Radioprotective Effects of Heat-Killed Mycobacterium Tuberculosis in Cultured Cells and Radiosensitive Tissues

Yuanyuan Chen a, Yang Xu a, Jicong Du a, Jiaming Guo a, Xiao Lei a, Jianguo Cui a, Cong Liu a, Ying Cheng a, Bailong Li a, Fu Gao a, Jintao Ju b, Jianming Cai a, Yanyong Yang a

a Department of Radiation Medicine, Faculty of Naval Medicine, Second Military Medical University, P.R. China
b Faculty of Naval Medicine, Second Military Medical University, Shanghai, P.R. China

Key Words
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Abstract
Background: Exposure to ionizing radiation (IR) often causes severe damage to radiosensitive tissues, which limits the use of radiotherapy in cancer patients. Novel safe and effective radioprotectant is urgently required. It has been reported toll like receptor 2 (TLR2) plays a critical role in radioresistance. In this study, we demonstrated the protective effects of Heat-Killed Mycobacterium tuberculosis (HKMT), a potent TLR2 agonist, against IR. Methods: Cell survival and apoptosis were determined by CCK-8 assay and Annexin V assay, respectively. An immunofluorescence staining assay was used to detect the translocation of nuclear faktor-kappa beta (NF-kB) p65. Tissue damage was evaluated by Haematoxilin-Eosin (HE) staining assay. We also used a flow cytometry assay to measure the number of nucleated cells and CD34+ hemopoietic stem cells in bone marrow. A western blot assay was used to detect the changes of proteins involving TLR signaling pathway. Results: We found that HKMT increased cell viability and inhibited cell apoptosis after irradiation. HKMT induced NF-kB translocation and activated Erk1/2, p38 signaling pathway. HKMT also protected bone marrow and testis from destruction. Radiation-induced decreases of nucleated cells and CD34+ hemopoietic stem cells in bone marrow were also inhibited by HKMT treatment. We found that radiation caused increase of inflammatory cytokines was also suppressed by HKMT. Conclusion: Our data showed that HKMT exhibited radioprotective effects in vivo and in vitro through activating NF-kB and MAPK signaling pathway, suggesting a potential of HKMT as novel radioprotector.

Y. Chen, Y. Xu and J. Du contributed equally to this work.

Department of Radiation Medicine, Faculty of Naval Medicine, Second Military Medical University; 800, Xiangyan Road, 200433, Shanghai (P.R. China)
E-Mail jujt0827@sina.com / cjmb820003@aliyun.com / yyyang2010@163.com
Introduction

Exposure to ionizing radiation (IR) often leads to severe damage to radiosensitive tissues, immunosuppression, which affects human health and limits the use of radiotherapy in cancer patients [1-3]. Cells and tissues like the ones present in the hematopoietic system, immunocytes, male reproductive system are very sensitive to IR, exhibiting severe injuries and dysfunction post-irradiation [4, 5]. In cancer therapy, ionizing radiation passes through and causes damages to normal tissues, even when accurate physical techniques are applied, such as Stereotactic Radiotherapy. Radiation may also cause a shift of Th1 to Th2 immune response, and thus caused immunosuppression [6, 7]. The only FDA approved radioprotector is amifostine, which is not free of toxicity [8]. It is urgently required to find novel effective and safe radioprotective compounds.

In 2008, Burdelya et al. reported that the ligand of toll like receptor 5 (TLR5) exerted radioprotective effects in mice through activating TLR5 and NF-kB signaling [9]. Later, our group demonstrated that TLR2, TLR4, and TLR9 ligands play critical roles in radioresistance [10-12]. We also showed that TLR2 ligand PAM3CSK4 protects mice from radiation damage [10]. However, traditional ligand of TLR2 showed severe toxicity when high doses were applied. TLRs recognized a variety of microbiota including bacterial, virus, and play critical roles in innate immunity [13, 14].

Previous studies have revealed that heat-killed Mycobacterium tuberculosis (HKMT) is a potent agonist of TLR2 signaling pathways [15, 16]. In pulmonary tuberculosis patients, heat-killed mycobacterium vaccine (SRL 172) reduced the influence of Th2 and enhancing Th1 to the benefit of the patients [17]. Several studies have also proved the safety of HKMT when used as adjuvant [18, 19]. We hypothesized whether HKMT could alleviate radiation damages as a potent TLR2 agonist with low toxicity. In present study, we demonstrated that HKMT protected mice and cultured cells against irradiation, induced NF-kB translocation and activated MAPK signaling pathway, indicating a potential role in radioprotection.

