Anti-Oxidative and Immuno-Protective Effect of Camel Milk on Radiation-Induced Intestinal Injury in C57BL/6 J Mice

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Abstract

Purpose: The main objective is to investigate the protective effect of camel milk (CM) on radiation-induced intestinal injury.

Methods: The C57BL/6 J mice in 2 experiments were assigned into control group (Con), irradiation group (IR), and CM+irradiation group (CM+IR). After receiving the CM via gavage for 14 days, the mice in the first experiment were exposed to 6 Gy X-ray whole body irradiation, and survival rate was compared among the groups. Mice in the second experiment were exposed to 4 Gy irradiation and sacrificed at day 7. The small intestines were collected to examine the histopathological changes and to determine the anti-oxidative index and HMGB1/TLR4 inflammatory pathway. Fasting blood was used to measure serum pro-inflammatory factors.

Results: Compared with the IR group, the survival time was prolonged, and survival rate was increased in the CM+IR group. CM increased levels of SOD and GSH and decreased MDA in the jejunum. Furthermore, intestinal protein expression of HMGB1/TLR4 pathway (TLR4, NF-κB, and HMGB1) was up-regulated by CM intervention. CM decreased the serum levels of TNF-α and IL-1β and increased IL-10 level.

Conclusions: CM extended the survival time and had a protective effect against radiation-induced jejunum injury by regulation of antioxidant capacity and HMGB1/TLR4/NF-κB/MyD88 inflammatory signaling pathway.

Introduction

With the increasing incidence of cancer, radiotherapy has become one of the most important treatments. Although this technology has made great progress, radiation-related injury restricts its application in clinical practice.[1] Small intestine is one of the organs that are most sensitive to radiation.[2] The main symptoms of radiation-induced intestine injury include anorexia, vomiting, diarrhea, dehydration, systemic infections, and in extreme cases, septic shock, and death.[3,4] These conditions seriously affect the prognosis of patients with abdominal or pelvic tumors and ultimately reduce their quality of life.[5] Amifostine is the only radioprotective agent currently approved by the U.S. Food and Drug Administration (FDA) for the prevention of severe radiation-induced toxic reactions. However, it could cause hematotoxicity and gastrointestinal toxicity.[6] Therefore, the development of novel radio-protective products with low toxicity from functional food is urgently needed.[7]

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Camel milk (CM), which has a special healthy function, has received recent research attention.[8] Compared with traditional cow milk, CM has lower fat, and is richer in protein and lactoferrin content and contains more polyunsaturated fatty acids and linoleic acid, which are necessary for human nutrition.[9] In addition, CM has unique antioxidant and anti-inflammatory properties due to its biologically active protein and vitamin components.[10,11] It can effectively alleviate the symptoms of rheumatoid arthritis,[12] diabetes,[13,14] and fatty liver,[15] and even promote wound healing.[16] Whether CM influences radiation-induced intestine injury is rarely reported. In this study, the protective effect of CM on radiation-induced intestinal injury model was determined. This work provided a novel adjuvant method for patients with abdominal tumors undergoing radiation therapy.

Materials and Methods

Reagents and Antibodies

CM was purchased from Xinjiang Nanshan Pasture (Urumqi, China). The milk was collected from *Camelus bactrianus.* After traditional pasteurization, it was transported to the laboratory via food cold chain logistics within 48 h. Cytokines TNF-α, IL-1β, IL-6, and IL-10 kits were obtained from Wuhan ColorfulGene Biological Technology Company. SOD, MDA, and GSH kits were acquired from Nanjing Jiancheng Bioengineering Institute. Antibodies for Western blot, namely, HMGB(166525-1-Ig) and TLR4 (19811-1-AP) were purchased from ProteinTech Group (Chicago, IL, USA). MyD88 (YT2928) was purchased from ImmunoWay Biotechnology Company (Plano, TX, USA). IKKβ (D30C6) was obtained from Cell Signaling Technology (Shanghai, China). NF-κB (ARG65677) was acquired from Bio-Platform Technology Company (Shanghai, China). β-actin (BM 0627) was bought from Boster Biological Technology (Wuhan, China). BeyoECL Plus (P0018) and BCA Protein Assay Kit were purchased from Beyotime Company (Shanghai, China).

