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Genistein sensitizes sarcoma cells *in vitro* and *in vivo* by enhancing apoptosis and by inhibiting DSB repair pathways

X.X. Liu[1,2,3], C. Sun[1,2,3], X.D. Jin[1,2,3], P. Li[1,2,3], X.G. Zheng[1,2,3,4], T. Zhao[1,2,3] and Q. Li[1,2,3]*

[1]Institute of Modern Physics, Chinese Academy of Sciences, 509 Nanchang Road, Lanzhou 730000, Gansu Province, China
[2]Key Laboratory of Heavy Ion Radiation Biology and Medicine, Chinese Academy of Sciences, Lanzhou 730000, China
[3]Key Laboratory of Basic Research on Heavy Ion Radiation Application in Medicine, Gansu Province, Lanzhou 730000, China
[4]University of Chinese Academy of Sciences, Beijing 100049, China
*Corresponding author: Institute of Modern Physics, Chinese Academy of Sciences, 509 Nanchang Road, Lanzhou 730000, Gansu Province, China.
Tel: +86–931–496–9316; Fax: +86–931–827–2100; Email: liqiang@impcas.ac.cn
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ABSTRACT

The aim of this work was to investigate the radiosensitization effects of genistein on mice sarcoma cells and the corresponding biological mechanisms *in vitro* and *in vivo*. Using the non-toxic dosage of 10 μM genistein, the sensitizer enhancement ratios after exposure to X-rays at 50% cell survival (IC_{50}) was 1.45 for S180 cells. For mice cotreated with genistein and X-rays, the excised tumor tissues had reduced blood vessels and decreased size and volume compared with the control and irradiation-only groups. Moreover, a significant increase in apoptosis was accompanied by upregulation of Bax and downregulation of Bcl-2 in the mitochondria, and lots of cytochrome *c* being transferred to the cytoplasm. Furthermore, X-rays combined with genistein inhibited the activity of DNA-PKcs, so DNA-injured sites were dominated by Ku70/80, leading to incompleteness of homologous recombination (HR) and non-homologous end-joining (NHEJ) repairs and the eventual occurrence of cell apoptosis. Our study, for the first time, demonstrated that genistein sensitized sarcoma cells to X-rays and that this radiosensitizing effect depended on induction of the mitochondrial apoptosis pathway and inhibition of the double-strand break (DSB) repair pathways.

KEYWORDS: sarcoma, radiosensitivity, genistein, DNA damage repair, apoptosis

INTRODUCTION

Sarcoma is a very rare and heterogeneous tumor; however, the disease is especially complex because each of the sarcoma subtypes is biologically and molecularly distinct [1]. Despite aggressive treatment modalities such as adjuvant chemotherapy or limb-sparing surgery, the prognosis of sarcoma patients remains unfavorable, and most patients die from sarcoma with metastatic disease [2, 3]. Radiotherapy is an alternative treatment modality for use against cancers so as to achieve local control and reduce the risk of recurrence. However, radioresistance limits its use in the treatment of some cancers, such as osteosarcoma [4]. Currently, various radiosensitizing molecular agents have been found to enhance radiotherapy effects on sarcoma, by inhibiting tumor cell growth or by promoting apoptosis [5, 6].

One of the most important mechanisms in the effect of radiotherapy on cancers is damage to DNA, leading to DNA double-strand breaks (DSBs). There are two major pathways for DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ) [7]. NHEJ is initiated by the association of the Ku70/80 heterodimer with DNA ends [8]. After end processing, the two tethered DNA termini are ligated and NHEJ is complete. HR repair is dependent on the RecA homologue *Rad51*. *Rad51* is expressed at higher levels in tumor cells compared with its expression in normal cells [9]. In addition, *p53* is a tumor suppressor gene that plays a pivotal role in apoptosis. *p53* has been extensively investigated as a promising tool for highly specific anticancer therapy in chemo- and radiotherapeutics [10]. Wild-type *p53* can downregulate the expression of

Rad51, which has been ascribed to non-transcriptional dependent mechanisms [10, 11].

Although the two DSB repair pathways can act in parallel [12, 13], the initial binding of repair factors to the DNA break site may affect the choice of HR or NHEJ. The binding of the Ku proteins, but not other components of NHEJ, to DSBs may to some degree interfere with the initiation of HR [14, 15]. Once Ku is bound and DNA-PKcs have been recruited to the broken ends, the protein kinase activity of the DNA-PKcs is activated and it phosphorylates itself and other targets, including Ku [16]. Only phosphorylated Ku can dissociate from DNA-bound Ku [17]. Thus, inhibitors of the DNA-PKcs could have potential uses as radiosensitizing agents to increase the effectiveness of radiation-induced cell death during cancer treatment.

Both *in vivo* and *in vitro* studies have shown that genistein can induce apoptosis of various cancer cells, including leukemia, hepatoma, prostate and breast cancer cells [18–21]. Moreover, previous studies suggest the involvement of DNA damage and DSB repair pathways in genistein anticancer action, though the molecule mechanisms have not been well understood [22–24]. In this paper, mouse sarcoma 180 cells and S180 tumor-bearing Balb/c mice were used to evaluate whether genistein can augment the response of sarcoma to X-rays and to investigate the biological mechanisms in terms of DSB repair.

MATERIALS AND METHODS

Chemicals

Genistein, dimethyl sulfoxide (DMSO) and polyethylene glycol 400 (PEG-400) were all purchased from Sigma, St Louis, USA. For the *in vitro* experiments, genistein was dissolved in DMSO as 15 mM stock solution and stored at –20°C. The concentrations of DMSO in the cell culture media were <0.5% in all experiments. For the *in vivo* experiments, genistein was solubilized in PEG-400 on the day of the experiments by 20 s of sonication (KQ-500E, Jiangsu, China). Genistein and the 0.1 ml PEG-400 vehicle were injected intraperitoneally.

