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Mangiferin Aglycone Attenuates Radiation-Induced Damage on Human Intestinal Epithelial Cells

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ABSTRACT

Recent studies suggest that mangiferin aglycone (norathyriol) has great potential as a novel radioprotector without any known toxic side effects. In this study, we assessed the protective effects of mangiferin aglycone against radiation-induced injuries on normal human intestinal epithelial cells (HIECs), while using mangiferin as a reference compound. The in vitro experiments showed that pretreatment of either mangiferin aglycone or mangiferin could inhibit cytotoxic effects of ionizing irradiation (IR) on HIECs. Cellular changes were estimated by measuring cell viability, clonogenic surviving rate, and apoptotic rate. Compared to mangiferin, we found mangiferin aglycone had greater radioprotective effects of mangiferin aglycone on HIECs. It has been demonstrated that the cytotoxicity of ionizing radiation relates to its capacity to induce DNA damage. In view of this, we monitored DNA double-strand breaks (DSBs) using γH2AX foci formation to test whether mangiferin aglycone and mangiferin could modulate genotoxic effects of radiation. It shows that mangiferin aglycone could eliminate 46.8% of the total DSBs of the cells exposed to 2 Gy IR, which is significantly better than mangiferin. Complementing earlier results from our group, it appears possible to conclude that mangiferin aglycone presents potential useful effects on IR-induced damage and may be a better radioprotective agent than mangiferin therapeutically. J. Cell. Biochem. 113: 2633–2642, 2012.

KEY WORDS: HIECS; MANGIFERIN; MANGIFERIN AGLYCONE; RADIOPROTECTION; γH2AX

Damage to normal cells and tissues is a consequence of both therapeutic and accidental exposures to ionizing radiation, such as radiation therapy, X-ray imaging, or radiation exposure from nuclear power plants accidents. Total body radiation exposure could result in death due to severe hematopoietic malfunction, intestinal damage, and central nervous system damage. Various damage to living organs or cells could be induced by radiation either by a direct way or an indirect way. Directly, it transfers energy to the biomolecules. While indirectly, it reacts with diffusible water [O'Neill and Fielden, 1993] and generates reactive oxygen species (ROS) during the radiolysis of water. ROS, such as hydrogen peroxide, superoxide anions and hydroxyl radicals, could react with most cellular macromolecules, including DNA, lipids, and proteins [Anderson et al., 2001; Bianchini et al., 2001]. It is known that hydroxyl radicals attack DNA resulting in single-strand breaks (SSBs), double-strand breaks (DSBs), and oxidative damage to sugar and base residues, which later could be converted to strand breaks [Wallace, 1988]. Among all these deleterious effects of radiation, DSBs is the predominant cause of cell death which will result in a diversity of cell responses, such as chromosomal aberrations leading to genomic instability, cell cycle arrest, apoptosis, and mutation [Natarajan et al., 1986]. γH2AX foci formation has gained attention for its relationship with DNA damage, particularly double strand breaks. It is widely used as a method for measuring the induction and

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Jixiao Lei and Chuanfeng Zhou contributed equally to this work.

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rejoining of DNA DSBs (Sedelnikova et al., 2002; Rothkamm and Lobrich, 2003; Daniel et al., 2004). Using this method, we mainly investigated the formation of DNA DSBs induced by ionizing radiation and its subsequent repair in human intestinal epithelial cells (HIECs) after different treatments.

Much attention has been paid to different radioprotective agents and their underlying mechanisms. Mangiferin (MGN), a xanthone glucoside, purified from the stem bark of *Mangifera indica L.*, shows a wide variety of biological activities, such as antioxidant, antiinflammatory, antiviral analgesic, and immunomodulatory properties [Rouillard et al., 1998; Sánchez et al., 2000; García et al., 2002]. Aglycone (norathyriol), one of the derivatives of mangiferin, was also tested in vitro as an inhibitor of the formylmethionylleucyl-phenylalanine induced respiratory burst in rat neutrophils [Hsu et al., 1997]. Due to presence of a phenolic group with an aromatic conjugation in mangiferin, Professor Yoshimi's study shows that mangiferin has a potent activity for scavenging hydroxyl radicals (·OH) and hypochlorous acid, a significant inhibitory effect on the peroxidation of rat brain phospholipids [Yoshimi et al., 2001]. The study of plant extracts and phytochemicals as modifiers of radiation effects is a new area of research. Some reports are available regarding the protective effect of mangiferin against radiation induced cellular damage in animal systems. It was reported that mangiferin could increase the survival of animals exposed to different doses of γ-radiation and reduce radiation-induced micronuclei formation and initial DNA damage in the HPBLs [Jagetia and Baliga, 2005]. However, the radioprotective effect of mangiferin aglycone, which can be artificially synthesized, has not been investigated. In our previous study, mangiferin aglycone shows a better effect than mangiferin in some aspects which was supported by Hsu MF's research. They reported mangiferin aglycone could reduce intestinal neoplasms in rats [Hsu et al., 1997], therefore, it may have special protective sensitivity on intestinal cells. Testis was chosen to study the underlying radioprotective mechanism on HIECs. No work has been carried out on its role on protecting cells against radiation induced DNA DSBs. Taking all above into account, the present study investigated the radioprotective effects of mangiferin and mangiferin aglycone on cultured HIECs by detecting radiation-induced DSBs as well as the survival of target cells exposed to various doses of γ-radiation.

METHODS AND MATERIALS

REAGENTS

Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan), Annexin V-FITC Kit, propidium iodide (PI), foetal calf serum (FCS), minimum essential Medium (DMEM), dimethyl sulfoxide (DMSO), EtOH, methylene blue, 4,6-diamidino-2-phenylindole (DAPI), were purchased from Sigma (St. Louis, MO). α-γH2AX rat monoclonal antibody (Upstate, Lake Placed, NY), GAM 488 goat-anti-rat antibody (Molecular Probes, Leiden, The Netherlands), goat blocking serum were obtained from (Invitrogen, Grand Island, NY), and other analytical reagents such as Giemsa dye, Triton X-100, acetone, and methanol. All the used chemical reagents were of analytical purity from commercial sources.

CELL CULTURES AND IRRADIATION PROCEDURE

Human intestinal epithelial cells (HIECs) were obtained from ATCC (CRL-1592) and maintained in DMEM with 1.5 g/L sodium bicarbonate 10% foetal bovine serum, 1% penicillin/streptomycin (Life Technologies, Gaithersburg, MD) and were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Exponentially growing HIECs were treated with mangiferin aglycone or mangiferin prior exposure to ^{60}Co γ-rays with a delivery rate of 2 Gy/min in the irradiation (IR) center (Faculty of Naval Medicine, Second Military Medical University, China), and then exposed to different radiation doses to assess their radioprotective effects and the best radioprotective dose.

