Protective effects of rosmarinic acid against radiation-induced damage to the hematopoietic system in mice

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ABSTRACT

Rosmarinic acid (RA) is an ester of caffeic acid and 3, 4-dihydroxyphenyl lactic acid. It is a potent antioxidant that functions by scavenging free radicals. Here, we used a 30-day survival assay to investigate the radioprotective effects of RA. Mice were treated with RA once per day for 10 consecutive days starting at 3 days before gamma irradiation at 7.5 Gy until 7 days post irradiation. Mice treated with 100 and 200 mg/kg body weight (bw) of RA had 30-day survival rates of 89% and 72%, respectively, compared with 32% in the control group, and the differences were statistically significant (P = 0.0008 and 0.0421, respectively). Spleen colony-forming units (CFU-S), the number of nucleated cells in the bone marrow (BMNC), bone marrow DNA content, and hematological parameters of the peripheral blood were measured to investigate the radioprotective effect of RA on the hematopoietic system. The treatment groups that received RA at 50, 100 and 150 mg/kg bw and whole-body exposure to 5.5 Gy of 137Cs γ-radiation had significantly higher CFU-S, BMNC and DNA content than the irradiation-only group. Assessment of hematological parameters in the peripheral blood showed that the treatment groups receiving RA at doses of 50, 100 and 150 mg/kg bw had higher white blood cell counts, hemoglobin and platelets than the radiation-only group. These results suggested that the administration of RA promoted the recovery of peripheral blood cells in irradiated mice.

KEYWORDS: rosmarinic acid, radioprotection, hematopoietic system

INTRODUCTION

Direct exposure to ionizing radiation poses a risk to all living organisms. Damage to DNA can occur directly, although genetic damage is mostly mediated by reactive oxygen species (ROS). The term ROS refers to a group of molecules (such as peroxides and free radicals) that are highly reactive toward biomolecules. Free radicals are any atom or molecule that contains one or more unpaired electrons. Ionizing radiation produces bursts of ROS by reacting with the aqueous environment of the cell. Hence, scavengers of free radicals form the principal group of radioprotective agents [1–4]. Antioxidants may decrease free radical attack on biomolecules and mitigate damage induced by irradiation. Antioxidants such as superoxide dismutase [5], nitroxide compounds [6], vitamins [7], melatonin [8], and phenolic compounds have been reported as potential radioprotective agents [9,10].

Rosmarinic acid (RA) is a water-soluble, naturally occurring ester of caffeic acid and 3, 4-dihydroxyphenyl lactic acid (Fig. 1) [11]. It is isolated from many species of the families Lamiaceae and Boraginaceae and is one of the active components of several medicinal plants in these families (e.g. Salvia officinalis, Mentha x piperita, Thymus vulgaris, Melissa officinalis, and Symphytum officinale) [12]. Various biological activities are attributed to RA, including antioxidant [13], antimutagenic [14, 15], anti-inflammatory [16, 17], antiangiogenic [18], anticancer [19], antimicrobial [20], and antineurodegenerative activities [21,22]. Here, we investigated the protective effect of RA in mice exposed to radiation. Survival rates were measured to evaluate...
the radioprotective effects of RA in mice after whole-body exposure to 7.5 Gy of $^{137}$Cs gamma-irradiation. Hematological parameters in the peripheral blood, spleen colony forming units (CFU-S), bone marrow DNA content and bone marrow nucleated cell (BMNC) counts were used to investigate the radioprotective effects of RA on the hematopoietic system after whole-body exposure to 5.5 Gy of $^{137}$Cs gamma-irradiation. The aim of the present study was to identify potential radioprotective agents.

**MATERIALS AND METHODS**

**Materials**

RA was purchased from Sigma–Aldrich, Co. (St Louis, MO, USA). WR-2721 was purchased from Dalian Meiluo Pharmaceutical Co. Ltd (Dalian, Liaoning, China).

