Original Article

Beneficial Effect of Rosuvastatin Therapy on Spleen Injury Induced by Gamma Irradiation in Rats: Targeting Nrf2/EPRE Pathway

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Abstract

Purpose: The present study investigates the new approach of rosuvastatin (RUV) administration as a drug for the management of spleen injury induced by gamma irradiation.

Main Methods: Forty rats were used and divided equally into 4 groups: control group, irradiated group, IRR + rosuvastatin group (10 mg/Kg b. wt), and IRR + rosuvastatin group (20 mg/kg b. wt) for 7 days orally.

Results: The possible curative effect can be illustrated via the improvement of hematopoietic cell count (Hb, RBCs, and WBCs) and oxidative stress markers (MDA and GST) in addition to biochemical parameters including [heme oxigenase-1 (HO-1), nuclear erythroid 2-related factor (Nrf2), NOD-, LRR- and pyrin domain- containing protein 3 (NLRP3) inflammasome] and immune assay of nuclear factor kappa beta (NF-kB P65) and inducible nitric oxide synthase (iNOS). Histological pictures emphasize the biochemical findings. Rosuvastatin treatments by using two different doses improve the tested parameters. High-dose administration of RUV (20 mg/kg p.o.) recorded better results than the low dose (10 mg/kg p.o.).

Conclusion: Our results suggested that rosuvastatin reversed the radiation-induced spleen-damaging effects. So, RUV can be introduced to the market as a new therapy for the management of spleen damages.

Keywords

Gamma radiation, Spleen, Rosuvastatin, NF-kB, Nrf2

High Lights

- Effect of high-dose gamma irradiation on spleen.
- Effect of rosuvastatin 10 mg/Kg b. wt. against spleen damage.
- Effect of rosuvastatin 20 mg/Kg b. wt. against spleen damage.
- Focus on Nrf2/EPRE pathway.

Introduction

Ionizing radiation (IR) has received a lot of interest because of both its positive and potentially harmful effects on humans. The majority of ionizing radiation's harmful effects are caused by the production of reactive oxygen species (ROS) by radiolysis of water, which results in the synthesis of various reactive intermediates.[1] To combat oxidative stress,

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the body has a defense system that includes enzymatic and non-enzymatic radical scavengers that can either directly detoxify reactive oxygen species or regulate their levels indirectly.[2] Overproduction of ROS causes uncontrolled chain reactions and lipid peroxidation, resulting in a variety of severe diseases affecting numerous body organs.[3] Endogenous antioxidant enzymes, which are thought to operate as a first-line defensive mechanism to maintain redox equilibrium and normal metabolic processes, are reduced by radiation exposure. As a result, antioxidant supplementation to boost radiation efficacy is a recent proposed method, as antioxidants can scavenge free radicals produced by radiolysis of water and protect cells from harm.[4] As a result, cellular antioxidant capacity declines, making organs more vulnerable to the harmful effects of ROS.[5] Total body irradiation has also been shown to cause oxidative tissue damage in rats.[6]

The blood is essential for removing waste products (carbon dioxide, lactic acid, and urea) from the same cells and tissues, as well as the immune system, clotting, and body temperature regulation, among other things.[7] Changes in RBC count are still considered the most sensitive biological evidence for both internal and external radiation exposure. The types and amounts of cells in the blood, particularly RBCs, are revealed by a complete CBC. Anemia, infection, and a variety of other illnesses can all be diagnosed with complete blood picture.[8,9] In medicine, there is a lack of understanding of hematological indices and the changes caused by various gamma-radiation dosages.

Statins have been proven in several trials to have benefits other than decreasing cholesterol. These non-lipid pleotropic effects include antioxidant effects,[10] anti-inflammatory effects,[11] upregulation of endothelial nitric oxide synthase,[12] osteogenic effect,[13] inhibition of platelet adhesion and aggregation,[14] and normalization of sympathetic outflow.[15] Furthermore, statins have showed encouraging outcomes in the treatment of primary biliary cirrhosis when combined with ursodeoxycholic acid.[16] Rosuvastatin (RUV), a relatively novel HMG-CoA reductase inhibitor, has shown a stronger affinity for HMG-CoA reductase's active site than other statins. Furthermore, rosuvastatin's cytoprotective effect against ischemia injury has been well established.[17] In vitro and in vivo animal studies also show that rosuvastatin has vasculoprotective benefits in addition to its lipid-lowering capabilities, which are known as statin pleiotropic effects. Endothelial function is improved, atherosclerotic plaque stability is improved, oxidative stress and inflammation are reduced, and the thrombogenic response is inhibited. As a result, we discovered that rosuvastatin is a promising target for research into its protective benefits in biliary obstruction-induced damage and spleen inflammation. So this study aimed to evaluate the effect of rosuvastatin by two doses against gamma irradiation-induced spleen injury in female rats.