Cell culture

The mouse sarcoma 180 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and preserved in our laboratory. S180 cell suspensions were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, USA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco) and 10% fetal bovine serum (FBS, Biowest, Nuaillé, France) at 37°C in a humidified atmosphere containing 5% CO_2.

Mouse treatment

Eight-week-old female Balb/c mice (20 ± 2 g) of Specific Pathogen-Free (SPF) grade were purchased from Lanzhou Medical College (Lanzhou, China) for this study. Mice were maintained on a 12-h light–dark cycle at temperature of 22 ± 1°C. All animal experiments were carried out according to the requirements of the Animal Care Committee at the Institute of Modern Physics (IMP) at the Chinese Academy of Sciences. During the experiments, mice were provided with sterilized food and water *ad libitum*. After being fed in our laboratory for one week, 20 mice were inoculated with S180 cells according to the method of Wu *et al.* (with some modifications) [25]. All mice were subcutaneously implanted with 1×10^6 ascites cells/mouse at the backside. Ten days after inoculation, the animals were randomly divided into the following four groups (each group containing five mice). The first group, the control group (***C***), was treated with the vehicle; the second group, the drug group (***D***), was given an intraperitoneal injection of genistein (200 mg/kg body weight). The third group of mice, the irradiated group (***IR***), was administered an intraperitoneal injection of the vehicle and received fractionated whole-body irradiation. The fourth group (***D+IR***) was administered an intraperitoneal injection of genistein (200 mg/kg body weight), and 24 h later, the mice were given fractionated whole-body exposure to X-rays in the same manner as the third group. The mice were sacrificed by cervical dislocation 24 h after the last irradiation. Tumor tissues were immediately removed and frozen at –80°C for biochemical examination. For histological evaluation, fresh samples were initially fixed in 4% formaldehyde–phosphate buffer solution.

Irradiation

After genistein pretreatment, cells and mice were irradiated with X-rays, which were generated with an X-ray machine (FAXITRON RX-650, Faxitron Bioptics, LLC, Tucson, USA) operated at 50 kVp. The dose rate was ~0.5 Gy/min. All irradiations were carried out at room temperature. S180 cells received single-fraction irradiation of 2 Gy X-rays, whereas 5-fraction irradiations at 2 Gy/fraction were given to each mouse once every two days, i.e. equivalent to a 10 Gy single dose.

Cell survival and cytotoxicity assay

Cell survival and cytotoxicity were measured by a cell counting method using a particle counter (Bechman Counter Z1, Thermo, USA). Briefly, exponentially growing S180 cells were seeded at 2×10^4 cells/well in a 12-well tissue culture plate with a total volume of 2 ml per well. Genistein concentrations ranging from 5–80 μM were added immediately. The cell number was counted at 24, 48 and 72 h after inoculation.

Detection of cell apoptosis *in vitro*

Cell apoptosis was quantified by a combined staining of Annexin V and propidium iodide (PI) using an Annexin V-FITC Apoptosis Detection Kit (BestBio, Shanghai, China). Cells were prepared according to the manufacturer's instructions. Briefly, ~1×10^6 cells per experimental condition were harvested, washed with cold PBS twice, and resuspended in 400 μl binding buffer. After adding 5 μl of Annexin V-FITC solution and 10 μl of PI solution, the cells were incubated for 15 min at room temperature in the dark. After incubation, 10 000 cells were analyzed in the flow cytometer.

TUNEL assay

In situ cell apoptosis was evaluated using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) kit (Roche, Mannheim, Germany) following the manufacturer's protocol. Briefly, paraffin sections were deparaffined with xylene and rehydrated in a graded series of ethanol solutions. Then the slides were washed and permeabilized by 5 min microwave irradiation (350 W) in 0.1 M citrate buffer (pH 6.0) and then incubated with blocking solution (0.1 M Tris-HCl, pH 7.5, and 3% bovine serum albumin (BSA)) for 30 min. Afterwards, the label solution (labeled

nucleotides and TdT enzyme) was added for 60 min at 37°C in the dark. Samples were rinsed, dried and incubated with converter-POD for 30 min at 37°C. Finally, the sections were visualized using DAB (DAKO, Carpenteria, CA, USA). Stained slides were assayed using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). Negative control staining was performed without the TdT mixture.

Western blot analysis

Western blot analysis was performed following the standard methods. Briefly, proteins lysates were resolved by SDS-PAGE under reducing conditions at a concentration of 30 μg of each protein sample per lane and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Blots were blocked with 5% skim milk in Tris-Buffered Saline and Tween 20 (TBST) for 1 h and then incubated overnight with primary antibodies (Bax, Bcl-2, cytochrome *c*, p53, β-actin, COXIV antibody, Cell Signaling Technology); DNA-PKcs, Ku70+80 antibody, Abcam; Phospho-DNA-PKcs antibody, ImmunoWay). They were then washed and incubated with a secondary peroxidase-conjugated antibody (1:5000 dilution, Abcam). Bound secondary antibody was detected using a chemiluminescence (ECL, Roche, Shanghai, China) system according to the manufacturer's protocol. Gray levels of the acquired western blotting data were measured using Quantity One Version 4.6.2 software.

Cell fraction

S180 cells and ground sarcoma tissues were lysed in buffer A (0.25 M sucrose, 10 mM Tris-HCl (pH7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) with a homogenizer. Homogenates were centrifuged at 750×*g* for 10 min at 4°C, and the supernatants were collected and centrifuged at 10 000×*g* for 20 min at 4°C. The supernatants were used as the cytosolic fraction, and the pellet was used as the mitochondrial fraction. The pellets were resuspended in buffer B (0.25 M sucrose, 10 mM Tris-HCl (pH7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and 1% NP 40).

