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Original Article

Oxidative damage of DNA induced by X-irradiation decreases the uterine endometrial receptivity which involves mitochondrial and lysosomal dysfunction

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Abstract: X irradiation may lead to female infertility and the mechanism is still not clear. After X irradiation exposure, significantly morphological changes and functional decline in endometrial epithelial cells were observed. The mitochondrial and lysosomal dysfunction and oxidative DNA damage were noticed after X irradiation. In addition, pretreatment with NAC, NH_4Cl or Pep A reduced the X irradiation induced damages. These studies demonstrate that the oxidative DNA damage which involved dysfunctional lysosomal and mitochondrial contribute to X irradiation-induced impaired receptive state of uterine endometrium and proper protective reagents can be helpful in improving endometrial function.

Keywords: X irradiation, endometrial receptivity, DNA damage, oxidative stress, mitochondrial dysfunction, lysosomal membrane instability

Introduction

The incidence of infertility increases in recent years worldwide. A successful pregnancy demands the ability of uterine endometrium to accept and accommodate mature embryo, such ability is termed as "endometrial receptivity" [1]. It is considered that ionizing radiation exposure is one of the important etiologies for infertility. People are consistently exposed to natural background X irradiation, and some population may also be exposed to X irradiation from working environment (such as radiologists), medical diagnosis tests and treatments or even some radiation accidents. Among the residents in high natural background X irradiation, the female primary infertility rate is 8% higher than those in the ordinary natural background radiation areas [2]. Medical X irradiation exposure (such as CT scan) to the mother may lead to unintended effect on the embryo/fetus which results in abortion [3]. *In vivo* studies have shown the chromosome aberrations induced by X irradiation in mouse oocytes and zygote in the early stages of mouse fertilization [4]. By exposing adult female nonhuman primates to targeted radiotherapy, ovarian failure and infertility were detected [5]. Thus far, the underlying basis of radiation induced infertility, especially on the endometrium is still largely unknown.

DNA damage induced by X irradiation includes DNA strand breaks, alterations of deoxyribose and bases. DNA damage affects the fertility ability in both males and females. High level of sperm DNA fragmentation was observed in infertility men [6]. Lipopolysaccharide (LPS)-induced DNA damage in pre-implanting embryonic and uterine cells during pre-implantation period of pregnancy ultimately inhibits the process of implantation in mice [7]. In mouse endometrial cells and uterine cells, damaged DNA integrity may lead to embryo implantation disorder when exposed to carbon disulfide [8]. Although X irradiation is well known to induce DNA damage in various cells and tissues, how DNA damage in uterine endometrial cells con-

tributes to the X irradiation induced infertility is still not clear.

The declined molecular and cellular functions can be caused by mitochondrial dysfunction and lysosomal membrane instability [9]. Mitochondrial dysfunction involves decreased MMP which is a biomarker of oxidative environmental stress [10], and elevated release of cytochrome C (e.g., Cyt C). *In vitro* studies suggested that the disruption of MMP and release of Cyt C were related to the reduction of sperm fertilizing capacity [11]. As major intracellular source of reactive oxygen species (ROS), mitochondrial dysfunction leads to the occurrence of oxidative stress which is known to be an important pathological factor in infertility among males and females [12]. ROS, a byproduct of the normal metabolism of oxygen produced in the mitochondria of the cells, have multifaceted roles in pathological processes induced by ionizing radiation [13]. 8-hydroxy-2-deoxyguanosine (8-OHdG), one of the most specific markers of oxidative DNA damage, is induced by the action of free radicals on the DNA base deoxyguanosine (dG) and DNA strand breakage [14]. Oxidative stress leads to the lysosomal protease leakage [15]. Activation and release of lysosomal proteases may be an important pathological event of acute radiation syndrome [16]. Cathepsin D, a ubiquitous acidic protease in lysosome, is up-regulated by ionizing radiation in breast cancer cells (MCF-7) and melanocytes [17, 18]. The concentration of cathepsin D is higher in patients with endometriosis which lead to infertility [19]. Thus far, whether mitochondrial and lysosomal dysfunction contributes to the X irradiation induced low endometrial receptivity remains unclear.

In the present study, we demonstrated that X irradiation affected the adhesiveness of embryonic cells to the endometrial epithelial cells by generating the oxidative DNA damage which involves depolarization of mitochondria and lysosomal protease leakage, the beneficial effects of antioxidant and lysosome protective agents on endometrial receptivity was represented.

Methods and materials

Cell culture, pretreatment and X irradiation

Human endometrial epithelial RL95-2 cell line and human embryonic JAR cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). RL95-2 cells were grown in DMEM/F12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 0.005 mg/mL insulin (Sigma-Aldrich), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C under 5% CO_2 in humidified air. The JAR cells were maintained in RPMI 1640 (Gibco) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C under 5% CO_2 in humidified air.