**Animals**

Institute of cancer research mice (6–8 weeks old), weighing 20 ± 2 g, were obtained from the Animal Center of the Chinese Academy of Medical Sciences, Beijing. They were maintained under controlled laboratory conditions at a temperature of 23–27°C and a humidity of 50–60%, with a controlled light cycle (14 h of light and 10 h of darkness). The mice were fed standard animal food pellets and water ad libitum. All animal experiments were performed according to the guidelines of the Institutional Ethics Committee.

**Irradiation of animals**

Total body gamma irradiation (TBI) was performed using a $^{137}$Cs Gamma Tissue Irradiator at a dose rate of 0.711 Gy/min (cammacell-40, Atomic Energy of Canadian Inc.) during the experimental period. Animals in all groups were kept in a perforated plastic container, and were placed on a rotating platform to ensure even dose delivery to all tissues when irradiated.

**Administration of RA**

RA was dissolved in normal saline for administration at the desired concentrations, and the dose was expressed in mg/kg body weight (bw). RA was administered to mice through an oral route in a maximum volume of 0.3 ml. Control animals received 0.3 ml of normal saline.

**Animal survival**

The effects of the administration of different concentrations of RA and irradiation on survival were investigated. Mice were randomly divided into six groups ($n = 18$ each). The control group and the radiation group were treated with saline once per day for 10 consecutive days from 3 days before irradiation until 7 days post irradiation. The treated group included 50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg RA-treated subgroups, and a 200 mg/kg WR2721-treated subgroup. The mice received RA administered orally once per day for 3 consecutive days at the indicated body-weight doses, and on Day 3 they were irradiated with gamma rays at a dose of 7.5 Gy 30 min after the administration of RA, followed by RA treatment for 7 consecutive days. Mice received 0.2 ml WR2721 at a dose of 200 mg/kg by intraperitoneal (ip) administration 30 min before radiation. Survival was observed daily up to Day 30 post irradiation, and data were expressed as percentage survival and average survival days.

**Radioprotective effects on the hematopoietic system**

Animals were randomly divided into seven groups ($n = 10$ each).

The control group was treated with saline administered orally once per day for 10 consecutive days. The radiation group was treated with saline administered orally once per day for 10 consecutive days from 3 days before irradiation at a dose of 5.5 Gy until 7 days post irradiation. The treated group included 50 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg RA-treated subgroups, and a 200 mg/kg WR2721-treated subgroup. The mice received RA administered orally once per day for 3 consecutive days at the indicated body-weight doses, and on Day 3, they were irradiated with gamma rays at a 5.5-Gy dose 30 min after administration of RA, followed by RA treatment for 7 consecutive days. Mice received 0.2 ml WR2721 at a dose of 200 mg/kg through ip administration 30 min before radiation.

Mice were sacrificed by cervical dislocation on Day 9 post-irradiation in all groups. Their spleens, bones and blood were collected. The endogenous CFU-S, BMNCs, and hematological parameters in the peripheral blood were investigated to estimate the radioprotective effects of RA on the hematopoietic system.

**Endogenous spleen colony forming unit measurement**

Spleens were removed from mice on Day 9 post irradiation and fixed in Bouin’s solution for 24 h. Macroscopic colonies (CFU-S) were scored in each spleen [23].

**Bone marrow nucleated cells count**

Mouse femoral bones were collected and the bone marrow was flushed out with 10 ml 3% acetic acid. The number of BMNCs was counted using a light microscope [23].

**Bone marrow DNA content detection**

Animals in all groups were sacrificed on Day 9 after irradiation. The femur of each mouse was removed. The bone marrow was flushed into tubes with 10 ml of a 0.005-mol/l CaCl$_2$ solution. Cell suspensions were incubated at 4°C for 30 min, and then centrifuged at 2500 rpm for 15 min. The pellet was resuspended in 5 ml of a 0.2-mol/l HClO$_4$ solution. The suspension was mixed and incubated at 90°C for 15 min, then filtered through a 0.22-μm membrane after cooling. The absorbance at 260 nm was detected using a 752-UV spectrophotometer [24] (Shanghai APL Instrument Co. Ltd).