Materials and Methods

Cells and treatments

Human embryonic hepatocytes (L02 cells), and human umbilical vein endothelial cells (HUVEC) (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 (PAA Laboratories) medium with 10% fetal bovine serum (PAA Laboratories) and 1% penicillin-streptomycin-glutamine (Hyclone, Logan, UT, USA) at 37°C in a 5% CO₂ humidified chamber. After HKMT (Invivogen, US) treatment, cells were exposed to 8Gy of γ-irradiation at the dose rate of 1Gy/min and used in next experiments.

Irradiation

60Co-γ rays in Irradiation Center (Faculty of Naval Medicine, Second Military Medical University, China) were used for the irradiation purpose. The average energy of 60Co-γ rays is 1.25MeV. Mice with and without HKMT treatment were exposed to 7Gy of total body irradiation with a dose rate of 1Gy/min. The mice received total-body irradiation in a holder designed to immobilize unanesthetized mice such that the abdomens were exposed to the beam. The environment light intensity and temperature in irradiation center are kept the same as those in the animal house.

Mice and treatments

All the experiments were approved by the Second Military Medical University, China in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (publication No. 96-01). Male wild-type BALB/c mice, 6-8 weeks old, were purchased from China Academy of Science (Shanghai, China). Mice were housed in individual cages in a temperature-controlled room with a 12h light/dark cycle. Food and water were provided ad libitum. HKMT (500μg/Kg) was delivered through intragastric administration at 12h before irradiation. At different time post-irradiation, mice were killed by cervical dislocation. For each time point, femurs, testis tissues and bone marrow cells were isolated from at least 6
mice. We used femur and testis for sectioning and HE staining, and bone marrow cells for flow cytometry analysis.

CCK8 assay
HUVEC and L02 cells were seeded in 96-well plates in triplicate and then treated with HKMT (10 μg/ml). After 12 h, cells were subjected to γ-irradiation at the dose of 8 Gy. 48 h after irradiation, cell viability was measured by using a CCK8 kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions.

Apoptosis assay
At 24 h post-irradiation with and without HKMT treatment, apoptosis of HUVEC and L02 cells were determined by double-staining with Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and Propidium Iodide (PI) by Apoptosis Detection Kit (Invitrogen, Carlsbad, California, USA) and analyzed by flow cytometry (Beckman Cytoflex) according to the manufacturer’s instructions.

Immunofluorescence analysis
We used an immunofluorescence assay to detect the location of NF-kB p65 protein. Briefly, HUVEC cells were seeded on cover glasses in 6-well plates at the concentration of 2 × 10^5 per well. At 0.5 h and 2 h post radiation treatment, cells were fixed and stained with NF-kB p65 primary antibody (Santa Cruz, US; 1:200) and then with the secondary antibody (1:1000). Cellular images were obtained using an Olympus BX60 fluorescent microscope (Olympus America Inc., Center Valley, PA, USA) equipped with a Retiga 2000R digital camera (QImaging Inc., Surrey, BC, Canada). Fluorescent signals were quantified for 100 cells for each group by using ImageJ software (version 1.44p, Wayne Rasband, National Institute of Health (NIH), USA).

Flow cytometry analysis of bone marrow cells
On the first day post irradiation, bone marrow cells were isolated and analyzed by using flow cytometry. For bone marrow cells calculation, cells from each femur of one mouse were collected and the red blood cells were dissociated and removed. The total number of nucleated cells was calculated with a flow cytometry. For CD34+ hemopoietic stem cells (HSCs) detection, nucleated cells from bone marrow were stained with CD34-FITC and B220-PE antibodies and subjected to flow cytometry analysis. [20].

Tissues isolation and Haematoxilin-Eosin (HE) staining
On day 1, 3, 7 post total body irradiation, murine femur and testis tissues were isolated, fixed and subjected to sections. A HE staining method was applied to detect tissue damages as previously described [21].