Antioxidant N-acetylcysteine (NAC, Beyotime, China), alkalizing reagent of lysosome ammonia cholotide (NH_4Cl, Sigma-Aldrich, USA), and the lysosomal cathepsin D inhibitor pepstatin A (Pep A, Sigma-Aldrich, USA) were used as protective reagents in this study. The concentration of NAC, NH_4Cl and Pep A were 5 mM, 2.5 mM, 100 μM, respectively, and the protective reagents were added 1 hr before X irradiation.

X irradiation was performed at dose of 2 Gy at the intensity of 100 cGy/min on a CLINAC 2300C/D-SN 27 X-ray unit.

CCK-8 assay

Cell Counting Kit (Doindo, Japan) was used to assess the cell viability according to the manufacture's protocol. All groups were treated with CCK-8 at 37°C for 1 hr, the absorbance was obtained at 450 nm using a microplate reader (Thermo, USA) and accessed.

Transmission electron microscopy

The TEM was used as previously reported [20]. The images were taken in an analytical transmission electron microscope in (Hitachi, S-4800 TEM).

Scanning electron microscopy

The SEM was used as previously reported [21]. The images were taken in a scanning electron microscope (JEM-2000 EX).

Adhesion of JAR cells to RL95-2 cell monolayer

RL95-2 cells were grown on the 96-well plate to form confluent monolayers. The JAR cells (1×10^4) were cultured with cytoplasmic dyes Cell Tracker Green (CMFDA) for 30 min then harvested, counted, and gently delivered onto the confluent monolayer of RL95-2 cells in JAR growth medium. After 1 hr, unadhered JAR cells were removed by washing with medium. The

