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Full Length Research Paper

Zingiber officinale extract modulates γ-rays-induced immunosuppression in mice

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Zingiber officinale **extract (ZOE), has been demonstrated to ameliorate the symptoms of radiation sickness and mortality induced by the gamma-irradiation in mice. In this study, we further investigated the immune-modulator potential of ZOE against the immunosuppression. Compared to mice irradiated alone, ZOE treatment significantly increased the spleen relative weight of the mice. Moreover, ZOE treatment enhanced the cellular immune response characterized by higher macrophage, splenocyte survival, splenocyte proliferation and higher ratio of CD4+ and CD8+. In addition, ZOE improved the humoral response represented by the higher antibody titers of IgG, IgA and IgM and up-regulated the secretion of cytokines such as IL-1β and IL-3. Furthermore, ZOE treatment significantly reduced the numbers of micronuclei (MN) in bone marrow polychromatic erythrocytes (PCEs). Collectively, these finding indicated that ZOE has the regulatory effect against γ-rays-induced immun-suppression by means of protecting DNA against the radiation.**

Key words: *Zingiber officinale* extract, γ-rays, immunosuppression, IL-1β, IL-3, micronuclei.

INTRODUCTION

The radiation can directly damage the structure and functions of the bioactive molecules in cells and tissues, eliciting disorder and apoptosis (Jensh, 1985). Besides, the radiation may cause bone marrow suppression and depletion of peripheral blood lymphocytes, and lead to severely inhibit the function of immune system such as making the exposed animals susceptible to opportunistic pathogens, more easily to be infected, and sometime to be lethal (Monje and Palmer, 2003; Dillman, 2006). Survival rate, after whole-body irradiation, depends on the critical number and immunocompetent function of haemopoietic stem cells (D'Amico et al., 1997; Wright, 2007). Recently, several immunostimulators such as GM-CSF, IL-2, IFN-γ and IL-6 have been reported to render radio protective effect on the mice, but, the deleterious effects of these immunomodulators, for example, headache, diarrhea, abdominal pain and so on , has severely restrained their use in clinics (Neta et al., 1992; Neta, 1997; Patchen, 1995; Singh and Yadav, 2005). Therefore, the development of the suitable, non-toxic immunostimulatory agents is an inevitable necessity to mitigate the radiation injuries, subsequent infections and haemorrhage.

Plants have been used to treat various ailments since the advent of human history, because the herbals have been usually considered to be safe and nontoxic compared to synthetic compounds. So, there are abundant studies about plant pharmacological properties (Yuan et al., 2002; Gubaev et al., 1996; Liu et al., 2009). The immunosuppression induced by radiation is an important factor for radiation mortality (Damian et al., 2008). Therefore, in the current context of the development of radioprotectors, the exploration of immunomodulatory effect of plant products has become a

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subject of active research at present (Arora et al., 2005).

Zingiber officinale roscoe is widely used as one of the important spices and traditional herbs in the world. Many reports have confirmed that the ginger or its extracts has some pharmacological activities, including anti-inflammation, antiemesis, analgesic effect, anti-tumor and anti-oxidation (Penna et al., 2003; Sharma and Gupta, 1998; Young et al., 2005; Habib et al., 2008; Stoilova, 2007). Liu et al have demonstrated that the *Zingiber officinale* extract (ZOE) increased the thymus index, spleen index, percentage of phagocytosis, and the titer of IgM in the mice with tumor (Liu and Zhu, 2002). Furthermore, ZOE has been shown to improve cellular immune response against the tumor. Recently, ZOE could ease the symptoms of radiation sickness and decreased mortality when mice were pretreated with ZOE (Jagetia et al., 2004).

In this study, we examined the immunomodulatory effect of ZOE as a radio proctor on the irradiated mice. Our results have demonstrated that ZOE could induce the higher cellular and humoral immune response in the γ-rays irradiated mice, and up-regulate the secretion of IL-1β and IL-3, through decreasing the number of MN in bone marrow PCEs.

MATERIAL AND METHODS

Reagents and animals

Bovine serum albumin, streptomycin, gentamycin, penicillin, trypan blue dye, and DMSO were purchased from Sigma (St. Louis, MO). Fluorescent conjugated anti-mouse monoclonal antibodies including anti-CD4-PE and anti-CD8-FITC were purchased from BD PharMingen (San Diego, CA, USA). Female Balb/c mice, 6 - 8 weeks old, 28 ± 2 g, were obtained from the Laboratory Animal Center of Sichuan University (Sichuan, China). The animals were housed under environmentally controlled conditions with pathogen-free food and water.

