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Reduced High-Dose Radiation-Induced Residual Genotoxic Damage by Induction of Radioadaptive Response and Prophylactic Mild Dietary Restriction in Mice

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Abstract

Radioadaptive response (RAR) describes a phenomenon in a variety of in vitro and in vivo systems that a low-dose of priming ionizing radiation (IR) reduces detrimental effects of a subsequent challenge IR at higher doses. Among in vivo investigations, studies using the mouse RAR model (Yonezawa Effect) showed that RAR could significantly extenuate high-dose IR-induced detrimental effects such as decrease of hematopoietic stem cells and progenitor cells, acute radiation hematopoietic syndrome, genotoxicity and genomic instability. Meanwhile, it has been demonstrated that diet intervention has a great impact on health, and dietary restriction shows beneficial effects on numerous diseases in animal models. In this work, by using the mouse RAR model and mild dietary restriction (MDR), we confirmed that combination of RAR and MDR could more efficiently reduce radio-genotoxic damage without significant change of the RAR phenotype. These findings suggested that MDR may share some common pathways with RAR to activate mechanisms consequently resulting in suppression of genotoxicity. As MDR could also increase resistance to chemotherapy and radiotherapy in normal cells, we propose that combination of MDR, RAR, and other cancer treatments (i.e., chemotherapy and radiotherapy) represent a potential strategy to increase the treatment efficacy and prevent IR risk in humans.

Keywords

adaptive response, dietary restriction, radiogenotoxicity, ionizing radiation, mice

Introduction

Ionizing radiation (IR) is a carcinogen. It is capable of inducing genotoxicity and genomic instability (GI), in particular, at high doses. Radiation-induced genotoxicity and GI are characterized by varied endpoints such as chromosomal rearrangements and aberrations, micronucleus formation and gene mutation, having a big impact on radiocarcinogenesis.[1,2] GI is central to carcinogenesis[3] and radiation-induced GI is the driving force responsible for radiocarcinogenesis.[1,4,5] As humans are unavoidably exposed to IR at higher doses in some circumstances such as long-term spaceflight mission and radiotherapy, limiting cancer risk from exposure to IR is of great public concern.

A lot of factors could modify IR-induced biological effects including carcinogenesis, such as pre-exposure to IR at low doses, and dietary and life-style related factors. Radioadaptive

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response (RAR) manifests as a decrease of radiosensitivity in which a low-dose of priming IR reduces the detrimental effects of subsequent challenge IR exposure at a higher dose. As an evolutionarily conserved phenomenon, RAR has been demonstrated in a variety of in vitro and in vivo biosystems from simple prokaryotes to higher eukaryotes including mammalian animals.[6] In vivo investigations showed that RAR could reduce challenge dose-induced DNA damage, micronucleus formation, chromosomal aberrations, cell transformation, cell death, hematopoietic death, and carcinogenesis.[6-12] In a series of previous studies using the RAR mouse model (Yonezawa Effect) which could rescue bone marrow death through induced resistance in the hematopoietic system,[13,14] we confirmed that RAR could reduce radiation-induced genotoxcity and GI measured as decreased frequency of micronucleus erythrocytes in bone marrow cells and reduced frequency of delayed homologous recombinant cells in bone marrow nucleated cells and splenocytes.[15,16] On the other hand, dietary and lifestyle-related factors could influence health in many species and play key roles in modulating the risk of developing cancer. As a fact, certain cancers are primarily dependent on dietary habits.[17,18] It has been known since the early work that dietary restriction, i.e. calorie restriction of either calories or macronutrients and fasting, would be a potent intervention against development of cancer including its initiation, progression and metastasis,[19] act synergistically with other treatments,[20] and be capable of decreasing significantly the incidence of both spontaneous and induced neoplasms in experimental carcinogenesis.[19,21-24] For example, on prevention of radiocarcinogenesis, investigations showed that food or caloric restriction decreased dramatically gamma- or X-ray-induced solid tumors and/or leukemias in mice and rats.[25-27] Caloric restriction onset either pre- or post-exposure to X-rays could extend latency of myeloid leukemia, and prevent radiation-induced myeloid leukemia and life shortening in mice.[28-31] Furthermore, post-exposure onset of dietary intervention (calorie restriction) in animal models showed extended lifespan, reduced frequencies of radiocarcinogenesis (late-occurring tumor) and mutations.[32,33] These studies demonstrated that dietary restriction could generally prevent incidence of radiocarcinogenesis, irrespective of calorie restriction onset timing in terms of irradiation, in experimental models.

