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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 ^{137}Cs 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

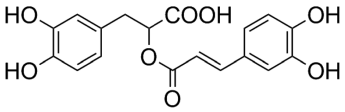

Fig. 1. Chemical structure of rosmarinic acid.

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 100 mg/kg, 200 mg/kg, and 400 mg/kg RA-treated subgroups. The animals received RA or saline 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.

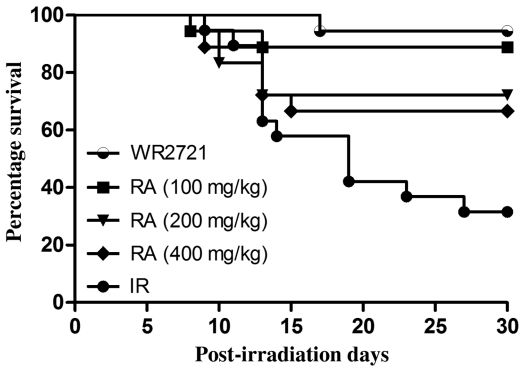

Fig. 2. The effects of the administration of different concentrations of RA and irradiation on survival were analyzed using a 30-day survival curve. The animals received RA or saline administered orally once a day for 10 consecutive days. On Day 3 after RA administration, they were exposed to 7.5 Gy whole-body gamma irradiation. Survival was monitored until the 30th post-irradiation day. The significance of the differences between the survival curves was analyzed by Kaplan–Meier survival analysis along with a log-rank test. The difference in survival between the irradiation-only and treatment groups at 100 and 200 mg/kg bw of RA was statistically significant ($P = 0.0008$ and 0.0421, respectively).

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

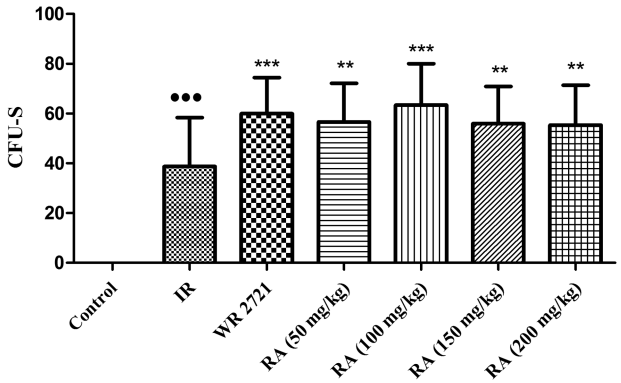

Fig. 3. Endogenous spleen colony–forming units in experimental mice. Spleens were removed from mice on Day 9 post irradiation and fixed in Bouin's solution for 24 h. CFU-S visible to the naked eye were scored in each spleen. The data are presented as the mean value of three independent sets of experiments each having 10 animals per group. A 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 irradiation group.

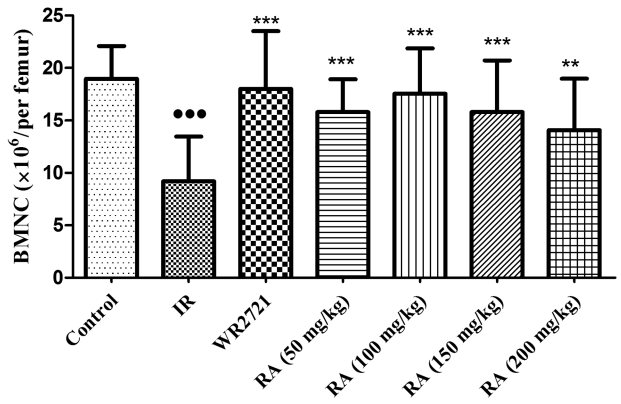

Fig. 4. The counts of bone marrow nucleated cells in experimental mice. Femoral bones were collected, and the bone marrow was flushed out with 3% acetic acid. The number of BMNCs was counted using a microscope. Results are presented as the mean ± SD ($n = 30$). The Student's *t*-test was used for statistical comparisons between the groups. Three dots: $P < 0.001$ vs the control group; $^{**}P < 0.01$, $^{***}P < 0.001$ vs the radiation-only group.

BMNC counts compared with the radiation-only group. These results suggested that RA had a protective effect on BMNCs in irradiated mice.

Bone marrow DNA content

The DNA content of bone marrow cells in experimental mice is shown in Fig. 5. The DNA content of bone marrow cells decreased significantly in the irradiated-only group on Day 9 after irradiation 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.

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 treatment groups receiving WR2721 and 50, 100 and 150 mg/kg bw of RA showed markedly higher WBC counts than the radiation-only group, and the difference was statistically significant ($P < 0.01$ or $P < 0.001$). These results indicated that the administration of RA improved peripheral blood WBC counts in irradiated mice.

Hemoglobin content

Changes in the HGB amount 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.