Administration of *Zingiber officinale* extract

ZOE (Xiaocao Botanical Development Co. Ltd., Xi'an, China) was dissolved in triple distilled water and each mouse received a dose of 400 mg/kg b.w. of ZOE orally once daily for 7 consecutive days before exposure to 5 Gy of γ-rays irradiation. Control mice only were given the normal triple distilled water. The animals were randomly divided into 4 groups: (n=15 each), (1) untreated control group, (2) ZOE treated, unirradiated group, (3) ZOE treated, irradiated group, (4) irradiated control group.

Irradiation

Each mouse, in a plastic container, was put in the ^{60}CoGamma irradiator (Model 220, Atomic Energy Commission, China), and irradiated with a dose rate of 0.6 Gy/min and exposed to 5 Gy of radiation for Dosimetry was carried out with Baldwin Farmer secondary dosimeter and Fricke dosimeter.

Relative spleen weight

After 48 h post-irradiation, the body weight of mice in all groups was determined, and then sacrificed by cervical dislocation. The thymus and spleen were excised from adhering tissues and weighed individually. The relative lymphoid organ weights were calculated according to the following equation:

Relative lymphoid organ weight= organ weight (mg)/body weight (g).

Detection of the survival rate of peritoneal macrophages and splenocytes

The mouse peritoneal macrophages were isolated as described previously (Edelson, 1976). Briefly, 4% Brewer's thioglycolate medium (Sigma, St. Louis, MO.) was injected into the peritoneal cavity of mice. After 72 h, the peritoneal cavity of the mice was flushed with RPMI 1640 medium(Hyclone, Logan, Utah, USA), and the peritoneal exuded cells were collected and centrifuged at 1500 rpm (10 min, 4^{O}C).The cell pallet was resuspended in compete RPMI 1640 medium plus 10% FCS. The cells were counted and adjusted to 1×10^6 cells/ml. The single splenocyte suspension was prepared as previously (Jin et al., 2004). The survival rate of macrophages and splenocyte was determined by trypan blue dye exclusion method (Yu et al., 2003).

T lymphocyte proliferation

Mice were sacrificed on day 2 after irradiation, and lymphocytes in the spleen were prepared as described previously (Du et al., 2007). Suspension of the lymphocytes was seeded in each well of 96-well microtiter plates at 4.0×10^5 cells/ml in the RPMI 1640 medium plus 10% FCS, cultured at 37°C in a 5% CO_2 incubator, and stimulated with 5 µg/ml of the concanavalin A (Sigma, St. Louis, MO) for 72 h. T cells proliferation was examined by MTT colorimetric assay. The OD values were read on an ELISA reader (BIO-TEK INSTRUMENT®, USA) at 570 nm. The data were expressed by the proliferation index (SI), and calculated as the mean reading of triplicate wells stimulated with concanavalin A, divided by the mean reading of triplicate wells stimulated with medium.

Detection of ratio of $CD4^+$ and $CD8^+$ T cell

The mice were sacrificed on day 2 after irradiation, and the single splenocytes suspension was prepared. CD4+ and CD8+ T cell populations in mice splenocytes were tested by FACS scan as described previously (Zhao et al., 2006). Briefly, cells were blocked with 1 µl of Fcy mAb (0.5 µg/ml) at 4°C for 30min, washed with PBS one time, stained with isotype controls, and double stained with anti-CD4-FITC and anti-CD3-PE, or with anti-CD3-FITC and anti-CD8-PE at 4°C for 30 min. The cells were measured by FACS Calibur and the ratio of CD4/CD8 was calculated by Cell Quest software.