In this work, for the first time in the research fields of RAR and dietary intervention, the impact from combination of mild dietary restriction (MDR) and induction of RAR on high-dose IR-induced genotoxicity was investigated in a mouse model measured as micronucleus erythrocytes in the bone marrow.

Materials and Methods

Animals

Three-week-old C57BL/6 J Jms strain female mice, wean just from breastfeeding, were purchased from SLC, Inc. (Japan). To avoid possible effects from the developmental condition of the animals, any mouse with a significantly different body weight (more or less than the mean $\pm$ 2 SD) upon arrival was omitted from this study. The selected mice were randomly assigned to 2 experimental groups either allowed free access to a standard laboratory chow MB-1 (Funabashi Farm Co., Japan) or under a MDR. The animals under MDR were given daily (around 9:30 am) 85% of the amount (weight in gram) of the chow consumed by the animals that were allowed to free access to the diet. Ingredients of the diet MB-1 contained 24.2% crude protein, 4.4% crude fat, and 54.4% carbohydrate. The metabolizable energy was 354.0 kcal/100 g. As the mean amount of chow consumed by per mouse allowed free access to the diet was 2.92 g per day, each of the mice under MDR was given daily 2.48 g of the chow. Thus, the weekly metabolizable energy was 72.36 kcal and 61.45 kcal respectively for each of the mice with no diet restriction and under diet restriction. All animals were maintained in a conventional animal facility under a 12 h light-12 h dark photoperiod, housed in autoclaved cages (1 mouse per cage) with sterilized wood chips, and allowed access to acidified water (pH = 3.0 $\pm$ 0.2) *ad libitum*.

Animals in each of the experimental groups were further divided into 3 subgroups, namely, the control group (without exposure to radiation), the RAR induction group (receiving both a priming dose and a challenge dose), and the high-dose exposure group (receiving only a challenge dose). Based on our previous studies and preliminary trials, in the present study at least 20 mice were used in each experimental subgroup and the experiment was repeated once. All experimental protocols (Experimental Animal Research Plan No. 09-1049 -1) involving mice were reviewed and approved by The Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology (QST-NIRS). The experiments were performed in strict accordance with the QST-NIRS Guidelines for the Care and Use of Laboratory Animals.

Irradiation

X-rays were generated with an X-ray machine (Pantak-320 S, Shimadzu, Japan) operated at 200 kVp and 20 mA, using a 0.50 mm Al + 0.50 mm Cu filter. An exposure-rate meter (AE-1321 M, Applied Engineering Inc, Japan) with an ionization chamber (C-110, 0.6 ml, JARP, Applied Engineering Inc, Japan) was used for the dosimetry. The dose rate for delivering the priming dose and the challenge dose was at about 0.30 Gy/min and 0.90 Gy/min, respectively. The mice held in acryl containers were exposed to total body irradiation (TBI) at room temperature.

Mouse Model for Induction of Radioadaptive Response

The mouse model for induction of RAR measured as significant rescue of bone marrow death (Yonezawa Effect) established by Yonezawa and colleagues.[13] In this model there were basically 2 types of combination for the latitude of priming dose and the interval of irradiation timing (between the priming dose and the challenge dose). Both types of combination were

adopted, verified and confirmed in C57BL/6 J Jms female mice under the experimental conditions in our research facilities. The type of combination for 0.5 Gy of the priming dose and 2-week interval of irradiation timing was finally applied to the present work. Namely, the timing for delivery of the priming dose and challenge dose was on postnatal ages of 6 and 8 weeks of the mice, respectively. A dose of 0.5 Gy was used as the priming dose to verify the existence of RAR in the 30-day survival test and to investigate the incidence of micronucleus erythrocytes in femur bone marrow. For the challenge dose, a lethal dose at 7.5 Gy was used in the 30-day survival test and a non-lethal dose at 4.0 Gy was used in micronucleus test.

