Original Article

Tetrahydrobiopterin Protects against Radiation-induced Growth Inhibition in H9c2 Cardiomyocytes

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Abstract

Background: Tetrahydrobiopterin (BH4) is an essential cofactor of nitric oxide synthases (NOSs) for the synthesis of nitric oxide (NO). BH4 therapy can reverse the disease-related redox disequilibrium observed with BH4 deficiency. However, whether BH4 exerts a protective effect against radiation-induced damage to cardiomyocytes remains unknown.

Methods: Clonogenic assays were performed to determine the effects of X-ray on H9c2 cells with or without BH4 treatment. The contents of lactate dehydrogenase (LDH), superoxide dismutase (SOD), and malondialdehyde (MDA) in H9c2 cells were measured to investigate oxidative stress levels. The cell cycle undergoing radiation with or without BH4 treatment was detected using flow cytometry. The expression levels of proteins in the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT)/P53 signaling pathway, inducible NOS (iNOS), and endothelial NOS (eNOS) were examined using Western blotting.

Results: X-ray radiation significantly inhibited the growth of H9c2 cells in a dose-dependent manner, whereas BH4 treatment significantly reduced the X-ray radiation-induced growth inhibition (control group vs. X-ray groups, respectively, $P < 0.01$). X-ray radiation induced LDH release, apoptosis, and G0/G1 peak accumulation, significantly increasing the level of MDA and the production of NO, and decreased the level of SOD (control group vs. X-ray groups, respectively, $P < 0.05$ or $P < 0.01$). By contrast, BH4 treatment can significantly reverse these processes (BH4 treatment groups vs. X-ray groups, $P < 0.05$ or $P < 0.01$). BH4 reversed the X-ray radiation-induced expression alterations of apoptosis-related molecules, including B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein, and caspase-3, and molecules of the PI3K/Akt/P53 signaling pathway. BH4 enhanced the production of NO in 2 Gy and 4 Gy radiated groups by upregulating eNOS protein expression and downregulating iNOS protein expression.

Conclusions: BH4 treatment can protect against X-ray-induced cardiomyocyte injury, possibly by recoupling eNOS rather than iNOS. BH4 treatment also decreased oxidative stress in radiated H9c2 cells.

Key words: Endothelial Nitric Oxide Synthase; Oxidative Stress; Proliferation; Radiation-induced Heart Disease; Tetrahydrobiopterin

Introduction

Advances in radiation therapy for malignant neoplasms and cardiovascular diseases have significantly improved the survival of many patients with breast cancer, Hodgkin's lymphoma, cardiovascular diseases, and others.[1] However, radiation therapy can cause many complications[1] such as second malignant neoplasms, endocrine disorders, cardiopulmonary dysfunction, cardiovascular complications, and cognitive and psychosocial problems,[2] among which radiation-induced heart disease (RIHD) is one of the most difficult complications. RIHD may manifest as pericardial disease, myocardial disease, valvular disease, conduction abnormalities, coronary artery disease, or cardiovascular death. RIHD is more common than previously believed and involves controversies regarding risks associated with

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modern radiation diagnosis techniques and the existence of a minimum threshold radiation dose. Recent evidence suggests that patients who have received radiation therapy carry a greater risk for developing cardiomyopathy, which increases the risks for myocardial infarction and heart failure.[3]

Radiation may result in the generation of excessive reactive oxygen species by cells and tissues, which causes oxidative stress.[4] The sustained generation of reactive oxygen species is the primary cause of apoptosis.[5] Studies in mice demonstrated that radiation-induced acute injury on cardiomyocytes can lead to apoptosis.[6] The loss of myocytes causes the thinning of the myocardium with a resulting increase in wall stress. As a result, the myocardium becomes deficient due to defective regenerative and adaptive pathways in the remaining myocardial cells. This ultimately results in symptoms of congestive heart failure. However, the mechanism of RIHD remains largely unknown and there is currently an urgent need to elucidate the mechanism of RIHD, in order to develop effective therapies for this condition.

Tetrahydrobiopterin (BH4) is an essential cofactor of nitric oxide synthases (NOSs) and is critical for nitric oxide (NO).[7] BH4 deficiency tends to induce endothelial NOS (eNOS) uncoupling *in vivo*.[8] In addition, BH4 is highly sensitive to oxidation and can be degraded within minutes.[8] BH4 therapy can reverse the disease-related redox disequilibrium observed with BH4 deficiency. For example, incubation with BH4 has been shown to improve oxidant stress levels in arteries in spontaneously hypertensive rats and streptozotocin-induced diabetic rats.[9,10] At present, whether BH4 exerts a protective effect against radiation-induced damage to cardiomyocytes remains unknown. In the present study, we evaluated the impact of X-ray radiation on the growth, necrotic death, cell cycle progression, apoptosis, and oxidative stress of H9c2 cells (a rat cardiomyoblast cell line). Moreover, this study is the first to explore the potential protective effect of BH4 on radiation-induced cardiomyocyte damage and the possible mechanisms involved.