Material and Methods

Animals

The rats utilized in the experiment were adult female albino rats weighing 130–140 g. The National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Cairo, Egypt, housed rats in an animal house. The animals were given normal food pellets and water *ad libitum* and were kept in good ventilation and lighting conditions. The animals' care, handling, and treatment regimen were carried out in accordance with NCRRT's animal ethics in Cairo, Egypt.

Radiation Process

The National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Cairo, Egypt, used a Canadian Gamma Cell -40, (^{137}Cesium) to undertake whole-body gamma irradiation. The rate of dosing was .61 Gy/min. Rats were given a single dose of 7 Gy administered as an acute dosage.

Drugs. Rosuvastatin (RUV) (10 and 20 mg/kg p.o.). All doses were obtained from literature.[18]

Experimental Design

Rats (n = 40) were divided into 4 groups each consisting of 10 rats. Group I: served as control (received saline orally). Group II: irradiated group (rats were exposed to whole-body gamma radiation as a single dose 7 Gy). Group III: rats were exposed to radiation as in group II then treated with low dose of RUV (10 mg/kg b.wt) for 7 consecutive days. Group IV: rats were exposed to radiation as in group II then treated with high dose of RUV (20 mg/kg b.wt) for 7 consecutive days.

Preparation of Samples

Rats were anesthetized using urethane (1.2 g/kg, i.p.). Blood samples were collected by heart puncture; the blood was placed on ethylenediaminetetra-acetic acid (EDTA) from Sigma Aldrich Chemical Co. (St Louis, Missouri, USA), for hematological analysis. Animals were dissected and the spleens were quickly removed. The tissue was divided into two parts, first was used for biochemical analysis and the second was used for histological examination. For biochemical analysis, the homogenates were prepared using saline solution 1: 10 w/v and were spun down with universal

16 R centrifuge, at 4°C and 3999 r/min for 29 min. Aliquots of supernatants were separated and used for the further analysis.

Hematological Analysis

The hemocytometer (Improved Neubauer counting chamber, Germany) was used to assess the hematological parameters, red blood cell (RBC) count, and white blood cell (WBC) count, as previously described by Dacie and Lewis.[19] According to Van Kampen and Zijstra,[20] haemoglobin (Hb) was measured using a commercial kit (Diamond Diagnostics, Egypt).

Biochemical Analysis

Assessment of Spleen Inflammatory and Oxidative Stress Biomarkers. NLRP3, Nrf2, and HO-1 was determined using ELISA Kit (BioSource, San Diego, CA, USA). All procedures were assayed according to manufacturer's instructions. In addition, malondialdehyde (MDA) was determined according to the method of Yoshioka et al,[21] and GST was estimated according to the method of Habig et al.[22]

Histopathology Examinations of Spleen Tissues

Spleen tissues were fixed in 10% formalin, then cut, cleaned, and dehydrated in progressively higher alcohol concentrations. Following that, the dehydrated specimens were cleaned in xylene, fixed in paraffin blocks, and sectioned at a thickness of 4–6 m. For histological analysis using an electric light microscope, the acquired tissue slices were deparaffinized with xylol and stained with hematoxylin and eosin (H&E).[23]

Statistical Analysis

Data were reported as mean ± S.E. They were analyzed using one-way ANOVA followed by Tukey–Kramer multiple comparison tests. The significance levels were set at $P < .05$.

Results

Effect of RUV Treatment on CBC Components

Exposure of rats to gamma radiation showed a decrease in Hb (3%), RBCs (12%), and WBCs (82%) as compared to the normal control group. While treatment with rosuvastatin (10 & 20) normalized RBCs and slight ameliorated WBCs when compared to the irradiated group (Figure 1).

Effect of RUV Treatment on Oxidative Stress Markers

Gamma radiation caused significant elevation in the levels of MDA (Fig: 2; 211%) as compared to normal control. On the other hand, the treatment with rosuvastatin (10 mg/kg) led to a significant decrease in the MDA level as compared to the irradiated group (Figure 2). While the treatment with rosuvastatin (20 mg/kg) recorded better results than RUV_{10} group comparing to control and irradiated groups (Figure 2). Radiation significantly decreased GST and HO-1 activities by 57% and 67%, respectively, as compared to normal control (Figure 2). RUV treatment ameliorated GST and HO-1 activities as compared to the irradiated group. Previous study data showed that RUV (10 and 20 mg) significantly improved the oxidative stress status.

