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Indigowood root extract protects hematopoietic cells, reduces tissue damage and modulates inflammatory cytokines after total-body irradiation: Does Indirubin play a role in radioprotection?

Weir Chiang You\textsuperscript{a,b}, Wen Chuan Lin\textsuperscript{c}, Jia Tsz Huang\textsuperscript{e}, Chang Chi Hsieh\textsuperscript{d,f,*}

\textsuperscript{a} Department of Radiation Oncology, Lin Shin Hospital, Taichung, Taiwan
\textsuperscript{b} Institute of Biomedical Science, National Chung Hsing University, Taichung, Taiwan
\textsuperscript{c} Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan
\textsuperscript{d} Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan
\textsuperscript{e} Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan
\textsuperscript{f} Department of Animal Science and Biotechnology, Tunghai University, No. 181, Section 3, Taichung Harbor Road, Taichung 40704, Taiwan

Keywords:
Anti-inflammation
Hematopoiesis
Indigowood root
Isatis indigotica
Radioprotection
Indirubin

\textbf{Abstract}

Radix of \textit{Isatis indigotica} (indigowood root, IR) has been used in traditional medicine for its potential anti-inflammatory effect. The purpose of this study is to investigate the radioprotective effects of radiation caused damages in hematopoietic system and normal tissues in mice. A total of 57 BALB/c mice were randomized into six treatment groups: control, IR treatment (0.195, 0.585 and 1.170 g/kg, p.o. daily), L-glutamine (0.520 g/kg) and sham group. All mice except the sham group were irradiated and then administered for one week. The radioprotective effect on hematopoietic system, serum cytokines, and intestinal toxicity was studied. Protective effects on spleen and thymus are found in IR-treated groups. IR assisted in restoration of leukocytopenia after whole mice irradiation with significant reduction of serum TNF-\textgamma, IL-1\textbeta, and IL-6. These enhancements of hematopoietic effects are due to an increase in the serum G-CSF concentration in IR treated groups. In histopathological assessment, significant improvement of intestine toxicity is observed in high-dose IR and L-glutamine group. Evidences show that IR has potentials to be a radioprotector, especially in recovery of hematopoietic system, reduction of inflammatory cytokines and intestinal toxicity. Indirubin may play a crucial role, but the underlying mechanism is not very clear and warrants further studies.

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\textbf{Introduction}

Ionizing radiation is one of the most important modalities for the treatment of human malignancies. However, its acute and late toxicity on normal tissue often result in severe mobility during treatment course. This disability also limits the role of radiotherapy in cancer treatment. Radiation toxicity occurs either by direct or indirect damage to the DNA (\textcite{Schulte-Frohlinde 1986}). Indirect radiation reaction, which occurs mainly through oxidative free-radicals mechanism, contributes to a large extent to radiobiological effect (\textcite{Zaider et al. 1994}). Damages caused to DNA by ionizing radiation can result in the loss of viability of the cells exposed to radiation. The factors that influence the response of living cells to radiation are tissue type, DNA repair status of damaged cells, local circulation, and physiological status (\textcite{Pasupathy et al. 2001}). In Chinese medicine, radiation could cause so called “toxic heat”, which can increase inflammatory fever, cause blood damage, loss of intercellular liquid and energy.

Radioprotection is important and can prevent acute mobility and late symptoms caused by accident exposure to ionizing radiation in humans. In clinical situation cancer patients need radiotherapy, radioprotector may aid in reducing radiation toxicity of normal tissues without increasing tumor radioresistance. Many previous studies have reported the radioprotective effect of several natural substances which are mediated through free-radical scavenger pathway (\textcite{Gandhi et al. 2004; Arora et al. 2005}). However, until now no single medicine is reported to fulfill all the characteristics required of an ideal radioprotector.

Indigowood root (radix of \textit{Isatis indigotica} Forr) named “Ban-Lan-Gan” in Chinese is a medicinal plant belonging to the Cruciferae family. It is different from \textit{Isatis tinctoria} (European wood) which is used for production of the blue dye indigo (\textcite{Gilbert et al. 2004}). In Chinese medicine, IR is traditionally used to reduce...
inflammatory factors in blood, and to relieve convulsion. IR is one of the most popular herbal medicines and has been recorded in Chinese Pharmacopoeia since 1985. Purified extracts of IR have been formulated for clinical use. Many chemical compounds are found in IR, including indigotin, indirubin, isatin, isaindigotidione, organic acids, and amino acids (Wu et al. 1997). It is widely studied and reported to have anti-viral, fever detoxification, and anti-inflammatory efficacy. The purpose of this study is to evaluate the radioprotective effect of IR, to discuss the underlying mechanism, and then to explore the possibility of clinical applications.