Immunofluorescence assay

S180 cells were pretreated with 10 μM genistein for 24 h, followed by exposure to 2 Gy X-rays. At 2 h and 12 h post irradiation, the cell suspensions were collected and fixed (methanol:acetone = 3:1), then dripped onto glass slides and allowed to dry. The fixed cells were permeabilized with 0.1% Triton X-100 and subsequently blocked with 5% BSA at room temperature for 1 h. The cells were then incubated overnight at 4°C with primary monoclonal antibodies against Ku70/80 (mouse monoclonal to Ku70/80), Rad51 (rabbit monoclonal to Rad51) and DNA-PKcs (rabbit monoclonal to DNA-PKcs) (Abcam, Cambridge, USA, 1:200 dilution). After primary antibody incubation, the cells were washed with PBST and incubated with donkey anti-mouse IgG-R secondary antibody and donkey anti-rabbit IgG-FITC secondary antibody (Santa Cruz, Dallas, USA, 1:500 dilution) for 1 h at room temperature. Nuclei were counterstained with mounting medium DAPI (1.5 μg/ml, VECTASHIELD Mounting Medium, Vector Lab. Inc., Burlingame, USA). Finally, Ku70/80, DNA-PKcs and Rad51 foci were detected with a laser scanning confocal microscope (LSM 700, Zeiss, Oberkochen, Germany). Mean values of foci were determined by 50 cells.

Statistical analysis

Statistical analysis was performed using either Student's *t*-test (for two-group comparison) or one-way ANOVA (for multiple-group comparison). Data were reported as mean ± S.E.M. Differences with a *P*-value of <0.01 were considered statistically significant.

RESULTS

Toxicity of genistein on S180 cells

In Fig. 1, the cytotoxic effects of genistein on S180 cells are shown. The day of drug exposure was designated Day 0, and cell counting was performed every 24 h for 3 days. Genistein inhibited the proliferation of S180 cells in a time- and concentration-dependent manner. However, when the concentration was <10 μM, genistein had little impact on cell survival. Therefore, the concentration of 10 μM was thought to be a mild dose and selected for examining the radiosensitizing effects of genistein for *in vitro* study.

Genistein increased the radiosensitivity of S180 cells *in vitro*

Cells were pretreated with 10 μM genistein for 24 h and then exposed to X-rays. Three days later, the cell numbers at the various doses were measured. As shown in Fig. 2, the surviving fractions of the cotreated cells decreased significantly, and the sensitizer enhancement ratio (SER) of genistein was 1.45 at 50% cell survival (IC_{50}). Clearly, genistein showed a radiosensitizing effect on mouse sarcoma *in vitro*.

Genistein increased the radiosensitivity of mouse sarcoma in vivo

Figure 3A shows the variation in body weight of the mice with time. One day before irradiation was defined as Day 0. The results indicate

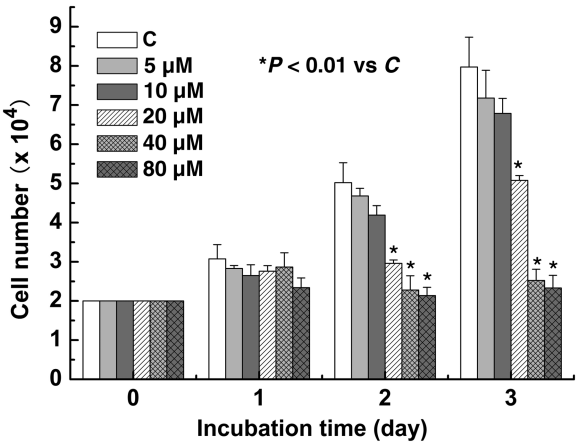

Fig. 1. Toxicity of genistein *in vitro*. Cells in the logarithmic growing period were incubated with genistein at various concentrations and the proliferation of the cells was determined each day by the cell counting assay for 3 days. *$P < 0.01$ versus control group. All data are presented as mean ± S.E.M from three independent experiments.

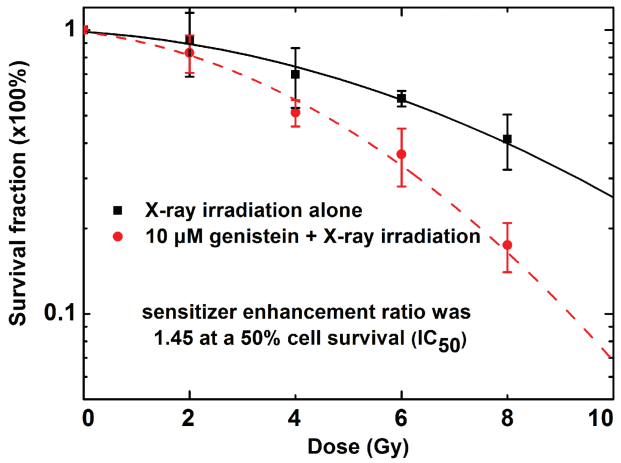

Fig. 2. Sensitization by genistein *in vitro*. Cells in logarithmic growing period were pretreated with 10 μM genistein for 24 h, then treated with X-ray irradiation. After incubation for 3 days, the cell number was counted. All data are presented as mean ± S.E.M from three independent experiments.

that there was a slight weight difference (all <10%) between the ***C*** and ***D*** groups of mice, demonstrating that genistein was well tolerated at the dosage level under investigation. However, there was a significant difference between the ***C*** and ***D+IR*** groups. This was probably related to a loss in tumor weight in the ***D+IR*** group. Figure 3B exhibits typical photographs of the excised sarcomas from all the groups, providing a direct visual representation of the radiosensitizing effect of genistein. Clearly, the tumor tissues in the ***C*** group had an irregular shape, with ill-defined borders and scarlet color. However, in the ***D+IR*** group, the excised tumor shape was more regular and had a pale-red color. Moreover, the weight and volume of the tumor tissues in the ***D+IR*** group were significantly less than those in the other groups ($P < 0.01$), as shown in Fig. 3C and D; significantly smaller tumor volumes (1.8-fold) were observed in the ***D+IR*** group compared with the ***IR*** group (0.38 ± 0.14 cm^3 vs 0.68 ± 0.16 cm^3, $P < 0.01$, Fig. 3D). The results indicate that genistein enhances *in vivo* radiosensitivity in the Balb/c xenograft model.