Effect of RUV treatment on inflammatory markers

Irradiation caused a significant decrease in Nrf2 (Figure 3; 70%) and a significant increase in NLRP3 (Fig: 3; 500%) as compared to the control group. Meanwhile, rosuvastatin (10 & 20 mg) treatment ameliorated Nrf2 (Figure 3; RUV_{10}: 133.33% & RUV_{20}: 200%, respectively) and NLRP3 (Figure 3; RUV_{10}: 50% & RUV_{20}: 30%, respectively) as compared to the irradiated group.

Histopathological findings

The histological sections of spleens of control rats showed normal splenic capsule and normal architecture of white pulp follicles with germinal centers surrounded by lighter marginal zones and the red pulp (Figures 4(a)-(c)). The irradiated group showed thickening of the splenic capsule which appeared irregular in some areas with marked congestion of splenic blood vessels and prominent hemorrhage. The red pulp showed congested blood sinusoids. Marked white pulp follicles atrophy (Figures 4(d),(e)) was a conspicuous finding with an obvious increase in the red pulp area in relation to the white one. The white pulp follicles showed thickening of the central arteriole and severe lymphocytic depletion in the periarteriolar sheath, follicle, and the marginal zone (Figures 4(f)) with appearance of the underlining reticular mash and increased number of tingible body macrophages. Rosuvastatin at both low (Figures 4(g)-(i)) and high (Figures 4(j)-(l)) doses almost restored the normal organization of the spleen particularly at the high-dose administration. The splenic follicle structure and size were markedly restored and showed mild-to-moderate degree of lymphocyte depletion with appearance of tangible body macrophages particularly in the high-dose group. Total scoring of histopathological data presented in Figure 5.

Immunohistochemistry results

The immune expression of NFκβ p65 and iNOS within the splenic tissue of various groups is presented in Figure 6 which showed intense diffuse immune-expression of NFκβ p65 and iNOS in the splenic tissue of the irradiated group, compared to the control and rosuvastatin treated groups. The latter groups showed a dose-related significant decreased expression of both markers as estimated by the image analysis software.