PREPARATION OF DRUGS

Mangiferin aglycone and mangiferin (synthesized by College of Pharmaceutical Sciences, Second Military Medical University, China) [Hu et al., 2007] were dissolved at a concentration of 10 mg/ml in DMSO when it was used. This stock was furtherly diluted within double distilled water (DDW) or diluted by DMEM medium as it was used. DMSO was used as vehicle control.

EXPERIMENTAL DESIGN

A fixed number (5×10^5) of HIECs were inoculated into several individual culture flasks and cultured. We selected the optimum protective dose and compared radioprotective effects of mangiferin aglycone with mangiferin by dividing the cells into following groups:

1. Control group: Cells were treated with 0.5% DMSO before gamma IR.
2. IR-alone group: Cells were exposured to various doses (0–16 Gy) of gamma-radiation and served as a negative control.
3. The mangiferin aglycone/mangiferin-alone group: Cells were treated with various concentrations (0–100 μg/ml) of mangiferin aglycone or mangiferin.
4. The mangiferin aglycone/mangiferin + IR group: Cells of this group were treated with different concentrations (0–100 μg/ml) of mangiferin aglycone or mangiferin for 1.5 h before exposured to radiation.

Final DMSO concentrations of all groups were significantly $<1\%$. For untreated controls, 0.5% DMSO was used. Cells were treated with the inhibitors 1.5 h prior to and during IR treatment. Cells from above groups were dislodged by mild trypsin EDTA treatment, and all the following assays were carried out using the same stock of cells.

HYDROXYPHENYL FLUORESCEIN IDENTIFIES ·OH

To detect ·OH that mangiferin aglycone or mangiferin reduces, we produced ·OH by radiolysis of H_2O. We exposured DDW with or without drugs to ^{60}Co γ-rays with a delivery rate of 2 Gy/min in the IR center. The process produced free radicals such as ·OH, atoms or molecules containing unpaired electrons. Levels of ·OH in cell-free systems was semiquantified by probe hydroxyphenyl fluorescein 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF; Daiichi Pure Chemicals, Tokyo, Japan) [Setsukinai et al., 2003] in

which the non-fluorescent HPF are specifically oxidized by $^{\bullet}OH$ and turned into strongly fluorescent. Fluorescence images were acquired under fluorescent microscopy to represent the production of $^{\bullet}OH$. Fluorescence were determined at wavelength 515 nm with excitation at 490 nm and the intensities of tests were compared between groups [Tomizawa et al., 2008].

γH2AX FOCI FORMATION

This experiment was conducted according to description of Sedelnikova OA (Sedelnikova, 2008). In short, 1×10^5 cells were seeded into 6-well culture plate containing a glass cover slip in each well and the cells were then pre-incubated with various concentration of drugs 1.5 h before exposed to IR. The control group was sham-irradiated and the irradiated group was treated in the same way. After IR the cells were allowed a recovery period (30 min ~ 8 h) prior to further preparation for immunocytochemistry as previously described.

Cells were fixed in 4% paraformaldehyde for 15 min, washed in PBS, permeabilized for 5 min on ice in 0.2% Triton X-100, and blocked with blocking serum for 1.5 h at room temperature. After that, samples were incubated with a mouse monoclonal anti-γH2AX antibody (1:100) over night at 4°C, followed with FITC-conjugated goat-anti-mouse secondary antibody (1:200) for 2 h. To stain the nuclei, DAPI was added to the cells and incubated for another 15 min. The coverslip was then mounted with 20 ml mounting medium to a slide and sealed with nail polish for γH2AX foci observation using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan). To prevent bias in the selection of cells that display foci, all cells were counted in the field of vision (at least 50 cells). Image Pro Plus (Media Cybernetics, Silver Springs, MD) was used to count the γH2AX foci in each cell, so that all of the visible foci and bands in a nucleus or mitotic figure were recorded. In addition, to exclude relatively weak foci and background spots, we used a setting as a standard for quantification in all the cells selected for analysis [Daniel et al., 2004].

FITC–ANNEXIN V STAINING

As one of the most reliable early apoptotic markers, the externalization of phosphatidylserine was detected in HIECs using Annexin V-FITC Kit (Sigma–Aldrich, St Louis, MO) to differentiate between viable apoptotic and necrotic cells. Briefly, a fixed number of 10^5 treated cells were incubated with Annexin V-FITC Kit and PI for 15 min at room temperature. Cells were then analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ), with CELLQUEST software and were further analyzed with the model fitting software (Mod-Fit LT 2.0, Becton Dickinson). For each treatment, the average fold-increase of apoptotic cells over control (±SEM) was calculated.

CCK-8 ASSAY

The viability of cells under the influence of mangiferin aglycone or mangiferin on IR was measured by the WST assay using a Cell Counting kit-8 (CCK-8). HIECs cultured in a 96-well plate were treated with various concentrations (0–100 μg/ml) of mangiferin aglycone or mangiferin and then exposed to 8 Gy gamma radiation and further incubated for 48 h. A separate experiment was carried out to study the radioprotective effects of mangiferin aglycone or mangiferin, HIECs were treated with 70 μg/ml of both compounds for 1.5 h before exposure to different doses (0, 4, 8, 12, or 16 Gy) of gamma radiation and the cells were allowed to grow for 48 h. Immediately after IR treatment, the media containing the chemical drugs was discarded and replaced with fresh medium. The modified methodologies for this entire assay were described in detail in an earlier paper [Satish Rao et al., 2009].

CLONOGENIC SURVIVAL

The colony-forming assay was performed as previously described [Yazlovitskaya et al., 2006]. Briefly, calculated numbers of cells were plated to enable normalization for plating efficiencies. The cultured cells were pretreated with various concentrations of DMSO, mangiferin aglycone or mangiferin (0–100 μg/ml) for 1.5 h before exposure 8 Gy gamma IR. A separate experiment was carried out to study its radioprotective effect, the cultures of this group were treated with 70 μg/ml of mangiferin aglycone or mangiferin 1.5 h before exposure to different doses (4–16 Gy) of gamma radiation. After 7 to 10 days incubation plates were fixed with 70% EtOH and stained with 1% methylene blue. Colonies consisting of >50 cells were counted under microscope. The survival fractions were calculated as

$$\frac{(\text{number of colonies}/\text{number of cells plated})}{(\text{number of colonies for corresponding control}/\text{number of cells plated})}$$

STATISTICAL ANALYSES

The mean and standard error of the mean (SEM) for each treatment group was calculated for all experiments. Statistical analysis was performed using Kruskal–Wallis one way analysis of variance (ANOVA). All pairwise comparison procedures including calculations of P values were done using the Student–Newman–Keuls method. A P value of <0.05 was considered to be statistically significant.