Detection of IgG, IgA and IgM

Mice were bled on day 2 after irradiation, the level of IgG, IgGA and IgM isotypes were analyzed by ELISA. Briefly, the 96-well plate was coated with anti-mouse IgG, IgA and IgM (Xiaocao Biotech Co. Ltd., Xi'an, China) at 1:1000, and held at 4^{L} overnight. Each well was blocked with 3% of BSA-PBST at 37°C for 1 h, and incubated with the mouse serum at 1:100. A secondary goat anti-mouse antibody of IgG, IgA and IgM conjugated with horseradish peroxidase (Xiaocao Biotech Co. Ltd., Xi'an, China) at 1:1000 was added into each well and incubated at 37°C for 1 h.100 µg/ml of TMB (Sigma, St. Louis, IL) solution was added each well for the color develop-

ment. The reaction was stopped by 2 M of H_2SO_4, and the plate

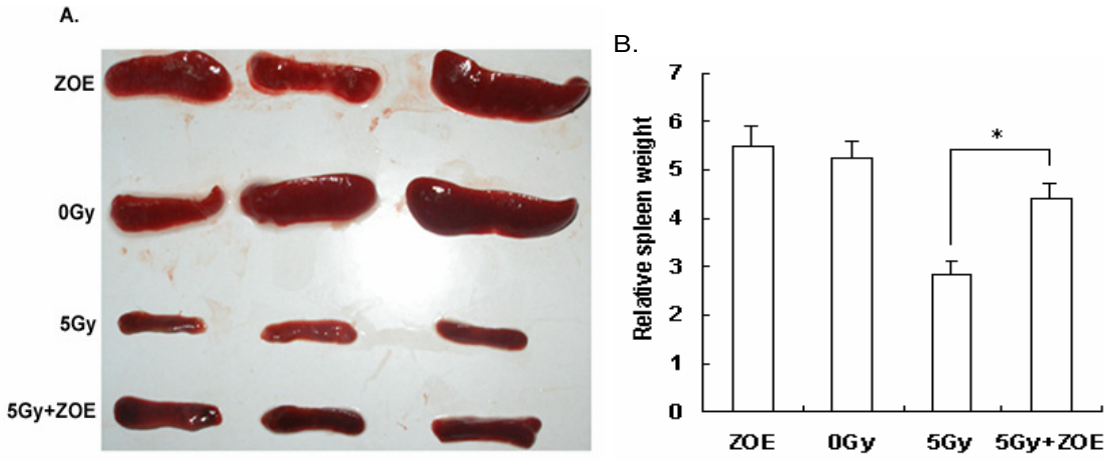

Figure 1. Analysis of the relative spleen weight. (A) The photograph of the spleen excised from the mice after γ-rays irradiation. (B) The weight of mouse and the spleen in the all groups was examined after radiation. The relative weight was expressed in the ratio of weight of spleen/mouse. * $p < 0.05$.

plate was read with a plate reader (Magellan, Tecan Austria GmbH) at 450 nm. Data are reported as optical density (OD) values.

Detection of the secretion of cytokine

Mice were bled on day 2 after irradiation, and the concentration of IL-1□ and IL-3 in the serum was determined by ELISA kit (Hendabeisheng Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. The concentration of cytokine wascalculated according to the standard curve plotted by IL-1beta or IL-3 standard sample from the kit, and the mean concentration was determined from at least three points of the linear portion of the titration curve.

Detection of bone marrow MN

The mice were sacrificed on day 2 after irradiation , and the bone marrow polychromatic erythrocytes (PCEs) suspension were prepared as described previously (Khan et al., 2003). The cells suspension from femur and tibia bones prepared in 5% BSA-PBS solution was centrifuged at 1000 r.p.m. and the cell pellet was resuspended. A drop of cells suspension was taken on a clean glass slides, and air-dried at room temperature. The slides were fixed in methanol for 5 min, and stained with Giemsa solution (Nanjing Jiancheng Biotech Co. Ltd., Nanjing, China) for 10 min. About 2000 PCEs per mouse were scored for the presence of MN.

Statistics

All experiments were repeated at least three times, and the results of representative experiments are presented. Data were analyzed using the one-sided Student's t test. Differences were considered statistically significant with $P < 0.05$.

RESULTS

Effect of *Zingiber officinale* extract on the relative spleen weight

To evaluate whether the ZOE influenced the immune organ, the spleen were weighed on day 2 after irradiation. As shown in Figure 1, compared to the irradiated group, the higher relative spleen weight was observed in the ZOE treated group. Particularly, the relative spleen weight of the ZOE treated and irradiated group was significantly higher than the irradiated group. This result suggested that ZOE could improve the protect effective of the immune organ against the irradiation.