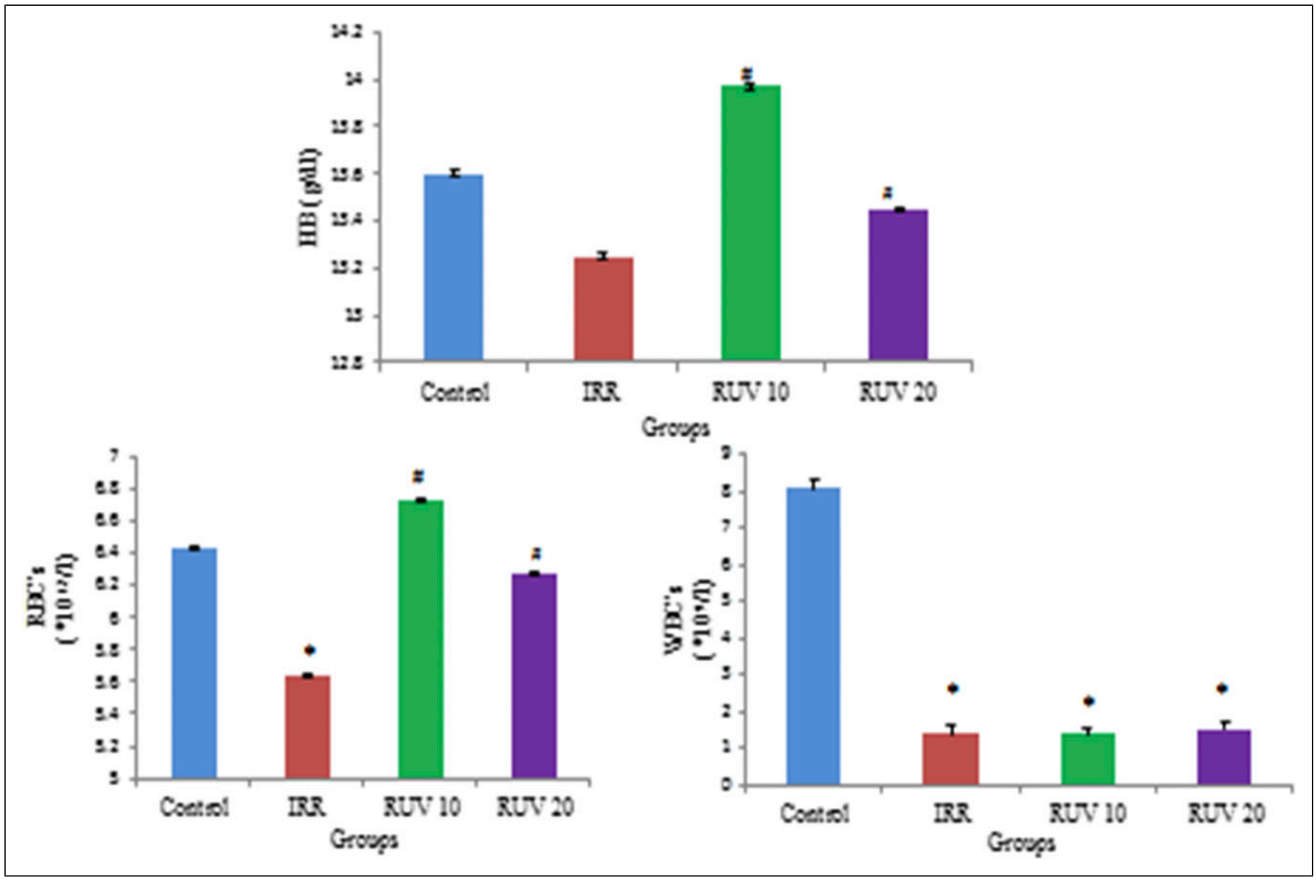

Figure 1. Effect of rosuvastatin (10 mg and 20 mg) on Hb, RBCs, and WBCs in spleen tissue of irradiated rats. Each value represents mean ± SE of the mean. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison tests. * significantly different from normal control group, # significantly different from the irradiated group. RUV, rosuvastatin; IRR, radiation.

Discussion

Chemical changes in biological molecules as a result of energy absorption are responsible for the biological effects of ionizing radiation.[24] The pathophysiology of irradiation-induced tissue injury is complicated by the production of reactive oxygen metabolites.[25] Gamma rays cause cells to produce an exponential burst of ROS as well as an exponential increase in intracellular Ca^{2+} levels.[26] Both oxidative damage and Ca^{2+} influx grow exponentially, resulting in huge, abrupt cell death.[27] Any one of these stages can be blocked to prevent cell death.[28]

The amount of radiation absorbed, the kind of radiation, and the susceptibility of different tissues all influence tissue damage.[29] The immunological organs of rats can be severely damaged by radiation. Meanwhile, the spleen, as an important part of the immune system, plays a role in blood cell creation as well as blood filtering, eliminating old blood cells, and fighting infection. Antioxidant systems and adaptive response capability influence cellular and tissue resistance to ionizing radiation. The spleen is one of the most radiation-sensitive organs.[30]

The RBCs of irradiated rats were found to be significantly lower in the current study. The drop in RBCs' count in our study could be explained by a study that found a decrease in erythropoietin production and spleen hematopoietic stem cell viability.[31]

Leucocytes are highly radiosensitive to radiation and are used to diagnose radiation injuries as a sensitive biological signal.[32] According to *Lee and Ducoff*,[33] radiation inhibits bone marrow's ability to form mature, highly differentiated blood cells.

Rosuvastatin (10 mg and 20 mg) resulted in significantly normalized RBCs and somewhat improved WBCs. The production of free radicals as a result of water radiolysis causes the majority of radiation-induced damage.[34] Radiation toxicity investigations have shown that reactive oxygen species-mediated cascading chain reactions and redox imbalances occur.

MDA is a sensitive biomarker for oxidative stress that occurs as part of the pathophysiology of numerous diseases and is produced by free radical attack on cell membrane phospholipids and circulating lipids.[35] The current study found that following post-radiation exposure, there was a large increase in MDA levels and a significant decrease in GST activity in the spleen. Similarly, elevated MDA levels in irradiated rats could be