RESULTS

HYDROXYL-RADICAL SCAVENGING ACTIVITY

To verify that mangiferin aglycone and mangiferin can protect against $^{\bullet}OH$ induced by IR, we assessed the accumulation of $^{\bullet}OH$ by fluorescence signal emitted by the oxidized form of HPF. We compared the fluorescence intensities of the mangiferin aglycone group in different concentrations (1–10 μg/ml) with the mangiferin group at 4 Gy HPF was given 20 min before IR Mangiferin aglycone or mangiferin was given 1.5 h before IR. The fluorescence of mangiferin aglycone group and mangiferin group were both significantly less than that of control group. Figure 1 shows that fluorescence signals of the oxidized HPF were reduced by these two compounds at all doses and a maximum reduction was observed at 7 μg/ml. However, there was no significant difference between them. While the concentration reached 10 μg/ml fluorescence signals reduced almost to the same level of $^{\bullet}OH$ at 7 μg/ml.

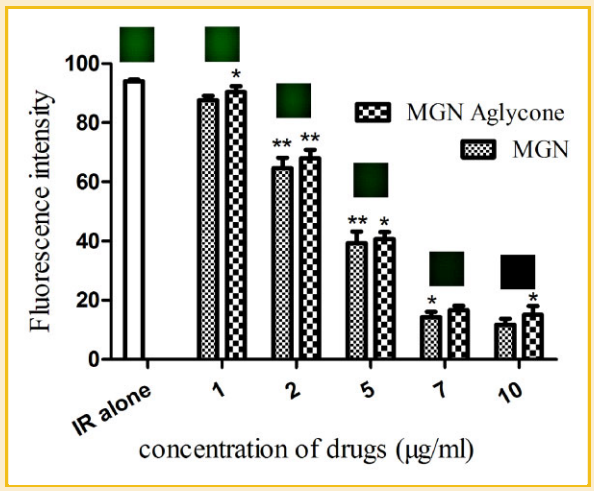

Fig. 1. Mangiferin aglycone scavenges the ·OH generated by radiolysis of H_2O using HPF. Relation between different concentrations (0–10 μg/ml) of mangiferin or mangiferin aglycone and fluorescence increase in radiolysis. HPF probe (final 10 μM, 0.1% DMF as a cosolvent) were added tophosphate buffers solution at 23°C and pH 7.4 in cell-free systems and then mangiferin or mangiferin aglycone with different concentrations were added in the system, treated with 4 Gy γ-radiation. Fluorescent images were obtained with a fluorescent microscope. The fluorescence intensities were semiquantified from the testes of each independent groups. At least six replicates were performed. Values are presented as means ± SEM of fluorescent intensity in relation to IR-alone group. The significant levels $^{*}P$, 0.05, $^{**}P$, 0.01, $^{***}P$, 0.001, and no symbol = non-significant, when compared with IR-alone group.

DSBS CAUSED BY IR

While exposed to ionizing radiation or other stimuli in response to DNA DSBs, thousands of H2AX molecules would be phosphorylated (termed γH2AX) and forms "foci" which can be later detected by immunofluorescence. We investigated the relationship between radiation dose(ranging from 0 to 4 Gy) and γH2AX focus formation in HIECs. In all cases, the DNA damage of control cells was constant, indicating that preparation and subsequent processing of the HIECs did not introduce significant damage to cellular DNA, as shown in Figure 2E. When cells were exposed to 1 Gy, the image of γH2AX foci was detectable ranging from 0 to 4 Gy, there was a linear relationship between doses and the γH2AX signals (Fig. 2A). For immunocytochemistry, we selected foci per cell as the primary endpoint because this corresponded well with DSBs per cell [Rothkamm and Lobrich, 2003].

Double-strand breaks are generally accepted to be the most biologically significant lesion caused by ionizing radiation. γH2AX foci formation in HIECs provide a good measure of IR exposure with clear reproducibility and high sensitivity. Changes in H2AX phosphorylation after combining radiation and drug therapy were readily detected using immunofluorescence. Within 30 min after IR, formation of γH2AX foci occurred and the formation of γH2AX was also dose-dependent (Fig. 2B and D). Compared to IR-alone group, fluorescence signals of cells pre-treated with mangiferin aglycone before exposed to 1, 2 and 4 Gy IR reduced by 41.3%, 46.8%, and 43.4% relatively, while the fluorescence signals of cells pretreated with mangiferin reduced by 36.2%, 40%, and 37.5%. Figure 2C showed the kinetics of γH2AX foci loss in different treated HIECs after 4 Gy IR. The numbers of γH2AX foci are known to decrease with increasing time post-exposure and they decreased to one-half of their maximum about 2 h post-IR. Cells pre-treated mangiferin aglycone or mangiferin significantly ($P < 0.05$) slowed the formation of γH2AX foci within 2 h post-IR. Pre-treatmeant with mangiferin aglycone or mangiferin significantly ($P < 0.05$) reduced the formation of foci per cell compared with IR-alone group and Figure 2D–F showed the images of some typical cases.

APOPTOSIS OF HIECS

To assess the type of cell death induced by gamma radiation, HIECs were stained by Annexin V-FITC and PI, and apoptotic rate of HIECs were assessed by flow cytometry. AnnexinV-FITC-positive cells representing apoptosis in lower right quadrant were not significantly increased either in the mangiferin aglycone-alone treated groups or mangiferin-alone treated groups in contrast to the control group (Fig. 3A). However, in the IR-alone group, the apoptotic cells increased to 21.85%, while the AnnexinV-FITC-positive/PI-positive cells which represent necrosis and late apoptotic cells in upper right quadrant increased to 2.64% (Fig. 3B). As shown in Figure 3B, pretreatment with 70 μg/ml mangiferin aglycone or mangiferin prior to radiation either the apoptotic or the necrosis cells were reduced. Compared to IR-alone group, late apoptosis of the group pretreated with mangiferin aglycone or mangiferin reduced by 65.4% and 57.39%, respectively. These data suggest that radioprotective effect of mangiferin aglycone or mangiferin in HIECs is due to the attenuation of radiation-induced apoptosis.

CELL VIABILITY OF HIECS

Cultured with different concentrations of mangiferin aglycone or mangiferin did not alter the cell viability of HIECs at 24, 48, and 72 h significantly ($P > 0.1$), whereas cell viability decreased after exposed to radiation as expected. As shown in Figure 4A, the cell viability (CCK-8 assay) of 8 Gy IR-alone group was only 33%. Treatment of HIECs with various concentrations of mangiferin aglycone or mangiferin (0–100 μg/ml) before exposure to 8 Gy gamma radiation resulted in a gradual increase in the cell viability (WST formazan formation). When treated with 50, 70, and 100 μg/ml, mangiferin cell viability were 73.17%, 73.2%, and 70.23%, respectively (Fig. 4A). While the best radioprotective effect of mangiferin aglycone was found at 70 μg/ml with a maximum cell viability of 78.3%, significantly higher than others.