Methods

Cell culture

Rat cardiomyoblast cell line H9c2 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) and were maintained at 37°C in a humidified atmosphere with 5% CO_2. The medium was replaced every 2–3 days, and the cells were subcultured or subjected to experimental procedures. The cells were passaged as they grew to 80% confluence.

Irradiation procedure

Cells were refed with fresh culture medium 2 h before radiation. A single dose of 2, 4, 6, or 8 Gy of X-rays was administered to the cells using a 160-kVp X-ray high energy linear accelerator (Faxitron Bioptics, LLC, Tucson, AZ, USA), which was operated at a distance of 100 cm. After irradiation, the cells were incubated in an incubator at 37°C with 5% CO_2 for up to 72 h. The medium was replaced every 24 h.

Clonogenic survival assay

Clonogenic assays were performed to determine the effect of BH4 treatment on radiation-induced apoptosis of H9c2 cells. Briefly, cells were plated in 60-mm dishes at 1×10^3 cells/well in triplicate and exposed to different doses (0–8 Gy) of irradiation 2 h after pretreatment with BH4 using an X-ray irradiator (J. L. Shepherd and Associates, Glendale, CA, USA) at a rate of 2.1 Gy/min. After irradiation, culture media were replaced with fresh complete media or complete media with 10 μg/ml of BH4, followed by incubation at 37°C for 14 days to allow for colony formation. Colonies were fixed in methanol for 20 min and stained with 0.5% Giemsa (Sigma-Aldrich Corp. St. Louis, MO, USA) for 30 min. The number of colonies (≥50 cells) was counted under a microscope.

Lactate dehydrogenase release assay

Cells were pretreated with BH4 for 2 h and received 2, 4, 6, or 8 Gy of radiation. Seventy-two hours after BH4 treatment, the supernatants of the culture media were collected. Lactate dehydrogenase (LDH) activity was measured by colorimetric assay using a commercial LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Morphological evaluation of apoptotic cells

After treatment with BH4 for 72 h, H9c2 cells were fixed with 1% glutaraldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, washed in PBS, and stained with 1 mmol/L of Hoechst 33342 for 20 min at 37°C. The cells were then observed under a fluorescence microscope (IX-81; Olympus, Tokyo, Japan).

Cell cycle analysis

H9c2 cells in the logarithmic growth phase were harvested and seeded in culture flasks at a density of 2×10^6 cells per flask. After allowing cells to adhere, the supernatant was removed, and the cells were grouped and underwent radiation and BH4 treatment. After experimentation, cells were harvested and washed once with cold PBS (0.1 mol/L). Then, the cells were resuspended in 1 ml of PBS and fixed with 2 ml of dehydrated alcohol for 30 min. Afterward, all cells were collected, washed once with PBS, and stained with 50 g/ml of propidium iodide (Boehringer Mannheim, Indianapolis, IN, USA) in the dark for 30 min at room temperature. Then, the cells were analyzed using a flow cytometer (Coulter XL; Beckman Coulter, Inc., Fullerton, CA, USA) to determine cell cycle distribution. All tests were performed in triplicate.

Antioxidant and lipid peroxide assay

The activity of superoxide dismutase (SOD) and the content of malondialdehyde (MDA) in H9c2 cells that had

undergone radiation and BH4 treatment were determined using commercial kits according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western blot analysis

Cells at 80% confluence were washed three times with PBS. RIPA buffer was added to extract the total protein, and equal amounts of protein were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for protein separation. Then, proteins were transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk for 3 h and incubated with primary antibodies (p53 at 1:1000, p-AKT [CST, USA] at 1:800, total AKT [CST, USA] at 1:800, B-cell lymphoma-2 [Bcl-2] [Abcam, UK] at 1:1000, Bcl-2 associated X protein [Bax] [Abcam, UK] at 1:1000, caspase-3 [Abcam, UK] at 1:1000, and iNOS [CST, USA] at 1:1,000) overnight at 4°C. After being washed in Tris-buffered saline (pH 7.2) containing 0.05% Tween 20, the membranes were incubated with the secondary antibody for 2 h at room temperature. Finally, the membranes were washed, and ECL chemiluminescence reagents (Amersham Pharmacia Biotech, Japan) were used to detect the proteins. The quantification of band intensity was performed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Detection of endothelial nitric oxide synthase dimer