Materials and methods

Experimental animals

A total 57 BALB/c strain mice of 5–6 weeks were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. The mice were housed in polycarbonate cages with ventilated micro isolator system (VMIS), at the Animal Center in China Medical University (Taichung, Taiwan), under pathogen-free and controlled conditions. A 12 hour light/dark cycle was set and the mice were provided with sterile Purina rodent chow and water. In order to minimize the effects of stress due to shipping, all mice were acclimatized for a minimum of 72 hours prior to irradiation. The protocol was approved by the Standing Committee on Animal Use of the China Medical University.

Preparation of the drugs and regimen dosing

IR extract powder was purchased from Sun-Ten Pharmaceutical Co. Ltd., Taiwan. The powder was composed of IR concentrated extract with an absorption ratio of 2:1 to IR powder. L-glutamine powder was purchased from Baxter Cooperation (Illinois, USA). IR and L-glutamine was dissolved in double-distilled water immediately before use and was administrated orally using curved needles.

Chromatographic conditions

Indirubin is the major pharmacological index content in IR. To determine the amount of indirubin in IR extract powder, high-performance liquid chromatography (HPLC) was used for quantitative determination of indirubin. In brief, each 8.0 g IR extract powder was dissolved in 100 ml of methanol to prepare the sample solutions. The combined methanol extract was evaporated under reduced pressure at 40°C for 4 hours, and the resulting residues were individually redissolved in 5 ml methanol. The analysis was performed on a Agilent 1100 HPLC system (Santa Clara, USA), consisting of a Quaternary pump, an Autosampler, a Thermostated column compartment and an diode array detector. The analytes were separated on a Merck Lichrocard Purospher STAR RP-18e column (250 mm × 4.6 mm, i.d., 5 μm, Merck KGaA, Germany) protected by a guard column (Merck Lichrocard Purospher STAR RP-18e column, 4.0 mm × 4.0 mm, i.d., 5 μm, Merck KGaA, Germany). The analysis was carried out at a flow rate of 0.8 ml/min with the detection wavelength set at 280 nm at temperature 35°C. The injection amount was 20 μl. The solvent gradient was composed of acetonitrile and 0.0015% triethylamine for HPLC. The gradient elution start at 30% acetonitrile/70% triethylamine for ten minutes, then progress to 45% acetonitrile/55% triethylamine in fifteen minvutes, 55% acetonitrile/45% triethylamine in fifty minutes and 100% acetonitrile/0% triethylamine in 55 minutes. The results were calculated by linear relationship and area under the curve (AUC) obtained from indirubin sample HPLC.

Experimental design and irradiation

A total of 57 BALB/c mice were randomized into six groups: sham (DD water 10 ml/kg/day), three IR groups with low (IR-L, 0.195 g/kg/day), moderate (IR-M, 0.585 g/kg/day) and high dose (IR-H, 1.170 g/kg/day), L-glutamine group (1.950 g/kg/day), and naive group. Each group was assigned the different test regimens. Treatment began immediately after irradiation for seven days before the mice were sacrificed. All mice except the naive group were restrained in polycarbonate cages and exposed to whole body irradiation with 1.8 Gy/day at a dose rate of approximately 300 cGy/min for three consecutive days before the treatment. High-energy electron beam 9 MeV was applied using Source to Surface Distance technique (SSD, 110 cm). A total of 5.4 Gy (Eleka Precision LINAC) was used in this study. The mice in the sham group were also placed in cages on the table but no radiation was delivered.