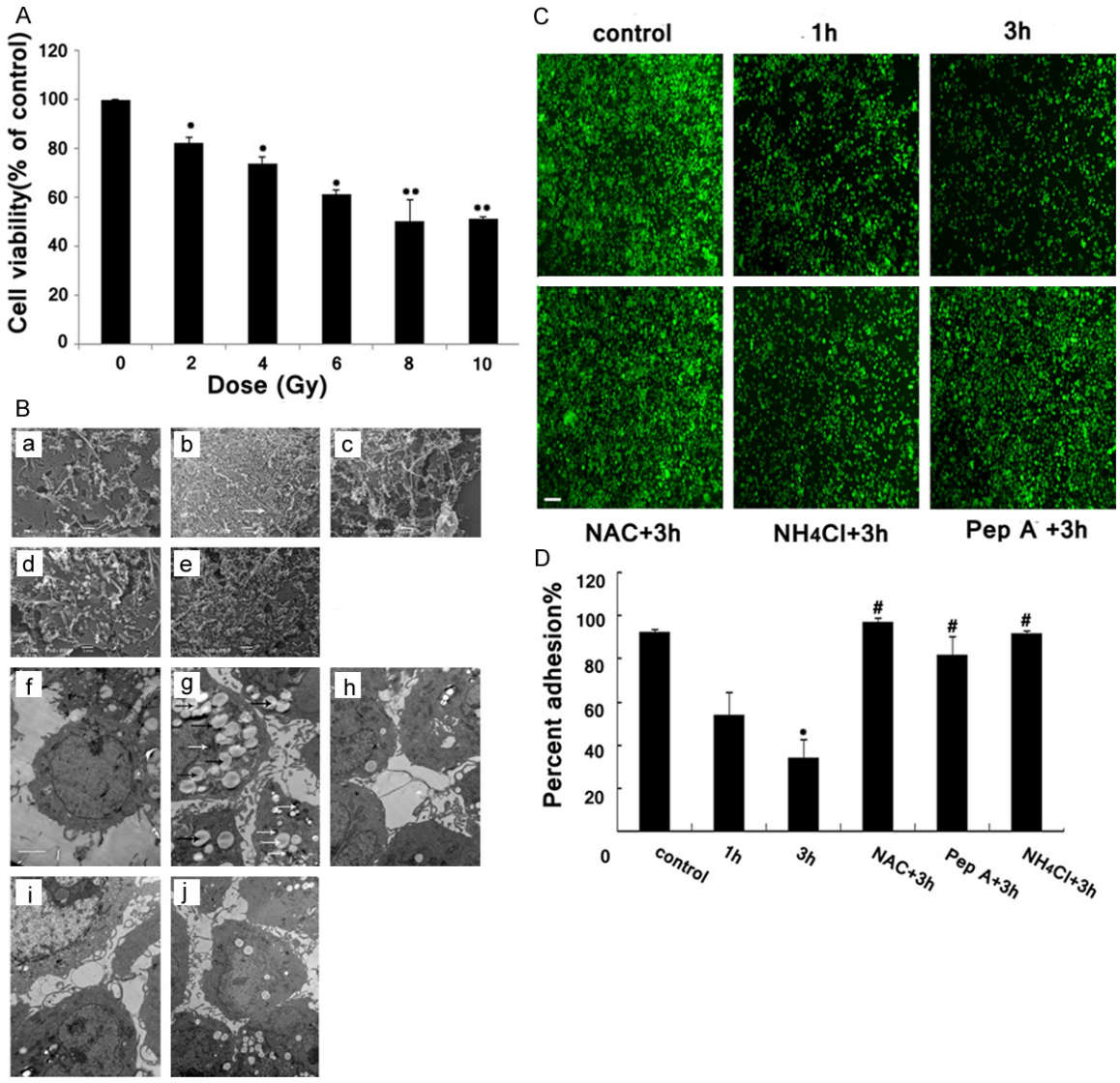

Figure 1. Alterations of cell viability and morphology in RL95-2 cells post-radiation and analysis of adhesiveness of JAR cells to X irradiated RL95-2 cell monolayer. A. Viability of RL95-2 cells after different dose of X irradiation (2, 4, 6, 8 and 10 Gy) 48 hrs post-radiation. B. Images of SEM and TEM. Image a~e showed microvilli on the surface of RL95-2 cells, Bar=1 μm. Images f~j showed the intracellular vacuoles (black arrow), secondary lysosome (white arrow) in RL95-2 cells, Bar=2 μm. (a, f: non-irradiated; b, g: 3 hrs post-irradiation; c, h: NAC+X irradiation; d, i: NH_4Cl+X irradiation; e, j: pepstatin A+X irradiation). C. Representative fluorescent microscopy images showed adhesion of CMFDA-labeled JAR cells (green) to RL95-2 cells monolayer. (1 h:1 hr post-irradiation; 3 h:3 hrs post irradiation). Bar=100 μm. D. The percentage adhesion was calculated by the fluorescent intensity. Error bars represent S.D. $^{*}P<0.05$ *vs*. control, $^{\#}P<0.05$ *vs*. 3 h, N=3.

images were captured under a fluorescence microscope (Nikon, Japan). Adhesion potential was calculated as the percentage of the attached JAR cells compared with control by gray analysis of the images.

Mitochondrial membrane potential (MMP) assay

The monolayer cells were then washed twice with PBS, labeled with 10 μM rhodamine 123 (Sigma-Aldrich, USA) for 1 hr in serum-free DMEM/F12 under 5% CO_2 at 37°C . After briefly washed with PBS, Images were obtained using the fluorescence microscope.

Lysosomal membrane stability assay

After washing twice with PBS, the monolayer RL95-2 cells were labeled with 50 nM Lyso-Tracker Red (Beyotime Institute, China) for 30 min in serum-free DMEM /F12 under 5% CO_2 at