Cell lysates were prepared by homogenization in ice-cold CelLytic M buffer (Sigma-Aldrich Corp., St. Louis, MO, USA) containing protease inhibitors (Roche Applied Science, Germany). Lysates were centrifuged at 12,000 r/min for 5 min at 4°C, and the samples were prepared using Laemmli sample buffer (Sigma-Aldrich Corp., St. Louis, MO, USA). Then, SDS-PAGE was performed to detect eNOS dimer. Protein lysates were resolved using 8% Tris-glycine gels (Invitrogen, USA) under reducing conditions. All gels and buffers were preequilibrated to 4°C before electrophoresis, and the buffer tank was placed in an ice bath during electrophoresis to maintain the gel temperature below 15°C. Standard blotting techniques were used, and membranes were incubated with rabbit anti-eNOS polyclonal antibody (CST, USA), as previously described.[11,12]

Determination of cyclic guanosine monophosphate by ELISA

Cyclic guanosine monophosphate (cGMP) was measured using a cGMP ELISA kit according to the manufacturer's protocol (Shanghai Enzyme-linked Biotechnology, Shanghai, China), and values are presented as fold changes compared with the control.

Nitric oxide assay

The levels of NO in H9c2 cells that received radiation and BH4 treatment were determined using commercial kits according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

Data are expressed as means ± standard deviation (SD). Two-way analysis of variance (ANOVA) was used to evaluate the various levels of radiation with or without BH4 treatment, and comparisons between same levels of radiation with versus without treatment were performed using a paired *t*-test; comparisons between groups were tested by one-way ANOVA and least significant difference test. A value of $P < 0.05$ was considered statistically significant.

Results

BH4 protects against the anti-proliferative and anti-apoptotic effects of X-ray radiation in H9c2 cells

To determine the optimal dose of BH4 for treating radiated H9c2 cells, a 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-2H-tetrazolium bromide assay was performed. The proliferation rate of H9c2 cells treated with 10 μg/ml of BH4 for 72 h was 1.10 ± 0.06 (data not shown), showing no statistically significant difference compared with controls ($P > 0.05$). Based on these results, a BH4 concentration of 10 μg/ml was selected for subsequent experiments.

The anti-proliferative effect of X-ray radiation and the protective effect of BH4 (10 μg/ml) in H9c2 cells were investigated using a clonogenic survival assay. These assays demonstrated that X-ray radiation significantly suppressed the growth of H9c2 cells in a dose-dependent manner, compared with the control, after cells were treated with X-rays at doses of 2–8 Gy for 12 days [Figure 1a and 1b]. Compared with the radiation groups, BH4 reduced the radiation-induced growth inhibition of H9c2 cells. Hoechst 33342 staining revealed that typical apoptotic changes, such as the formation of apoptotic bodies, appeared in cells that underwent radiation for 72 h (data not shown), and the number of cells was decreased. BH4 reduced apoptosis induced by radiation (data not shown) and increased the number of cells (data not shown).

BH4 reduces X-ray radiation-induced G0/G1 peak accumulation in H9c2 cells

Flow cytometric analysis was performed to determine the mechanism responsible for radiation-mediated cell growth inhibition and the protective effect of BH4. After radiation with increasing doses of X-ray and BH4 treatment for 72 h, the distribution of H9c2 cells at each phase of the cell cycle was analyzed. X-ray radiation-induced G0/G1 peak accumulation in a dose-dependent manner compared with the control, and BH4 reduced G0/G1 cell cycle arrest compared with the radiation groups [Figure 2]. Compared with treatment with X-ray at 2, 4, 6, or 8 Gy alone, BH4 treatment significantly decreased the percentage of cells in the G0/G1 phase in each radiation group (radiation groups vs. BH4 treatment groups, 68.20 ± 1.45%, 76.75 ± 1.54%, 82.30 ± 0.60%, and 85.05 ± 0.33% vs. 64.20 ± 1.04%, 69.75 ± 1.26%, 77.22 ± 0.74%, and 79.41 ± 1.23%,