Effect of *Zingiber officinale* extract on the survival rate of peritoneal macrophages and splenocytes

Macrophage and splenocyte are the very important immune cells in the immune system and play critical function on the immune response. To confirm the ability of ZOE in increasing number of the immune cells, the survival rate of the peritoneal macrophages and splenocyte was tested by the trypan blue dye method. As showed in the Figure 2A, the survival rate of macrophages in the ZOE treated plus irradiated group was significantly higher than the irradiated group. For splenocyte, the similar pattern was observed in the ZOE treated plus irradiated group (Figure 2B). The data indicated that ZOE significantly prevented the death of the immune cell after irradiation.

Lymphocytes proliferation response

To determine whether ZOE enhanced the cell-mediated immunity, the single splenocyte suspension was prepared from the mice after irradiation to perform the lymphocytes proliferation assay. As shown in the Figure 3, the level of proliferation response in the ZOE treated plus irradiated

mice were significantly higher than that in the irradiated mice. The results indicated that ZOE increased the function of lymphocyte proliferation response after irradiation.

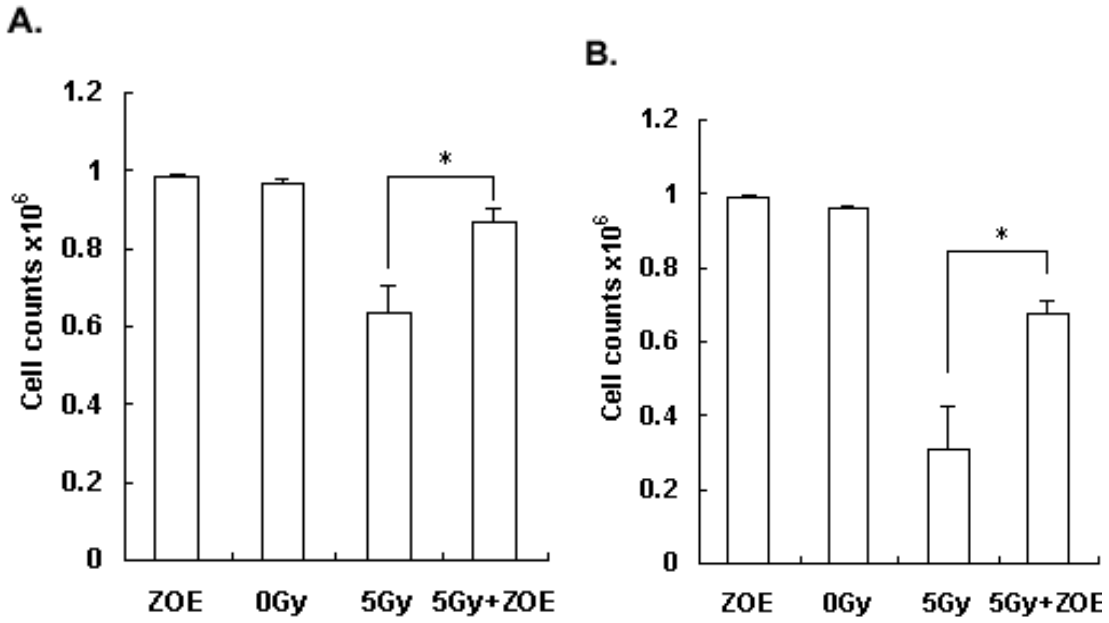

Figure 2. The survival rate of peritoneal macrophages and splenocytes. Peritoneal macrophages and splenocytes were isolated from mice at 48h after radiation. The survival rate of macrophages and splenocytes was tested by the trypan blue dye staining. The percentage of the survival cells was the mean of the three different repeats of experiments.(A) macrophages survival rate, (B) splenocytes survival rate * $p < 0.05$.