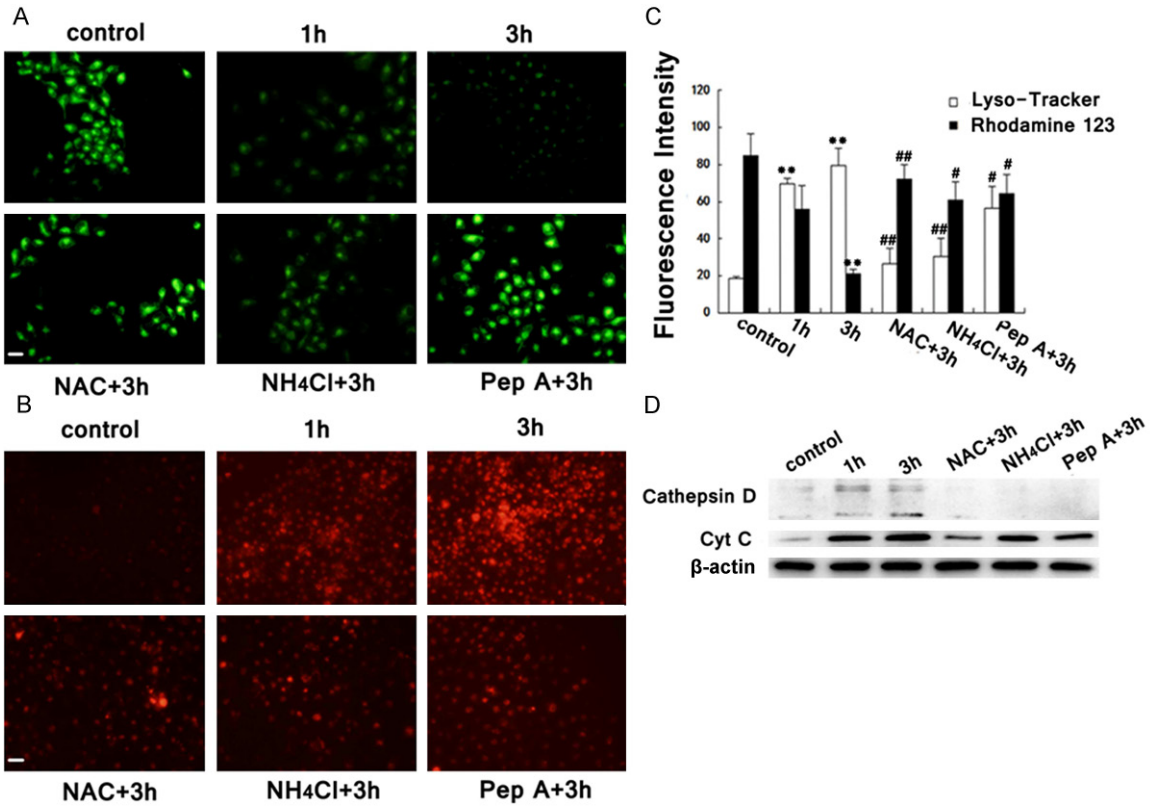

Figure 2. X irradiation induced MMP decrease and lysosomal membrane instability in RL95-2 cells. (A) Images of rhodamine 123 staining. Bar=50 μm. (B) Images of lyso-tracker red staining. Bar=50 μm. (C) Statistical analysis of fluorescence intensity of rhodamine 123 and lyso-tracker red in (A and B). Error bars represent S.D. $^{*}P<0.05$; $^{**}P<0.01$ *vs.* Control; $^{\#}P<0.05$; $^{\#\#}P<0.05$ *vs.* 3 h, N=3. (D) Detection of cytosolic cathepsin D and Cyt C by Western blot.

37°C . Images were obtained using the fluorescence microscope.

Measurement of intracellular ROS

Cells were twice-washed with PBS, then incubated with 5 μM DCFH-DA (Beyotime, China) for 30 min to allow intracellular conversion of DCFH, the absorbent was obtained using a microplate reader (λex=495, λem=520) and analyzed.

Subcellular fractionation and immunodetection for cathepsin D and cytochrome C

2×10^6 RL 95-2 cells were washed twice with sucrose buffer (250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1 g/mL aprotinin, 1 g/mL leupeptin), resuspended in 20 μl of sucrose buffer and incubated on ice for 15 min. Then cells were homogenized with a Dounce (40 strokes) and centrifuged at 1000×g for 10 min at 4°C . The resulting supernatant was subjected to 20,000×g for 20 min at 4°C . Then supernatants were used to conduct western blot with anti-cathepsin D rabbit monoclonal antibody (1:1000) (Bioworld Technology, USA) or anti-cytochrome C rabbit polyclonal antibody (1:1000) (ProteinTech, USA).

Western blot for γH2AX

Western blot was performed according to the previous report [22]. Cell lysate (50 μg) was loaded onto a SDS-PAGE and transferred onto NC membranes and revealed with anti-γH2AX (Ser139) rabbit monoclonal antibody (1:1000) (Cell Signaling Technology, USA).

Comet assay

Comet assay was assessed according to Zhang's protocol [23] with slight modification. The comet images were taken by fluorescent