Animals

Six-week-old C57BL/6 J mice (20 ± 2 g body weight) were obtained from Shanghai Shrek Company Chinese Academy of Sciences and housed under a 12 h/12 h light/dark cycle at a constant temperature of 22 ± 2°C and 60 ± 5% relative humidity. All experimental procedures were performed in accordance with the Guidelines in the Care and Use of Animals and with the approval of Soochow University Animal Welfare Committee.

Experiment to Assess Animal Survival

After 1 week of acclimatization, 18 animals were assigned randomly to the control group (Con), 6 Gy group (IR), and CM+6 Gy group (CM+IR). Mice were administered 0.2 ml of CM through gavage twice every day for 14 days before irradiation.[8] The mice in the Con and IR groups were administered distilled water. At day 15, mice in the IR and CM+IR groups were exposed 6 Gy of whole-body X-ray irradiation by using a RS-2000 biological X-ray irradiator (Rad source technology, Suwanee, GA, USA) at a dose rate of 1.283 Gy/min.[17,18] Animals were observed twice a day until all mice died. Death time and number of mice were recorded. The percentage of surviving animals was used for survival analysis. This experiment ended at 21 days after radiation.

Experiment to Observe Intestinal Injury

For the observation of intestinal injury, a total of 18 animals were divided randomly into the control group (Con), 4 Gy group (IR), and CM+4 Gy group (CM+IR). The same methods involving CM intervention and irradiation exposure (except irradiation dose) were used to assess animal survival. All the mice were observed for any signs of radiation sickness, and body weight was recorded daily.

Collection of Sample

The mice were sacrificed 7 days after 4 Gy irradiation. Portions of the jejunum were fixed immediately in 10% formalin for future histological observation, and the remaining portions were stored at −80°C until further use. The thymus and spleen were immediately dissected and weighed. Blood samples were collected from the retrobulbar vein. Serum was separated by centrifugation and stored at −80°C.
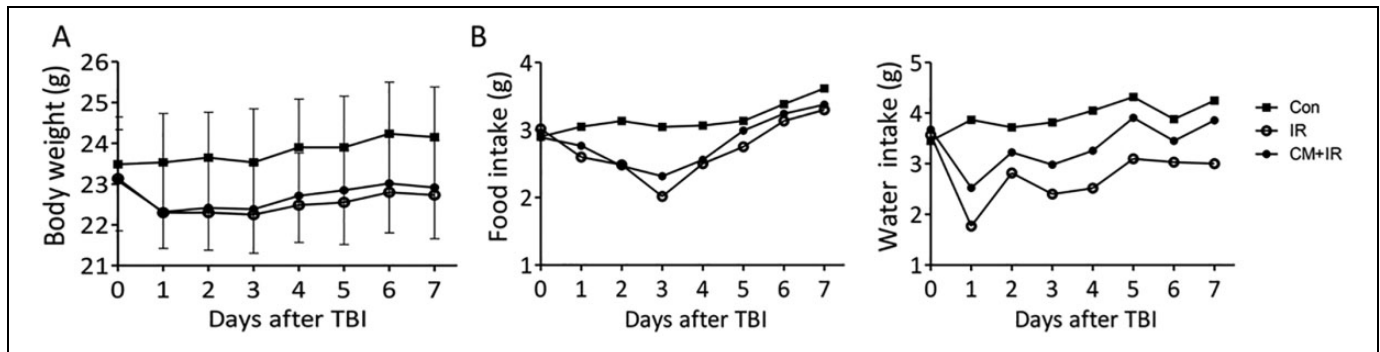
Pathological Observation of Intestinal Tissue