The radioprotective effect of the two drugs is dose-dependent. Pretreatment with either mangiferin aglycone or mangiferin significantly ($P < 0.05$) increased cell viability compared to IR-alone group (Fig. 4B). A significant elevation of cell viability (1.52-, 2.37-, 2.41-, and 2.40-fold) was observed in mangiferin aglycone-pretreated groups in comparison with IR-alone groups (4, 8, 12 and 16 Gy). This radioprotection effect of mangiferin aglycone was comparable with mangiferin at doses ranging from 4 to 16 Gy, while at the dose of 8 Gy mangiferin aglycone demonstrated significantly ($P < 0.05$) better protection on HIECs.

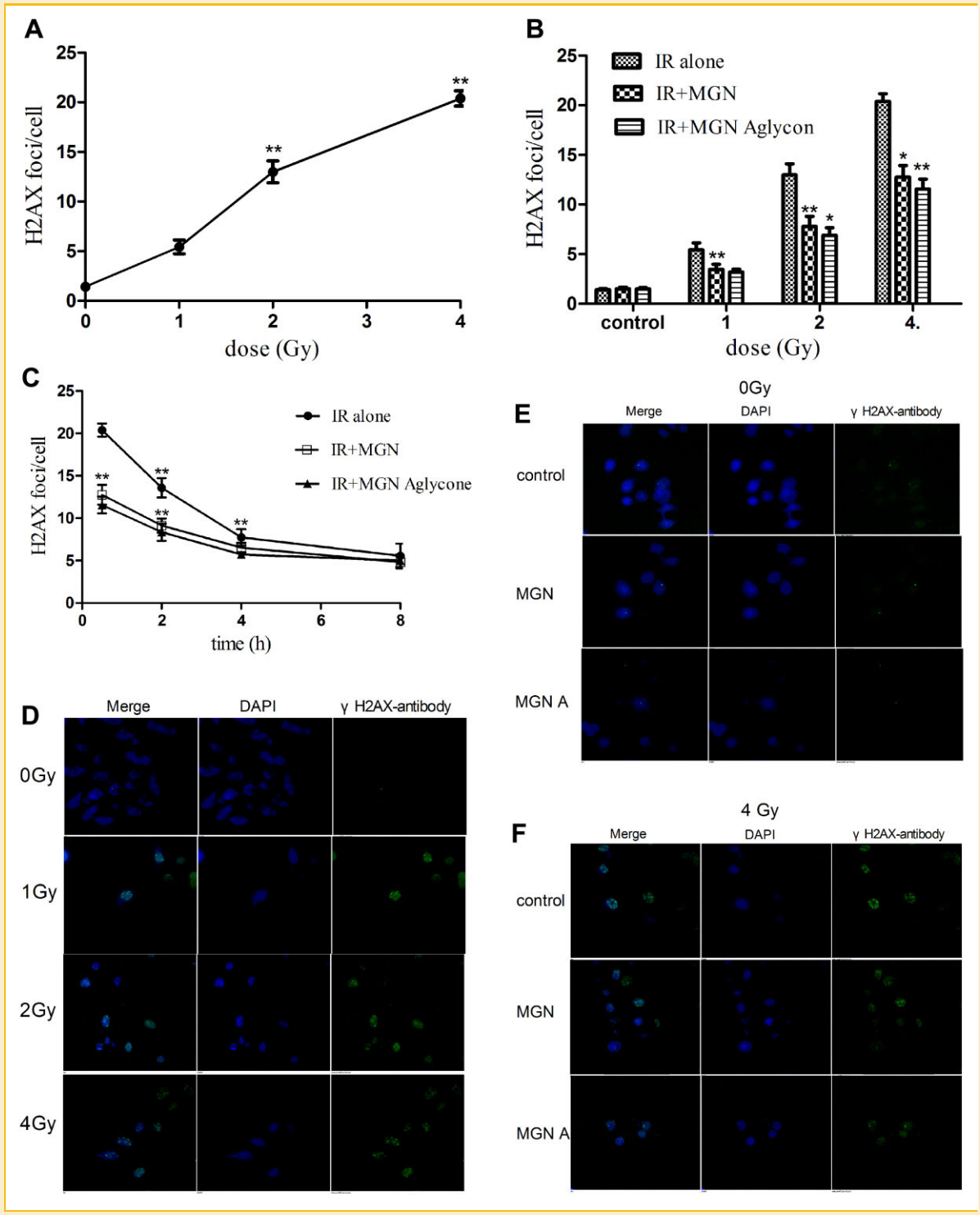

Fig. 2. Mangiferin aglycone attenuates the number of γH2AX foci caused by IR. A,B: HIECs were incubated with 0.5% DMSO, 70 μg/ml mangiferin or mangiferin aglycone for 1.5 h before exposure to different doses of gamma radiation. Cells were purified 30 min post-IR, stained for γH2AX detection and then the mean number of γH2AX foci per cell (foci/cell) were counted. Values are shown as mean SEM from the data of a minimum of three separate experiments. The significant levels $^{*}P$, 0.05, $^{**}P$, 0.01, $^{***}P$, 0.001, and no symbol = non-significant, when compared with IR-alone group. C: Kinetics of γH2AX foci loss in different groups of cells after 4 Gy gamma radiation and incubated at 37°C. At indicated times HIECs were stained for γH2AX detection and then the mean number of γH2AX foci per cell (foci/cell) were counted. D: Representative laser-scanning confocal images of the fluorescence of the γH2AX foci distribution in the HIECs were taken 30 min after exposure to 0, 1, 2, and 4 Gy. Green, γH2AX; blue, DNA stained with PI. The two images were superimposed (merge). E: Representative laser-scanning confocal images of the fluorescence of the γH2AX foci distribution in the HIECs after treatment with 0.5% DMSO, 70 μg/ml mangiferin or mangiferin aglycone for 1.5 h. Green, γH2AX; blue, DNA stained with PI. The two images were superimposed (merge). F: Representative laser-scanning confocal images of the fluorescence of the γH2AX foci distribution in the HIECs after treatment with 0.5% DMSO, 70 μg/ml mangiferin or mangiferin aglycone for 1.5 h before exposure to 4 Gy. Green, γH2AX; blue, DNA stained with PI. The two images were superimposed (merge).