Micronucleus Test

The bone marrow micronucleus test was carried out accordingly.[15] Induction of micronucleus erythrocytes in bone marrow by TBI was used as an index to evaluate both acute radiogenotoxicity and radiation-induced GI depending on early and late timing of measurement after exposure. Mice were sacrificed the following day after the 30-day survival test. Bone marrow smears prepared from both femurs were processed for the enumeration of micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic erythrocytes (MNNCEs). The slides were coded to avoid observer bias. The micronuclei were scored using a light microscope at a magnification of 1000 $\times$. At least 5000 PCEs and 5000 NCEs per mouse were counted and the data for each experimental point were from at least 5 mice.

Physiological Endpoints

Physiological conditions were comparatively studied in mice that were allowed free access to the diet and being under MDR. The assessments included evaluating changes in body mass and main organ/tissue weights, and measurements of peripheral hemogram and bone marrow cellularity (number of nucleated cells). Body weight gain was monitored weekly from onset of MDR at postnatal age 4 weeks to the end of experiment at postnatal age 13 weeks. Main organ and intra-abdominal fat weights, peripheral hemogram, and femur bone marrow cellularity were measured at the end of the experiment. For analysis of hemogram, peripheral blood was collected with a heparinized syringe in vacutainer blood collection tubes containing EDTA (Venoject II, Terumo Co., Japan), then samples were immediately subjected to a differential blood cell count and hemoglobin concentration measurement using a blood cell differential automatic analyzer (SYSMEX K-4500, Sysmex Corporation, Japan). The data for each experimental subgroup were from at least 5 mice.

Statistical Analysis

Statistical evaluation of the data was done using the Chi-squared test for the 30-day survival and micronucleus test, and Student's *t*-test for the other endpoints. The statistical significance was assigned to $P < 0.05$.

Results

Validation of Physiological Effects From Mild Dietary Restriction

Physiological effects were assessed by evaluating changes in body mass and main organ/tissue weights, peripheral hemogram, and bone marrow cellularity. Body mass measurements of animals under MDR pointed to a general significantly lower body weight gain 1 week after onset of MDR until the end of the experiment compared to that of animals allowed free access to the diet (Figure 1A). No significant changes in the weight of brain, liver, spleen, and kidney except for a markedly decreased intra-abdominal fat weight were observed in mice being under MDR (Figure 1B). No changes were observed in peripheral hemogram measured as cell count and hemoglobin concentration (Figure 1C) and bone marrow cellularity measured as number of nucleated cells (Figure 1D). In addition, all mice being under MDR looked normal and healthy throughout the whole monitoring period. Results indicated that there is no significant detrimental physiological effect from MDR on mice during the whole period of diet regimen in our experimental setup.

Validation of Radioadaptive Response Induction in Mice Under Mild Diet Restriction

Reproducibility of the RAR mouse model (Yonezawa Effect) was verified in mice allowed free assess to the diet (Figure 2A) and being under MDR (Figure 2B) using 30-day survival test. For mice allowed free assess to the diet, the survival rate was 16.7% and 80.0% respectively for animals receiving a challenge dose of 7.5 Gy at postnatal 8 weeks and animals receiving both a priming dose of 0.5 Gy at postnatal 6 weeks and a challenge dose of 7.5 Gy at postnatal 8 weeks. For mice being under MDR, the survival rate was 13.3% and 76.7% respectively for animals receiving a challenge dose of 7.5 Gy and animals receiving both a priming dose of 0.5 Gy and a challenge dose of 7.5 Gy. Results demonstrated that the priming dose could successfully induce a RAR in mice regardless of the diet regimen. Furthermore, neither marked differences in survival rate after exposure to the challenge dose alone, nor significant differences in efficacy for induction of RAR were observed regardless of the diet regimen. These results clearly demonstrated that there is no significant modifying effect from MDR on responses to the high challenge dose alone and induction of RAR measured as the survival rate in 30-day survival test.