Hematopoietic system evaluation

After consecutive feeding with the testing regimens for seven days, all the 57 mice were sacrificed immediately. Spleen and thymus were dissected from mice with careful removal of connective tissue. The weight of the spleen and thymus was determined, washed separately and splenocytes were allowed to pass through a 200-mesh stainless filter to make a single cell suspension. The samples were given a hypotonic shock to lyse erythrocytes under gentle hypotonic conditions while preserving the leukocytes. Cell suspension was washed in cold buffer (Dulbecco’s modified phosphate-buffered saline with 0.1% of sodium azide) and then centrifuged for 5 minutes. Staining was achieved by incubation of 1 × 10⁶ leukocytes for 30 minutes at 4°C with fluorochrome-conjugated mAbs (CD45, CD3, CD19, CD14, ebioscence, San Diego, CA, USA) and washed again with wash buffer. After centrifugation, the supernatant was removed. Samples were run on the Becton Dickinson FACScan Flow Cytometry System (BD Bioscience, San Jose, CA, USA). The lymphocyte population was gated for analysis on the basis of forward scatter and side scatter. Splenocytes were also cytoospin to slide and was differentially counted by staining with Liu’s stain.

Estimation of serum cytokines

To study the effect of IR on production of serum cytokines in irradiated mice, a double-antibody sandwich enzyme linked immunosorbent assays (ELISA) was performed according to the manufacturer’s recommendations (ebioscence and R&D Systems). G-CSF kit was purchased from R&D Systems (DuoSet ELISA Development kit, Minneapolis, MN, USA) and TNF-α, IL-1β, IL-6 kits were purchased from ebioscence (ELISA Ready-SET-Go! San Diego, CA, USA). In summary, capture antibodies were added to 96-well plates and then incubated overnight at 4°C. Each sample well was washed three times, blocked for 60 minutes at room temperature, and washed again three times. Standards and samples at a dilution of 1/20 were then added to the wells. After 2 h incubation at room temperature, the wells were washed five times, and the detection antibodies were added and then washed seven times. Substrate solution (Tetra Methyl Benzidine) was added to each well and incubated in the dark for 30 minutes Stop solution was added to terminate the enzyme activity and the
absorbance was measured at 450 nm with reference to 570 nm by ELISA reader (Multiskan Spectrum, Thermo Electron Corporation, San Jose, CA, USA).

Assessment of intestinal toxicity

The specimens of jejunum from the sacrificed mice were embedded in Paraffin wax. 5 μm thick sections were bathed in Ehrlich’s haematoxylin for 5 minutes and rinsed for 5–10 minutes in running water. The sections were subsequently differentiated in 1% acid alcohol and then washed again for 5–10 minutes in running water. The slides were dipped in 1% eosin for 5 min and then placed under running water until the nuclei appeared blue. The sections were again dehydrated by the addition of serially concentrated alcohol (70%, 80%, 90%, and 100%). The H&E stained sections were examined by a pathologist. The number and height of crypt and villi of intestine was measured under a light microscope.

Statistical analysis

Parameters were documented at the end of the study and analyzed with SPSS software (Version 13, Illinois, USA) to determine the significances. We analyzed the variance to compare the weight of spleen and thymus, differential count of leukocytes, serum cytokines, number and height of intestinal villi by means of one-way analysis of variance (ANOVA). P-values less than 0.05 were considered to indicate statistical significance.

Results

Quantitative measurement of indirubin using HPLC

The linear relationship and AUC of indirubin standard solutions are calculated and is shown in Fig. 1. Indirubin is identified using HPLC (280 nm UV) at retention time of 31.560 minutes and purification factor of 998.594 (Fig. 2). The results from our research indicate that the amounts of the three analytes of IR extract powder are $4.2 \pm 0.1$ μg/g.

Radioprotective effect of IR in hematopoietic system

The weight of spleen and thymus shows a significant decrease in the irradiated sham group compared with the naive group ($p<0.001$). The result shows the impact of irradiation on the hematopoietic system in this animal model. Radioprotective effect with better conservation of spleen and thymus is observed in IR-treated and L-glutamine groups. Increased weight of thymus is found in IR-M (0.585 g/kg/day), IR-H (1.170 g/kg/day) ($p<0.01$). With regard to the weight of spleen, the same phenomenon is noted in the IR-M ($p<0.05$) and IR-H ($p<0.001$) groups. Furthermore, the weight of thymus and spleen in the IR groups demonstrates an increase in a dose-dependent manner (Table 1).

