Diallyl Disulfide Mitigates DNA Damage and Spleen Tissue Effects After Irradiation

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Background: Several factors found in foods are beneficial to human health and they may contribute to radiation protection. Taking food factors could be an easy way to reduce the effects of radiation after nuclear accidents, as well as secondary radiation risks after cancer radiotherapy or space missions. Here, diallyl disulfide (DADS), a component of garlic oil, was studied for its ability to mitigate radiation damage.

Material/Methods: We investigated the effects of DADS on micronucleus (MN) formation and apoptosis in HepG2 cells by use of 4-Gy X-ray irradiation. We also assessed the effects of DADS on radiation damage *in vivo* by evaluating MN formation in bone marrow cells in mice (BALB/c, 8-week-old females) after oral intake of DADS prior to irradiation with 4 Gy. Several tissue effects were also investigated.

Results: The presence of DADS inhibited MN formation, whereas DADS had no influence on the radiation-induced inhibition of cell cycle progression in HepG2 cells. An increase in apoptosis in HepG2 cells was induced after irradiation, and this effect was stronger in the presence of DADS than in its absence. In mice, when DADS was administered daily for 3 days prior to irradiation, MN formation in irradiated mice was decreased. The decrease in MN formation in mice was greater with 0.5% DADS compared to 1% DADS. Moreover, an increase in spleen weight observed 3 weeks after irradiation was suppressed in mice administered DADS.

Conclusions: DADS is a potential radiation-protective agent that effectively mitigates DNA damage, and its effects in the spleen observed after irradiation may be related to inflammation and carcinogenesis.

MeSH Keywords: DNA Damage • Garlic • Radiation Protection • Radiation-Protective Agents • Spleen

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Background

Modern society provides increased risk of exposure to radiation. For example, a variety of medical treatments using radiation have been developed, and humans are exposed to cosmic radiation during space activities while in long-duration low-earth orbit. Radiation exposure in low-earth orbit can be of many different types, including those induced by solar particle events [1]. Protection against radiation exposure is important to various aspects of human life. Many compounds have been tested for their radiation-protective effects. For example, amifostine is a radio-protective agent approved for human use by the United States Food and Drug Administration (FDA) [2]. However, amifostine has been reported to have adverse effects, and research is continuing to minimize its adverse effects as well as those of other compounds. Radiation is known to induce oxidative stress and to be genotoxic. Food factors or natural products are potential protective agents against radiation and carcinogenic chemicals [3–6]. Kennedy et al. demonstrated that dietary supplements containing antioxidant agents suppress space radiation-induced carcinogenesis [7]. Furthermore, it was reported that moderate drinking of wine mitigates radiotherapy-induced adverse effects in patients undergoing radiation therapy [8]. Radio-protective agents should be easily-administered and safe [7–11]. Foods, beverages, and supplements are easily consumed and their potential beneficial functions have attracted much attention. In particular, natural dietary components of foods or beverages hold promise as radiation-protective agents [5,6,12–14]. Garlic and garlic oil contain phytochemicals and have been reported to have radiation-protective properties [15–17]. Diallyl disulfide (DADS) is a component of garlic oil that exhibits many functions, including the prevention of genotoxicity [18,19]. The effects of DADS on radiation damage, and particularly radiation-induced DNA damage, remain unclear, and it is also unclear whether DADS mitigates the damage observed long after exposure to radiation. In the present study, we show that DADS reduces both acute radiation damage and long-term damage observed up to 3 weeks after the irradiation of mice with sublethal doses of X-rays.

Material and Methods

Materials

HepG2 cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, and 10% fetal bovine serum.

DADS was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). DADS was used as received (80% purity) and this purity was used for concentration calculations since the concentration was reported to be above 80% [20].