Next, we performed histological staining of the excised tumors from the mice 24 h after the last irradiation. Representative microphotographs are shown in Fig. 3E. The tissues of the control group revealed a dense population of cells on H&E staining. However, the space between the cells increased and the cell number reduced significantly in the ***D+IR*** group, indicating that genistein combined with X-ray irradiation induced lots of cell death. These results further confirmed that genistein could enhance the radiosensitivity of mouse sarcoma *in vivo*.

X-rays combined with genistein increased S180 cell apoptosis

S180 cells were incubated with 10 μM genistein for 24 h, followed by exposure to 2 Gy X-rays. Figure 4A shows the S180 cell apoptotic rates at 12 h and 24 h post irradiation. The apoptotic rate in the ***D+IR*** group not only showed a highly significant difference from all the other groups ($P < 0.01$), but also increased gradually with time.

In situ cell apoptosis was detected by TUNEL staining. The apoptotic index of the sarcoma cells was defined as the percentage of apoptotic cells out of the total number of cells counted. As shown in Fig. 4B, a large number of TUNEL-positive nuclei were observed, and the cell apoptotic rate was remarkably elevated in the ***D+IR*** group. In particular, the apoptotic index in the combined treatment group increased by 49.4 ± 2.1% relative to that in the ***IR*** group.

X-rays combined with genistein activated the cell apoptosis–associated proteins

Figure 5A and B show the expression levels of several apoptosis-related proteins *in vitro* and *in vivo*, respectively. Both results indicate that X-rays combined with genistein caused a significant increase in the expression of Bax, a decrease in the expression of Bcl-2 in mitochondria, and lots of cytochrome *c* release from the mitochondria into the cytosol. COXIV, a specific marker of mitochondria, was detected in mitochondrial fractions, indicating perfection of mitochondrial preparations.

In addition, as shown in Fig. 5C, from 2–12 h after irradiation, the level of γ-H2AX hardly changed in the combined treatment group, S180 cells with a wild- type of p53 gene [26], while the increased expression of p53 downregulated the Rad51 both *in vitro* and *in vivo*.

X-rays combined with genistein inhibited NHEJ and HR repair pathways

As shown in Fig. 6A, in the irradiation-only group, lots of DNA-PKcs and Ku70/80 were expressed and recruited to DSB site at 2 h post irradiation, but the number of DNA-PKcs and Ku70/80 foci reduced significantly at 12 h post irradiation. In the co-treatment group, whatever 2 h or 12 h post irradiation, the Ku70/80's red fluorescence was strong, but the green fluorescence of the DNA-PKcs foci was weak. In Fig. 6B, the colocalization of Ku70/80 and Rad51 foci in S180 cells is shown. Both Ku70/80 and Rad51 foci were recruited to DSB sites at 2 h post irradiation in irradiation-only cells. However, the number of Ku70/80 and Rad51 foci decreased significantly at 12 h post irradiation. In the co-treatment group, whatever 2 h or 12 h post irradiation, lots of red fluorescence (Ku70/80 foci) was recruited at DSB sites, but the green fluorescence (Rad51) was faint. These results demonstrate that genistein inhibited DNA-PKcs binding to Ku70/80, thereby interfering in the combination of Rad51 with the DSB sites.

To further confirm this phenomenon, we detected the activity of DNA-PKcs. Phosphorylation of DNA-PKcs at S2056 is mediated by DNA-PKcs and is therefore widely used to detect the activation of DNA-PKcs [27, 28]. As observed in Fig. 6C, in the irradiation-only group, although the expression of DNA-PKcs protein hardly altered at any time, the phosphorylated-DNA-PKcs and the expression of Ku70/80 was significantly enhanced at 2 h post irradiation, compared with in the control group. However, in the co-treatment group, both 2h and 12h post irradiation, Ku70/80 was highly expressed, but DNA-PKcs was not activated. These results further indicate that genistein inhibited DSB repair via inactivation of DNA-PKcs, leading to incompleteness of both NHEJ and HR repairs.