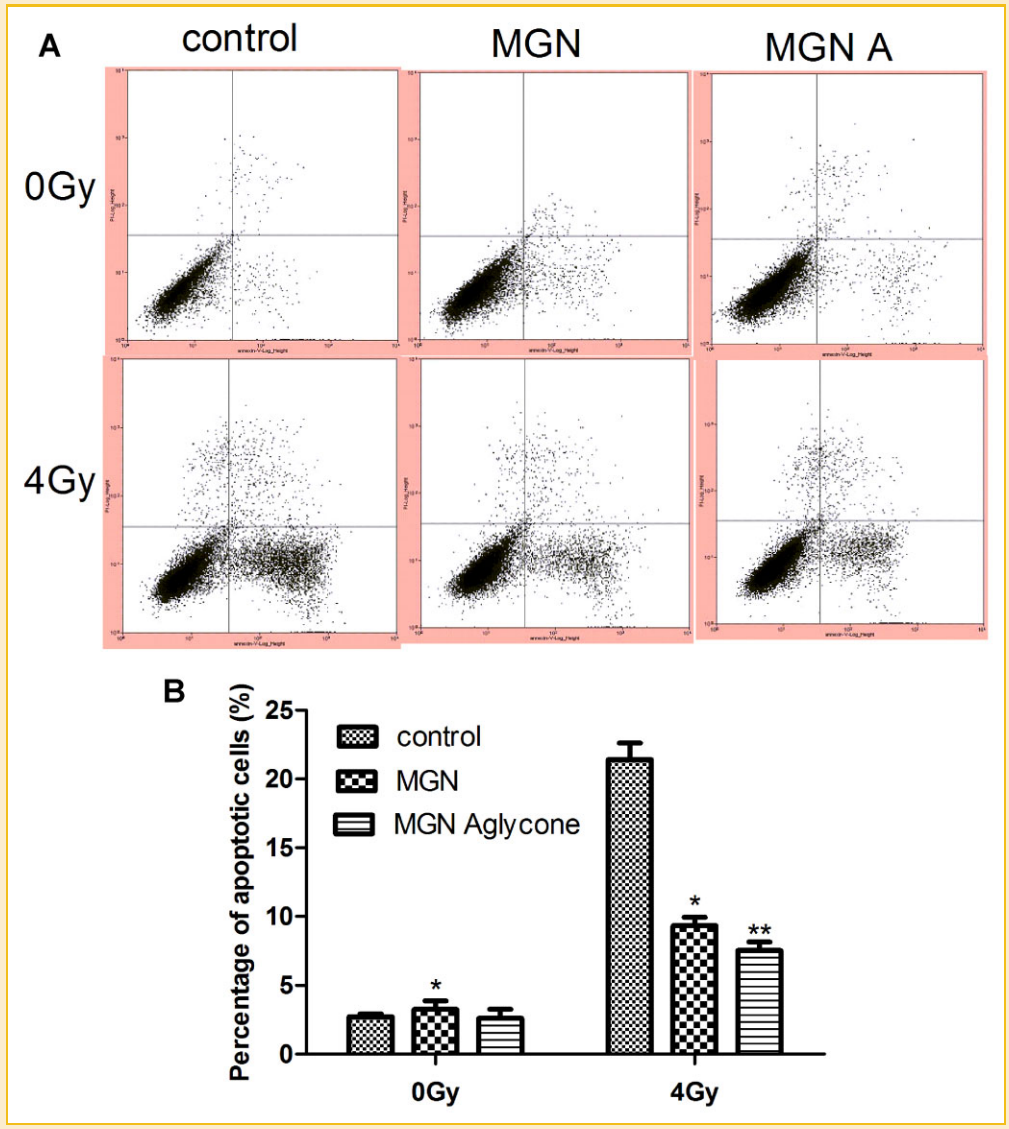

Fig. 3. Mangiferin aglycone reduces apoptosis of HIECs against IR. A,B: HIECs were treated with 0.5% DMSO, 70 μg/ml mangiferin or mangiferin aglycone 1.5 h prior to IR with 4 Gy. Cells were collected 24 h after IR, stained with Annexin V-APC and PI and analyzed by flow cytometry. Shown are representative diagrams of distribution of stained cells (A) and a bar graph of apoptotic cells expressed as a percent of total cells for each treatment with SEM from three experiments (B). In (A), cell staining was evaluated with fluorescein isothiocyanate (FITC)-labelled Annexin V (green fluorescence, X axis), simultaneously with dye exclusion of PI (negative for red fluorescence, Y axis). As the test described, discriminates intact cells (FITC−/PI−), apoptotic cells (FITC+/PI−) and necrotic cells (FITC+/PI+). In Figure 3B, the significant levels $^{*}P$, 0.05, $^{**}P$, 0.01, $^{***}P$, 0.001, and no symbol = non-significant, when compared with IR-alone group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

CLONOGENIC SURVIVAL RATE OF HIECS

Figure 5A shows the clonogenic survival rate. Compared to IR alone group at a dose of 8 Gy, pretreatment with different concentrations of mangiferin aglycone or mangiferin (0–100 μg/ml) for 1.5 h significantly ($P < 0.01$) increased clonogenic survival of HIECs. Similar to the results of CCK-8, the optimum protective concentration of mangiferin aglycone was 70 μg/ml higher than that of mangiferin (50 μg/ml). However, no significant differences were found between these two compounds.

Treating HIECs with different doses (0–16 Gy) of gamma radiation resulted in a dose-dependent decline in the cell viability as evidenced by the significant reduction in surviving fraction (Fig. 5B). Treating HIECs with 70 μg/ml mangiferin aglycone for 1.5 h before exposure to different doses of gamma radiation resulted in 2.11-, 2.46-, 3.71-, and 4.83-fold increase in clonogenic survival in comparison with 4, 8, 12, and 16 Gy IR-alone group, and closely reproduced by mangiferin.

DISCUSSION

There is an obvious need to develop efficient counter-measurable agents for use in nuclear emergency or as adjuncts to radiotherapy to protect healthy tissues from the damage of IR. Searching for