Detection of micronuclei and apoptosis in HepG2 cells after X-ray-irradiation

Cells were seeded in 35-mm dishes with coverslips (0.4×10^6 cells) and the cells were used 1 day after seeding. One hour before irradiation (a total dose: 4 Gy, a dose rate: 0.85 Gy/min), the cells were cultured in the presence of DADS (5 µM) [22,23]. The HepG2 cells were irradiated at a dose rate of 0.85 Gy/min using a TITAN 320S machine (Shimadzu, Kyoto, Japan) equipped with a 0.50-mm Al+0.50-mm Cu filter and operated at 200 kVp and 20 mA. An exposure rate meter (AE-1321M; Applied Engineering, Inc., Tokyo, Japan) was used for dosimetry measurement. Immediately after irradiation, the medium was changed to a medium containing cytochalasin B (6 µg/ml) with DMSO (0.1%) or DADS (5 µM) and cultured for 48 h. The cells were then washed with PBS, fixed with ethanol (–20°C), and stained with Hoechst 33342 (Dojindo, Kumamoto, Japan). The population ratio of binucleated (2N) cells to the observed cell population containing 1 nucleus (1N), 2N, and >2N was evaluated by counting more than 500 cells. The number of micronuclei in the binucleated cells were counted. At least 1000 binucleated cells were observed to evaluate micronucleus (MN) formation. The scoring criterion used was as described previously for hepatocytes [24]. Apoptosis was evaluated by culturing cells for 1 day after irradiation without cytochalasin B, then staining the cells as described above. Cells with chromatin condensation and fragmentation were evaluated as apoptotic cells and counted (at least 1000 cells). Cells with micronuclei and apoptotic cells stained with Hoechst 33342 were observed and photographed using a bio-imaging analysis system comprising a BX53 fluorescence microscope and DP73 digital microscope camera (OLYMPUS, Tokyo, Japan) controlled by Lumina Vision (MITANI CORPORATION, Tokyo, Japan).

Mice

Eight-week-old female BALB/c mice were obtained from Japan SLC Co. (Hamamatsu, Japan). Mice were housed for 10 days to allow for adaptation before performing the experiments. The mice were divided into 4 groups and 4 or 5 mice in each group were used in the experiments. The effects of administering 1%DADS on irradiated mice were evaluated in 2 independent experiments. The DADS concentration (1% or 0.5% in mineral oil) used was determined in reference to a report that 1 administration of 1% DADS has no effect on the survival of mice [21]. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS), and were done in strict accordance with the NIRS Guidelines for the Care and Use of Laboratory Animals.

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Oral administration of DADS to mice and subsequent irradiation

DADS oral administration was performed following the method described previously [21].

DADS (200 µl, 0.5% or 1% DADS in mineral oil) was administrated to mice using a feeding needle every morning for 3 days before irradiation. The same volume of mineral oil alone was administered to the control group. Before administering DADS in the morning, food was withheld from the mice from the previous evening. X-ray irradiation was performed 1 day after the final administration of DADS. The mice were irradiated with a total dose of 4 Gy at a dose rate of 0.85 Gy/min using a Pantak 320S machine (Shimadzu) equipped with a 0.50-mm Al+0.50-mm Cu filter and operated at 200 kVp and 20 mA. An exposure rate meter (AE-1321M; Applied Engineering, Inc.) was used for dosimetry measurements.

Evaluation of tissue weights and detection of micronuclei in bone marrow cells in mice after X-ray irradiation

Mice were anesthetized by inhalation of gaseous isoflurane (Pfizer, Tokyo, Japan) and then euthanized by cervical dislocation. Liver, thymus, and spleen tissues were collected for weight measurement. The pictures of spleen were taken using an LCD digital microscope DIM-03 (Alfa Mirage, Osaka, Japan). Radiation-induced genotoxicity and the effects of DADS on genotoxicity were assessed using the standard bone marrow micronucleus test, as described previously [25]. Bone marrow smears were prepared from both femurs. Micronuclei were counted in immature polychromatic erythrocytes (PCEs) and in mature normochromatic erythrocytes (NCEs). The frequencies of micronuclei in PCEs and NCEs are referred to as MNPCEs and MNNCEs, respectively. The ratio of PCEs to mature NCEs (P/N ratio) is an indicator of the relative proliferation rate in the erythroid lineage. At least 6000 cells (PCE+NCE) per mouse were counted, and the data for each experimental point were obtained from at least 4 mice.

Statistical analysis

Statistical analysis of the HepG2 cell experimental results was performed using the paired *t* test. In the animal experiments, statistically significant changes were evaluated using the unpaired *t* test or the Mann-Whitney U test.