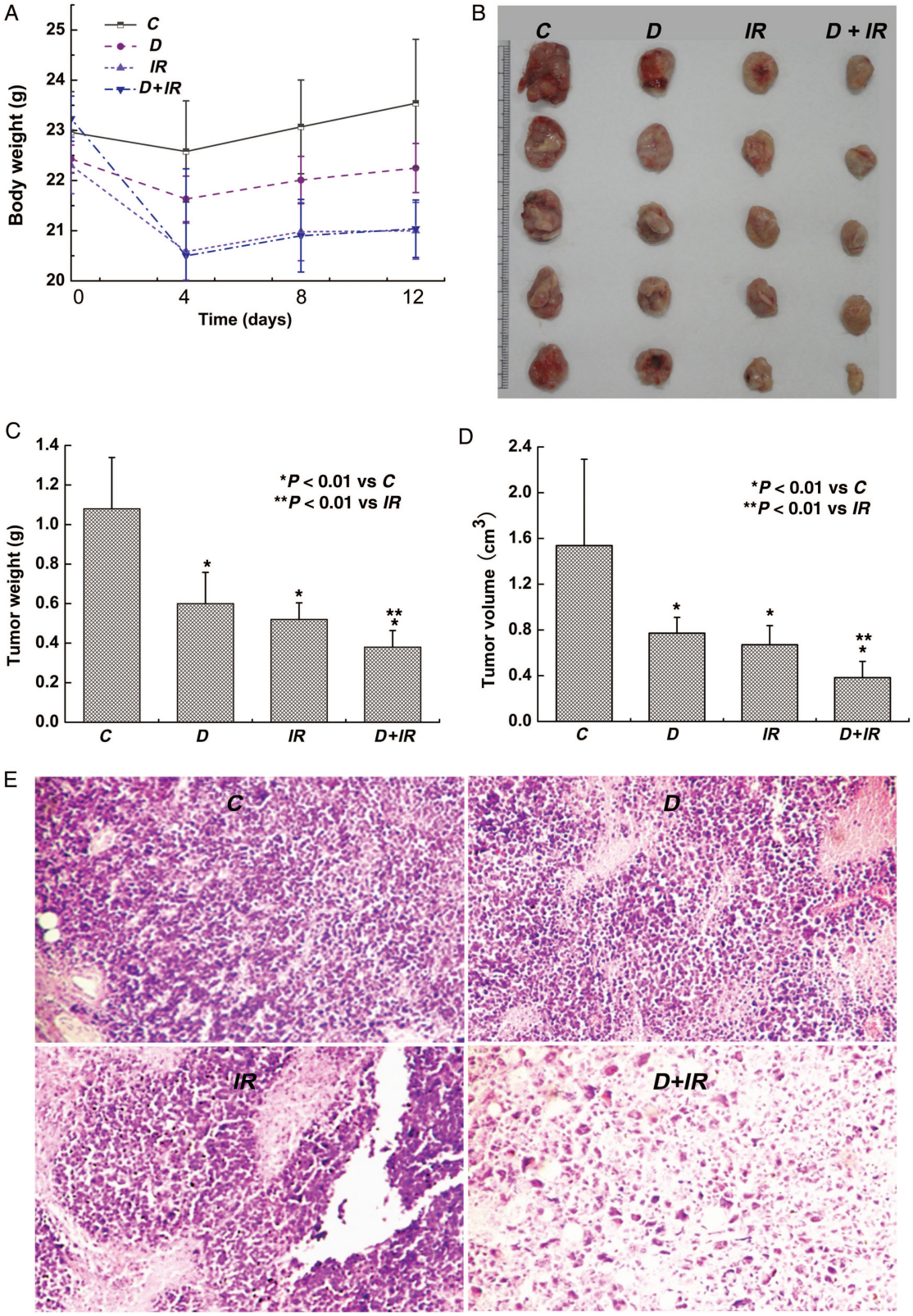

Fig. 3. Sensitization by genistein *in vivo*. Body weight – time profile of the S180 tumor-bearing mice (A) (one day before irradiation was defined as Day 0, etc.). Photograph of the excised tumors (B); weight (C) and volume (D) of the tumor tissues, *$P < 0.01$ versus C group; **$P < 0.01$ versus IR group; each value is expressed as mean ± S.E.M of five animals per group. The tumors were paraffin-sectioned at 4 μm, and the slides were stained with H&E for histopathological analysis (original magnification ×200): representative figures of five mice per group (E).