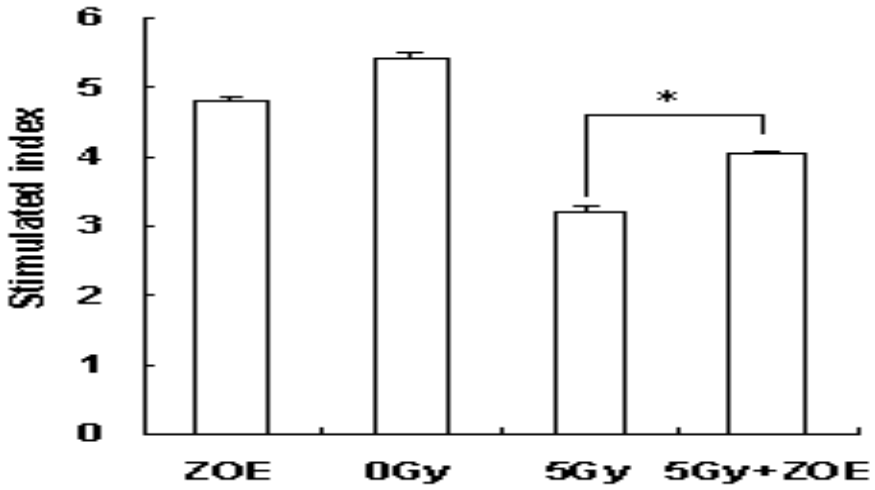

Figure 3. Analysis of the lymphocyte proliferation response. The splenocytes were isolated after irradiation, and stimulate with Con A for 72 h. The proliferation response was tested by MTT method, and expressed by the stimulated index. * $p < 0.05$.

Effect of *Zingiber officinale* extract on the ratio of $CD4^+$ and $CD8^+$ T cell

To further evaluate whether ZOE influenced the subset of T cells, the $CD4^+$ and $CD8^+$ T cells were analyzed by the FACS on day 2 after irradiation. As showed in the Figure 4A, B and C, the percentage of the $CD4^+$ subset T cell in the ZOE treated plus irradiated group was significant higher than that in the irradiated group. Moreover, the ratio of $CD4^+/CD8^+$ in the ZOE treated plus irradiated

group was significantly up-regulated compared to the irradiated group. Taken together, the data indicated ZOE increased the ratio of $CD4^+$ subset T cells in the T cell population after irradiation.