Validation of Micronucleus Incidence in Bone Marrow Erythrocytes

Incidence of MNPCEs (Figure 3A) and MNNCEs (Figure 3B) in bone marrow erythrocytes was assessed in mice allowed free

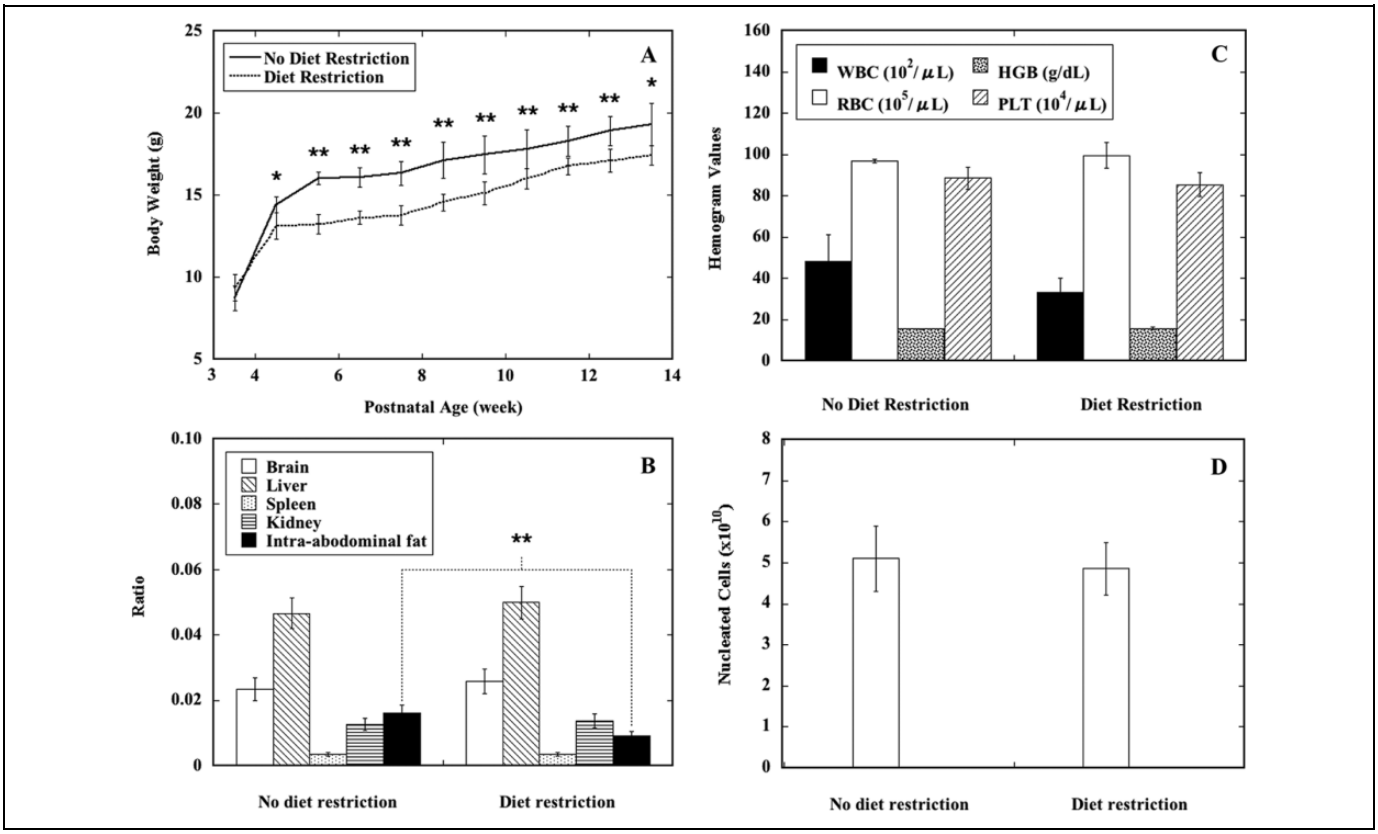

Figure 1. Physiological effects from diet restriction on mice. Body weight gain (A), ratio of organ or tissue weight to the body weight (B), peripheral hemogram (C), and bone marrow cellularity (D) were measured to comparatively study the effect. Body weight (g) is presented as mean ± SD (A). The solid line and dotted line stand for mice having free access to the diet and being under diet restriction, respectively. The ratio of organ or tissue weight (g) to the body weight (g) measured at postnatal age 13 weeks is presented as mean ± SD (B). The open bar, striped bar, dotted bar, transversely striated bar, and solid bar stand for brain, liver, spleen, kidney, and intra-abdominal fat, respectively. Peripheral hemogram was measured at postnatal age 13 weeks (C). Cell count or hemoglobin concentration is presented as mean ± SD. The solid bar, open bar, dotted bar and striped bar stands for white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), and blood platelet count (PLT), respectively. Bone marrow cellularity was measured as total cell count of nucleated cells in 2 femurs per mouse (D). One (*) and 2 asterisks (**) respectively indicate statistically significant differences at $P < 0.05$ and $P < 0.01$ between the 2 groups that were compared.

access to the diet and being under MDR. In general, regardless of the diet regimen, exposure of animals to the challenge dose (4.0 Gy) alone induced a markedly increased incidence of MNPCEs and MNNCEs, and induction of RAR by the priming dose (0.5 Gy) significantly reduced the incidence of MNPCEs