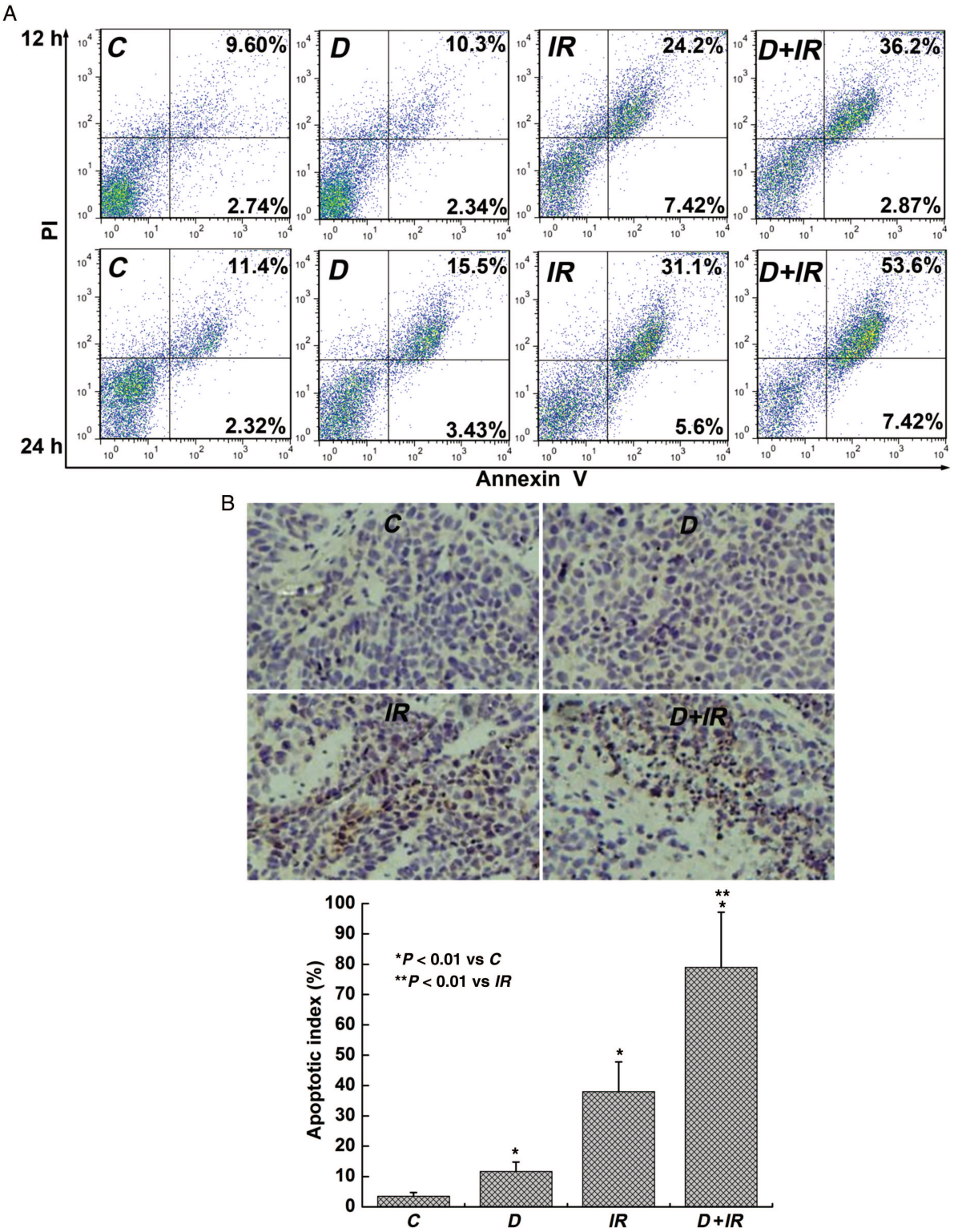

Fig. 4. Apoptosis by genistein *in vitro* and *in vivo* represented by S180 cell apoptotic rates at 12 h and 24 h post irradiation (A). TUNEL-stained histology of mouse sarcoma tissue sections and the apoptotic index in randomly chosen histological fields (B). Each value is expressed as the mean ± S.E.M of five animals per group. *$P < 0.01$ versus *C* group; **$P < 0.01$ versus *IR* group.

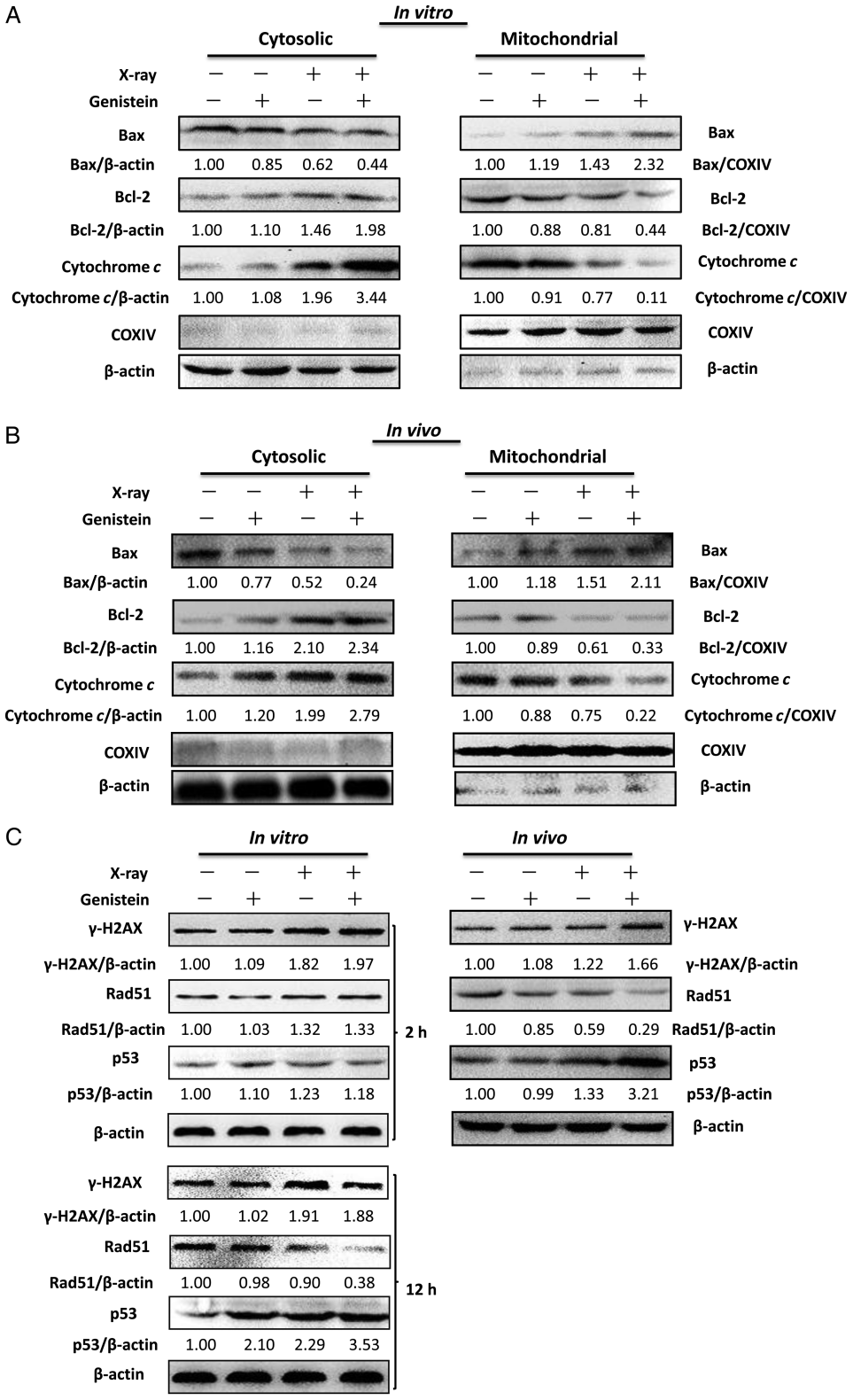

Fig. 5. The expression of apoptosis-related proteins: the Bax, Bcl-2 and cytochrome *c* expressions from the mitochondrial and cytosolic protein extract *in vitro* (A) and *in vivo* (B), and the γ-H2AX, Rad51 and p53 expressions from the whole cell protein extract *in vitro* and *in vivo*, respectively (C). Each blot represents three independent experiments. The β-actin level was used to confirm equal loading of cytosolic proteins, and COXIV was used to confirm equal loading of mitochondrial proteins.