**Hematological parameters in the peripheral blood assessment**

Blood was collected from the caudal vein into heparinized tubes on Day 9 post irradiation. White blood cell (WBC) counts, hemoglobin content (HGB) and platelet counts (PLT) were analyzed using a Coulter LH755 Hematology Analyzer.
Statistical analysis
Statistical analysis was performed using SPSS 17.0 for Windows. Data were expressed as the mean ± standard deviation (SD). A Student’s t-test was used for statistical comparisons between the groups. The significance levels were set at $P < 0.05$, $P < 0.01$ and $P < 0.001$. The significance of survival curves was analyzed by Kaplan–Meier survival analysis and the log-rank test.

RESULTS
Animal survival
Overall, 32% of irradiated animals that were not administered RA were alive at 30 days post irradiation (Fig. 2). The administration of WR2721 before 7.5 Gy whole-body gamma-irradiation resulted in a 30-day survival rate of 94.4%. The administration of RA at 100, 200 and 400 mg/kg bw before 7.5 Gy whole-body gamma-irradiation resulted in 89%, 72% and 67% 30-day survival rates, respectively. The significance between the survival curves was analyzed by Kaplan–Meier survival analysis and the log-rank test. The difference in survival between the radiation-only group and the 100 and 200 mg/kg bw RA treatment groups was statistically significant ($P = 0.0008$ and 0.0421, respectively). WR2721 treatment showed significant protective effects against radiation damage in mice compared with radiation only ($P = 0.0001$).

Endogenous spleen colony–forming units
Figure 3 shows that endogenous CFU-S were not observed in the control group, whereas they emerged when mice were exposed to irradiation. Treatment groups receiving WR2721 and 50, 100, 150 and 200 mg/kg bw of RA had significantly higher CFU-S than the irradiation-only group. The increase in the CFU-S indicates that RA may play a role in protecting the stem cells of irradiated mice.

Bone marrow nucleated cells
As shown in Fig. 4, the number of nucleated cells in the bone marrow in the radiation-only group decreased markedly compared with that in the control group. The treatment groups receiving WR2721 and 50, 100, 150 and 200 mg/kg bw of RA had significantly higher BMNCs compared with the irradiation-only group. The Student’s t-test was used for statistical comparisons between the groups. Three dots: $P < 0.001$ vs the control group; **$P < 0.01$, ***$P < 0.001$ vs the radiation-only group.
administration of RA improved peripheral blood WBC counts in irradiated mice. WR2721 and RA attenuated the damage. Bone marrow cells were damaged during irradiation, and administration of WR2721 and RA attenuated the damage.

**Hematological parameters in the peripheral blood**

**White blood cell counts**

The WBC counts are shown in (fig. 6A). The WBC count decreased sharply in the radiation-only group compared with that in the control group (P < 0.001). The DNA content in the four groups receiving WR2721 and 50, 100 and 150 mg/kg bw of RA increased significantly compared with that in the irradiated-only group (P < 0.001 or P < 0.01). These results suggested that bone marrow cells were damaged during irradiation, and that administration of WR2721 and RA attenuated the damage.

**Hemoglobin content**

Changes in the HGB content in the different groups are shown in (fig. 6B). The HGB content of the irradiation group was significantly lower than that of the control group (P < 0.001). The treatment groups receiving 200 mg/kg bw of RA did not exhibit obvious changes in the HGB content in comparison with the irradiation-only group; however, the groups treated with RA at doses of 50, 100 and 150 mg/kg bw and that treated with WR2721 had higher HGB levels than the radiation-only group, and the difference was statistically significant (P < 0.05 or P < 0.01). These results suggested that the administration of RA improved the peripheral blood HGB content in irradiated mice.

**Platelet counts**

(2C) shows that the PLT count in the irradiated groups was significantly decreased compared with that in the control group (P < 0.001). PLT counts in the treatment groups increased significantly compared with that in the irradiation group (P < 0.05, P < 0.01 or P < 0.001). These results suggested that irradiation decreased the count of peripheral blood PLT’s, and that the administration of RA effectively restored PLT levels.