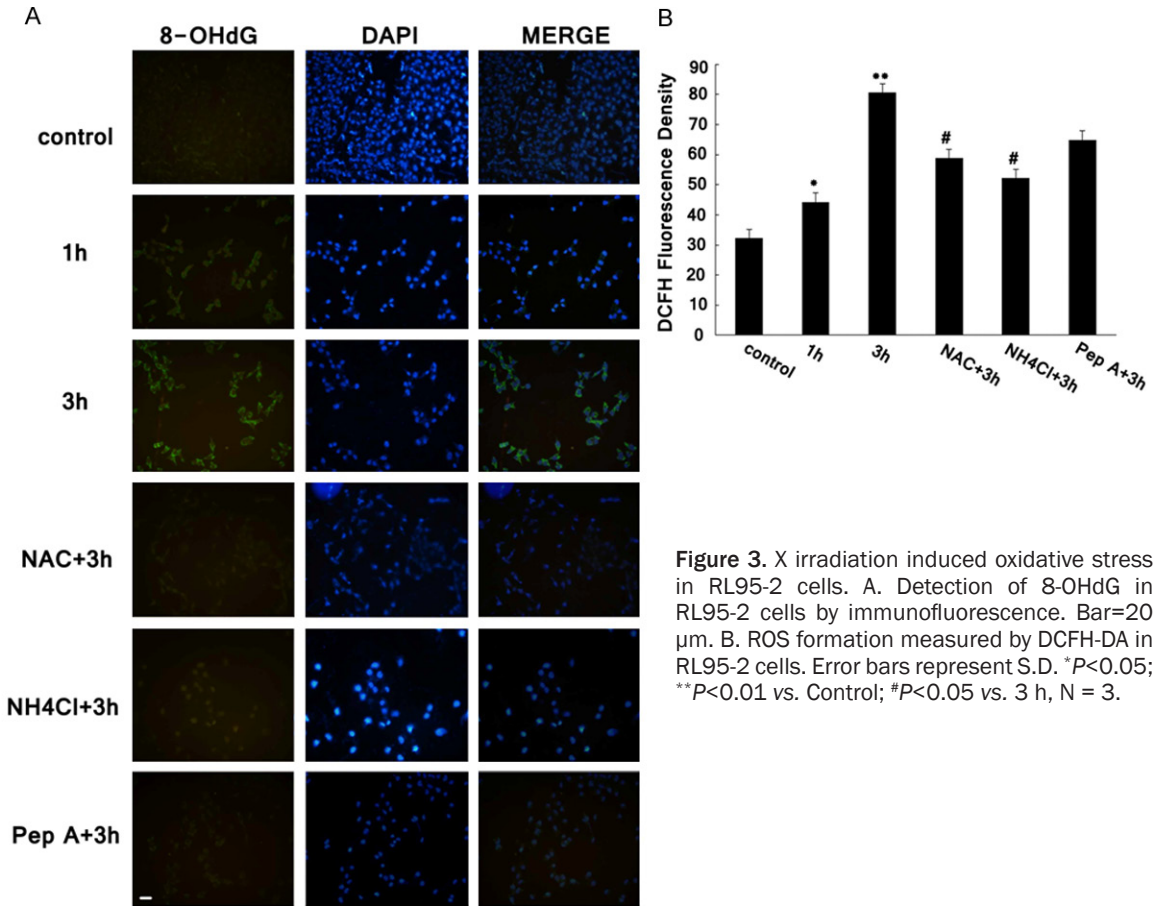

Figure 3. X irradiation induced oxidative stress in RL95-2 cells. A. Detection of 8-OHdG in RL95-2 cells by immunofluorescence. Bar=20 μm. B. ROS formation measured by DCFH-DA in RL95-2 cells. Error bars represent S.D. $^{*}P<0.05$; $^{**}P<0.01$ *vs.* Control; $^{\#}P<0.05$ *vs.* 3 h, N = 3.

microscopy (Nikon, Japan) with an excitation filter of 549 nm and barrier filter of 590 nm. Image analysis was performed using Comet Assay Software Project casp-1.2.2. Three independent experiments were performed, and 150 randomly selected comets were analyzed. The extent of DNA damage was quantified by tail DNA (%).

Immunofluorescence staining of γH2AX and 8-OHdG

RL95-2 cells of all groups were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by incubation with ice-cold methanol (-20°C, 10 min). After blocking with PBS containing 0.3% Triton-X-100 and 5% BSA (w/v) (1 hr, room temperature), cells were incubated with Anti-γH2AX (Ser139) rabbit monoclonal antibody (1:400) (Cell Signaling Technology, USA) and 8-OHdG mouse monoclonal antibody (1:100) (Santa Cruz, USA) overnight at 4°C. After incubation with the PE-conjugated anti-goat IgG (1:500, ProteinTech, USA) for 1 hr at room temperature in the dark, samples were visualized and images were captured on a confocal microscope (Olympus, Japan) and fluorescence microscope (Nikon, Japan).

Statistical analysis

All data were presented as mean ± standard deviation (S.D.). The data were analyzed by one-way analysis of variance (ANOVA) and Student's t-test, using SPSS 11.5 software. The level of significance was set at $P<0.05$, $P<0.01$ and $P<0.001$ for all statistical analyses.

