results are in agreement with previous reports showing that adriamycin treatment causes DNA damage in spermatozoa, as assessed by the alkaline comet assay[14]. Therefore, it can be concluded that adriamycin causes DSB in male germ cells of all developmental stages from early spermatogenic cells to late mature spermatozoa. Our findings may have some significance for clinical purposes, such as the evaluation of sperm damage and the risk of infertility in cancer patients receiving adriamycin-involved treatment. However, much needs to be done before the evaluation is used clinically.

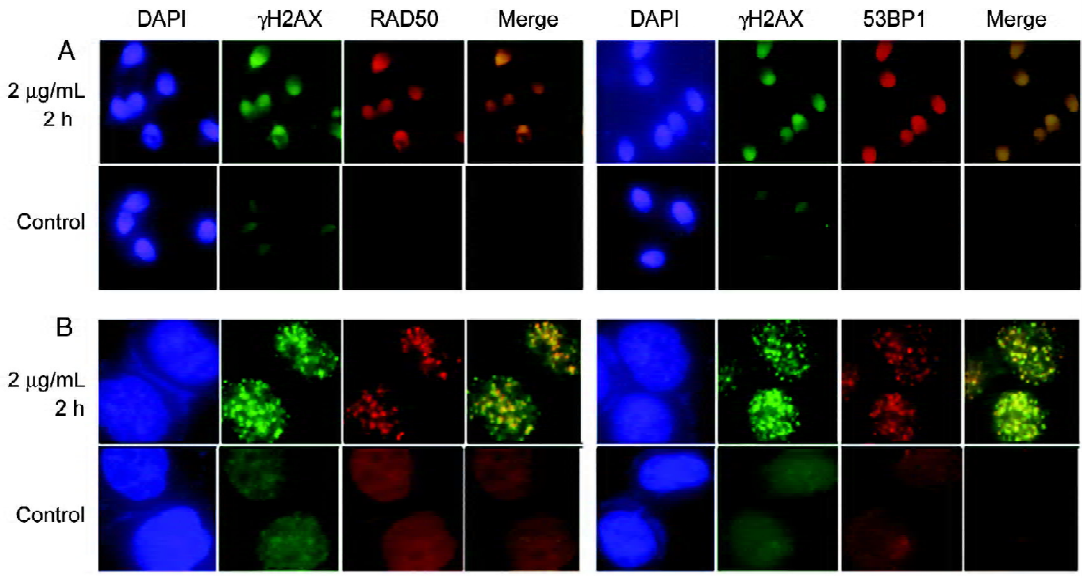

Figure 3. Adriamycin induces concurrent appearance of RAD50 and 53BP1 with γH2AX. After adriamycin treatment (2 μg/mL for 2 h), double immunofluorescent staining revealed concurrent appearance RAD50 (left panel) or 53BP1 (right panel) in human spermatozoa (A), as well as in FL cells (B). (Exposure time: 1.5 ms). DAPI, 4',6-diamidino-2-phenylindole.