Paraffin-embedded sections of intestinal tissues were dewaxed with 5 μm sections, stained with hematoxylin-eosin (HE) (Sigma-Aldrich, USA), and examined under a microscope for assessment. The number of surviving crypts per circumference of the jejunum was assessed quantitatively by pathologists under blinded conditions. Ten circular transverse sections per mouse were stained with HE and blindly analyzed from the photographs to measure the length of small intestinal villi. The depth of small intestinal crypts were also measured. The ratio of villus length to crypt depth (V/C) was calculated as an index to determine the comprehensive function of the small intestine.[18,19]

Radiation Injury Score (RIS)

The overall severity of structural radiation injury was assessed using the RIS system.[17] RIS is a composite histopathologic scoring system that provides a global measure of the severity of structural radiation injury. Seven histopathologic parameters of radiation injury, namely, mucosal ulceration, epithelial atypia, subserosal thickening, vascular sclerosis, intestinal wall fibrosis, ileitis cystica profunda, and lymph congestion, were assessed and graded from 0 to 3. RIS value is defined as the sum of individual alteration scores.

Figure 1. (A) The survival time of the 3 groups during the experiment. *P < 0.05, **P < 0.01, ***P < 0.005. (B) The survival curve of the 3 groups during the experiment. P = 0.04 compared with the IR group.

Determination of Organ Index

To give a quantitative assessment of the immune response, spleen and thymus indexes (Sx) were calculated as follows: Sx = [Weight of experiment organ (mg)]/[Weight of experiment animal (g)].[20,21]

ELISA Assay for Inflammatory Cytokines

The obtained tissue samples were homogenized with a tissue homogenizer at 4°C for 10 min and then centrifuged at 3000 rpm for 10 min. The supernatants were stored at −80°C. Inflammatory cytokines IL-6 and IL-10 and serum inflammatory factors TNF-α, IL-1β in the supernatants were tested according to kit instructions using quantitative sandwich assay to determine the concentrations. OD value was measured by multimode plate reader (Synergy NEO, BioTek, USA) at 450 nm, and the levels of each factor were calculated.

Measurement of SOD and GSH Activity and MDA Content

SOD activity in jejunum was examined using the xanthine oxidase method provided by the standard assay kit,[22] in which the xanthine–xanthine oxidase system was employed to produce superoxide ions that will react with 2-(4-iodophenyl)-3-(4-nitrophenol-5-phenlyltetrazolium chloride) to form a red formazan dye. Absorbance at 550 nm was determined. Protein concentration was determined by a BCA protein assay kit, and the values were expressed as units per mg protein. One unit of SOD was defined as the amount of SOD inhibiting the rate of reaction by 50% at 25°C. GSH activity in jejunum was tested according to the manufacturer's instructions and recorded using a microplate reader at 405 nm absorbance. Lipid peroxidation was evaluated by measuring the MDA concentrations using the thiobarbituric acid (TBA) method as commercially recommended. This technique was based on the spectrophotometric measurement of the color produced during the reaction of TBA and MDA. MDA content were calculated by the absorbance of TBA reactive substances at 532 nm.

Western Blot Analysis in Jejunum Tissues

Jejunum tissue samples were homogenized in ice-cold lysis buffer and centrifuged, and the supernatants were collected. Protein concentration was measured using the BCA protein assay kit. After denaturation, equal amounts of protein (50 μg) were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane. The membranes were blocked with 5% skim milk in Tris-buffered saline Tween-20 solution for 1 h and then incubated overnight with the following primary antibodies at 4°C: HMGB1 (1:1000), IKKβ (1:1000), TLR4 (1:400), MyD88 (1:1000), NF-κB (1:500), and β-actin (1: 1000). After washing thrice in PBST and incubated with appropriate secondary antibodies, the membranes were developed by chemiluminescence using Superstar ECL Western blot substrate reagents. Band intensities were quantified using Image J software. All experiments were conducted in triplicate, and the average value was obtained.