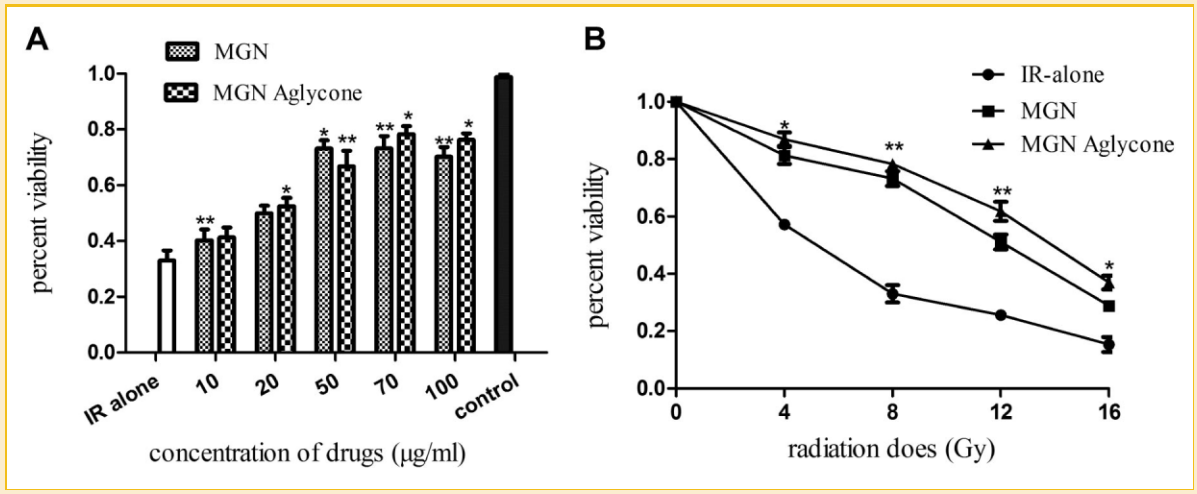

Fig. 4. Mangiferin aglycone preserves viability of HIECs against IR. A: Percent viability assessed by CCK-8 assay, HIECs treated with various concentrations (0–100 μg/ml) of mangiferin or mangiferin aglycone and then expose to 8 Gy gamma radiation and further incubated for 48 h. B: HIECs following 1.5 h treatment with 70 μg/ml mangiferin or mangiferin aglycone before exposed to different doses of gamma radiation and further incubated for 48 h. Values are shown as mean SEM from the data of a minimum of six separate experiments. The significant levels $^{*}P$, 0.05, $^{**}P$, 0.01, $^{***}P$, 0.001, and no symbol = non-significant, when compared with IR-alone group.

natural products that can be used to prevent the IR damage induced by free radicals is a feasible way. Polyphenols is considered as one of the most powerful natural antioxidants and a great deal of work has been carried out in an attempt to characterize the pharmacological effects such as affecting the cardiovascular, immune, and nervous systems, as well as those with antibiotic, antiviral, anti-inflammatory, and antidiabetic effects [Hahn et al., 1994]. Mangiferin, a polyphenolic compound, which is widely distributed in plants and known to exhibit antioxidant effects and cytoprotective effects against radiation-induced oxidative stress [Muruganandan et al., 2002; Jagetia et al., 2005]. Mangiferin and mangiferin aglycone in our studies did not show cytotoxic effects on HIECs, as the cell viability, clonogenic surviving rate, apoptotic rate, and DSBs are remained unaltered after mangiferin or mangiferin aglycone-alone treated in our study (Figs. 2–5). This finding is in accordance with the reported animal study with mangiferin in which the administration of 400 mg/kg Mangiferin induced 50% mortality [Jagetia and Baliga, 2005]. Mangiferin has been reported to possess many biological activities. In our previous study, we found the biological activity of mangiferin aglycone is better than mangiferin

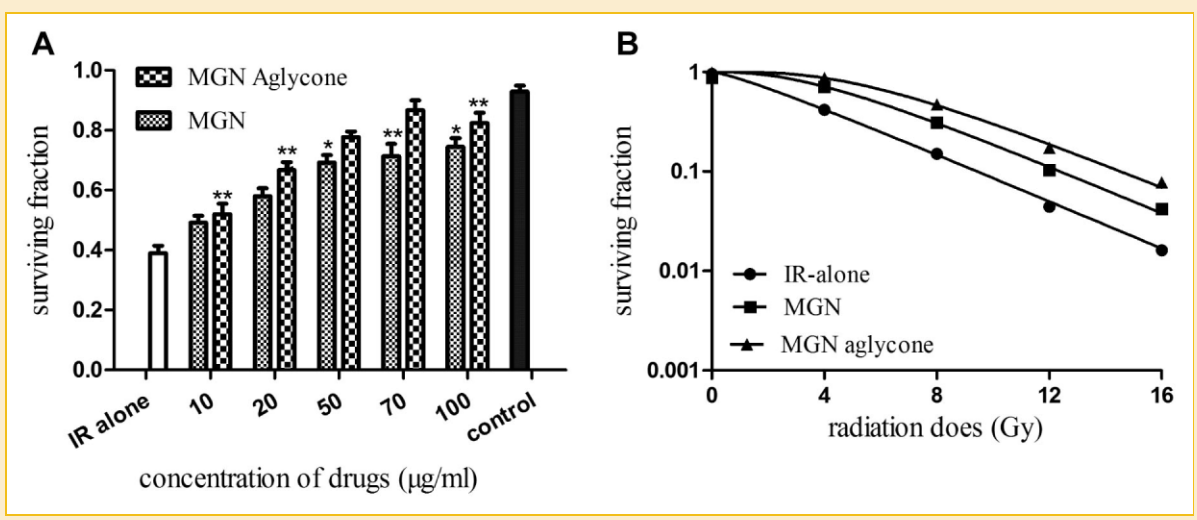

Fig. 5. Mangiferin aglycone increase clonogenic survival rate of HIECs after IR. A: Changes in HIECs survival after treatment with different concentrations (0–100 μg/ml) of mangiferin or mangiferin aglycone prior exposure to 8 Gy gamma radiation. B: Changes in HIECs survival after treatment with 70 μg/ml mangiferin or mangiferin aglycone for 1.5 h before exposure to different doses of gamma radiation. Values are shown as mean SEM from the data of a minimum of six separate experiments. The significant levels $^{*}P$, 0.05, $^{**}P$, 0.01, $^{***}P$, 0.001, and no symbol = non-significant, when compared with IR-alone group.

itself in several targets. What's more, structure-based drug discovery in the pharmaceutical industry benefits from cost-efficient methodologies that quickly assess the feasibility of specific, refractory protein targets to form well-diffracting crystals [Hosfield et al., 2003]. Thus, mangiferin aglycone has a small molecular structure which means better water-soluble and convenient synthesized. Thus we focus on the radioprotective effect of mangiferin and its aglycone. In the present study, we selected HIECs as the target cells based on the following reasons. Firstly, in the gastrointestinal (GI) tract, epithelial cells of the small intestinal crypts are highly radiosensitive cells which can be damaged by lower radiation doses [Ramachandran et al., 2000]. Secondly, after exposure to large dose (>1 Gy) ionizing radiations, the larger intestinal cell lesion is the main cause of death in humans and animals [Bowen et al., 2006]. How to enhance intestinal cell survival rates has become a problem to be solved. What's more, the ex vivo response of HIECs to either radiation exposure or radioprotective agents appears mimic to that of the in vivo response.