Results

The effects of DADS on DNA damage caused by X-ray irradiation were assessed using HepG2 cells, as this cell line is often used to evaluate the toxic or protective effects of natural or chemical compounds [22]. The frequency of MN formation increased after irradiation with 4 Gy and this increase was mitigated by the presence of 5 µM DADS, as shown in Figure 1A. In addition, the effects of DADS on cell cycle progression after irradiation was assessed by evaluating the ratios of 2N cells in cell populations observed in the present experiments. Radiation inhibited cell cycle progression and was unaffected by DADS (Figure 1B). However, it is interesting that DADS promoted radiation-induced apoptosis (Figure 1C). Typical images of MN in binucleated cells and an apoptotic cell are shown in Figure 1D.

We evaluated the effects of DADS on radiation-induced damage *in vivo* by orally administering DADS to BALB/c mice. We specifically investigated the protective effects of DADS observed 3 weeks after irradiation, as these effects could be related to long-term outcomes after irradiation. Radiation induced dramatic decreases in thymus weight and slight increases in liver weight. As shown in Figure 2, the administration of DADS did not affect mouse body or liver weights. DADS induced a slight increase in thymus weight but did not improve acute decreases in thymus weight after irradiation. Interestingly, irradiation induced an increase in spleen weight 3 weeks after irradiation, and this increase was inhibited in mice administered DADS prior to irradiation.

Increases in the frequencies of MN formation in PCE in bone marrow cells were observed 3 weeks after irradiation, but were lower in mice administrated 1% DADS (Figure 3A). Moreover, even when 0.5% DADS was administered to the irradiated mice, DADS decreased the frequency of MN formation in both PCE and NCE in bone marrow cells after irradiation (Figure 4A). In particular, 0.5% DADS appeared to reduce this frequency more clearly than did 1% DADS. The P/N ratios among the groups were essentially unchanged 3 weeks after irradiation (Figures 3B, 4B), although a slight decrease in the irradiated mice was observed (Figure 4B).

Radiation induces decreases in spleen weight early after irradiation [26,27]. We assessed the effects of radiation on spleen weight 1 week after irradiation and observed decreased spleen weights in irradiated mice (Figure 5A, 5C), showing that DADS had no effect on this decrease (Figure 5A). However, 3 weeks after irradiation, as indicated above (Figure 2), spleen weights increased in the absence of DADS administration (Figure 5B, 5C), and the administration of even 0.5% DADS inhibited this large increase in spleen weight (Figure 5B).

Discussion

There are several reports that sulfur compounds from garlic can protect against the acute effects of radiation [15,17,27]. DADS is a main component of sulfur-containing compounds in

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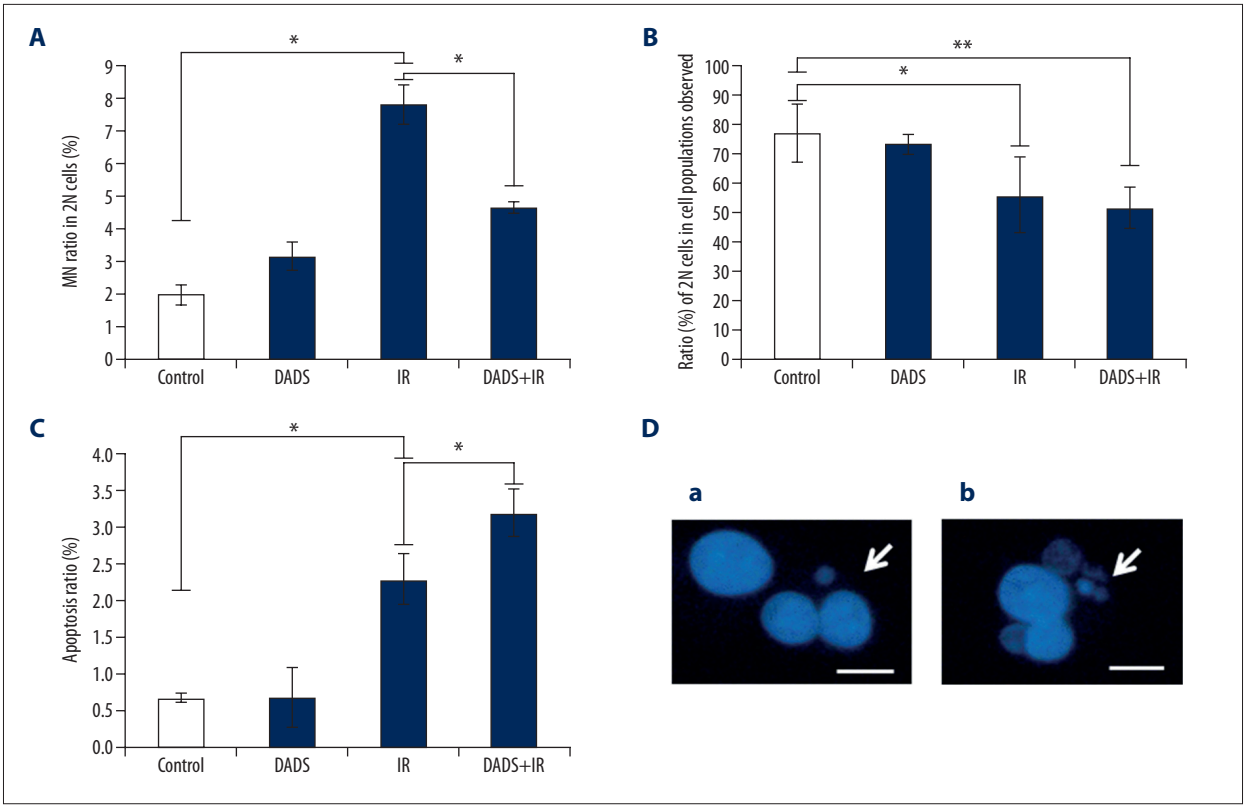