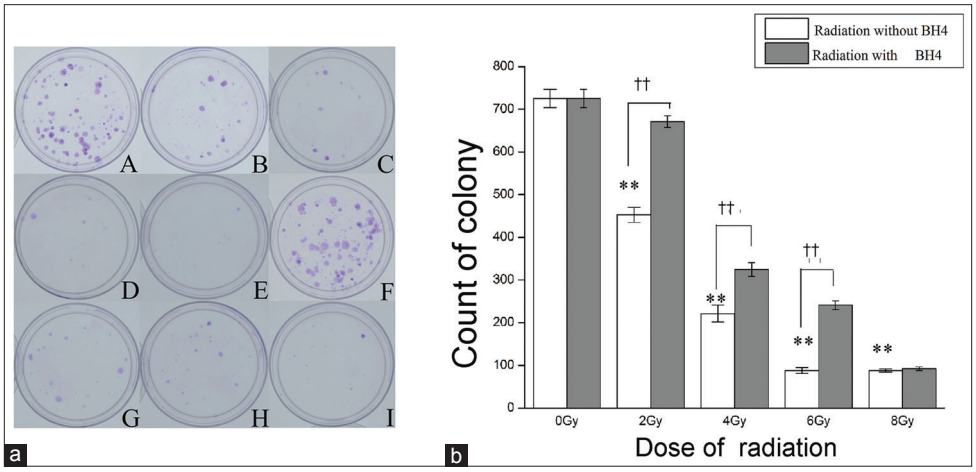

Figure 1: (a) Representative images showing colonies formed by control cells without radiation or BH4 treatment (A), cells treated with 2 Gy (B), 4 Gy (C), 6 Gy (D), and 8 Gy (E) of X-ray radiation alone, and cells treated with 2 Gy + BH4 (F), 4 Gy + BH4 (G), 6 Gy + BH4 (H), and 8 Gy + BH4 (I). (b) Quantitative analysis of the number of colonies formed by different groups of cells. Data shown are the means from three independent experiments (**$P < 0.01$ vs. Control, $^{\dagger\dagger}P < 0.01$ vs. BH4 treatment).

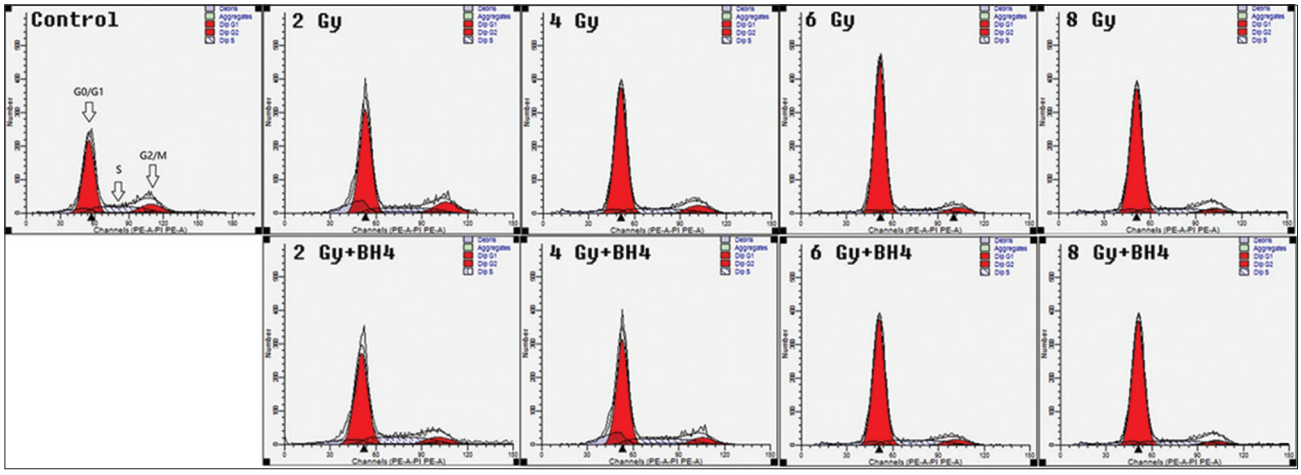

Figure 2: Effect of X-ray radiation alone or in combination with BH4 on cell cycle distribution in H9c2 cells. X-ray radiation-induced G0/G1 peak accumulation in a dose-dependent manner, and BH4 treatment reversed this trend.

respectively, $P < 0.05$). Compared with control cells, X-ray irradiation at doses from 2 Gy to 8 Gy significantly increased G0/G1 cell cycle arrest (59.97 ± 1.45% vs. 68.20 ± 1.45%, 76.75 ± 1.54%, 82.30 ± 0.60%, and 85.05 ± 0.33%, respectively $P < 0.05$).

BH4 protects against X-ray radiation-induced oxidative stress in H9c2 cells

To determine whether BH4 protects against X-ray radiation-induced oxidative stress in H9c2 cells, LDH release, antioxidant, and lipid peroxide assays were performed. As shown in Figure 3, X-ray radiation significantly upregulated the levels of MDA and LDH but downregulated the level of SOD. Compared with all radiation groups, BH4 treatment significantly downregulated the levels of MDA and LDH and upregulated the level of SOD [Figure 3a–3c].

Role of the phosphatidylinositol 3 kinase-Akt-P53 signaling pathway in the X-ray radiation-induced apoptosis of H9c2 cells

Hoechst 33342 staining showed that X-rays induced apoptosis in H9c2 cells and that BH4 reduced the radiation-induced apoptosis of H9c2 cells. We next detected X-ray radiation-induced expression alterations of apoptosis-related molecules and molecules of the phosphatidylinositol 3 kinase (PI3K)-Akt-P53 signaling pathway in H9c2 cells using Western blotting. X-ray radiation significantly upregulated the expression levels of Bax, P53, and caspase-3 but downregulated the levels of PI3K, Akt, phosphorylated Akt, and Bcl-2 [Figure 4a and Table 1]. In radiation-damaged cells, BH4 treatment significantly downregulated the expression levels of Bax, P53, and caspase-3 and upregulated the expression levels of PI3K, Akt, phosphorylated Akt, and Bcl-2 [Figure 4a and Table 1].