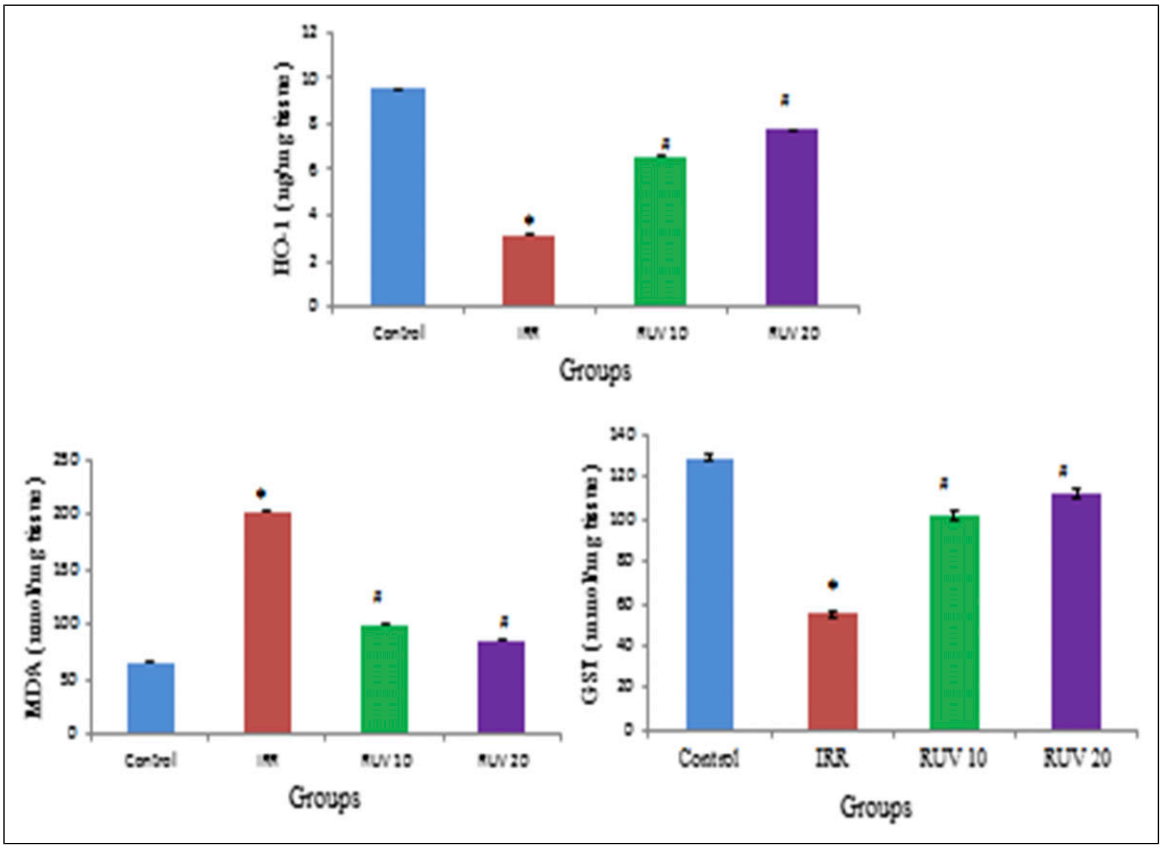

Figure 2. Effect of rosuvastatin (10 mg and 20 mg) on HO-1, MDA, and GST in spleen tissue of irradiated rats. Each value represents mean ± SE of the mean. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison tests. * significantly different from normal control group, # significantly different from the irradiated group. $p < 0.05$. RUV, rosuvastatin; IRR, radiation.

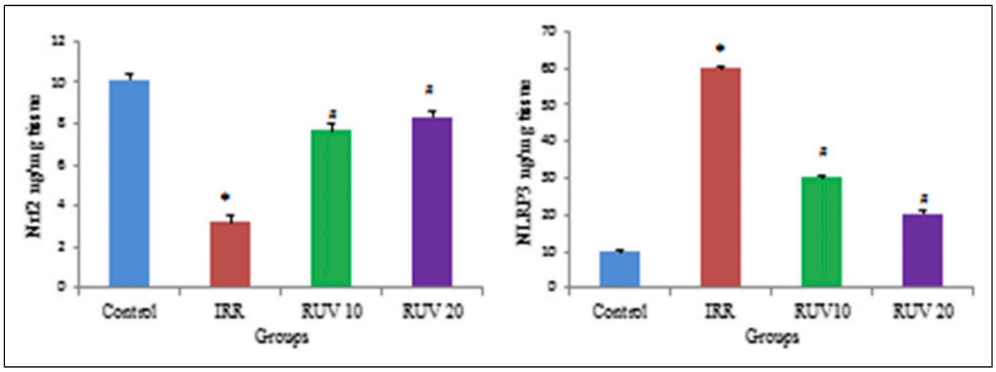

Figure 3. Effect of rosuvastatin (10 mg and 20 mg) on Nrf2 and NLRP3 in spleen tissue of irradiated rats. Each value represents mean ± SE of the mean. Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer multiple comparison tests. * significantly different from normal control group, # significantly different from the irradiated group. $p < 0.05$. RUV, rosuvastatin; IRR, radiation.