Statistical Analysis

All data were expressed as the mean ± standard (SD). Differences between groups were tested by 1-way ANOVA (SPSS24), followed by the LSD post hoc test. Differences were considered statistically significant at P < 0.05.

Results

CM Extended the Survival Time of Irradiated Mice

The mice in the IR group started to die at day 9 after irradiation and the last mouse died at day 11. In CM+IR group, the first death also occurred at day 9, and the last mouse died at day 21. The mean survival time was significantly longer in the CM+IR group than in the IR group (14.40 ± 4.09 vs. 10.60 ± 1.03 days, P < 0.05) (Figure 1A). Survival analysis showed that the survival rate was significantly higher in the CM+IR (P = 0.04) than in the IR group (Figure 1B).

Figure 2. Body weight (A) and daily food/water intake (B) of mice during the experiment.

Figure 3. Effects of CM on radiation-induced intestinal injury in mice. (A) Representative images of the HE stained sections of the jejunum; (B) radiation injury score; (C) villus length of small intestine; (D) crypt depth of small intestine; (E) V/C ratio of small intestine; (F) regenerated crypt/ circumference of small intestine. *P < 0.05, ***P < 0.005.

CM Ameliorated the General Status of Mice

Radiation decreased the food and water intake and the body weight (Figure 2). Although food and water intake recovered within 2-3 days, the body weight did not increase. No significant difference in the body weight and food/water intake was found between the IR and CM+IR groups.

CM Attenuated the Radiation-Induced Intestinal Injury in Mice

HE staining of the jejunum was performed to evaluate the protective effect of CM on mice at 7 days after radiation. Compared with the Con group, the intestinal villi in the IR group

were shorter, thicker, and more irregularly arranged. In the CM+IR group, the villi statue was gradually repaired, and they became regularly organized (Figure 3A). CM had a remarkable improvement effect on radiation-induced intestinal injury. RIS was used to evaluate the extent of intestinal injury. Compared with the IR group, the CM group had significantly decreased RIS (Figure 3B).