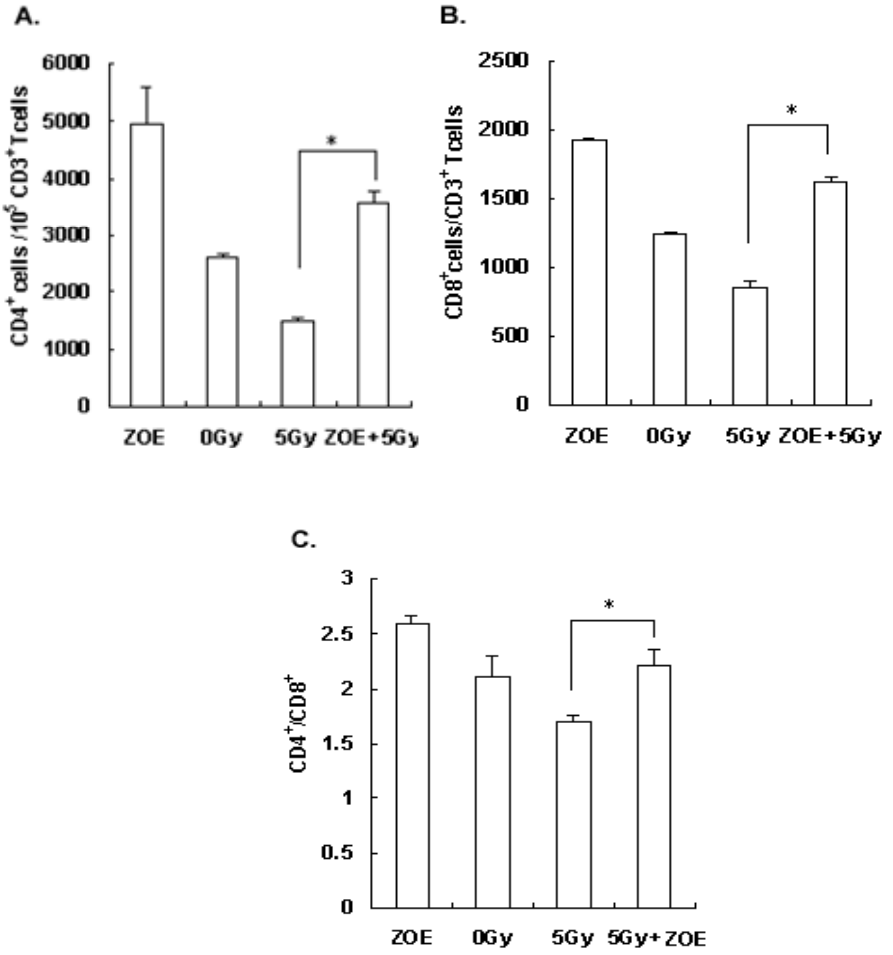

Figure 4. Analysis of the ratio of CD4+ and CD8+ subset T cell. T cells were isolated from spleen after irradiation, and double stained through anti-CD3 and anti-CD4, or by anti-CD3 and anti-CD8. CD4+ and CD8+ subpopulation were tested by FACS. (A),CD4+ in total T cells, (B) CD8+ in total T cells, (C) ratio of CD4+/CD8+.

Effect of *Zingiber officinale* extract on antibody isotype

To confirm if ZOE improved the humoral immune response, the Ab isotype, such as IgG, IgA and IgM, was tested by the ELISA on day 2 after irradiation. As showed in Figure 5A, B and C, the levels of IgG, IgA and IgM in the ZOE treated plus irradiated group were significantly higher than that in the irradiated group, besides, the levels of IgG isotype in ZOE treated mice were higher than that in the untreated group. The data indicated that ZOE enhanced the humoral immune response against the radiation.

Effect of *Zingiber officinale* extract on secretion of cytokines

To understand role of ZOE in secreting cytokines after irradiation, cytokines such as IL-1 and IL-3 were tested by ELISA kit. As shown in Figure 6A and B, the concentration of IL-1β and IL-3 in ZOE treated plus irradiated group were significantly higher than that in the irradiated mice, and levels of IL-1β and IL-3 in the ZOE treated group were also significantly higher than that in the untreated group. The results suggested ZOE up-regulated secretion of cytokines against the irradiation.

Effect of *Zingiber officinale* extract on number of bone marrow MN

To study further why ZOE broken the immunosuppression induced by irradiation, the bone marrow cells were

isolated after irradiation and MN was detected by Giemsa staining. As showed in Figure 7, the number of MN in the ZOE treated plus irradiated group was significantly decreased compare to the irradiated group, and MN of