Elisa assay
Exposure to IR often results in upregulation of multiple inflammatory cytokines, such as TNF-α, IL-6 and IL-1β etc. It was known that IR also causes an increase of Th2 response, while Th1 response is inhibited. To explore the influence of HKMT on radiation-induced inflammation, we examined the level of cytokines in blood serum. On the first day post-irradiation, blood serum was isolated and subjected to an Elisa assay according to the manufacturer’s instructions (Westang Tech., Shanghai, China).

Antibodies and Western blotting analysis
At 2, 8 h post-irradiation, the proteins from HUVEC cells were obtained by using ProtecJETTM Mammalian Cell Lysis Reagent (Fermentas, Vilnius, Baltic, Lithuania) according to manufacturer’s protocol, and then analyzed by western blotting to detect γH2AX (Cell Signaling Tech., 1:1000), TRIF (Proteintech, Wuhan, China; 1:1000), MyD88 (Abcam, US; 1:1000), p-Erk, p-Jnk, p38, and β-actin (sampler kit from Cell signaling technology, US; 1:1000). The secondary antibody (1:1000) was also purchased from Cell Signaling Technology.

Statistical analysis
Data are expressed as means ± the standard error of mean (SEM) for each experiment. The number of samples is indicated in the description of each experiment. We used an analysis of variance (ANOVA)
followed by a Student-Newman-Keuls post hoc test for statistical analysis. Experiments for quantification were conducted in a blinded fashion and all the experiments were repeated for at least 3 independent times.

**Results**

*HKMT improved cell viability and inhibited cell apoptosis caused by irradiation*

To evaluate the protective effects of HKMT, we measured the cell viability and apoptosis in human HUVEC and L02 cells. Our data showed that HKMT treatment significantly improved cell viability in both HUVEC and L02 cells (Fig. 1A, B). HKMT also inhibited cell apoptosis caused by irradiation in HUVEC and L02 cells (Fig. 1C, D, E, F).

*HKMT induced NF-kB translocation in cultured human cells*

NF-kB activation is a downstream effect of multiple TLRs, and it is also related to cell survival and radiation resistance [22]. We used an immunofluorescence staining method to detect the NF-kB translocation. It was found that in normal control groups, p65 was mainly located in the cytoplasm, while the level of p65 in nuclear increased hugely in the HKMT treated group. The ratio of p65 level inside and outside the nucleus was also quantified, indicating HKMT strongly activated NF-kB (Fig. 2A, B).

*Regulatory effects of HKMT on MAPK signaling pathway*

A western blot assay was used to detect the regulatory effects of HKMT on TLR2 and downstream signaling pathway. We found that HKMT reduced the phosphorylation of pJNK,

![Fig. 1.](image)

HKMT protected cultured cells from ionizing radiation. A, B: a bar graph of cell viability measured by a CCK8 assay in HUVEC and L02 cells. C, D: a bar graph of the percentage of Annexin V positive cells in HUVEC cells (C) and L02 cells (D). E, F: representative images of flow cytometry analysis of cell apoptosis in HUVEC cells (E) and L02 cells (F). *P<0.05, **P<0.01 Vs IR groups.
while enhancing the activation of p38 and pErk1/2. In addition, DNA damage is a main effect of radiation injury. Therefore, we tested the level of γH2AX and the phosphorylation of DNA-PKcs. Our data showed that HKMT reduced the level of γH2AX, marker of DNA double strand breaks. But no change in DNA-PKcs phosphorylation at 2056 was observed. (Fig. 3)

HKMT alleviated radiation damages on bone marrow and increased the number of CD34+ hemopoietic stem cells (HSCs)

Radiation caused severe damage to bone marrow, which exhibited reduced nucleated cells, congestion, and structural destruction. In HKMT treated group, the damages of IR on structure of bone marrow was alleviated, and the decrease of nucleated cells was significantly inhibited (Fig. 4). At day 7 post-irradiation, the bone marrow starts to recover, which was much faster than irradiation groups. We also found that HKMT treatment increased the total number of nucleated cells as well as the number of CD34+ HSC (Fig. 5A, B, C).