attributable to water radiolysis, which produces a hydroxyl group as a byproduct, which interacts with polyunsaturated fatty acids in the phospholipid component of cellular membranes.[36] Previous research suggested that IRR's lowering of GST activity was due to the antioxidant system's increased use in an attempt to detoxify free radicals created by radiation.[37]

The treatment of rosuvastatin, particularly the 10 and 20 mg doses, was able to considerably reduce the increase in TBARS and reactive oxygen species, as well as the decreased activity of GST in irradiated rats. However, it's vital to keep in mind the TBARS assay's limitations, such as its non-specific MDA reactivity and the likelihood of MDA formation from biological events unrelated to lipid peroxidation. These

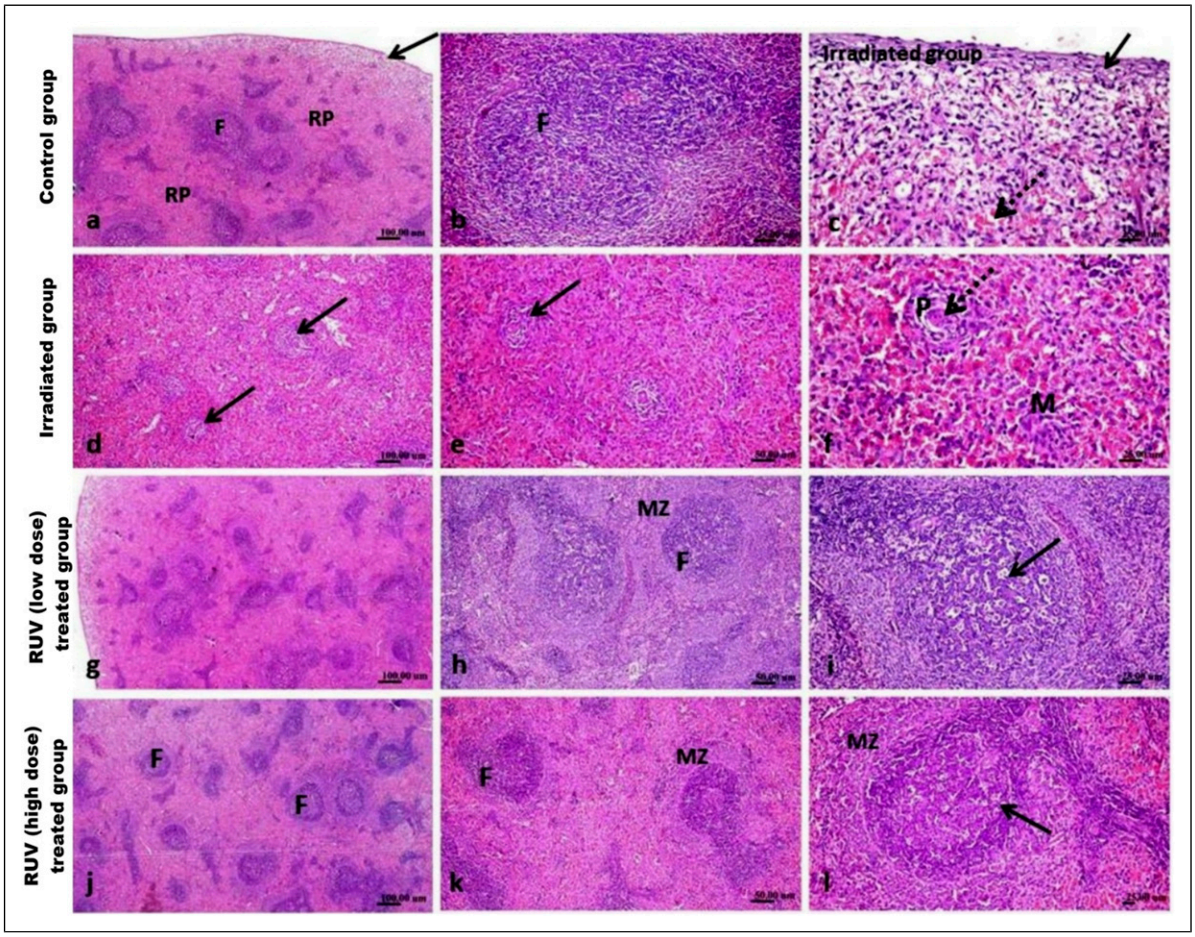

Figure 4. Photomicrographs of spleen sections stained with H&E. (a) and (b) Control rat showing normal splenic capsule (arrow) and normal histological structure of white pulp follicles (F) and red pulp structure (RP). (c–f) Irradiated rat showing (c) thickening of the splenic capsule (arrow), congested sinusoids and hemorrhage (dotted arrow) in red pulp, (d) marked white pulp follicles atrophy (arrow), thickening of the central arteriole (dotted arrow) and severe lymphocytic depletion in the peri-arteriolar sheath (P), follicle and the marginal zone (M) with appearance of the underlining reticular mash. (g–l) Rosuvastatin pre-treated groups at low dose (g–i) and high dose (j–l) showing marked restoration of the white pulp follicles' size (F) was markedly restored and showed mild-to-moderate degree of lymphocyte depletion with appearance of tingible body macrophages (arrow) and restoration of the marginal zone (MZ) particularly in the high-dose group.