Results

Cell viability, morphological and functional changes by X irradiation

The 2 Gy dose reduced cell survival in RL95-2 cells by 19% relative to non-irradiated controls, and was selected for further analysis. X irradiation induced increasing number of microvilli and secondary lysosome, as well as numerous intracellular vacuoles with different sizes in comparison to the control group at 3 hrs post-

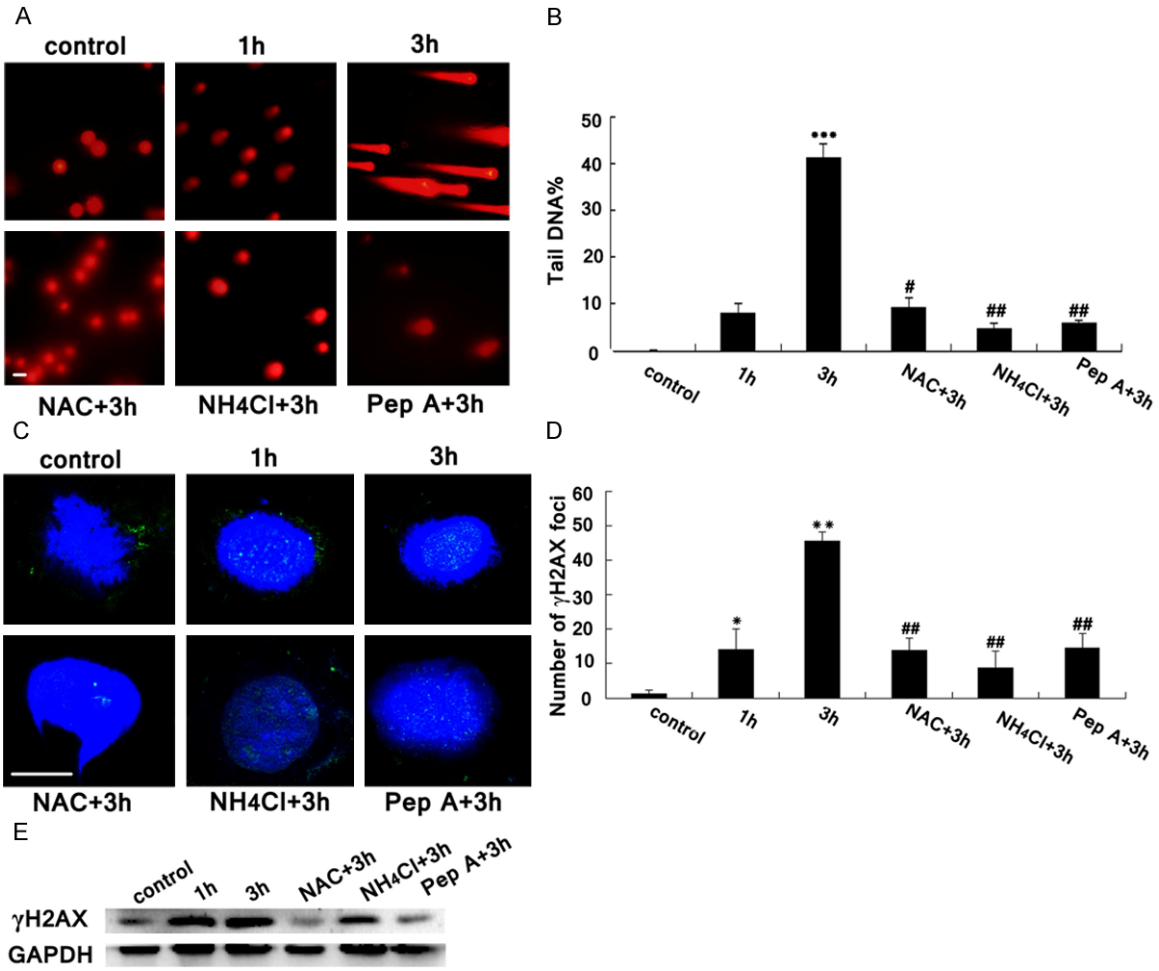

Figure 4. DNA damage induced by X irradiation in RL95-2 cells. A. Presentative images of DNA damage detected by comet assay 1 hr and 3 hrs post-irradiation without or with the pretreatment of NAC, NH_4Cl and Pep A. Bar=20 μm. B. The extent of DNA damage was measured quantitatively by the tail DNA (%) value of RL95-2 cells. C. Presence of nuclear γH2AX foci RL95-2 cells incubated with by anti-γH2AX antibody and followed with FITC-labelled exposure under confocal microscopy. Bar=25 μm. D. Number of nuclear γH2AX foci per cell. E. Detection of γH2AX by Western blot. Error bars represent S.D. $^{***}P<0.001$; $^{*}P<0.05$; $^{**}P<0.01$ *vs.* Control; $^{\#}P<0.05$; $^{\#\#}P<0.01$ *vs.* 3 h, N=3.

radiation, meantime, groups with the pretreatments of NAC, NH_4Cl and Pep A showed less microvilli and secondary lysosome (**Figure 1B**). The adhesion of JAR cells to RL95-2 cell monolayer was shown (**Figure 1C** and **1D**). Statistical analysis showed that the percentage adhesion of 3 hr-treatment group was significantly decreased in comparison to the control group ($P<0.05$). Compared with 3 hr-treatment group, the adhesion percentage of NAC+3 h, NH_4Cl+3 h and Pep A+3 h groups were significantly increased ($P<0.05$).