A significant decline in granulocyte, monocyte, and lymphocyte counts are observed in the sham compared with the naive group ($p<0.001$, Table 2). Immunosuppressant effect of irradiation is evident in this animal model. Significantly increased lymphocyte, monocyte and granulocyte counts are observed in the IR-M and IR-H groups when compared to the

![Fig. 1. The linear relationship and AUC of indirubin standard solutions.](image1)

![Fig. 2. The content of indirubin in IR extract is quantitatively measured using HPLC (280 nm UV). Note that the peak at retention time of 31.560 min identified indirubin (purification factor: 998.594).](image2)
irradiated controls. L-glutamine group also exhibited similar effects but the degree of effect is less compared with the IR groups. These enhancements of hematopoiesis effects are due to the increased serum G-CSF concentration in IR treated groups (p < 0.05, Fig. 3).

**Inflammatory cytokines assay**

The expression of serum cytokines, TNF-α, IL-1β, and IL-6 are quantitatively determined using ELISA assay. Significant elevation of serum TNF-α, IL-1β, and IL-6 is found in the sham compared with the naive groups (p < 0.001, Figs. 4–6). Activation of inflammatory cytokines after irradiation is obvious in this mice model. IR-treated and L-glutamine groups maintains significantly lower serum TNF-α levels (p < 0.01, Fig. 4). Meanwhile, significant reduction of serum IL-1β is observed in the IR-M and IR-H groups (p < 0.05, Fig. 5). Dose-dependent reduction of serum IL-6 is found in the IR groups and the reduction is significant in the IR-H group (p < 0.05, Fig. 6).

**Histopathological study of intestinal toxicity**

Significant reduction in the height and number of villi is observed in the radiation groups compared with the sham and the naive group by hematoxylin and eosin stain (Table 3 and Fig. 7). These results suggest that jejunal villi are destroyed by irradiation and this intestine toxicity model in mice is effective. IR-H group significantly shows an increase in villi number but not villi height when compared with the sham (p < 0.01). The same radioprotective effect is also found in the L-glutamine group (p < 0.01).

**Discussion**

The mechanisms of lethal damages caused by radiation, both in cancer cells and in normal tissue cells, are by apoptosis or clonogenic cell death (Ross 1999). The damages to DNA produced by radiation are single- or double-strand DNA breakage, DNA base damage, and inter/intra strand DNA linkage (Karran 2000). In normal tissues, radiation injury results from a sequence of simultaneous events that include activation of the immune system, inflammation process, regeneration of epithelium and endothelium, tissue fibrosis, and remodeling (Fajardo 1989). This phenomenon occurs as a result of complicated interactions of molecular signals, including cytokines, chemokines, and growth factors (Denham and Hauer-Jensen 2002).

The radioprotective effect of IR extract powder is studied in this whole body irradiated murine model. Acute exposure to elevated doses of ionizing radiation causes defects in hematopoiesis, resulting in leukocytopenia. In the present study, evidence of significant conservation in the weight of spleen and thymus, and hematopoietic system status shows the radioprotective effect of IR extract powder. The radioprotective ability of IR in the recovery of hematopoietic system is further confirmed by the increase in the spleen and thymus weights in IR-treated groups. In this study, mice exposed to whole body irradiation of 5.4 Gy in three consecutive days and then treated with IR are very effective in stimulating recovery of hematopoietic cells. The mechanisms by

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**Table 1**

Effect of IR on weight of spleen and thymus in mice.

| Group     | Body weight (g) | Thymus weight (mg) | Spleen weight (mg) |
|-----------|-----------------|--------------------|--------------------|
| Naive     | 23.0±1.1        | 49.8±7.6           | 113.1±19.4         |
| Irradiation |                 |                    |                    |
| Sham      | 22.1±0.8        | 25.6±32***         | 7.9±0.5***         |
| IR-L (0.195 g/kg/day) | 22.3±2.1 | 28.4±3.8           | 31.0±3.6           |
| IR-M (0.585 g/kg/day) | 20.4±0.5 | 30.1±3.0**         | 31.5±1.8**         |
| IR-H (1.170 g/kg/day) | 21.5±1.1 | 30.0±2.8**         | 317±3.0***         |
| L-glutamine | 21.8±1.2       | 28.5±2.0           | 30.4±1.6           |

Values are means ± S.D. ***p < 0.001, compared with the sham group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with sham group.