X-ray radiation-induced oxidative stress is associated with elevated nitric oxide concentration and inducible nitric oxide synthase expression in H9c2 cells

The expression levels of eNOS, iNOS, and cGMP in H9c2 cells after exposure to radiation for 72 h were assessed using Western blotting. X-ray radiation

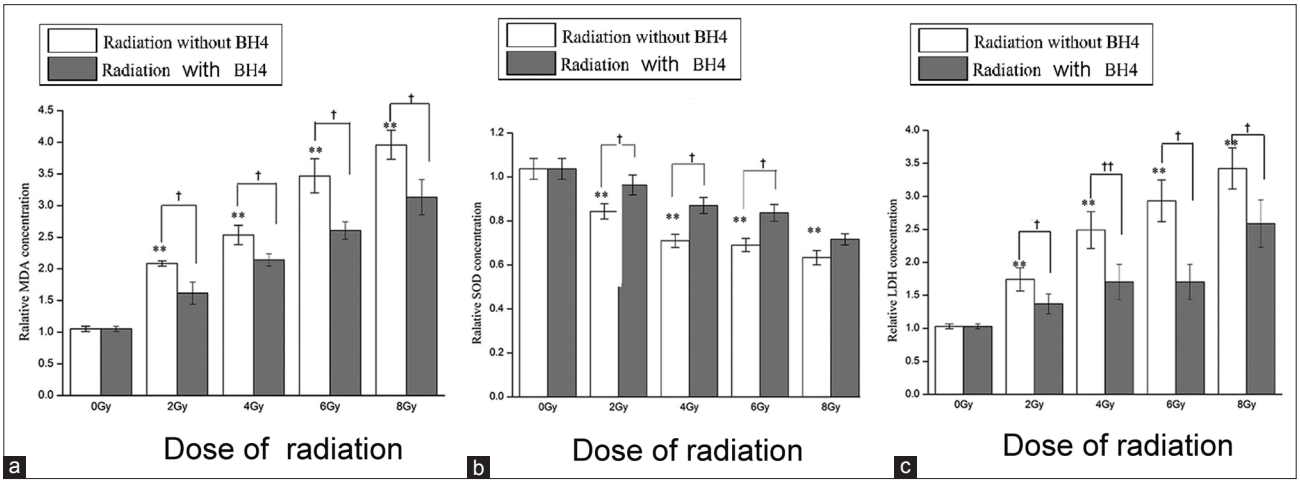

Figure 3: BH4 protects against X-ray radiation-induced imbalance of oxidative stress in H9c2 cells. The effects of X-ray radiation alone or combination with BH4 on MDA (a) and SOD (b) in H9c2 cells were determined using commercial kits, and the effects of X-ray radiation and BH4 treatment on LDH release were assessed using LDH release assay (c), as described in the Materials and Methods section. The data shown are the means of three independent experiments. $^{**}P < 0.01$ vs. Control, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. BH4 treatment. LDH: Lactate dehydrogenase; SOD: Superoxide dismutase; MDA: Malondialdehyde.