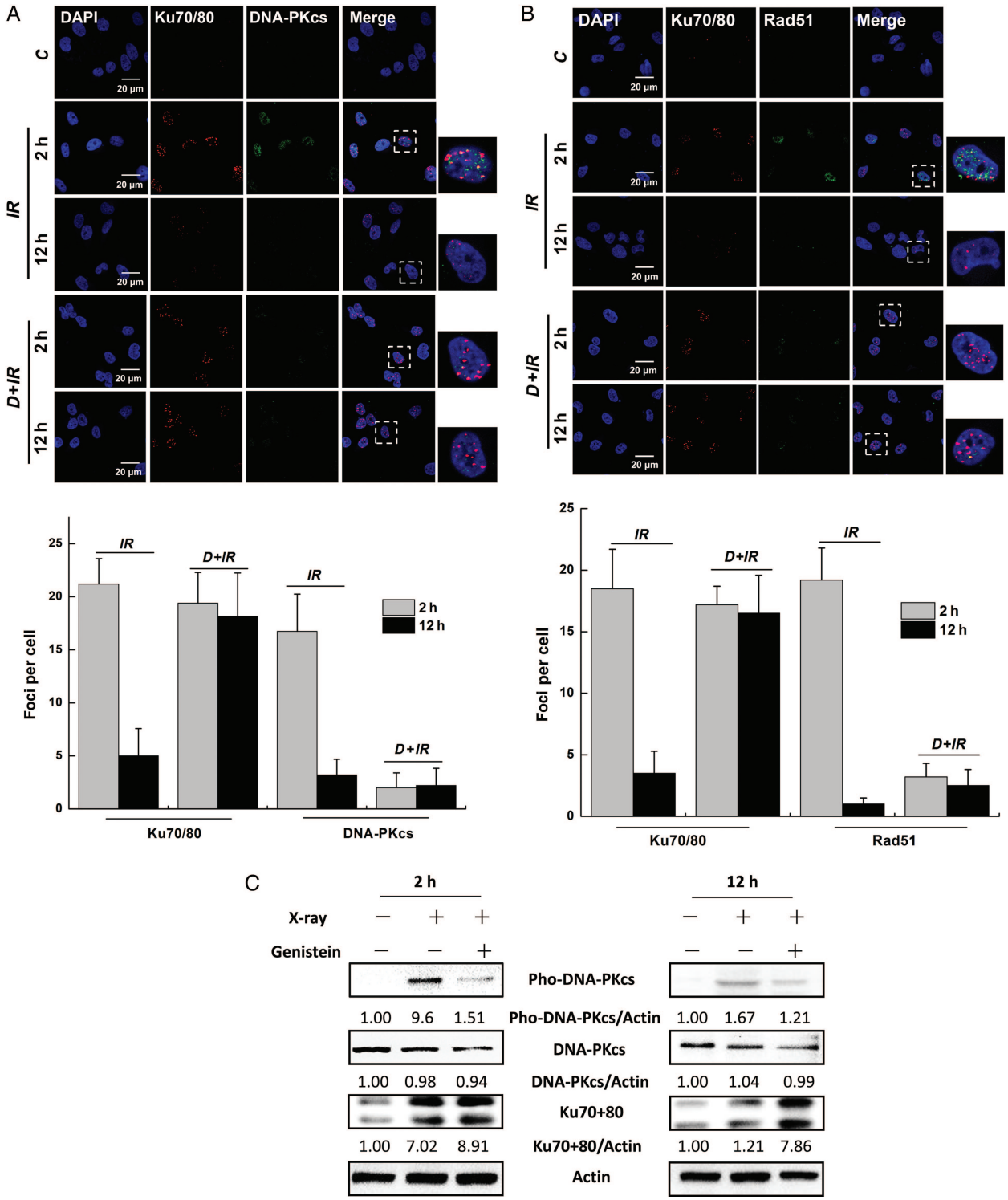

Fig. 6. The NHEJ and HR repair pathways were inhibited by the combined treatment. Cells received irradiation alone or were incubated with genistein for 24 h before irradiation. The confocal microscopy was used to visualize the intracellular colocalization of Ku70/80 red fluorescence and DNA-PKcs green fluorescence (A), the distribution of Ku70/80 red fluorescence and Rad51 green fluorescence (B), and the expressions of pho-DNA-PKcs, DNA-PKcs and Ku70+80 protein (C).

DISCUSSION

Overcoming tumor radioresistance is one of the major challenges for radiation biologists and oncologists. Much research has been done in order to solve this problem, including development of tumor-specific radiosensitizers. One of the ideal outcomes for developing radiosensitizers would be to specifically sensitize tumor cells while protecting normal tissues.