**Table 2**

Analysis of differential counts of leukocyte in spleen of mice.

| Group     | Cell numbers (1 × 10⁶) |
|-----------|------------------------|
|           | Granulocyte  | Monocyte  | Lymphocyte  |
| Naive     | 12.8±2.7     | 12.9±2.0   | 56.8±5.6    |
| Irradiation |            |          |            |
| Sham      | 3.4±0.5***   | 4.2±1.4*** | 13.0±3.6*** |
| IR-L (0.195 g/kg/day) | 5.6±0.9**  | 6.9±4.1*   | 27.8±1.6*** |
| IR-M (0.585 g/kg/day) | 7.5±3.3*** | 10.9±6.8*** | 36.3±10.8*** |
| IR-H (1.170 g/kg/day) | 7.4±1.7***  | 10.8±3.5*** | 39.8±6.9*** |
| L-glutamine | 2.9±0.6     | 2.7±0.8    | 16.5±1.6    |

Values are means ± S.D. ***p < 0.001, compared with the naive group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with sham group.

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Fig. 3. Significant increase in serum G-CSF concentration in IR treated groups (p < 0.05).
which IR stimulate the regeneration of hematopoietic cells might be due to the increase in G-CSF expression. Suzuki et al. reports that the possible mechanism may be attributed to the presence of the therapeutic content, namely, indirubin in IR (Suzuki et al. 2005). Our results show that the indirubin concentration in IR powder is $4.2 \pm 0.1 \text{ mg/ml}$, which is higher ($0.224–2.384 \text{ mg/g}$) than Liau et al. detected in botanical indigowood root (Liau et al. 2007).

During the outbreak of SARS (Severe Acute Respiratory Syndrome) in 2003, IR was widely applied in the treatment of SARS in China and was reported to have anti-SARS effect (Lin et al. 2005). The major cause of mortality in SARS patients are interstitial pneumonia, characterized with a condition of diffuse, chronic inflammation of the lungs beyond the terminal bronchioles (Fowler et al., 2003). Radiation pneumonia occur with similar clinical pictures—acute inflammatory stage and a late fibrotic change (Tsoutsou and Koukourakis 2006). Many chemical compounds in IR are reported in previous studies, including indigotin, indirubin, isatin, isaindigotidione, organic acids, and amino acids (Wu et al. 1997). Recently, indigotin and indirubin

**Table 3**

| Group              | Villi number/mm | Villi height (mm) |
|--------------------|-----------------|-------------------|
| Naive              | 12.67 ± 0.52    | 0.49 ± 0.09       |
| Irradiation        |                 |                   |
| Sham               | 9.17 ± 0.98***  | 0.32 ± 0.07**     |
| IR-L (0.195 g/kg/day) | 10.17 ± 0.75   | 0.36 ± 0.08       |
| IR-M (0.585 g/kg/day) | 10.00 ± 0.89   | 0.32 ± 0.08       |
| IR-H (1.170 g/kg/day) | 10.83 ± 0.75**  | 0.39 ± 0.07       |
| L-glutamine        | 11.00 ± 1.26**  | 0.35 ± 0.09       |

Values are means ± S.D. ***P<0.001, **P<0.01 compared with the naive group, *P<0.01 compared with sham group.
have been found as potent aryl hydrocarbon receptor (AhR) agonists (Peter Guengerich et al. 2004). Indirubin and alkaloid isaindigotone are part of water-insoluble components in IR. The methanol extract of IR is proven to have antinociceptive, anti-inflammatory, and antipyretic effects in animal study (Ho and Chang 2002). Alkaloid isaindigotone and its derivatives are found to have inhibitive function on leukocytes and act as a scavenger of superoxide either through the hypoxanthine/xanthine oxidase system or through stimulated human neutrophils (Molina et al. 2001). Indirubin is reported to be one of the most effective compounds in IR, which have anti-inflammatory and cyclin-dependent kinases (CDK) inhibitory reaction (Hoessel et al. 1999). Indirubin is also found to have inhibitory action on the expression of the chemokine regulated on activation, normal T-cell expressed and secreted (RANTES) mRNA expression in influenza-infected bronchial epithelial cells (Mak et al. 2004).