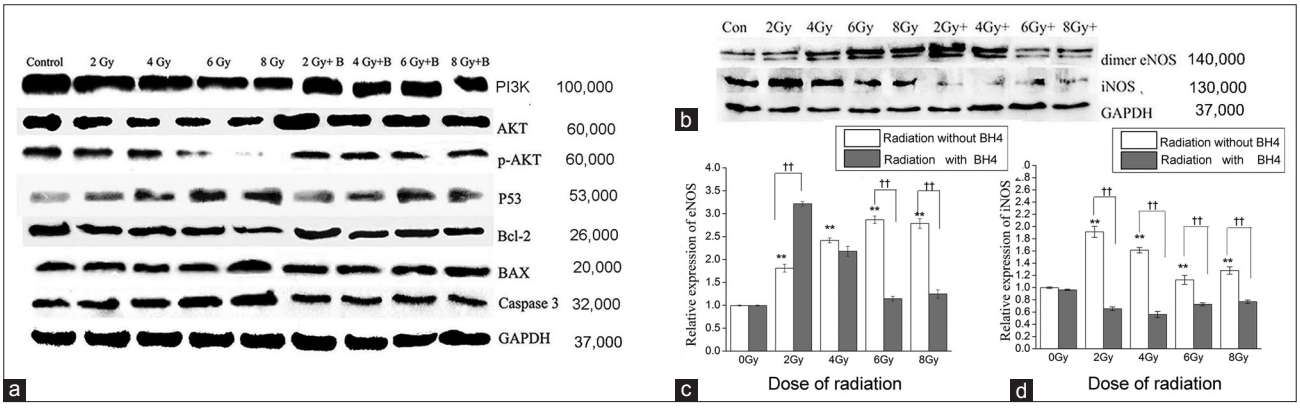

Figure 4: After treatment with 2, 4, 6, or 8 Gy of radiation with or without BH4 for 72 h. The expression levels of proteins were determined using Western blot. GAPDH was used as a loading control. Experiments were repeated three times. Representative images of the Western blots are shown. (a) Representative images of apoptosis-related molecules are shown; (b) representative images of eNOS and iNOS are shown; (c) relative expression of eNOS; (d) relative expression of iNOS. $^{**}P < 0.01$ vs. Control, $^{\dagger\dagger}P < 0.01$ vs. BH4 treatment. eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase.

significantly upregulated the expression levels of eNOS, iNOS, and cGMP [Figures 4b and 5b]. NO concentration also increased with the dose of X-ray radiation [Figure 5a]. BH4 treatment significantly downregulated the expression levels of iNOS in cells treated with 2 Gy or 4 Gy X-ray; however, the expression of eNOS was enhanced in the 2 Gy + BH4 and 4 Gy + BH4 groups and downregulated in the 6 Gy + BH4 and 8 Gy + BH4 groups. The NO concentration also exhibited the same trend after BH4 treatment [Figure 5a].

Discussion

Cardiac catheterizations for congenital heart disease, radiation therapies for cancer, and radiation exposures from all medical sources, during which the heart is unavoidably irradiated, can cause irreversible cardiac injury and even cardiac failure.[13] The severity of RIHD has been reported. However, most of these reports only described the mortality and morbidity of RIHD and its preventive strategy, and very few reports have shown its exact mechanism and treatment. In the present study, we assessed the impact of X-ray radiation on the biological behavior of H9c2 cells, the protective effect of BH4 treatment on X-ray radiation-induced alterations in the biological behavior of H9c2 cells, and the possible mechanisms involved. We found that X-ray radiation inhibited the growth of H9c2 cells in a dose-dependent manner, whereas BH4 treatment significantly reduced the X-ray radiation-induced growth inhibition of H9c2 cells, suggesting that BH4 may be a protective agent against X-ray radiation-induced damage to cardiomyocytes.

Limited cell growth may be due to increased necrotic death.[14] LDH leakage, a marker of necrotic cellular death, was therefore measured. Results obtained from the LDH assay were consistent with findings from the clonogenic

Table 1: Effect of X-ray and BH4 treatment on apoptosis of H9c2 cells, mean ± SD

Items	Akt	Bax	Bcl-2	Caspase-3	p53	p-Akt	PI3K	p-akt/akt
Radiation (Gy)								
Control	1.02 ± 0.01	1.01 ± 0.04	1.01 ± 0.05	1.02 ± 0.06	1.03 ± 0.02	1.02 ± 0.02	1.02 ± 0.02	0.99 ± 0.01
2	0.74* ± 0.05	1.10 ± 0.03	0.80* ± 0.02	1.14* ± 0.05	1.47* ± 0.02	0.54* ± 0.02	0.71* ± 0.02	0.73* ± 0.02
4	0.58* ± 0.05	1.14* ± 0.05	0.79* ± 0.02	1.18* ± 0.04	2.01* ± 0.02	0.33* ± 0.03	0.67* ± 0.05	0.57* ± 0.04
6	0.58* ± 0.05	1.29* ± 0.08	0.78* ± 0.03	1.36* ± 0.05	2.55† ± 0.01	0.23† ± 0.03	0.59* ± 0.05	0.40* ± 0.01
8	0.59* ± 0.07	1.42 ± 0.06	0.59* ± 0.02	1.60* ± 0.04	2.67† ± 0.03	0.03† ± 0.01	0.54† ± 0.04	0.05† ± 0.02
Radiation + BH4 (Gy)								
2	1.34§ ± 0.36	1.01 ± 0.06	0.94‡ ± 0.05	1.14 ± 0.05	1.53 ± 0.02	0.82§ ± 0.02	0.76 ± 0.05	0.61‡ ± 0.03
4	0.93‡ ± 0.02	1.07‡ ± 0.04	0.92§ ± 0.01	1.00‡ ± 0.04	1.58‡ ± 0.02	0.69§ ± 0.04	0.71 ± 0.07	0.74‡ ± 0.05
6	0.94§ ± 0.04	1.15‡ ± 0.03	0.72 ± 0.05	1.13‡ ± 0.05	1.80‡ ± 0.01	0.58§ ± 0.04	0.73 ± 0.06	0.61‡ ± 0.04
8	0.83‡ ± 0.04	1.27‡ ± 0.05	0.67 ± 0.04	1.24‡ ± 0.04	1.96‡ ± 0.03	0.57§ ± 0.02	0.49 ± 0.07	0.68§ ± 0.01

n = 3, each.*P<0.05; †P<0.01 radiation groups versus control (without radiation and BH4); ‡P<0.05, §P<0.01 treatment groups radiation with BH4 versus the same dose radiation (without BH4) groups. PI3K: Phosphatidylinositol 3 kinase; Bax: Bcl-2 associated X protein; Bcl-2: B-cell lymphoma-2; BH4: Tetrahydrobiopterin; SD: Standard deviation.