Figure 1. Effect of DADS on radiation damage in HepG2 cells. (**A**) Radiation-induced micronucleus production in binucleated cells. (**B**) Ratio of binucleated cells in cell distribution. (**C**) Radiation-induced apoptosis. (**D**) Photographs of binucleated cells with micronuclei (**a**) and apoptotic cells (**b**) stained with Hoechst 33342, indicated with an arrows (↓). Scale bars indicate 10 μm. Data are shown as means±SE from 3 independent experiments. * p<0.05, * *p<0.01.

garlic oil; therefore, DADS might be effective in reducing DNA damage or persistent effects after irradiation. DADS has been reported to have anti-cancer or anti-inflammatory functions and to induce apoptosis [19]. Here, we evaluated the effects of DADS on DNA damage induced by X-ray irradiation using HepG2 cells. DADS inhibited an increase in the frequency of MN formation caused by irradiation. The DADS concentration used in this study (5 μM) was appropriate because the maximum concentration of DADS and its metabolites, such as allylmercaptan (AM) and allyl methyl sulfide (AMS) measured *in vivo* after a single oral administration of DADS, was reported to be around 1–8 μM in rat plasma [22,23]. The reduction by DADS of radiation-induced MN formation in binucleated cells is indicative of efficient removal of cells with damaged DNA. Indeed, DADS-induced apoptosis appears to contribute to the reduction of MN, since increased radiation-induced apoptosis was observed in the presence of DADS. The anti-oxidative effects of DADS arise from its role in increasing glutathione (GSH) levels [28]. However, induced apoptosis was observed after irradiation, suggesting that the direct scavenging abilities of radiation-induced oxidative stress by GSH is not the main mechanism underlying the radio-protective effects of DADS.

MN induction was also inhibited by DADS in mice. The reduction of MN formation without a change in P/N ratio suggests that damaged cells are eliminated efficiently, although it is also possible that DADS influences not only apoptosis but also many other anti-cancer functions in cells [19].

It was reported that MN formation is induced 48–72 h after irradiation [29]. In the present study, induction was still observed 3 weeks after irradiation, as well as the inhibition of induction by DADS. These results suggest that DADS reduces DNA damage observed as late effects after irradiation, thus potentially preventing radiation-induced inflammation or carcinogenesis.