The intestinal villus length, crypt depth and number, and V/C ratio were measured (Figure 3C-F). The average villus height was shorter in the IR group than in the Con group (271.90 ± 37.09 μm vs. 411.14 ± 25.701 μm), and the V/C ratio was markedly reduced from 5.94 ± 0.65 in the Con group to 3.67 ± 0.61 in the IR group. All these data indicated that radiation seriously damaged the intestine. CM effectively
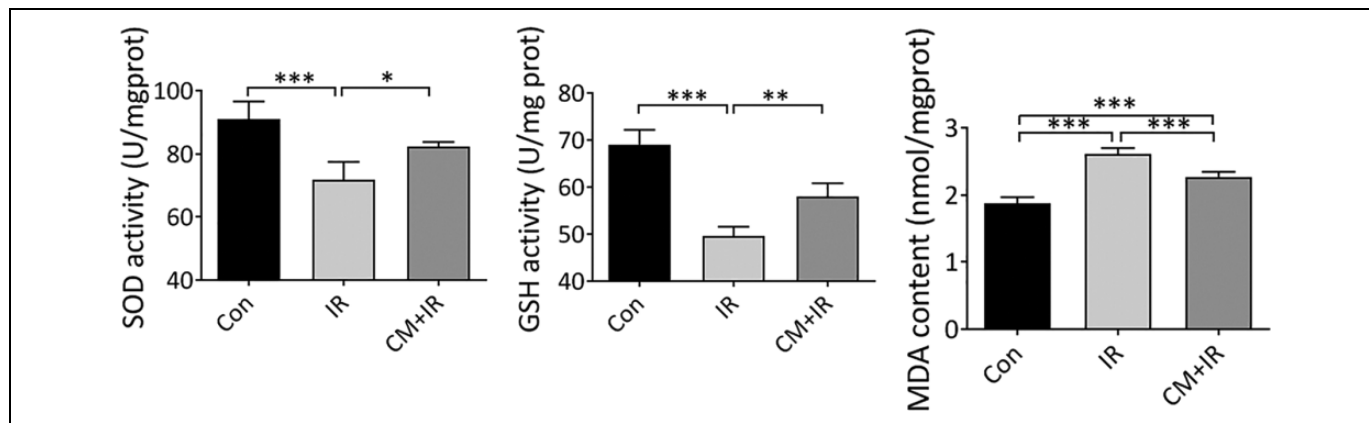
Figure 4. The effect of CM on anti-oxidative activity in jejunum of mice. (A) SOD activity; (B) GSH activity; (C) MDA content. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.
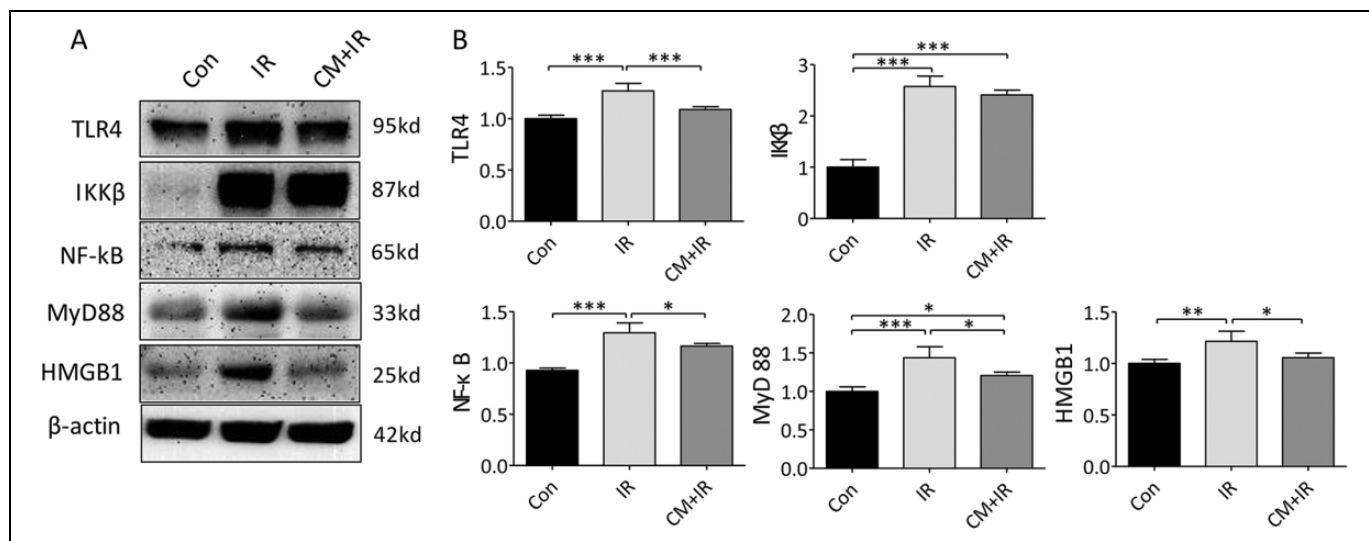
Figure 5. CM reduced inflammation response via HMGB1/TLR4 pathway. (A) Representative protein expression of Western blot. (B) Average band intensity of TLR4, IKKβ, NF-κB, MyD88, and HMGB1 in jejunum homogenate. The expression was normalized with respect to the Con group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.

improved the villus statue by increasing the length to 327.95 ± 26.08 μm and the V/C ratio to 4.66 ± 0.57 in the CM+IR group, indicating its protective effect on radiation-induced intestinal damage. Although no significant differences were found in crypt depth among the 3 groups, CM significantly increased the number of crypts.

CM Promoted Anti-Oxidative Activity in Jejunum Exposed to Radiation

SOD, GSH, and MDA levels were detected to evaluate the oxidative stress level of jejunum tissues. Compared with the Con group, SOD and GSH levels significantly decreased, and the MDA level significantly increased in the IR group. This finding indicated that radiation induced oxidative damage to mice jejunum. Pre-treatment with CM before radiation effectively alleviated this phenomenon. Compared with the IR group, the mice in the CM+IR group showed an increase in SOD and GSH levels and a decrease in MDA level (Figure 4).