and MNNCEs caused by the subsequent challenge dose. While for animals receiving the challenge dose, incidence of MNPCEs and MNNCEs was respectively 5.5 ± 1.1 and 4.0 ± 1.2 per mille (‰) in mice that were allowed free access to the diet. Incidence of MNPCEs and MNNCEs was respectively 3.1 ± 1.1 and 3.0 ± 1.0 ‰ in mice under MDR. On the other hand, for animals receiving both the priming dose and the challenge dose, incidence of MNPCEs and MNNCEs was respectively 1.8 ± 0.5 and 1.8 ± 0.5 ‰ in mice that were allowed free access to the diet. Incidence of MNPCEs and MNNCEs was respectively 1.0 ± 0.3 and 0.9 ± 0.4 ‰ in mice under MDR. Of note, in animals receiving both the priming dose and the challenge dose, incidences of both MNPCEs and MNNCEs in mice under MDR were significantly lower than that in mice that were allowed free access to the diet. Results clearly demonstrated that induction of RAR could relieve radiogenotoxicity caused by the high challenge dose, and increased efficacy for reduction of radiogenotoxicity could be further achieved in combination with MDR.

Discussions

Increased opportunity for exposure to IR due to such as cancer radiotherapy and long-term space mission is associated with an increasing risk of health effects in humans, in particular, carcinogenesis. Reducing health risk from unavoidable IR exposure of normal tissue in medical treatment and spaceflight crew is of great concern and significance. To modify the radioresistance of normal tissues and cancer cells whenever possible, triggering the mechanisms to selectively increase the radioresistance of the normal tissues and sensitize the cancer cells is of great importance. Many approaches such as development of radioprotective agents, pharmaceutical gene regulation, and intervening dietary