**DISCUSSION**

The results of the present study show that RA exerted protective effects against radiation damage in mice. The mice treated with RA and WR2721 had a higher survival rate than those receiving radiation alone. WR2721 is currently approved by the US FDA for use in radiation therapy. However, its use is not convenient because it requires intravenous administration [25]. RA showed radioprotective effects when administered orally, and could therefore be a promising agent for attenuating the effects of irradiation.

Our animal survival results indicated that RA was more effective when administered at a dose of 100 mg/kg than at 200 mg/kg and 400 mg/kg. A similar phenomenon was observed in mice regarding its protective effect on the hematopoietic system. In preliminary experiments, the dose–effect relation curve showed that RA was not dose dependent. This is consistent with a previous study by Pereira et al. [22]. This could be attributed to the fact that RA has an antioxidant effect at low concentrations, whereas it is a pro-oxidant at high concentrations. Several antioxidants such as β-carotene, α-tocopherol and ascorbic acid play antioxidant and pro-oxidant roles simultaneously [26].

Rapidly dividing tissues such as cells of the hematopoietic system are prone to radiation-induced damage. Our present study indicated that RA treatment increased the number of radiation-induced endogenous spleen colonies, the count of nucleated cells and the DNA content in the bone marrow compared with these parameters in the radiation-only no-treatment group. These findings suggested that RA treatment promoted the recovery of hematopoiesis after TBI. Hematological parameters in the peripheral blood such as WBC, HGB and PLTs were effectively restored after administration of RA.

Ionizing radiation is known to generate ROS, and RA may absorb and neutralize free radicals, quench singlet and triplet oxygen, and decompose peroxides owing to its redox properties. The radioprotective effect of RA was correlated with its ability to scavenge free radicals. In our study, RA administration ameliorated radiation injury in mice. RA was previously shown to reduce the frequency of micronuclei induced in human lymphocytes by gamma irradiation [27], which indicates that the protective effects can be extended from the cell to whole body.

The radioprotective effect of RA could be attributed to its potent antioxidant activity [28–30], which is related to its chemical structure.
As for most potent antioxidants, the antioxidant activity of RA is mainly due to the combination of conjugated structures in the polyphenolic skeletons, especially hydroxyl groups in the ortho position of the aromatic ring, and also to the presence of a carboxylic group. The catechol structures of RA are the most important structural elements for its antioxidant activity. Furthermore, the presence of two catechol structures conjugated with a carboxylic acid group in RA increases its antioxidant activity [31].

Obviously, the mechanism by which the RA acted as a radioprotector in this experiment would be due to its antioxidant and, probably, anti-apoptotic activity and DNA damage protection. Its antioxidant potential is not only related to its free radical scavenging capacity, but also to its capacity to regulate certain enzymatic activities involved in these processes. The cytoprotective effect of RA on ultraviolet B (UVB)-induced oxidative stress in HaCaT keratinocytes was reported by Fernando et al. [32]: RA exerted a significant cytoprotective effect by scavenging intracellular ROS induced by UVB. Furthermore, RA increased the expression and activity of superoxide dismutase, catalase, heme oxygenase-1, and their transcription factor Nrf2, which are decreased by UVB radiation. The protective effects of RA on apoptosis induced by hydrogen peroxide in astrocytes were studied by Gao et al. [33]. Pretreating cells with RA significantly

Fig. 6. Hematological parameters in the peripheral blood of experimental mice. The data are presented as the mean value of three independent sets of experiments with 10 animals in each group. (6A) WBC counts, (6B) HGB content and (6C) PLT counts. A Student’s t-test was used for statistical comparison between the groups. Three dots: *P < 0.001 vs the control group; **P < 0.05, ***P < 0.01, ****P < 0.001 vs the irradiation group.
increased cell viability and decreased the apoptosis rate induced by H₂O₂. The anti-apoptotic effect of RA was further confirmed by increase in the mitochondrial membrane potential and inhibition of caspase-3 activity. The potential of RA to protect against DNA damage was evaluated—RA showed protective activity in pBR322 plasmid DNA against the mutagenic and toxic effects of UV and H₂O₂. Thus, it is suggested that the mechanism for the radioprotective effect of RA may be related to the roles mentioned above; whether other mechanisms are involved requires further study.