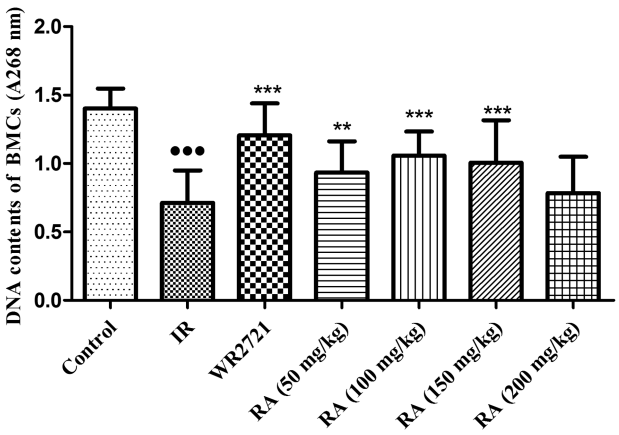

Fig. 5. DNA content of bone marrow cells in experimental mice. The bone marrow was flushed, DNA was extracted and its absorbance at 260 nm was detected using a spectrophotometer. The bar graph represents the mean ± standard deviation obtained by analyzing three independent sets of experiments each with 10 animals per group. A 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 group.

Platelet counts

(fig. 6C) 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 PLTs, 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 administrated 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.

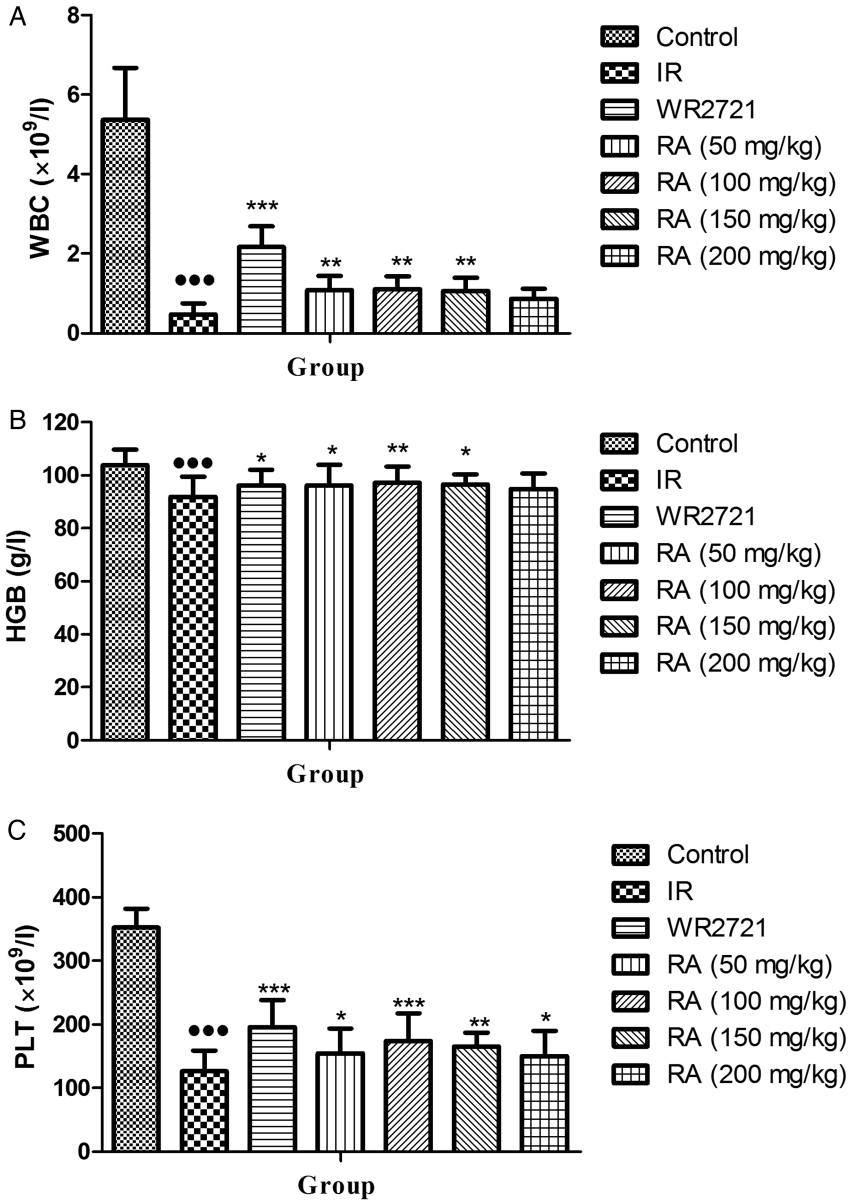

Fig. 6. Hematological parameters in the peripheral blood of experimental mice. The data are presented as the mean value of three independent sets of experiments with 10 animals in each group. (6A) WBC counts, (6B) HGB content and (6C) PLT counts. A Student's *t*-test was used for statistical comparison 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.

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

increased cell viability and decreased the apoptosis rate induced by H_2O_2. 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 H_2O_2 [28] 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 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.

FUNDING

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