The effects of DADS on damage induced by electron beam irradiation were reported in a recent study [27] in which spleen weight changes and DNA damage were evaluated 24 h after irradiation with 6 Gy. The dose of X-rays used in our study was 4 Gy, which is much less than LD50/30 (5.9 Gy) in the case of BALB/c mice [30]. The dose used in the present study (4 Gy) is often used for evaluating DNA damage caused by radiation [31] and also within the range of doses administered to normal tissues during cancer radiotherapy [32]. In addition, although Tenkanidiyoor et al. demonstrated the protective effects of

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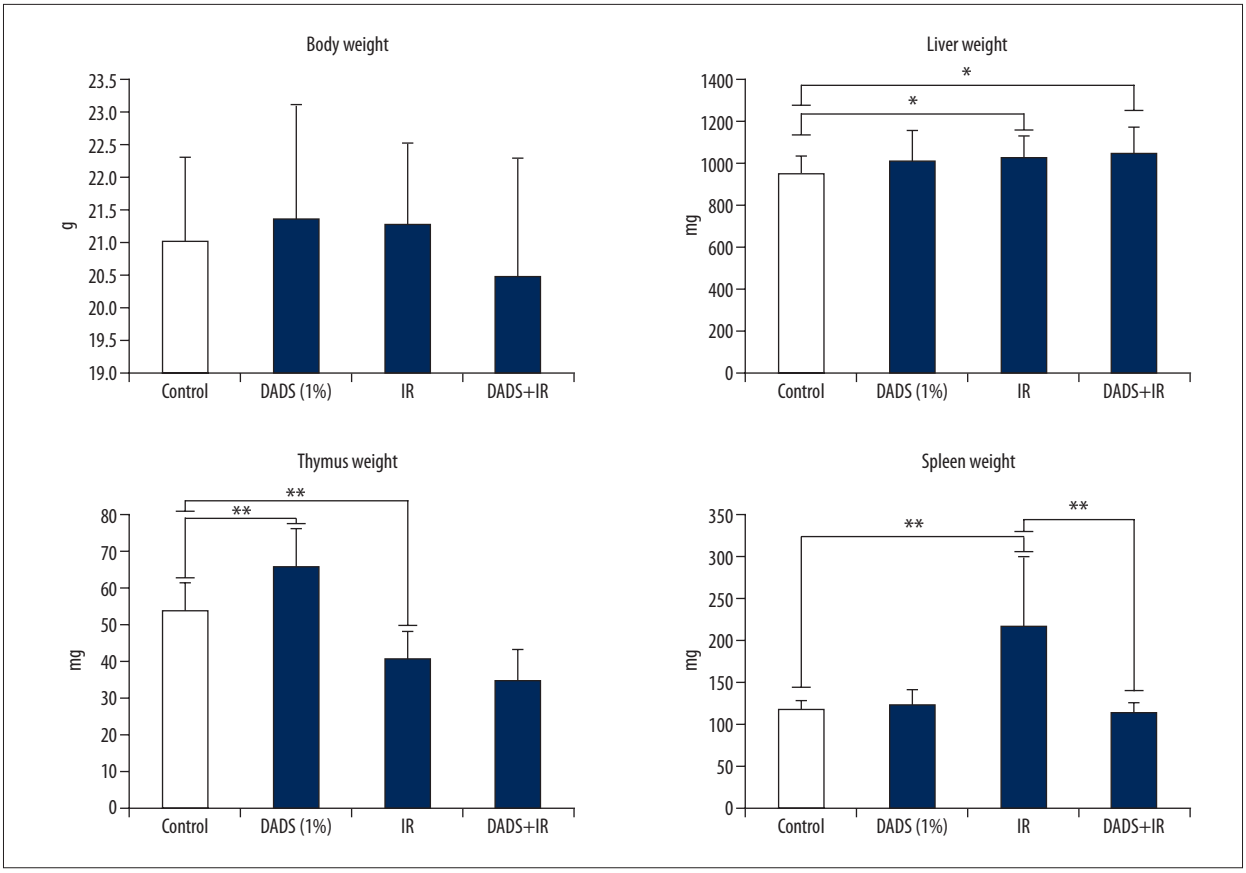

Figure 2. Effect of oral administration of 1% DADS on body weight, and the weight of the thymus, spleen, and liver in irradiated mice. Each weight was evaluated 3 weeks after irradiation. Data are shown as means±SD from 9–10 mice. * p<0.05, * *p<0.01.