RA possesses numerous biological activities. RA showed chemopreventive potential against 1, 2-dimethylhydrazine-induced rat colon carcinogenesis, and it is a possible chemopreventive agent against carcinogenesis, and it is a possible chemopreventive agent against colon cancer [34]. RA ameliorates cisplatin-induced oxidative stress, inflammation, and apoptosis in the kidneys [35]. In another study, RA was shown to effectively inhibit tumor metastasis in vitro and in vivo [36]. In the present study, we showed that RA has a radioprotective effect in mice. RA is therefore a promising anticancer agent with potential chemopreventive and radioprotective effects.

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**REFERENCES**

1. Maier P, Wenz F, Herskind C. Radioprotection of normal tissue cells. Strahlenther Onkol 2014;190:745–52.
2. Citrin D, Cotrim AP, Hyodo F, et al. Radioprotectors and mitigators of radiation-induced normal tissue injury. Oncologist 2010;15:360–71.
3. Greenberger JS, Clump D, Kagan V, et al. Strategies for discovery of small molecule radiation protectors and radiation mitigators. Front Oncol 2011;1:59.
4. Rosen EM, Day R, Singh VK. New approaches to radiation protection. Front Oncol 2014;4:381.
5. Huang XJ, Song CX, Zhong CQ, et al. Research progress in the radioprotective effect of superoxide dismutase. Drug Discov Ther 2012;6:169–77.
6. Soule BP, Hyodo F, Matsumoto K, et al. Therapeutic and clinical applications of nitrooxide compounds. Antioxid Redox Signal 2007;9:1731–43.
7. Bairati I, Meyer F, Gelinas M, et al. Randomized trial of antioxidant vitamins to prevent acute adverse effects of radiation therapy in head and neck cancer patients. J Clin Oncol 2005;23:5805–13.
8. Topkan E, Tuğan H, Yayuz AA, et al. Comparison of the protective effects of melatonin and amifostine on radiation-induced epiphyseal injury. Int J Radiat Biol 2008;84:796–802.
9. Weiss JF, Landauer MR. Radioprotection by antioxidants. Ann N Y Acad Sci 2000;899:44–60.
10. Gajowik A, Dobrynyska MM. Lycopene – antioxidant with radioprotective and anticancer properties. A review. Roczn Panstw Zakl Hig 2014;65:263–71.
11. Petersen M, Simmonds MS. Rosmarinic acid. Phytochemistry 2003;62:121–5.
12. Petersen M, Abdullah Y, Benner J, et al. Evolution of rosmarinic acid biosynthesis. Phytochemistry 2009;70:1663–79.
13. Lee HJ, Cho HS, Park E, et al. Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide–induced apoptosis. Toxicology 2008;250:109–15.
14. De Oliveira NC, Sarmento MS, Nunes EA, et al. Rosmarinic acid as a protective agent against genotoxicity of ethanol in mice. Food Chem Toxicol 2012;50:1208–14.
15. Furtado MA, de Almeida LC, Furtado RA, et al. Antimutagenicity of rosmarinic acid in Swiss mice evaluated by the micronucleus assay. Mutat Res 2008;657:150–4.
16. Osakabe N, Takano H, Sanbongi C, et al. Anti-inflammatory and anti-allergic effect of rosmarinic acid (RA); inhibition of seasonal allergic rhinoconjunctivitis (SAR) and its mechanism. Biofactors 2004;21:127–31.
17. Yang EJ, Ku SK, Lee W, et al. Barrier protective effects of rosmarinic acid on HMGB1-induced inflammatory responses in vitro and in vivo. J Cell Physiol 2013;228:975–82.