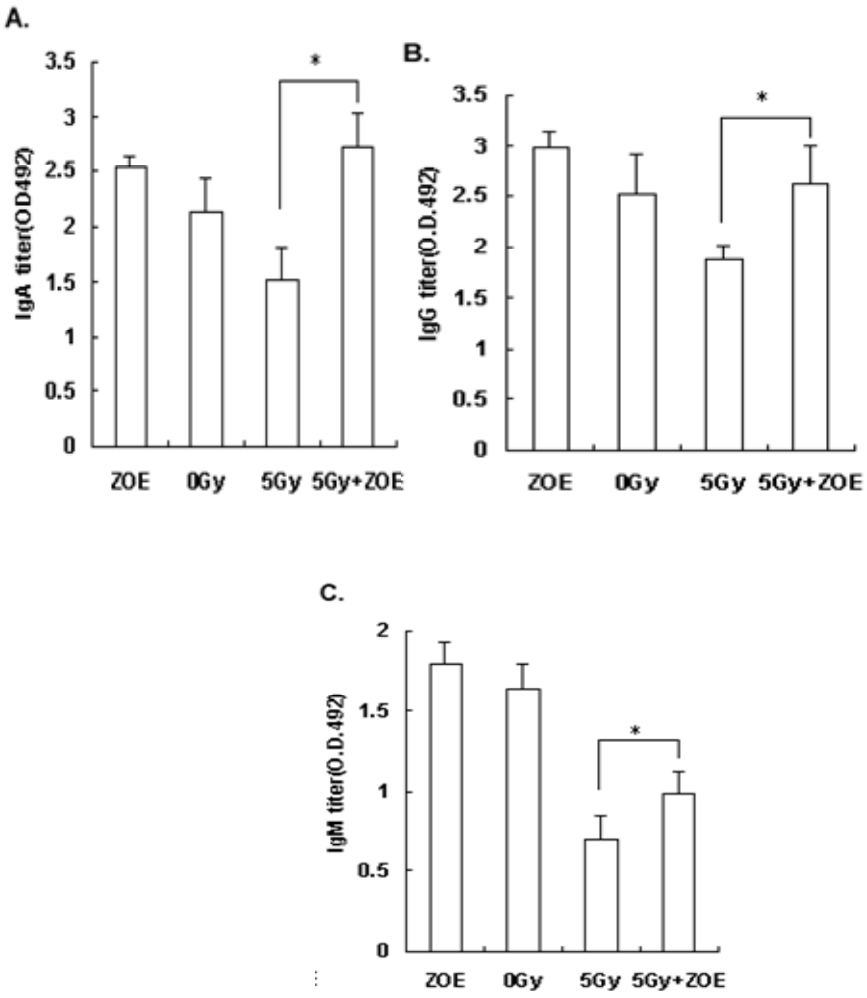

Figure 5. Analysis of the IgG's isotype by ELISA. The mice were bled after irradiation, and sera were isolated. The levels of IgG, IgA and IgM were tested by ELISA. (A) IgA, (B) IgG, (C) IgM. * $p < 0.05$.

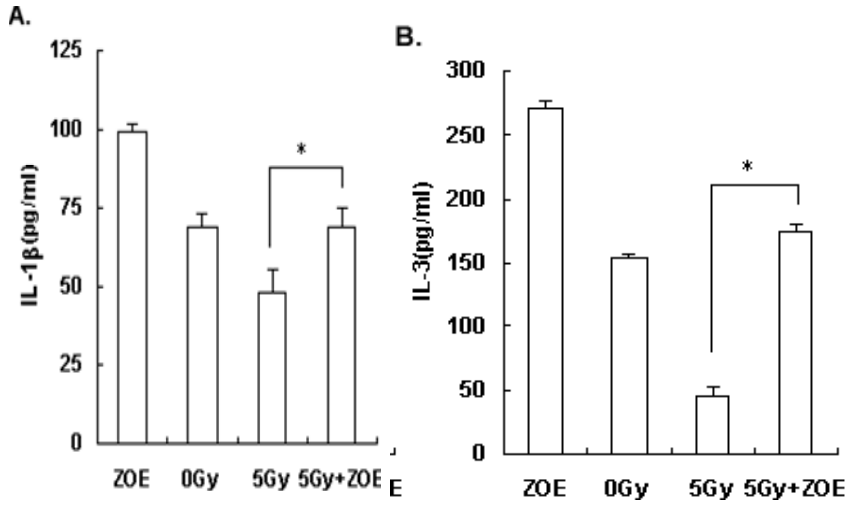

Figure 6. Analysis of Cytokines by ELISA. The mice were bled after irradiation, and

sera were isolated. The levels of IL-1β and IL-3 were tested by ELISA. (A)IL-1β , (B) IL-3 * $p < 0.05$.

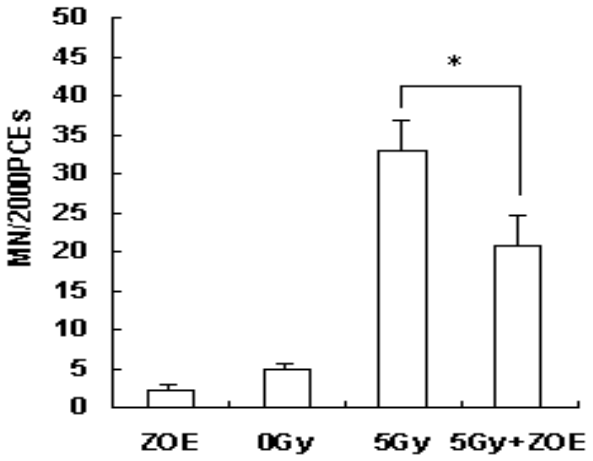

Figure 7. Analysis of the MN. The mice were sacrificed after irradiation, and bone marrow cells were isolated to test the number of MN by Giemsa staining. The number of MN in 200 bone marrow cells was expressed in the each group.* $p < 0.01$.

the ZOE treated group was decreased than the untreated group. The results suggested ZOE may regulate the immune response after irradiation by reducing DNA damage in the bone cells.