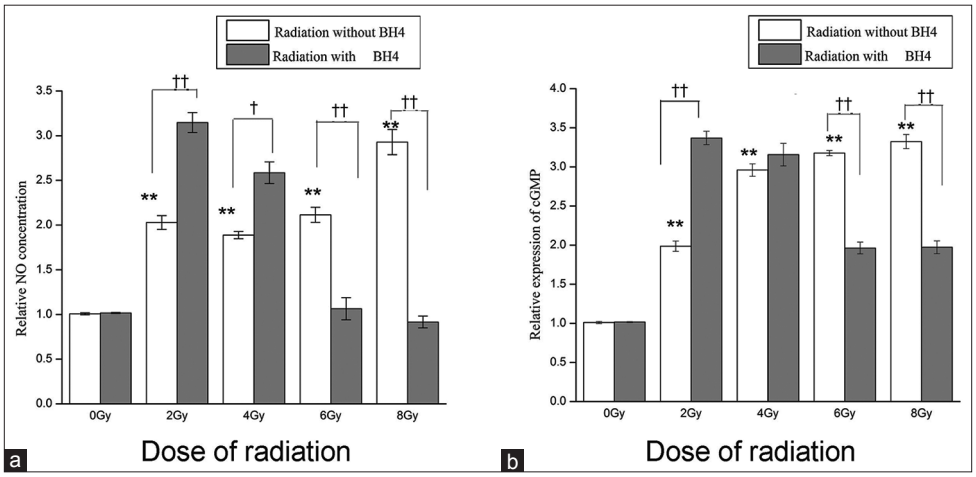

Figure 5: Effect of X-ray radiation alone or in combination with BH4 on NO production and cGMP concentration in H9c2 cells. After treatment with 2, 4, 6, or 8 Gy of radiation with or without BH4 treatment for 72 h, the NO production and cGMP concentration were determined. Control cells did not undergo either radiation or BH4 treatment. (a) NO production; (b) cGMP concentration. **P < 0.01 vs. control, †P < 0.05, ††P < 0.01 vs BH4 treatment. NO: Nitric oxide; cGMP: Cyclic guanosine monophosphate.

survival assay. There were significant differences in LDH levels between the control group and single radiation groups. X-ray radiation induced a typical dose-dependent increase in LDH release. However, BH4 treatment reversed the effect of X-ray-induced LDH release in H9c2 cells. Taken together, these findings suggest that BH4 treatment may decrease X-ray radiation-induced necrotic death in cardiomyocytes.

Radiation is widely known to cause DNA damage. Following DNA damage, cell cycle checkpoints that slow or arrest cell cycle progression are activated for cells to repair or prevent the transmission of damaged genome.[15] Consistent with this view, we noted a G0/G1 peak accumulation in H9c2 cells after exposure to X-ray radiation, suggesting that the retardation of cell cycle progression may be one of the mechanisms underlying the anti-proliferative effect of X-ray. After treatment with BH4, X-ray-induced sub-G1 peak accumulation was reversed. These findings indicate that BH4 may be a valuable tool for relieving X-ray induced G0/G1 peak accumulation in cardiomyocytes.

Oxidative stress plays a central role in the pathogenesis of radiation-induced damage, and organisms can rapidly eliminate anomaly-free radicals and reactive oxygen by antioxidants and enzymes under normal physiological conditions to maintain redox system homeostasis.[16] In radiation-induced cell injury, abundant reactive oxygen is constantly produced and accumulated, oxidative stress is induced, and the intracellular oxidant/antioxidant balance becomes upset.[17] Consequently, structural damage to cells and changes in enzyme activity can occur via chain reactions.[18] In this study, we discovered that radiation significantly increased intracellular MDA levels and decreased SOD content compared with control cells, whereas BH4 treatment significantly promoted the production of SOD and blocked MDA production in radiation-damaged H9c2 cells. These results suggest that the antioxidant activity

of BH4 may help protect cells from radiation-induced oxidative stress disequilibrium.