18. Sotnikova R, Okruhlicova L, Vlkovicova J, et al. Rosmarinic acid administration attenuates diabetes-induced vascular dysfunction of the rat aorta. J Pharm Pharmacol 2013;65:713–23.
19. Amusuya C, Manoharan S. Antitumor initiating potential of rosmarinic acid in 7,12-dimethylbenz(a)anthracene-induced hamster buccal pouch carcinogenesis. J Environ Pathol Toxicol Oncol 2011;30:199–211.
20. Moreno S, Scheyer T, Romano CS, et al. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic Res 2006;40:223–31.
21. Al-Dhabi NA, Arasu MV, Park CH, et al. Recent studies on rosmarinic acid and its biological and pharmacological activities. EXCLI J 2014;13:1192–5.
22. Pereira P, Tysca D, Oliveira P, et al. Neurobehavioral and genotoxic aspects of rosmarinic acid. Pharmacol Res 2005;52:199–203.
23. Xu W, Shen X, Yang F, et al. Protective effect of polysaccharides isolated from Tremella fuciformis against radiation-induced damage in mice. J Radiat Res 2012;53:353–60.
24. Jiang S, Shen X, Liu Y, et al. Radioprotective effects of Sipunculus nudus L. polysaccharide combined with WR-2721, rhIL-11 and rhG-CSF on radiation-injured mice. J Radiat Res 2015;56:515–22.
25. Johnke RM, Sattler JA, Allison RR. Radioprotective agents for radiation therapy: future trends. Future Oncol 2014;10:2345–57.
26. Zhang P, Omaye ST. Antioxidant and prooxidant roles for beta-carotene, alpha-tocopherol and ascorbic acid in human lung cells. Toxicol In Vitro 2001;15:13–24.
27. Del BM, Castillo J, Benavente-Garcia O, et al. Radioprotective–antimutagenic effects of rosemary phenolics against chromosomal damage induced in human lymphocytes by gamma-rays. J Agric Food Chem 2006;54:2064–8.
28. Sevgi K, Tepe B, Sarikurcu C. Antioxidant and DNA damage protection potentials of selected phenolic acids. Food Chem Toxicol 2015;77:12–21.
29. Mushfaq N, Schmatz R, Ahmed M, et al. Protective effect of rosmarinic acid against oxidative stress biomarkers in liver and kidney of streptozotocin-induced diabetic rats. J Physiol Biochem 2015;71:743–51.
30. Govindaraj J, Sorimuthu PS. Rosmarinic acid modulates the antioxidant status and protects pancreatic tissues from glucolipotoxicity mediated oxidative stress in high-fat diet: streptozotocin induced diabetic rats. Mol Cell Biochem 2015;404:143–59.
31. Sanchez-Campillo M, Gabaldon JA, Castillo J, et al. Rosmarinic acid, a photo-protective agent against UV and other ionizing radiations. Food Chem Toxicol 2009;47:386–92.
32. Fernando PM, Piao MJ, Kang KA, et al. Rosmarinic acid attenuates cell damage against UVB radiation-induced oxidative stress via enhancing antioxidant effects in human HaCaT cells. Biomol Ther 2016;24:75–84.
33. Gao LP, Wei HL, Zhao HS, et al. Antiapoptotic and antioxidant effects of rosmarinic acid in astrocytes. Pharmazie 2005;60:62–5.
34. Venkatachalam K, Gunasekaran S, Jesudoss VA, et al. The effect of rosmarinic acid on 1, 2-dimethylhydrazine induced colon carcinogenesis. Exp Toxicol Pathol 2013;65:409–18.
35. Domitrovic R, Potocnjak I, Crncevic-Orlic Z, et al. Nephroprotective activities of rosmarinic acid against cisplatin-induced kidney injury in mice. Food Chem Toxicol 2014;66:321–8.
36. Xu Y, Xu G, Liu L, et al. Anti-invasion effect of rosmarinic acid via the extracellular signal-regulated kinase and oxidation-reduction pathway in Ls174-T cells. J Cell Biochem 2010;111:370–9.