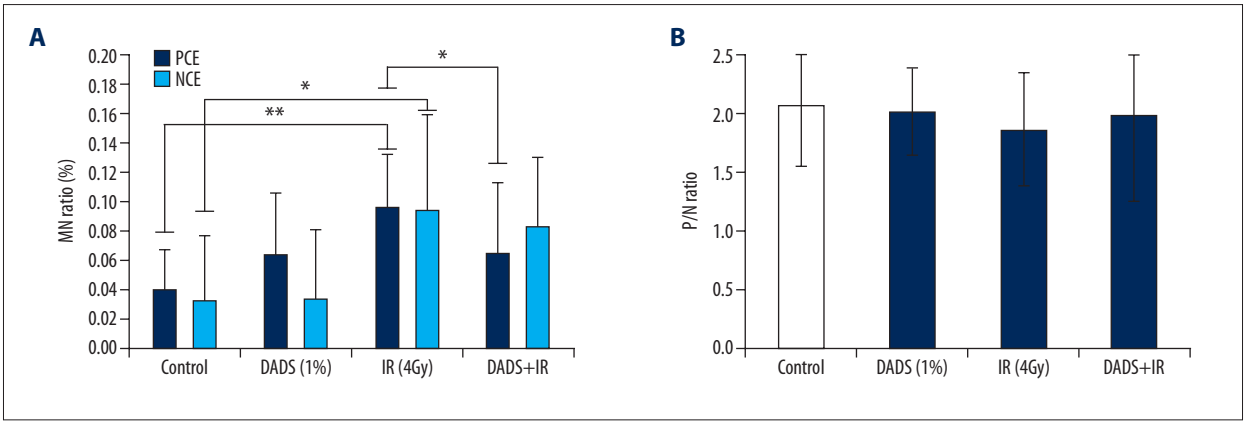

Figure 3. Effect of oral administration of 1% DADS in mice irradiated with X-rays. (**A**) Bone marrow micronucleus test. Frequency of micronuclei in the bone marrow cells of BALB/c mice. (**B**) Ratio of polychromatic erythrocytes to normochromatic erythrocytes (P/N ratio) in the bone marrow of BALB/c mice administered 1% DADS. Both data sets were evaluated 3 weeks after irradiation. Data are shown as means±SD from 9–10 mice. * p<0.05, * *p<0.01.

DADS on spleen damage 24 h after irradiation [27], we primarily evaluated the effects *in vivo* 3 weeks after irradiation. Many researchers have demonstrated that high-dose radiation causes decreased spleen weight [26,27]. Interestingly, irradiation with 4 Gy resulted in decreased spleen weight 1 week after irradiation, even in mice treated with DADS (Figure 5A). These results indicate that the protective effect of DADS spontaneously rescues the spleen from radiation damage shortly (at around 24 h) after irradiation, but subsequently leads to decreases in spleen weight. In addition, although acute recovery

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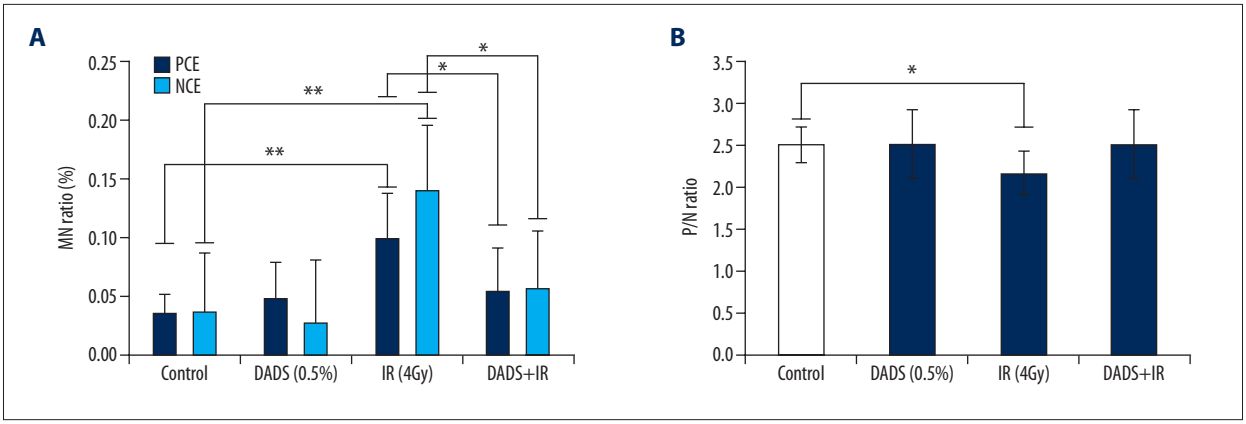

Figure 4. Effect of oral administration of 0.5% DADS in mice irradiated with X-rays. (**A**) Bone marrow micronucleus test. Frequency of micronuclei in the bone marrow cells of BALB/c mice. (**B**) Ratio of polychromatic erythrocytes to normochromatic erythrocytes (P/N ratio) in the bone marrow of BALB/c mice administered 0.5% DADS. Both data sets were evaluated 3 weeks after irradiation. Data are shown as means±SD from 4–5 mice. * p<0.05, * *p<0.01.