CM Inhibited the Expression of HMGB1/TLR4 Pathway Inflammation-Related Proteins in Jejunum Exposed to Radiation

The expressions of inflammation-related proteins were examined by using Western blot analysis to address the underlying mechanism of CM protective effect on radiation-induced intestinal injury. TLR4, IKKβ, NF-κB, MyD88, and HMGB1 levels increased significantly after irradiation but decreased after the pre-treatment with CM. These results showed that CM could mediate HMGB1/TLR4 pathway and inhibit radiation-induced inflammation in the jejunum (Figure 5).
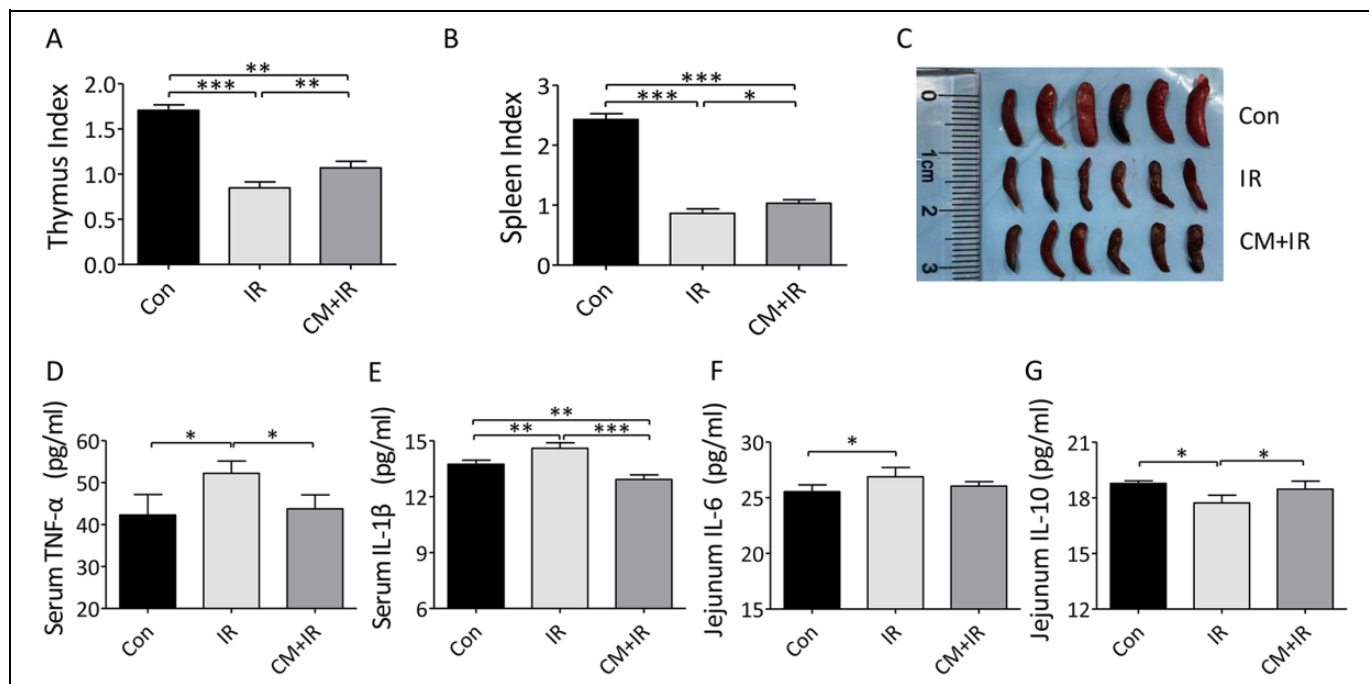
Figure 6. Effect of CM on the organ indexes and inflammatory cytokine levels in mice. (A) Thymus index. (B) Spleen index. (C) Spleen anatomy. (D) Serum TNF-α level. (E) Serum IL-1β level. (F) Jejunum IL-6 level. (G) Jejunum IL-10 level. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.