In this context, natural compounds acting as radiosensitizers have been preferred. Previous studies have shown that genistein can sensitize cancer cells to radiation. Hillman *et al.* found that the combination of genistein and radiation showed an enhanced inhibitory effect on DNA synthesis in and growth of prostate cancer cells [29]. Furthermore, they found that radiation combined with genistein caused greater inhibition in PC-3 colony formation and increased cancer cell death (leading to greater control of the primary tumor and of metastasis to lymph nodes than either genistein or radiation alone), suggesting that genistein enhanced the radiosensitivity of PC-3 prostate cancer cells [30–32]. A similar report revealed that genistein enhanced the radiosensitivity of cervical cancer cells through increased apoptosis, prolonged cell cycle arrest and impaired repair of DNA damage [33]. Additionally, genistein has also been shown to enhance the radiosensitivity of human breast and esophageal cancer cells *in vitro* [22, 34]. In this study, the radiosensitizing effect of genistein, for the first time, was investigated in mouse sarcoma *in vitro* and *in vivo*. As shown in Fig. 1, the concentration of genistein at 10 μM could be considered a mild dose at which genistein had no toxicity to S180 cells *in vitro*. The sensitizer enhancement ratio (SER) of 10 μM genistein for S180 cells exposed to X-rays at 50% cell survival (IC_{50}) was derived to be 1.45 from the *in vitro* experiments (Fig. 2). In the ***C*** group of S180 tumor-bearing Balb/c mice, the excised tumors received sufficient blood supply and their growths were not suppressed at all. However, in the ***D+IR*** group, the tumor color was palered as a consequence of an obvious suppression to the tumor vascular system (Fig. 3B); the excised tumors not only had a markedly smaller size and a more regular shape, but also their weight (Fig. 3C) and volume (Fig. 3E) decreased significantly. Moreover, the tissue sections with H&E staining showed much higher apoptotic rates in the ***D+IR*** group than in the ***C*** and ***D*** groups (Fig. 3E). These data definitely indicate that genistein promoted the radiosensitivity of mouse sarcoma *in vitro* and *in vivo*.

In this study, we confirmed that genistein increased either radiation-induced apoptosis of S180 cells *in vitro* (Fig. 4A) or the percentage of TUNEL positive cells *in vivo* (Fig. 4B). Therefore, the radiosensitizing effect of genistein on sarcoma cells was linked with the increased apoptotic rates observed in the *in vitro* and *in vivo* experiments. We then focused on potential molecular mechanisms for the radiosensitizing effect of genistein on sarcoma cells in terms of apoptosis. Previous studies showed that Bax is a monomeric and predominantly cytosolic protein in non-stressed cells [35]. Mitochondrial dysfunction induces Bax translocation from the cytosol to the mitochondria, and cytochrome *c* release from the mitochondria is a critical event that occurs during apoptotic processes [36, 37]. Our data reveal that Bax expression was upregulated significantly, whereas Bcl-2 was downregulated in the mitochondria, which induced a change in the mitochondrial outer membrane permeability and thus cytochrome *c* release to the cytoplasm in the ***D+IR*** group (Fig. 5A and B) and subsequent activation of cell apoptosis.

It is well known that cancer radiosensitivity is mainly determined by the ability of cancer cells to repair radiation-induced DNA damages. Sugimoto *et al.* reported that osteosarcoma cells have strong DNA repair systems to resist DNA damage [38]. Therefore, we hypothesized that genistein might sensitize S180 cells by affecting the DSB repair machinery. γ-H2AX is a key marker for DSB lesions and is routinely used to evaluate DNA DSBs repair activities [39]. We observed that X-rays combined with genistein induced the expression of more γ-H2AX, but that Rad51 was downregulated by a negative feedback role of p53 both *in vitro* and *in vivo* (Fig. 5C). These results indicate that the HR repair was partially inhibited by the co-treatment, whereas the cell damage persisted, resulting in the ultimate occurrence of cell apoptosis.

In addition, cells lacking DNA-PKcs or Ku are highly sensitive to the killing effect of ionizing radiation (IR). Since DNA-PKcs is only active when assembled at a DSB site, it is reasonable to assume that its physiological targets may also be present at, or recruited to, the DSB site. Previous studies have reported that small interfering RNA (siRNA) to DNA-PKcs increases the sensitivity of human cancer cells to IR, inhibiting DNA-PKcs activity and abrogating DNA DSB repair [40, 41]. In another study, transfection of a peptide corresponding to the carboxy-terminal DNA-PKcs-binding region of Ku80 was shown to inhibit DNA-PKcs activity, reducing DSB repair, and sensitizing breast cancer cell lines [42]. In the present study, as shown in Fig. 6A, in the irradiation-only group, lots of DNA-PKcs and Ku70/80 were recruited to the DSB site at 2 h post irradiation. Moreover, the phosphorylated-DNA-PKcs and the expression of Ku70/80 increased significantly at 2 h post irradiation, while reducing noticeably at 12 h post irradiation (Fig. 6C). These results indicate that the damaged DNA was repaired at 2 h post irradiation. However, in the co-treatment group, both 2h and 12h post irradiation, the Ku70/80's red fluorescence was strong, but the green fluorescence of DNA-PKcs foci was weak and the activity of DNA-PKcs was inhibited (Fig. 6C), suggesting that genistein inhibited the ability of DNA-PKcs to bind to Ku70/80, thus failing to form a DNA-PKcs-Ku70/80 complex at the DSB site, and thereby interfering with the NHEJ repair pathway. In addition, as shown in Fig. 6B, both Ku70/80 and Rad51 foci were recruited to the DSB site at 2 h post irradiation in the irradiation-only cells, demonstrating that the damaged DNA was repaired by HR, together with the NHEJ repair pathway. However, Ku70/80 and Rad51 foci decreased significantly at 12 h post irradiation. This indicates that the repair of damaged DNA had been completed. In the co-treatment group, both 2h and 12h post-irradiation, lots of Ku70/80 were expressed (Fig. 6C) and red fluorescence (Ku70/80 foci) was recruited to the DSB site, but green fluorescence (Rad51) was faint. These results imply that the damaged DNA ends were dominated by Ku70/80 for a long time and that Rad51 could not be recruited to the DSB site, leading to incompleteness of the NHEJ and HR repairs and the eventual occurrence of cell apoptosis.

CONCLUSION

Our study, for the first time, demonstrates that genistein enhanced the radiosensitivity of mouse sarcoma *in vitro* and *in vivo* and that this radiosensitizing effect was dependent on induction of the mitochondrial apoptosis pathway and inhibition of both the DSB repair

pathways. Genistein might thus be used as a potential radiosensitizer in radiotherapy for treatment of radioresistant sarcoma.

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