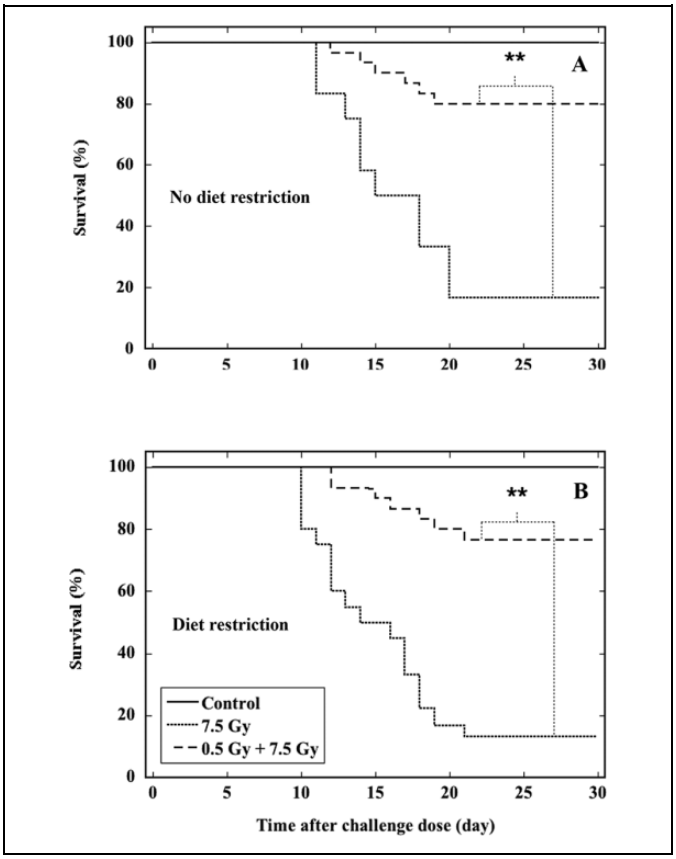

Figure 2. Induction of RAR in mice having free access to the diet and being under diet restriction. Induction of RAR (Yonezawa Effect) by a priming dose of 0.5 Gy X-rays at postnatal 6 weeks followed by a subsequent challenge dose of 7.5 Gy X-rays at postnatal 8 weeks was verified in mice having free access to the diet (A) and being under diet restriction (B). The solid line, broken line, and dotted line stand for the mice receiving no TBI (control), the mice receiving the challenge dose alone (7.5 Gy), and mice receiving both the priming dose and the challenge dose (0.5 Gy + 7.5 Gy), respectively. Two asterisks (**) indicate statistically significant differences ($P < 0.01$) between the 2 groups that were compared.

habit, psychological stress and life style were proposed.[34] When compared to the research and development of these therapeutic modalities, intervention of life-style factors is less time-consuming and with a low cost, which neither depends on the advancement of technology. Notably, dietary restriction, as one of the mostly used intervention of life-style factors, showed a great promise as a modifier to prevent incidence of radiation-induced myeloid leukemia in mice, irrespective of calorie restriction onset timing in terms of irradiation.[28-31] It is rational that initiatives to reduce the health risk from IR exposure should focus on the tenets of appropriate combination of different approaches. To date, there is no study on the efficacy of combination of induction of RAR and dietary restriction for reduction of radiogenotoxicity and IR-induced GI.

The mouse RAR model (Yonezawa Effect) is a well-designed system in investigating the late health effect *in vivo* such as IR-induced GI. In a series of our previous investigations, we demonstrated that induction of RAR could relieve IR-induced GI measured as micronucleus formation and delayed homologous recombination in the hematopoietic system.[15,16] Recently, we also verified the modifying effects from dietary factors on the biological response to IR exposure of mice. Our studies confirmed that there existed an interaction between life-style factors (i.e., high fat diet, low fat diet and alcohol drinking) and IR, which could lead to altered responses to IR in mice.[35-37] In the present work, combination of MDR and RAR was further verified in the mouse RAR model. Results indicated that MDR would not influence the phenotype of RAR measured as 30-day survival, and combined treatment with MDR and RAR could achieve a higher efficacy to relieve radiogenotoxicity and radiation-induced GI measured as reduced micronucleus frequency in the erythrocytes in the bone marrow.