CM Improved the Organ Index and Inhibited the Inflammatory Cytokine Levels of Irradiated Mice

Radiation significantly decreased the thymus and spleen indexes. Compared with the IR group, the thymus and spleen indexes significantly increased in the CM+IR group. CM protected the immune organs that were damaged by radiation (Figure 6A-C).

ELISA was used to detect the levels of pro-inflammatory factors TNF-α and IL-1β in the serum and the levels of inflammatory cytokines IL-6 and IL-10 in jejunum tissues (Figure 6D-G). The serum levels of TNF-α and IL-1β in the IR group were significantly higher than those in the Con group. After CM intervention, the levels of TNF-α and IL-1β were significantly restored ($P < 0.005$). The level of anti-inflammatory factor IL-10 significantly decreased in the IR group ($P < 0.05$) but significantly increased after CM intervention ($P < 0.05$).

Discussion

Radiation is commonly used in cancer therapy, especially in the treatment of gynecological and colorectal cancers. The high proliferative rate of intestinal epithelial cells and stem cells makes the bowel one of the organs that are most sensitive to radiation injury. Thus, methods to alleviate radiation-induced small intestine injury are needed to extend survival.[19,23]

In this study, CM was first examined to explore its protective effect on radiation-induced intestinal injury. CM intervention extended the survival time and improved the survival rate of irradiated mice. Survival extension was attributed to the alleviation of the injury of the small intestines, which are susceptible to radiation because they contain rapidly dividing transit cells.[24] Crypt areas were visualized by HE staining, and the findings showed that CM histopathologically attenuated the intestinal injury and quantitatively increased the villus length and V/C ratio. The level of oxidative stress in jejunum was also tested to further understand the underlying mechanism. Radiation substantially reduced SOD and GSH activities in jejunum tissues and increased MDA level. These adverse reactions were effectively improved by CM intervention. Arab also revealed that CM can inhibit renal oxidative stress and enhance antioxidant capacity.[25] The distinctive anti-oxidant properties of CM is likely due to its high content of vitamins C and E, zinc, and selenium.[12]