Cytokines including TNF-α, IL-1β, and IL-6 play a significant role in the development of radiation related toxicity (Müller and Meineke 2007). Indirubin is found to induce TNF-α which may exacerbate the cytotoxic effects of radiation. Interestingly, TNF-α is also found to enhance X-ray killing of human tumor cells in vitro and enhances tumor control when combined with radiotherapy in animal tumor models (Azria et al. 2004). Sakai et al used IL-6 knockout mice to study the cytokines related to radiation pneumonia (Sakai et al. 2008). They found that stable expression of inflammation (CD44) and apoptosis (Bak) markers are observed in IL-6 knockout mice after thoracic irradiation when compared with control group. Moreover, IL-6 is reported to be a biomarker to predict radiation lung toxicity (Kong et al. 2008). In our study, significantly lower serum TNF-α, IL-1β, and IL-6 levels are observed in the IR-treated groups when compared with the control group. These results strengthen the important role of IL-6 in radiation-induced inflammation and imply the potential use of IR in radioprotection.

The intestine is one of the highly radiosensitive and dose-limiting organs. Radiation enteropathy results from the death of rapidly proliferating crypt cells and the disruption of the epithelial barrier and mucosal inflammation. The pathogenesis of radiation enteropathy involves alterations of intestinal wall compartments and changes in the absorption function (Paris et al. 2001). Endothelial dysfunction and release of TNF-α and TNF-β occur during the process of radiation injury (Rubin et al. 1991; Zhou et al. 1992). In our study, mice administered with IR-H significantly maintained number of villi. It has been shown that IR treatment reduced the level of serum TNF-α, thereby preventing endothelial dysfunction and radiation-induced enterotoxicity. The above radioprotection to the intestinal epithelium may play a role in preventing defects in absorption.

Glutamine has been shown to be a regulator of glutathione (GSH), a ubiquitous antioxidant (Klimberg et al. 1990). GSH is an antagonist to prostaglandin E2 (PGE2) production, which is a strong inflammatory mediator (Coeffier et al. 2002). The depilating effect of radiation on epithelial cells also triggers the depletion of GSH (Saunders et al. 1991). Increased GSH level is the first line of defense in radiation cytotoxicity, which detoxifies and eliminates free radicals and electrophilic compounds from the cell (Soubra et al. 1990). In our study, treatment with L-glutamine is proved to significantly increase the number of irradiated mice villi. The mechanism by which glutamine decrease radiation induced enteropathy in mice may be partly by altering the inflammatory response.

Indirubin is reported to be a potent Glycogen synthase kinase-3β (GSK3β) inhibitor, which promotes inflammatory responses in peripheral immune cells (Leclerc et al. 2001). Yuskaits and Jope found that inhibitors of GSK3 attenuated microglia responses to inflammatory stimuli and reduced inflammatory cytokines production (Yuskaits and Jope 2009). Furthermore, Takadera et al. reported that glycogen synthase kinase-3 inhibitors prevent caspase-dependent apoptosis induced by ethanol in cultured rat cortical neurons (Takadera and Ohyashiki 2004). Indirubin is also used to inhibit inflammatory reactions in delayed-type hypersensitivity in cell cultures in mice (Kunikata et al. 2000). In this study,
the amount of interferon-γ in the culture supernatants of elicited mouse lymphocytes is inhibited by indirubin treatment. There is a significant drop of interferon-γ if the concentration of indirubin is higher than 5 ng/ml. In our study, the concentration of indirubin in IR is 4.2 ± 0.1 μg/g, which is quantitatively determined by HPLC. These results suggest that indirubin may play a great role in anti-inflammatory effects of IR.

IR is used for thousands of years in China and is recognized by ancient Chinese to have anti-inflammatory effect. In our animal model, evidence shows that IR can reduce tissue injury caused by radiation, demonstrates radioprotective effect in hematopoietic system recovery, modulation of serum inflammatory cytokines, and improvement of severe enteropathy in irradiated mice. Indirubin may play a therapeutic role, but the underlying mechanism is not very clear and warrants further studies.

Acknowledgments

This study is supported by grants from Committee on Chinese Medicine and Pharmacy, Department of Health, Executive Yuan, Taiwan, R.O.C. CCMP93-RO-07. We thank Dr. Juin-Wang Liao D.V.M in National Chung Hsing University for pathological scoring.

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