DISCUSSION

Recently, *Z. officinale* as a radioprotector has been investigated (Jagetia et al., 2004; Haksar et al., 2006). There are also several reports showing *Z. officinale* has the immun-modulator effect on tumor, autoimmune diseases, chronic inflammation, and so on (Chrubasik et al., 2005). However, few are known about that whether *Z. officinale* can maintain the normal immune function of immune system after radiation. In this study, we addressed this question by demonstrating that ZOE can elicit the cellular and humoral immune response in the γ-ray-irradiated mice. Administration of ZOE before the irradiation can increase the immune organ's weight (Figure 1). Compared to the irradiated mice, Administration of ZOE before the irradiation increase the survival rate of immune cells such as macrophages and splenocytes (Figures 2A and B), improved the lymphocyte proliferation response (Figure 3), and increased the ratio of T subset $CD4^+/CD8^+$ (Figures 4A, B and C). All those data suggest that ZOE can increase the celluar immune response. Moreover, this strategy can enhance the level of the humoral immune response because it increases the higher titer of antibody such as IgG, IgA and IgM (Figures 5A, B and C) compared to the irradiated mice. Besides, ZOE can up-regulate secretion of the cytokines such as IL-1β and IL-3 (Figures 6A and B). In addition, ZOE against the immunosuppression may be be relative to decreasing DNA damage (Figure 7) compared to the irradiated mice.

An important reason of radiation leading to the immune-suppression is that ionizing radiation can inhibit the function of the hematopoietic stem cells, which maybe differentiate into all kinds of immune cells such as lymphocytes, macrophages, dendritic cells, and so on (Norval, 2006). So, keeping normal cellular immune response is key part in recovery the function of immune system after irradiation. In the present study, we observed that ZOE increased the relative spleen weight, percentage of survival macrophages and splenocytes, lymphocyte proliferation response and the ratio of $CD4^+/CD8^+$ (Figures 1, 2, 3 and 4). We presumed that ZOE can prevent the hematopoietic stem cells from damaged by the γ-rays and protect the normal function against γ-rays. Thereby, equal number immune cells from mice without irradiation can home into the spleen maintaining normal spleen weight, and ZOE can keep the normal survival percentage of immune cells and ratio of T subsets.

Humoral immune response has an important role in fighting against the foreign pathogen (Myers, 1991). So, humoral immune can maintain the stability and balance of the immune system. Antibody, one of productions from the B lymphocyte, is an essential element of the humoral immune response (Ochi et al., 1976). Goel et al have demonstrated that Podophyllum hexandrum extract increased the levels of IgG isotype such as IgG1, IgG2b and IgM from day 1 to day 10 after γ-rays irradiation (Goel et al., 2007). In this study, we showed that ZOE improved the titer of IgA, IgG and IgM in the serum (Figure 5). So, we concluded that ZOE may help B lymphocyotes to escape from γ-rays irradiation, and increased the levels of IgG istotype.

Cytokine, secreting from the immune cells, have pivotal role in regulating of the immune response because they are responsible for the communication between immune cells (Singh and Yadav, 2005). IL-1βcan influence hematopoiesis by inducing the stem cells to secrete more hematopoietic factors such as G-CSF, M-CSF, GM-CSF, and IL-6 (Maisin, 1998). Previous studies had demonstrated that IL-1β is an important cytokine for radio-protection (Neta et al., 1994; Neta et al., 1986). IL-3 can regulate the radiation-induced the myelo-suppression by producing the platelets and neutrophils (Farese et al., 1993). In this study, ZOE increased levels of IL-1β and IL-3 in the sera after γ-rays irradiation (Figure 6), suggesting ZOE reduced the bone marrow cells injures induced by irradiation through enhancing secretion of cytokines for radio-protection.

It has been known that the ionizing radiation can directly cause DNA double-strand breaks and indirectly

(Bauerschmidt et al.). After radiation, the unrepaired or misrepaired DNA fragments form micronuclei (MN), and more MN can result in the cell death or chromosomal aberrations (Kaspler et al., 2009). In our study, ZOE

significantly decreased MN formation in the bone marrow cells (Figure 7). Therefore, we presumed that ZOE could prevent DNA damage of cells induced by γ-rays, which was an important reason that more T and B lymphocytes or other immune cells survived and had normal function. Thus, ZOE finally improved the celluar and humoral immune response after radiation.

In summary, the data presented here explicitly revealed that ZOE had amended γ-rays-induced immuno-supression by decreasing DNA damage. Moreover, the results we have presented suggested that the administration of ZOE is maybe an effective strategy to protect the immune system against the irradiation. However, the mechanism of ZOE decreasing DNA damage needs further investigation in the future.

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