There have been in vivo studies showing that mangiferin prevented the production of ROS and the oxidative tissue damage in vivo and it was more active than vitamin C, vitamin E, mangiferin, and [beta]-carotene [Pardo et al., 2005]. These free radical scavengers have been shown to protect cellular DNA against a significant proportion of the indirect effects of IR-induced hydroxyl radicals ($^{\bullet}OH$), which are believed to be the primary active species responsible for the damage, it is biologically important to timely eliminate the hazard during IR [Okata et al., 1983]. This study showed that the radioprotective effects of mangiferin aglycone and mangiferin were directly related to $^{\bullet}OH$ scavenging capacity for the first time. In this study, we detected and semiquantified the toxic ROS, $^{\bullet}OH$, using the HPF fluorescence probe. Both of these two compounds significantly decreased fluorescence signals and scavenged $^{\bullet}OH$ which was derived from radiolysis of H_2O. Radioprotective effects of mangiferin aglycone and mangiferin on DNA (Fig. 2) can be explained by in part due to its ROS scavenging capacity as evidenced by hydroxyphenyl fluorescein assay (Fig. 1). Some results showed that the fluorescence intensity was proportional to the dose of γ-radiation ($R_2 = 0.991$) [Yunhai et al., 2011]. Therefore, HPF could detect $^{\bullet}OH$ formed by radiolysis of H_2O in terms of a dose-dependent increase in fluorescence, confirming the reliability of this method to quantify the generation of $^{\bullet}OH$. There is considerable literature suggesting that free radical scavengers can be used to prevent oxidative damage and cell death, including apoptosis caused by ionising radiation [Hall and Giaccia, 2006]. In the present study, these two compounds effectively suppressed programmed cell death by decreasing apoptotic features (Fig. 3A). This process may involve certain caspase activation, increasing anti-apoptotic molecules (Bcl-2) and decreasing pro-apoptotic molecules (Bax).

Radioisotopes incorporated into DNA kill cells much more efficiently than radioisotopes in RNA or in proteins. Therefore, DSBs is the most highly cytotoxic form. If not correctly repaired, it can initiate chromosome aberrations, genomic instability, and may eventually lead to cancer and hereditary disease [Wyman and Kanaar, 2006]. There is an increasing use of γH2AX foci formation to detect the break site of DSBs produced by ionizing radiation in lymphocytes, oral cells, or skin biopsies [Rothkamm and Horn, 2009]. That may be due to that γH2AX foci formation assay is more sensitive to certain types of DNA damage and more convenient than other techniques that detect DNA damages. The numbers of γH2AX foci are known to decrease with increasing time post-exposure since some of DNA lesions are easy to repair [Horn et al., 2011]. In the present study, a maximum number of γH2AX foci was observed at 30 min post-IR treatment time and the average number of foci per cell per Gy was about 6.49 (Fig. 2A). We used different radiation doses for addressing the radioprotective potential of mangiferin aglycone on DNA. Because optimal radiation doses selected for these assays would generate an optimal level of specific desired damage. These radiation doses are suitable for screening the protective potential of the drug. Our results indicated that pretreatment of mangiferin aglycone (70 μg/ml) offered a greater radioprotection on HIECs in terms of foci per cell than the same concentration of mangiferin at different radiation doses (0–4 Gy). The most significant difference was found at 2 Gy. Mangiferin aglycone alleviated almost 47% of the total cell DSBs whereas mangiferin alleviated <40%. We may come to the conclusion that the difference between mangiferin aglycone and mangiferin is not obvious at low radiation doses but become significant at higher doses. A possible reason for these results is that mangiferin aglycone has a small molecule structure and may have ability to scavenge free radicals formed in the initial step of DSBs.

In this study, we focused on mangiferin aglycone, used mangiferin as a positive control. Mangiferin aglycone, used mangiferin evaluated the radioprotective effect of these two compounds on cell survival, colony formation ability, apoptosis, and DNA DSBs. As being anticipated, both mangiferin aglycone and mangiferin treatment before IR have good protective effects. This is in agreement with the earlier studies, the radiation protective effect on HPBLs evidenced by a reduction in the frequency of MNBNCs (micronucleated binucleate cells) and a reduction of DNA strand breaks [Jagetia et al., 2005]. What's more, our results showed that mangiferin aglycone can be a better radioprotector especially in increasing the ability of colony formation and decreasing DNA DSBs when compared with mangiferin. Notice that in our experiments the optimum radioprotective concentration of mangiferin on HPBLs was 50 μg/ml which was in accordance with earlier studies, while the optimum radioprotective concentration of mangiferin aglycone was 70 μg/ml. This may be due to different cell species have been reported to exhibit a variation in the drug sensitivities and radiosensitivities.

The sulphydryl compound amifostine, named WR-2721, which is the only radioprotectant registered for use in humans, has shown good radioprotective effects [Gosselin et al., 2002]. However, it is relatively high toxicity. Cytokines and immunomodulators should be used after IR or in combination with radical scavengers and antioxidants [Herodin and Drouet, 2005] and natural antioxidants, such as vitamin E, flavonoids, and others, have fewer toxic side-effects but also a lower degree of protection compared to thiol agents [Hosseinimehr, 2007]. Mangiferin has been tested in a broad set of toxicological tests with satisfactory results, including acute and subcronic toxicity, genotoxicity, and irritability and is classified as a nontoxic product [Sato et al., 1992]. Similar to mangiferin, exposure

to various concentrations of mangiferin aglycone did not significantly alter the cell viability of HIECs during all post-irradiation periods in our study. The development of radiation protectors is important not only to enhance the effectiveness of cancer treatment, but also for the study of the underlying mechanisms of radiation cytotoxicity [Yazlovitskaya et al., 2006]. Therefore, mangiferin aglycone is of potential interest in the setting of radiation protection. Additional modifications to this compound may theoretically allow improving radioprotective effect and targeting of specific organs for protection.

In conclusion, the present study has demonstrated that mangiferin aglycone, as well as mangiferin rendered a significant anti-cytotoxic, anti-apoptotic and anti-genotoxic plausibly ascribable to its antioxidant/free radical scavenging ability. We confirmed that mangiferin aglycone is a novel radioprotector. However, limitation of the current study is lack of in vivo data, more studies should be a necessity to identify the precise and underlying mechanism of mangiferin aglycone in biology. Furthermore, since the protective effects on the normal cells during radiation were observed at very low concentrations, mangiferin aglycone may have great potential for clinical applications.

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REFERENCES

Anderson FR, Fisher JL, Hara Y, Harris T, Mak BW, Melton DL, Packer EJ. 2001. Green tea catechins partially protect DNA from $^{\bullet}OH$ radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. Carcinogenesis 22:1189–1193.

Bianchini F, Jaeckel A, Vineis P, Martinez-Garcia C, Elmstahl S, Kappel AV, Boeing H, Ohshima H, Riboli E, Kaaks R. 2001. Inverse correlation between alcohol consumption and lymphocyte levels of 8-hydroxydeoxy-guanosine in humans. Carcinogenesis 22:885–890.

Bowen JM, Gibson RJ, Cummins AG, Keefe DM. 2006. Intestinal mucositis: The role of the Bcl-2 family, p53 and caspases in chemotherapy-induced damage. Support Care Cancer 14:713–731.

Daniel R, Ramcharan J, Rogakou E, Taganov KD, Greger JG, Bonner W, Nussenzweig A, Katz RA, Skalka AM. 2004. Histone H2AX is phosphorylated at sites of retroviral DNA integration but is dispensable for postintegration repair. J Biol Chem 279:45810–45814.