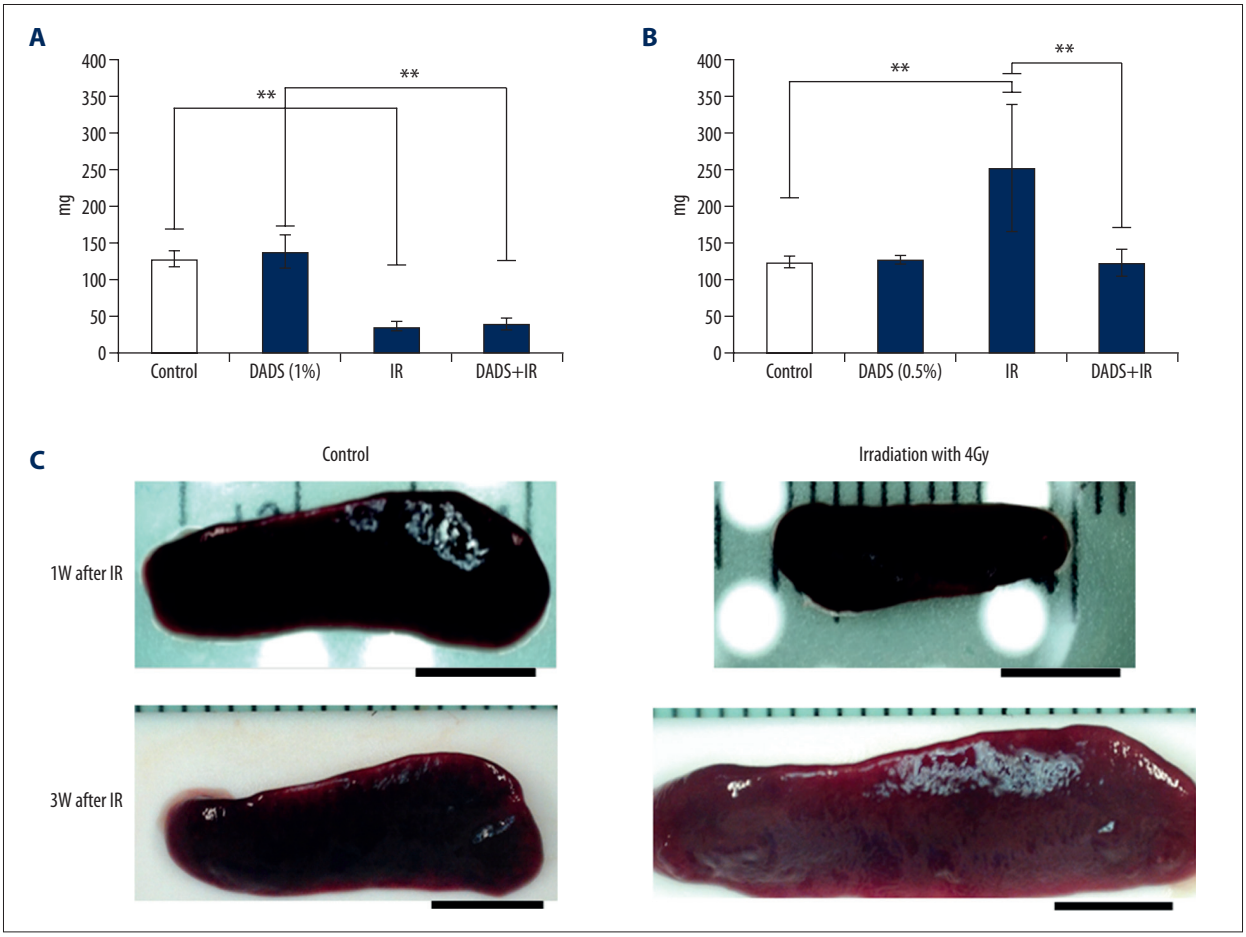

Figure 5. Effects of oral administration of DADS on spleen weights in mice after irradiation. (**A**) The weights of spleens from mice (1 week after irradiation) administered 1% DADS (N=4–5). ** p<0.01. (**B**) The weights of spleens from mice (3 weeks after irradiation) administered 0.5% DADS (N=4–5). ** p<0.01. (**C**) Representative pictures of spleens from mice (1 week and 3 weeks after irradiation with 4 Gy). Scale bars indicate 5 mm.

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of the spleen is observed several weeks after irradiation, this recovery is regulated to the control level in mice administered DADS (Figure 2). On the other hand, taking the results of the P/N ratios (Figures 3B, 4B) into consideration, the hematopoietic status in bone marrow appears to have essentially recovered 3 weeks after irradiation. The recovery process in the spleen after irradiation has been reported in C57BL/6 mice [33], and this earlier study demonstrated that recovery is accompanied by outstanding induction in the B cell population several weeks after irradiation. B cells induce the secretion of several cytokines that regulate effective immune responses [34–36], and DADS may mediate cytokine regulation [37,38]. DADS might ameliorate the large increase in spleen weight by regulating cytokine secretion. In addition, spleen weight is a potential sign of inflammation, and DADS may mitigate chronic inflammation in inflamed areas in mice, which can lead to carcinogenesis [39]. This inhibition of increased spleen weight after irradiation was also observed following the administration of 0.5% DADS (Figure 5B). DADS thus appears to influence spleen weight after irradiation, and further investigations, including pathological analyses, are needed to explain this effect.

Natural compounds exhibiting anti-oxidative functions have been reported as possible radio-protective agents [6,40]. Sulfur metabolism might be related to the protective activity of DADS [28]. DADS induces sulfur-metabolizing enzymes [41] and promotes anti-oxidative functions [42]. In addition, garlic oil and garlic organosulfur compounds were reported to mitigate radiation damage after high-dose irradiation [15,16]. The components in garlic oil involved in sulfur metabolism likely have radiation-protective