It is known that normal cells and tumor cells from both mice and humans respond differently to induction of RAR. For example, in normal cells RAR could be induced measured as increased cell survival and decreased micronucleus frequency in the cultured cells in vitro, and increased cell proliferation and reduced apoptosis in vivo. On the other hand, higher induction of apoptosis along with high expression of pro-apoptotic gene Bax and lower expression of anti-apoptotic gene Bcl-2 was induced in tumor cells in tumor-bearing mice under RAR induction condition.[38-40] These works demonstrated clearly that RAR could be induced in normal cells but not in tumor cells under both in vitro and in vivo conditions. Interestingly, diet intervention also has the potential to differently change the response of normal tissue and tumors, namely, to alleviate genotoxicity of normal tissue and enhance cytotoxicity of cancer cells. Thus, it is expected that innovative use of diet intervention as a novel therapeutic option would bring an additional clinical benefit in the treatment of cancer patients. These studies suggested a very important clinic-relevant phenomenon and implied the potential for application of RAR to protect normal tissues without diminishing the efficacy of tumor radiotherapy.[40] Collectively, both induction of RAR and appropriate dietary restriction could selectively protect normal cells from the side effects of IR at higher doses and sensitize cancer cells to IR. In this regards, clinically combined application of prophylactic MDR and RAR before cancer radiotherapy would have a great potential for benefiting cancer patients from the point of view of increasing the maximum tolerated dose and reducing the risk of secondary cancer.

It should be pointed out that appropriate dietary restriction without malnutrition is critical for induction of beneficial health effects. For examples, moderate food restriction could suppress aging rate.[41] MDR in the present work could enhance the efficacy of RAR-induced radiogenotoxicity, and in addition, with a concomitant decrease in body weight gain and intra-peritoneal fat weight in mice. Conversely, severe dietary restriction or malnutrition due to unbalanced diet could increase sensitivity of normal tissues to radiogenotoxicity, radiation-induced bone marrow death and life shortening in mice.[36,42]

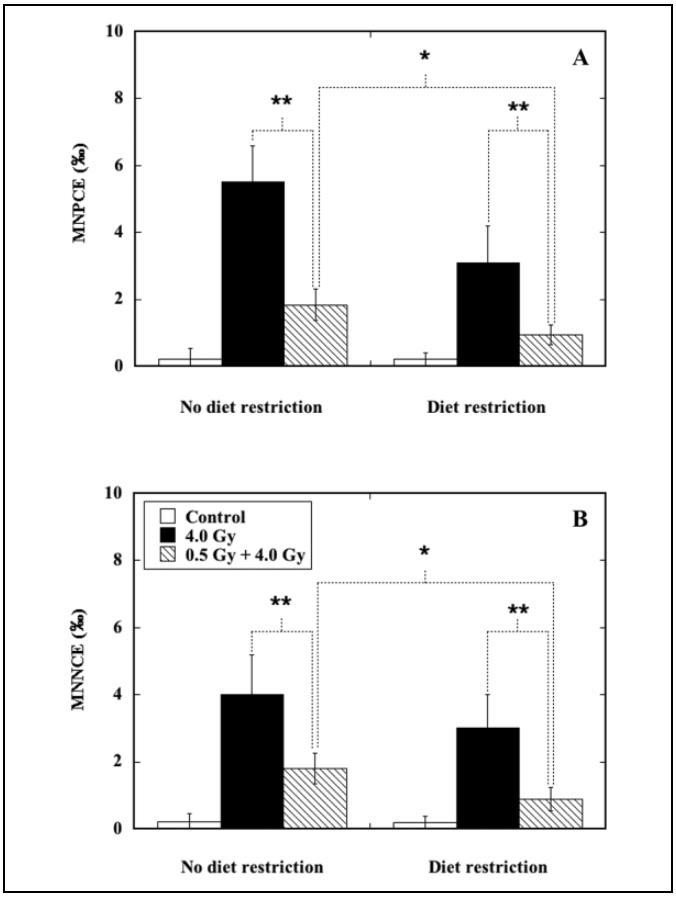

Figure 3. Incidence of MNPCEs and MNNCEs induced by TBI in femur bone marrow erythrocytes (RBC) in mice having free access to the diet and being under diet restriction. Incidence as the number of MNPCEs per 1000 PCEs (A) or of MNNCEs per 1000 NCEs (B) is presented as mean $\pm$ SD. The open bar, solid bar, and striped bar stand for samples from mice receiving no TBI (control), the mice receiving a challenge dose alone (4.0 Gy), and mice receiving both a priming dose and a challenge dose (0.5 Gy + 4.0 Gy), respectively. One (*) and 2 asterisks (**) respectively indicate statistically significant differences at $P < 0.05$ and $P < 0.01$ between the 2 groups that were compared.