CM protected the immune organs destroyed by radiation, decreased inflammatory factors TNF-α and IL-1β in the serum, and increased anti-inflammatory factor IL-10 in the jejunum. Inflammatory factors play an important role in the occurrence and development of radiation-induced intestinal injury.[26] CM has a potential anti-inflammatory effect that contributes to its immune-protective influence on radiation-induced intestinal injury. Zhu et al found that CM reduces the LPS-induced increase in neutrophil infiltration and TNF-α and IL-1β levels in rats.[27] By constructing a rheumatoid arthritis mouse model, Salama et al found that CM lowers TNF-α and augments the anti-inflammatory IL-10 levels in the sera and exudates of arthritic rats.[12] The antioxidant and immune regulation
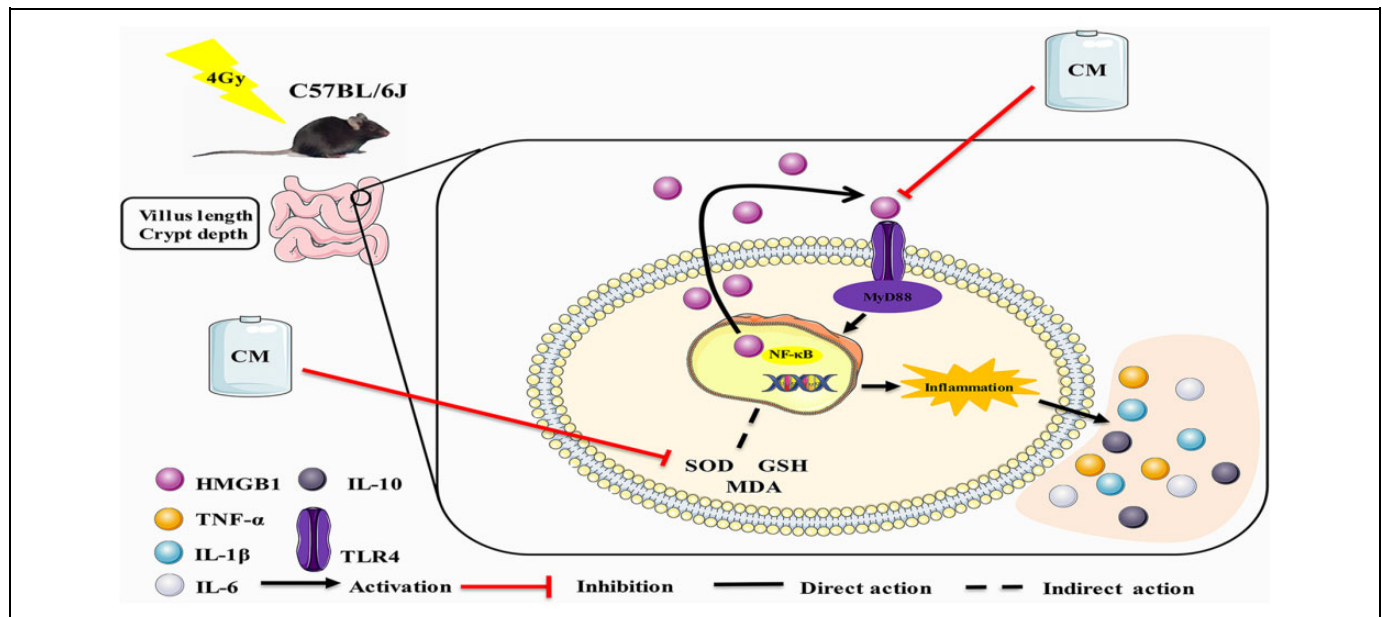
Figure 7. Mechanism of CM protective role in radiation-induced intestinal injury.

capacities of CM played important roles in its radio-protective effect.

Several studies have shown that CM has antioxidant capacity. Since HMGB1 plays pivotal roles in the regulation of the cellular response to stress. We further investigated whether the antioxidant effect of CM is mediated by HMGB1 and downstream signaling pathways, which included TLR4, IKKβ, NF-κB, and MyD88.[28] HMGB1 is a late-stage inflammatory factor that can be passively released by necrotic and damaged cells or activated through immune cell activation. Once released in the extracellular space and upon its interaction with a large panel of cell surface receptors, HMGB1 exerts a plethora of cell regulatory functions from maturation, proliferation, and motility to inflammation, survival, and cell death.[28-31] HMGB1 can also directly bind to TLR4 and induce the secretion of pro-inflammatory cytokines.[32] CM significantly reduced the irradiation-induced upregulation of HMGB1/TLR4 protein in intestinal tissues.

CM contains many nutrients, and the protective effect on radiation-induced intestinal injury is due to the combined actions of various proteins, vitamins, and nutritional factors. Lactoferrin, which exhibits various beneficial properties,[33] is used as an example. CM has higher lactoferrin content than milk from cows, goats, and buffaloes.[34] A study revealed that lactoferrin could increase the survival rate of mice exposed to X-ray irradiation by decreasing the levels of serum IL-6 and TNF-α and reducing the expression of radiation-induced IKKα/β and NF-κB.[19]

In conclusion, our findings demonstrated that CM reduces the level of inflammatory cytokines by regulating the HMGB/TLR4/NF-κB/MyD88 pathway and the antioxidant capacity to decrease radiation-induced intestinal damage (Figure 7). To elucidate the exact and comprehensive mechanism in the future, it is necessary to conduct local irradiation that is limited to the gastrointestinal tract.

Author Contributions

Yu-Zhong Chen and Chao Li contributed equally to the study.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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