functions in organisms exposed to radiation due to their involvement in sulfur metabolism. Here, the effects of DADS on MN formation as an indicator of DNA damage induced by X-rays were evaluated *in vivo* in mice. Given that DADS promotes apoptosis in HepG2 cells after irradiation, DADS may inhibit residual radiation-induced DNA damage *in vivo* by promoting apoptosis in damaged cells. The promotion of apoptosis by DADS might be useful for cancer therapy. DADS and several synthetic analogs have been evaluated for their anti-cancer properties in terms of apoptosis induction [19,43]. Interestingly, it has been demonstrated that carbon-ion radiation with DADS pretreatment promotes apoptosis in cervical cancer cells [44], whereas carbon-ion irradiation-induced apoptosis in testis was reported to be attenuated by DADS [17]. Thus, the effects of DADS on radiation-induced damage might be dependent on the tissue or cell type. In addition, it has been reported that the administration of more than 5% DADS affects the survival in BALB/cA mice, whereas 1% DADS administration has no effect [21]. In the present study, we administered 0.5% or 1% DADS; the administration of 0.5% DADS resulted in clearer protective effects than did 1% DADS, indicating that an appropriate concentration of DADS is required for maximum effect. Moreover, although DADS is metabolized to compounds such as allyl methyl sulphoxide (AMSO) and allyl methyl sulphone ($AMSO_2$), to the best of our knowledge, the participation of metabolites derived from DADS in radiation protection has not been studied [23] and thus both DADS and its metabolites might exhibit radio-protective abilities. Other natural compounds, such as kojic acid [14], flavonoids [13], and ferulic acid [12], have radiation-protective properties [6], and as we have demonstrated, DADS may also be a radiation-protective agent. In the future, we will further evaluate the effects of DADS on radiation-induced damage.

Conclusions

Treatment with DADS decreases the frequency of micronucleus formation in human cells and in mice after irradiation, and may also influence subsequent damage through its effects in the spleen after irradiation. DADS could be a natural compound that mitigates radiation damage.

Conflict of interests

None.

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