HKMT inhibited radiation injuries on testis tissues

Male reproductive system is very sensitive to IR, which could cause teratogenicity and carcinogenesis [23]. Irradiation resulted in severe structural damage and progressive germ cell loss. HKMT treatment protected germ cells from destruction in testis, and reduced condensation of nuclear chromatin (Fig. 6). HKMT also enhanced the recovery of sperm cells.
HKMT suppressed the inflammation induced by irradiation

IR induced a variety of inflammatory cytokines secretion, and in our study, we found that HKMT significantly suppressed the huge increase of IL-1β, IL-6, TNF-α in serum (Fig. 7A, B, C), which was all elevated in irradiation groups. The level of Th1 related cytokines IL-2, IL-12, IFN-γ was increased in HKMT treated group (Fig. 7 D, E, F). HKMT treatment also significantly reduced the level of Th2 related cytokine IL-13, while no obvious changes was observed in IL-4 and IL-10 (Fig. 7G, H, I). These showed that, in addition to anti-inflammation, HKMT also reversed the shift from Th1 to Th2 immune response caused by irradiation.
Discussion

In our present study, we demonstrated that a heat killed bacteria, HKMT, exerted protective effects in cells and mice against ionizing radiation. HKMT increased cell viability
which was reduced by irradiation, and inhibited radiation-induced apoptosis. IR also resulted in severe injuries on radiosensitive tissues such as bone marrow and testis, while in HKMT treated group, tissue damages was obviously mitigated in terms of tissues structure, nucleated cells and also inflammation. After HKMT treatment, the number of HSC was also protected. As it has been reported that as an adjuvant, HKMT exerted its function through regulating TLR2 signaling pathway, we checked and found that HKMT induced NF-kB translocation and activated MAPK signaling pathway.

To our knowledge, this is the first study demonstrating the radioprotective effects of HKMT on cells and animals. It has been reported that TLR2 was related to the basal resistance of IR [10]. IR directly or indirectly interacts with DNA and causes different types of isolated or clustered DNA damages including double strand breaks or single strand breaks [24]. In our present study, we found that HKMT increased cell viability in cells and inhibited radiation-induced apoptosis. These data provide a possibility that HKMT might exert protective effects in vivo.

Then we investigated the radioprotective effect of HKMT on bone marrow and testis. IR induces a huge decrease of nucleated cells in bone marrow and repression of hematopoiesis [25, 26]. We found that HKMT protected bone marrow structure and restored the number of nucleated cells. CD34+ hematopoietic stem cells (HSCs) were the source of almost all the blood cells and immunocytes [27]. Our data also showed that HKMT increased the number of HSCs in bone marrow after irradiation. On the other hand, we found that HKMT inhibited

Fig. 7. HKMT regulated inflammatory cytokines and Th1/Th2 balance. A-I: bar graphs of cytokines level in blood serum 24h after irradiation. *P<0.05, **P<0.01 Vs IR groups. (n=6.)
radiation injuries on testis, which are very sensitive to IR. As radiation induced testis damage increased the possibility of teratogenesis [28-31], HKMT provide novel therapeutic strategy for radioprotection on normal tissues.

IR often causes imbalance of immune response, one example of which is the shift from Th1 to Th2 response [6]. Radiation-induced Th1/Th2 imbalance also accounts for immunosuppression in irradiated animals and human [32]. Even after a long time, when the structure of immune organs is reconstructed, immunity remains suppressed [7]. In our study, HKMT reversed the radiation-induced Th1/Th2 imbalance, suggesting a role of HKMT in regulating immune response post-irradiation. HKMT also reduced of inflammatory cytokines which was upregulated by IR, including TNF-α, IL-6 and IL-1β, showing that HKMT might play an anti-inflammation role.

It was reported that HKMT is a potent TLR2 agonist [33-35]. In our study, we investigated the effects of HKMT on TRIF and MAPK signaling pathways. Our data showed that compared to single radiation group, HKMT significantly induced a translocation of NF-kB p65 protein and induced the phosphorylation of Erk and p38, providing a novel mechanism underlying the radioprotective effects of HKMT.

In conclusion, our data showed that HKMT effectively protected cells and mice against IR. HKMT inhibited cell apoptosis after irradiation and induced NF-kB translocation and activated Erk1/2, p38 signaling pathway. HKMT also protected bone marrow and testis from destruction. Radiation caused increase of inflammatory cytokines was also suppressed by HKMT. These data suggests a potential of HKMT as a safe and effective radioprotectant.

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**Disclosure Statement**

The authors have no interest of conflicts to disclose.

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