Effect of X irradiation on mitochondrial dysfunction and lysosomal membrane stability

As determined by rhodamine 123 fluorescence staining, the fluorescent intensity was significantly decreased in X irradiated cells, indicating decreased MMP. Meantime, pretreatment with NAC, NH_4Cl and Pep A rescued X irradiation-disrupted MMP (**Figure 2A** and **2C**). X irradiation also induced high release of Cyt C, which was also prevented by pretreatment with NAC, NH_4Cl and Pep A (**Figure 2D**).

Lyso-tracker red fluorescence intensity (**Figure 2B** and **2C**) and the release of cathepsin D (**Figure 2D**) were significantly increased by X irradiation at 1 and 3 hr post-radiation compared with non-treated group ($P<0.01$). Pretreatment with lysosomal protection compounds NH_4Cl and Pep A significantly increased the lysosomal membrane stability compared with the 3 h-treatment group, ($P<0.01$), Furthermore, We also proved that oxidative

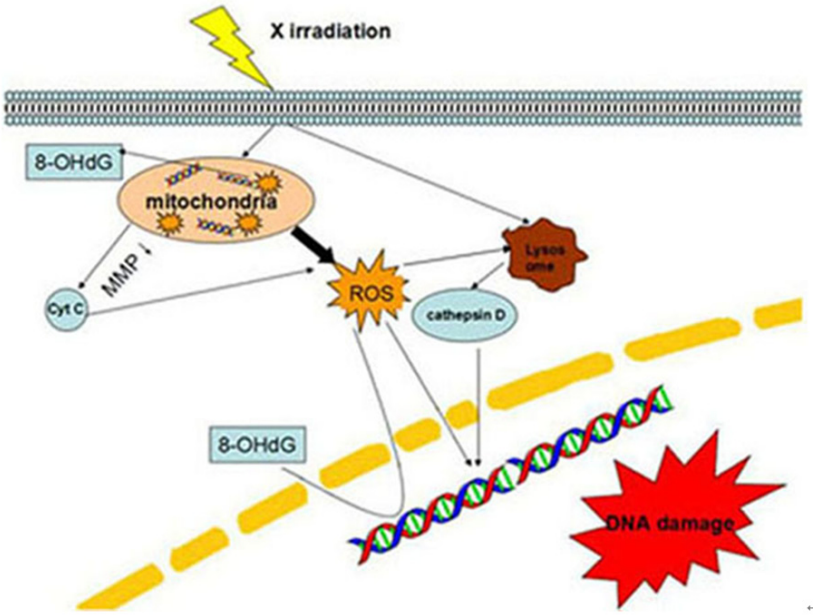

Figure 5. Potential mechanism of X irradiation induced low endometrial receptivity which involved mitochondrial and lysosomal dysfunction.

stress damaged the lysosomal membrane stability which was rescued by antioxidant NAC (P<0.05).

X irradiation induced oxidative stress

High fluorescent intensity of 8-OHdG was found in X irradiated cell cytoplasm when compared with the non-irradiated group and NAC pretreatment reduced 8-OHdG formation in RL95-2 cells (P<0.05) (**Figure 3A**). These results indicated X irradiation-induced oxidative stress, which was further verified by increased ROS generation in X irradiated RL95-2 cells which was significantly reduced by pretreatment with NAC (P<0.05). In addition, pretreatment with NH_4Cl also significantly reduced the oxidative effect of X irradiation (**Figure 3B**).

Induction of DNA damage in RL95-2 cells by X irradiation

X irradiation strongly increased DNA migration at 3 hr post-radiation compared with non-irradiated group (P<0.05) (**Figure 4A** and **4B**). In addition, DNA migration was significantly inhibited by NAC (P<0.05), NH_4Cl and Pep A (P<0.01). Induction of γH2AX expression in RL95-2 cells was observed in response to X irradiation by immunofluorescence staining and analysis 1 hr (P<0.05) and 3 hr (P<0.01) (**Figure 4C** and **4D**), and was further confirmed by Western blot (**Figure 4E**). The number of nuclear γH2AX foci was significantly decreased by pretreatment of NAC , NH_4Cl and Pep A (P<0.01) (**Figure 4C** and **4D**).