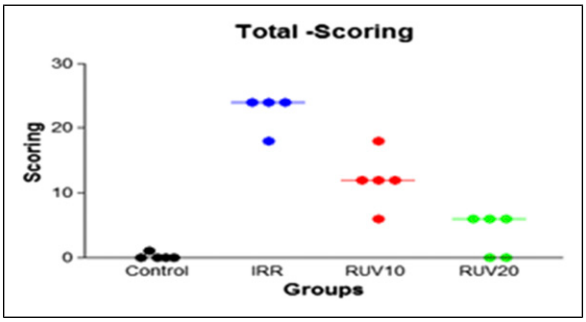

Figure 5. Total scoring of histopathological examination of the spleens of different groups. Data are presented as median. * significantly different from control. # significant different from the irradiated group. RUV, rosuvastatin; IRR, radiation.

findings suggest that rosuvastatin has a potent antioxidant effect and could be useful in the treatment of radiation damage.

NF-kB is a protein complex that is involved in a variety of activities including inflammation, apoptosis, and the immunological response. It is found in practically all types of animal cells and is responsible for DNA transcription. The NF-kB dimers are sequestered in the cytoplasm of unstimulated cells by a class of inhibitors known as IBs (inhibitor of IBs). The signal-induced degradation of IB proteins by a kinase called the IB kinase (IKK) through the phosphorylation and ubiquitination pathways activates the NF-kB. Pro-inflammatory cytokines are among the most potent NF-kB activators, acting on extracellular receptors and eventually causing intracellular degradation of IB proteins, allowing the NF-kB complex to

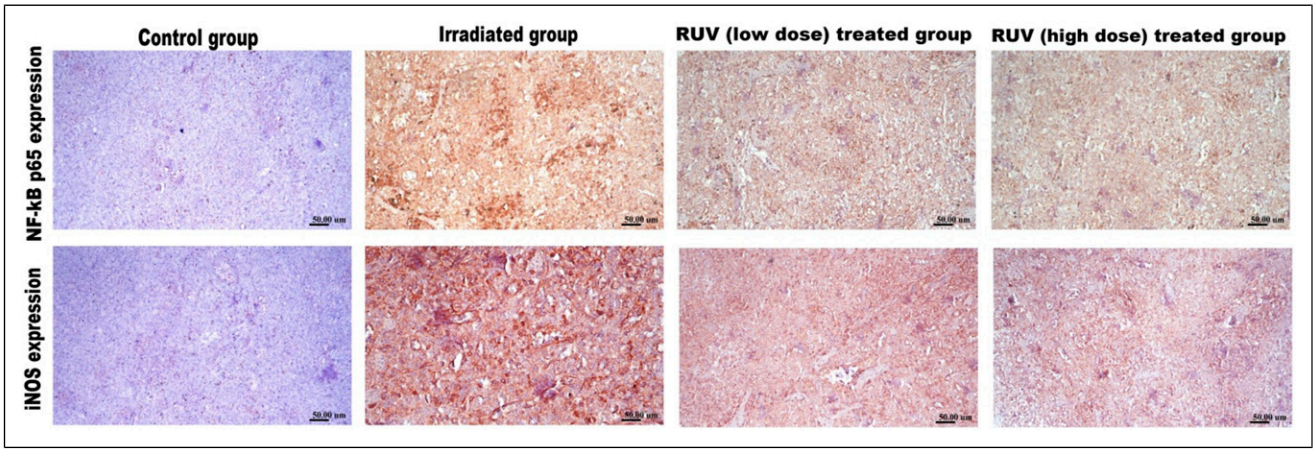

Figure 6. Photomicrographs of NF-κβ p65 and iNOS immune-stained spleen sections showing; Negative expression in control group, marked expression in the irradiated group, significant dose-related decreased expression in rosuvastatin pre-treated groups. Image analysis of the optical density of the positive brown color. Values are expressed as mean ± SE. Data were analyzed by using one-way ANOVA followed by Tukey post hoc test.