A complete understanding of the underlying mechanisms is imperative in reducing health risk due to IR exposure in humans. Protection against radiocarcinogenesis could be achieved by different mechanisms (i.e., free radical scavenging, caloric restriction, anti-inflammation, and humoral factors), and the efficacy could be enhanced by targeting multiple mechanisms at the same time.[43] For dietary intervention in the animal models, it is firmly established that the effect depends on many variables, such as diet composition, feeding regimen, age of onset and genetics, in addition to the calories provided in the diet.[44] For caloric restriction, the anticancer and genomic effects could be established more rapidly in mitotic tissues. Acute caloric restriction showed reduced detrimental effects and aging alterations in the organs in both animal models and in humans, as possibly the highly conserved mechanisms for health enhancement.[45,46]

MDR may share some common pathways with RAR to activate mechanisms consequently resulting in suppression of genotoxicity. IR could cause both acute tissue damage and late effects including long-term or residual bone marrow injury. As a carcinogen, IR poses a significant cancer risk in part by induction of GI, for example, development of leukemia in hematopoietic system. Exposure of mice to a sublethal dose of TBI could induce a persistent increase in ROS production in hematopoietic stem cells. Studies showed that increased the generation and accumulation of mitochondrial ROS was via overexpression of miR-22 or nicotinamide adenine dinucleotide phosphate oxidase and led to induction of hematopoietic GI.[47,48] Exposure of mice to TBI could induce persistent oxidative stress in hematopoietic stem cells at least in part via up-regulation of nicotinamide adenine dinucleotide phosphate oxidase and associated with sustained increases in oxidative DNA damage, DNA double-strand breaks, and inhibition of clonogenic function.[47,49] On the other hand, a majority of studies in animal models and humans showed that dietary restriction-induced reduced mutations and radiocarcinogenesis are underlying mechanisms of increased antioxidant glutathione level, improved redox state, decreased mitochondrial ROS production, reduced oxidative stress and damage, and decreased chronic inflammation, leading to an overall reduction of steady-state oxidative damage to macromolecules including proteins, lipids and DNA in animals.[23,32,33,50-54] Meanwhile, RAR is also known as through mechanisms of increased antioxidant capability, decreased ROS production, reduced oxidative stress and damage, increased DNA repair and decreased chronic inflammation.[7-12]

As diet and metabolism link to cancer, for cancer treatment, it was demonstrated that dietary restriction could simultaneously target many signal pathways that were targeted by anticancer drugs[20] and cancer radiotherapy. For example, in mouse model, investigation showed that calorie restriction could augment efficacy of radiotherapy in breast cancer.[55] In humans, study showed that caloric restriction coupled with radiotherapy could decrease metastatic burden in triple negative breast cancer.[56] These studies indicated that diet intervention could be used in conjunction with chemotherapy and radiotherapy to render cancer cells more susceptible to treatment, which could lead to new cancer therapies using the metabolism-involved mechanisms. Dietary restriction could increase DNA repair in mice.[57] Equally interesting and important, increasing convincing evidence in animal models showed that post-exposure onset of calorie restriction could also reduce frequencies of radiocarcinogenesis. Pre-exposure caloric restriction could prevent the initiation of direct genotoxic leukemogenesis, and post-exposure could improve mitochondrial function, reduce production of reactive oxygen species, and relieve indirect and epigenetic leukemogenesis.[28-33,58]

In summary, the present work demonstrated that combined treatment with RAR and MDR could more efficiently relieve high-dose IR-induced genotoxicity in mice. These findings indicated that diet intervention could be a potential modifiable factor for reducing risk from IR exposure and provide a clear direction for initiating such studies prior to clinical implementation in humans. As a potentially promising strategy for reducing long-term IR health risk from cancer radiotherapy, combination of RAR induction and lifestyle management (i.e., MDR) could maximize medical utilization of IR, via

increasing the organ tolerance dose and reducing radiogenotoxicity, radiation-induced GI and radiocarcinogenesis.

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Declaration of Conflicting Interests

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