Discussion

In this study, we found significant morphological alterations, including numerous intracellular vacuoles of the different sizes and the increasing number of secondary lysosomes in X irradiated RL95-2 cells (**Figure 1B**). X irradiation leads to similar changes in hematopoietic stem/progenitor cells and such alterations also occurred in cells with senescence-associated biological changes [24] which directly correlated to the altered cellular function, as shown in present study that the adhesiveness of the X irradiation damaged RL95-2 cells to embryonic cells was decreased (**Figure 1C** and **1D**). The adhesiveness of uterine cells to the trophoblast comes with the loss of microvilli which are developed in atrophic and hyperplastic endometrium [25], the increasing microvilli is also observed in the endometrium of women with spontaneous abortion after receiving sorbent and bioresonance (extremely high-frequency) therapy [26]. We observed the increasing microvilli on the surface of RL95-2 cells after X irradiation exposure which was considered as pathological change (**Figure 1B**). These results suggest that X irradiation causes the remarkable ultrastructure damage and is correlated with the decreased endometrial receptivity by which leading to endometrium entering into the non-receptive state and resulting in implantation failure and infertility.

Mitochondrial and lysosomal dysfunction can be involved in oxidative DNA damage, and the released lysosomal enzyme can attack mitochondria and promote the oxidative stress [27] which was considered as the “The mitochondrial-lysosomal axis theory” [28]. Here we demon-

strated this theory by using the antioxidant NAC and lysosomal inhibitor NH_4Cl and pep A (**Figures 2**, **3**). Mitochondrial dysfunction plays an important role in the intrinsic pathway of genotoxic-agent-induced apoptosis [32]. Exposure to ionizing radiation can lead to mitochondrial dysfunction followed by increased release of Cyt C and decreased MMP [30].The disruption of mitochondria was found in reproduction related cells from people who had reduced fertilizing capacity [31]. We found here, that mitochondrial dysfunction in endometrial correlated with the decreasing of endometrial receptivity when exposed to X irradiation. Oxidative stress plays a critical role on X irradiation damaged endometrium receptivity. As shown by us that X irradiation exposure significantly increased ROS and the formation of 8-OHdG (**Figure 3**). Environmental pollutants were found to trigger oxidative stress by which to lead to the female infertility. Aitken and colleague had demonstrated that oxidative damage in sperm plasma membrane was causally involved in the etiology of male infertility [32]. Studies from Shahin and colleague showed that oxidative stress induced by exposure to low-level microwave (MW) irradiation led to decreases in implantation and pregnancy in mice [33]. Oxidative stress might further induce lysosomal disruption because the intralysosomal iron-catalyzed peroxidative reactions led to the decreased lysosomal membrane stability and leakage of protease [23]. In addition, ionizing radiation led to lysosomal membrane destabilization [34] which facilitated the release of cathepsin D. In women with endometriosis, increased expression of cathepsin D was observed [19]. We found increased release of the lysosomal protease cathepsin D and low lysosomal membrane stability were involved in X irradiation induced endometrium damage (**Figure 2**). DNA damage is associated with infertility in reproductive cells and organs [7, 8, 35], we verified DNA damage in X irradiated human uterine endometrial RL95-2 cells (**Figure 4**), Interestingly, the lysosomal membrane instability, mitochondrial dysfunction and oxidative stress are involved in DNA damage as shown in our result that the antioxidant and lysosomal protective reagents successfully rescued the DNA damage (**Figure 4**). In short, the "mitochondrial-lysosomal axis" had close connection with oxidative DNA damage induced by X irradiation and finally lead to low endometrial receptivity.

Accumulating evidences have shown that X irradiation impaired cellular functions by which to lead to infertility. Searching for proper protections against radiotoxicity is essential for females who have high risk of exposing to X irradiation. In the present study, adhesiveness of embryonic cells to endometrial cells was successfully rescued by three compounds: NAC, a well known antioxidant, safe drug without major side effects; NH_4Cl, a lysosomal inhibitor, neutralizes the acidic endo-lysosome compartments thus decreases the protease leakage [36]; pep A, specifically inhibit the lysosomal protease cathepsin D activity [37]. These three reagents are known to show beneficial effect against genotoxicity and cytotoxicity, but their protective effect on endometrial receptivity still remains unclear. The present study successfully demonstrated potential protective effect of these reagents on endometrial receptivity from the damage of X irradiation.

In conclusion, the lysosome and mitochondria dysfunction are involved in X irradiation induced oxidative DNA damage which leads to the declined endometrial receptivity. The present study also evaluated the protective potential of antioxidants and lysosome protectors on the endometrial receptivity from X irradiation damage (**Figure 5**).

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Disclosure of conflict of interest

None.

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