reach the nucleus.[38] However, the effect of radiation on NF-kB/NLRP3 signaling, adhesion molecule upregulation, and monocyte/macrophage recruitment is dependent on the types and fractions of radiation used, as well as the types of endothelial cells exposed to radiation.[39] Radiation-induced spleen dysfunction can happen in both NLRP3-dependent and NLRP3-independent ways. This is consistent with our findings, which showed that an increase in nuclear NF-kB and NLRP3 after gamma irradiation can be explained as a major contributor to the high levels of pro-inflammatory cytokines and NF-kB, as well as prior studies by *Sores et al*.[40]

HO-1 is the most essential Nrf2 target gene because it catalyzes the breakdown of heme into antioxidant biliverdin, anti-inflammatory carbon monoxide, and iron. HO-1 has been demonstrated to protect against a number of illnesses.[41] In the current investigation, we discovered a significant decrease in HO-1 levels and protein expression in irradiated rats' spleen tissues, which could contribute to free hem overload and tissue injury. Free heme is a rich source of redox-active iron implicated in the Fenton reaction, which acts as an iron detoxifying mechanism by increasing membrane permeability, allowing cells to lyse and die more quickly. Furthermore, free hem impairs the membrane integrity of blood cells, resulting in hemolysis.[42] Our findings, which showed a drop in the concentration of all cellular components in the blood, back this notion.

Nrf2 is a transcription factor that regulates cellular redox equilibrium and acts as a protective antioxidant. Kelch like-ECH-associated protein 1 (Keap1) and Cullin 3, which ubiquitinate Nrf2, keep it in the cytoplasm. Nrf2 has a half-life of only 20 minutes in typical conditions.

The Keap1-Cul3 ubiquitination pathway is disrupted in response to oxidative stress, resulting in an increase in free Nrf2 molecules, which are then translocated into the nucleus to bind to a DNA promoter and activate transcription of anti-oxidative genes and proteins.[43]

In this study, we observed a significant fall in the level and protein expression of Nrf2 after radiation exposure and the result is in line with Jung et al[44] and Marina et al.[45] The decrease might be explained by the disruption of Nrf2 system, whereas post γ-radiation exposure, ROS might activate Nrf2 in order to increase ARE-dependent gene expression, but with the persistent rise of ROS, a decrease of Nrf2 level is observed.

Furthermore, rosuvastatin therapy decreased nuclear NF-kB contents, as well as pro-inflammatory cytokines. We believe that increasing HO-1 levels protects the spleen tissue by lowering the contents of NF-kB and pro-inflammatory cytokines. Several investigations have shown that HO-1 and its metabolite CO may block LPS-induced endothelial injury to protect splenic endothelial cell injury, implying that HO-1-mediated CO synthesis protects rosuvastatin-treated splenic endothelial cells against radiation toxicity.[46] Additionally, the HO-1 metabolite bilirubin has the ability to attenuate endothelial activation and dysfunction by blocking endothelial leukocyte transmigration via adhesion molecule.[47] Our findings, like those of *Nyane et al*,[48] indicated that rosuvastatin has an antioxidant impact via inducing Nrf2 and HO-1, which operate as lipid peroxidation scavengers, protecting vascular cells from damage. In another study, rosuvastatin increased membrane fluidity along the hydrophilic surface of erythrocytes, preventing them from rupturing.

Conclusions

We can conclude that rosuvastatin has a radio protective activity against radiation-induced spleen injury through decreasing ROS. RUV illustrated a new approach in the

management of spleen-damaging effects in patients receiving radiotherapy.

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Thanaa M. Fahim, MarwaAbd EL-Hameed Mohamed, SaharSM Abdelrahman, and Dina M. Lotfy. The first draft of the manuscript was written by ThanaaM. Fahim, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Consent to publish

All authors and their institute approved the publication.

Consent to participate

All authors participated equally in this work.

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