Radiation-induced cardiomyocyte apoptosis has been reported.[6] The mitochondrial pathway is one of the two main pathways leading to apoptosis.[19] In this pathway, Bcl-2 is a major protein that inhibits apoptosis by protecting cells from cell death, whereas Bax members (e.g., Bax and Bad) induce apoptosis; these proteins also regulate a common cell death pathway and function at a point where various signals converge. Executors are similarly important for apoptosis. Caspase-3 is essential for DNA fragmentation and morphological changes associated with apoptosis.[20] In the present study, many apoptotic bodies were observed in X-ray-treated H9c2 cells. Compared with the control group, significantly more X-ray radiation-treated cells underwent apoptosis, as shown by Hoechst 33342 staining, which demonstrates that X-ray radiation promotes cardiomyocyte apoptosis. Western blot analysis revealed that X-ray radiation upregulated the expression levels of Bax and caspase-3 but downregulated the expression of Bcl-2, whereas the treatment of X-ray-damaged H9c2 cells with BH4 significantly decreased the number of apoptotic bodies, upregulated the expression level of Bcl-2, and downregulated the expression levels of Bax and caspase-3. Collectively, these findings suggest that BH4 can protect H9c2 cells from X-ray-induced apoptosis.

Apoptosis pathways may be under the control of multiple signaling pathways, in which the PI3K/AKT signaling pathway has been associated with decisions related to cell death, survival, and apoptosis.[21] A previous study revealed that the PI3K/Akt pathway plays a critical role in promoting cell survival in the heart.[22] In this study, X-ray radiation was found to downregulate the PI3K/Akt signaling pathway, whereas BH4 treatment significantly increased the expression of molecules of the PI3K/Akt signaling pathway. Notably, the expression of the PI3K/Akt-regulated downstream signal p53 was also altered after both X-ray radiation and BH4 treatment. These results suggest that the PI3K/Akt signaling pathway may be involved in the X-ray radiation-induced growth inhibition and the protective effect of BH4 against radiation-induced myocardial injury in cardiomyocytes.

NO may exhibit both beneficial and harmful effects in conditions of oxidative stress imbalance.[23] NO derived from iNOS exerts a toxic effect in ischemic myocardium.[24] Previous studies demonstrated that NO and O_2^- easily react to produce $ONOO^-$, which has been proven to result in cell death and tissue injury (even apoptosis) through multiple signaling pathways. In contrast, NO derived from eNOS has myocardial protection effects in the same condition.[25,26] Our data revealed that X-ray radiation-induced the expression of eNOS and iNOS, which resulted in an increase in NO concentration in H9c2 cells. Even in radiation (2 Gy and 4 Gy) plus BH4 treatment groups, eNOS expression and NO concentration were maintained at high levels, suggesting that the PI/3K-Akt-eNOS-cGMP signaling pathway may be involved in this process. In contrast, the expression level of iNOS was downregulated. These results suggest that BH4 exhibited a protective effect due to the recoupling of eNOS rather than of iNOS. This finding is in line with the studies of Li *et al.*[27] and Storling *et al.*

Goryacheva *et al.* reported that neuronal NOS protein expression remained unchanged after adaptation to hypoxia.[28,29] Our finding demonstrated that oxidative stress induced by X-ray radiation depended on the dose of X-ray radiation. High-dose X-ray radiation-induced severe oxidative stress resulted from high NO concentration catalyzed by eNOS and iNOS. BH4 at 10 μg/ml only exhibited a protective effect against injury induced by 2 Gy and 4 Gy of X-ray radiations by recoupling eNOS and reducing iNOS expression. Regarding the 6 Gy and 8 Gy X-ray radiated groups, oxidative stress was too severe and BH4 was not able to exhibit a protective effect because BH4 is easily converted to BH2[30] and loses the bioavailability of recoupling eNOS. Collectively, our data show that iNOS-derived NO had detrimental effects on the myocardium; this finding is in line with that of Lee *et al*.[28]

There are two major limitations in this study. First, instead of primary human cardiomyocytes, the H9c2 cell line used in this study is derived from embryonic rat heart with characteristics of heart muscle myoblasts and with no ability to beat spontaneously.[31] Second, this study is an observational study without mechanistic insight. Future studies should carefully address these problems.

In summary, our study indicated that the inhibition of cell cycle progression and induction of necrotic cellular death, apoptosis, and oxidative stress may contribute to the anti-proliferative effects of X-ray in H9c2 cells, and BH4 treatment can protect against X-ray-induced cardiomyocyte injury possibly by reversing the above processes. Additionally, the PI3K/Akt signaling pathway may be involved in the effects of both X-ray radiation and BH4 treatment, and BH4 exhibits a protective effect by recoupling eNOS rather than iNOS. From the above, we would like to further evaluate BH4 as a promising agent for treating radiation-induced cardiomyocyte injury.

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Conflicts of interest

There are no conflicts of interest.

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