García D, Delgado R, Ubeira FM, Leiro J. 2002. Modulator effects of rat macrophage function by *Mangifera indica* L extract (Vimang) and mangiferin. Int Immunopharmacol 2:797–806.

Gosselin TK, Mautner B, Wilkinson K. 2002. Amifostine as a radioprotectant. Clin J Oncol Nurs 6:175–176 180.

Hahn SM, Krishna MC, Samuni A, Degraff W, Cuscela DO, Johnstone P, Mitchell JB. 1994. Potential use of nitroxides in radiation oncology. Cancer Res 54:2006s–2010s.

Hall EJ, Giaccia AJ. 2006. Radiobiology for the Radiologist, 6th edition. Philadelphia: Lippincott Williams & Wilkins. p. 192.

Herodin F, Drouet M. 2005. Cytokine-based treatment of accidentally irradiated victims and new approaches. Exp Hematol 33:1071–1108.

Horn S, Barnard S, Rothkamm K. 2011. Gamma-H2AX-based dose estimation for whole and partial body radiation exposure. PLoS One 6(9): e25113.

Hosfield D, Palan J, Hilgers M, Scheibe D, McRee DE, Stevens RC. 2003. A fully integrated protein crystallization platform for small-molecule drug discovery. J Struct Biol 142(1):207–217.

Hosseinimehr SJ. 2007. Trends in the development of radioprotective agents. Drug Discov Today 12:794–805.

Hsu MF, Raung SL, Tsao LT, Lin CN, Wang JP. 1997. Examination of the inhibitory effect of norathyriol in formylmethionyl-leucyl-phenylalanine-induced respiratory burst in rat neutrophils. Free Radic Biol Med 23:1035–1045.

Hu HG, Wang MJ, Zhao QJ, Yu SC, Liu CM, Wu QY. 2007. Synthesis of mangiferin derivates and study their potent PTP1B inhibitory activity. Chin Chem Lett 11(18):1323–1326.

Jagetia GC, Baliga MS. 2005. Radioprotection by mangiferin in DBAxC57BL mice: A preliminary study. Phytomedicine 12(3):209–215.

Jagetia GC, Venkatesha VA, Reddy TK. 2005. Effect of mangiferin on radiation-induced micronucleus formation in cultured human peripheral blood lymphocytes. Environ Mol Mutagen 46(1):12–21.

Muruganandan S, Gupta S, Kataria M, Lal J, Gupta PK. 2002. Mangiferin protects the streptozotocin-induced oxidative damage to cardiac and renal tissues in rats. Toxicology 176:165–173.

Natarajan AT, Darroudi F, Mullenders LH, Meijers F. 1986. The nature and repair of DNA lesions that lead to chromosomal aberrations induced by ionizing radiations. Mutat Res 160:231–236.

O'Neill P, Fielden EM. 1993. Primary radical process in DNA. Adv Radiat Biol 17:53–120.

Okata S, Nakamura N, Sasaki K. 1983. Radioprotection of intracellular genetic material. In: Nygaard OF, Simic MG, editor. Radioprotectors and anticarcinogens. New York: Academic Press. pp. 339–356.

Pardo AG, Delgado R, Velho J, Inada NM, Curti C, Vercesi AE. 2005. *Mangifera indica* L extract (Vimang) inhibits Fe^{2+}-citrate-induced lipoperoxidation in isolated rat liver mitochondria. Pharmacol Res 51:427–435.

Ramachandran A, Madesh M, Balasubramanian KA. 2000. Apoptosis in the intestinal epithelium: Its relevance in normal and pathophysiological conditions. J Gastroenterol Hepatol 15:109–120.

Rothkamm K, Horn S. 2009. gamma-H2AX as protein biomarker for radiation exposure. Ann Ist Super Sanita 45:265–271.

Rothkamm K, Lobrich M. 2003. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low X-ray doses. Proc Natl Acad Sci USA 100:5057–5062.

Rouillard LF, Thiais AJ, Robin JR. 1998. Cosmetic or pharmaceutical composition containing, as active ingredient, mangiferine or its derivatives, in pure or in plant extracts. US Patent 5:824 320.

Sánchez GM, Re L, Giuliani A, Núñez-Sellés AJ, Davison GP, León-Fernández OS. 2000. Protective effects of *Mangifera indica* L. extract, mangiferin, and selected antioxidants against TPA-induced biomolecules oxidation and peritoneal macrophage activation in mice. Pharmacol Res 42:565–573.

Satish Rao BS, Sreedevi MV, Nageshwar Rao B. 2009. Cytoprotective and antigenotoxic potential of Mangiferin, a glucosylxanthone against cadmium chloride induced toxicity in HepG2 cells. Food Chem Toxicol 47:592–600.

Sato TAK, Tamura A, Tatsum Y, Fuiji T. 1992. Mechanism of antioxidant action of pueraria glycoside (PG)-1 (an isoflavonoid) and mangiferin (a xanthonoid). Chem Pharm Bull 40:721–724.

Sedelnikova OA, Rogakou EP, Panyutin IG, Bonner WM. 2002. Quantitative detection of ^{125} IdU-induced DNA double-strand breaks with gamma-H2AX antibody. Radiat Res 158:486–492.

Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. 2003. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J Biol Chem 278:3170–3175.

Tomizawa S, Imai H, Tsukada Simizu T, Honda F, Nakamura M, Nagano Urano Y, Matsuoka Sedelnikova OA, Horikawa I, Redon C, Nakamura A, Zimonjic DB, Popescu NC, Bonner WM. 2008. Delayed kinetics of DNA double-strand break processing in normal and pathological aging. Aging Cell 7:89–100.

Wallace SS. 1988. Detection and repair of DNA base damages produced by ionizing radiation. Environ Mol Mutagen 12:431–477.

Wyman C, Kanaar R. 2006. DNA double-strand break repair: All's well that ends well. Annu Rev Genet 40:363–383.

Yazlovitskaya EM, Edwards E, Thotala D, Fu A, Osusky KL, Whetsell WO, Jr, Boone B, Shinohara ET, Hallahan DE. 2006. Lithium treatment prevents neurocognitive deficit resulting from cranial irradiation. Cancer Res 66:11179–11186.

Yoshimi N, Matsunaga K, Katayama M, Yamada Y, Kuno T, Qiao Z, Hara A, Yamahara J, Mori H. 2001. The inhibitory effects of mangiferin, a naturally occurring glucoxanthone, in bowel carcinogenesis of male F344 rats. Cancer Lett 26:163–170.

Yunhai C, Fu G, Bailong L, Luqian Z, Liren Q, Fei C, Lei W, Xuejun S, Jianguo C, Jianming C. 2011. Hydrogen-rich saline attenuates radiation-induced male germ cell loss in mice through reducing hydroxyl radicals. Biochem J (in press).