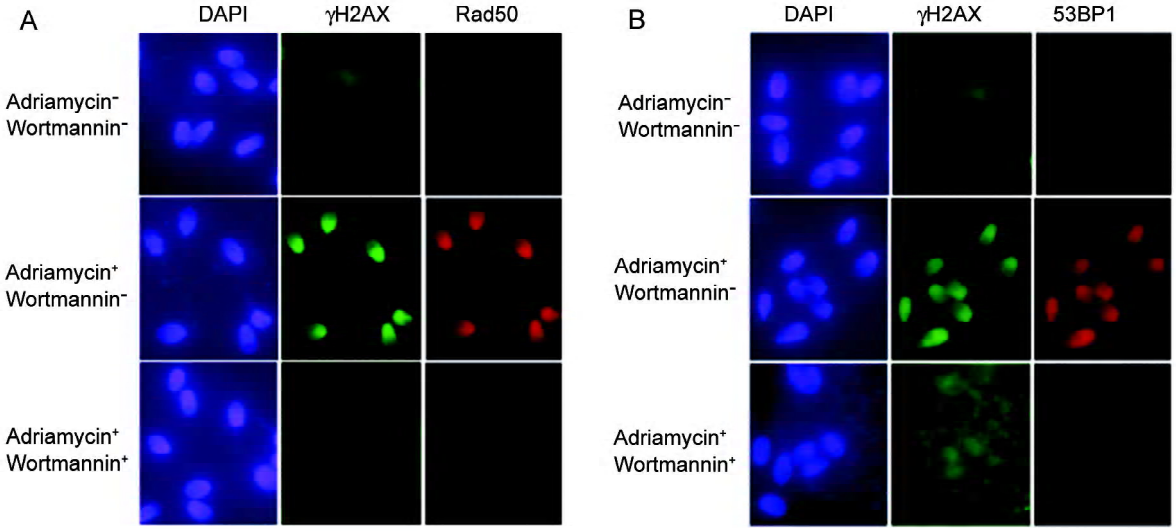

Figure 4. Wortmannin inhibits the phosphorylation of γH2AX and concurrently abolishes RAD50 and 53BP1appearance. Pretreatment of cells with wortmannin (50 mmol/L) for 30 min prior to adriamycin addition completely attenuated the adriamycin (2 μg/mL for 2 h)-induced phosphorylation of H2AX and subsequent accumulation of RAD50 (A) or 53BP1 (B) in the nuclei of spermatozoa. (Exposure time: 1.5 ms). DAPI, 4',6-diamidino-2-phenylindole.

In somatic cells, H2AX phosphorylation has been regarded as an early indicator for DNA DSB and, more importantly, such modification is essential for subsequent recruitment and colocalization of other proteins, including RAD50 and 53BP1, to the damaged sites [6]. The formation of such a protein complex is believed to initiate,

or at least take part in, a series of cellular stress responses, including DNA repair, cell cycle arrest and apoptosis [10]. In the present study, we have reported for the first time that adriamycin treatment could induce H2AX phosphorylation via the PI3K family in a dose-dependent and time-dependant manner in mature human spermatozoa. In addition, with an increased concentration or incubation time, the immunofluorescent intensity also increased. The expression of γH2AX as indicated by the immunofluorescent intensity was correlated with the results of the neutral comet assay. This suggests that γH2AX could also be used as a specific indicator of sperm DNA damage induced by genotoxic insults.

It was noted that γH2AX displayed homogeneous staining in sperm nuclei, which is different from the typical foci observed in somatic cells. Similar patterns were also observed for RAD50 and 53BP1. Such discrepancy might be due to the different biological characters between the non-dividing mature spermatozoa and actively dividing somatic cells. In fact, we have previously found that homogeneous staining also exists in human FL cells after exposure to high doses of genotoxic agent [18]. Recently, Marti *et al.* [19] reported that ultraviolet irradiation mainly induces H2AX phosphorylation in somatic cell as a diffuse staining pattern, which depends on nucleotide excision repair, but not DNA DSB. It is of interest to investigate further the underlying mechanism for such differences in γH2AX staining between spermatozoa and somatic cells.

Our observations on the colocalization of γH2AX and 53BP1 or RAD50 imply the presence of DNA repair in mature human spermatozoa after adriamycin exposure. Nonetheless, it is generally believed that DNA repair can function only in meiotic spermatocytes through early elongating spermatid stages, whereas effective DNA-repair capacity is thought to be lost from elongated spermatids and mature spermatozoa [20]. Thus, H2AX phosphorylation, together with 53BP1 and RAD50 coappearance, might not function in DNA repair in spermatozoa, but probably has other biological roles, such as activating the apoptotic pathway as there is an increasing body of evidence showing that apoptosis occurs in mature human spermatozoa under a variety of conditions [21]. In addition, the colocalization of 53BP1 with γH2AX and hyperphosphorylation of 53BP1 at damaged sites can activate p53-dependent and independent apoptotic pathways if DNA repair fails [12]. This hypothesis in spermatozoa is currently under investigation in our laboratory. However, even if this is the case, it does not mean that all the damaged spermatozoa will undergo apoptosis before fertilization. There is accumulating evidence that some spermatozoa with DNA damage still have the opportunity to complete fertilization and that very soon DNA repair may occur in the fertilized egg [22].

In conclusion, we have shown that adriamycin can induce H2AX phosphorylation and the subsequent appearance of RAD50 and 53BP1 in human spermatozoa through action of members of the PI3K family. Because the mature spermatozoa play an important role in keeping the correct transmission of genetic material to the next generation, it is of great importance to identify DNA damage in spermatozoa. On the basis of our results, it is likely that γH2AX could be used as an indicator for the screening of abnormal spermatozoa with DNA damage, which will definitely benefit those couples who are seeking assisted reproduction therapy.

Acknowledgment

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