Journal of Food and Drug Analysis, Vol. 20, No. 2, 2012, Pages 516-523 doi:10.6227/jfda.2012200213

Modulation of Proinflammatory Cytokines by Red Mold Dioscorea Ethanol Extract in Radioactive Cobalt-60 Exposure

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(Received: June 24, 2011; Accepted: November 7, 2011)

ABSTRACT

Monascus-fermented products offer valuable therapeutic benefits and have been extensively used in East Asia. Secondary polyketide metabolites of *Monascus*, including monacolin K (MK), ankaflavin (AK) and monascin (MS), have been reported to have antioxidation, antiinflammation and tumor metastasis suppression effects. This study investigated the attenuation of radiation-induced proinflammatory cytokines overexpression by ethanol extract of red mold dioscorea (RMDE). We used the B16-F0 melanoma cell line to establish an animal radiotherapy model and serum proinflammatory cytokines (i.e. TNF-a, IL-1b and IL-6) were analyzed. RAW 264.7 cells were pre-treated with different pure compounds [AK, MS and citrinin (CT)] of RMDE and irradiation of 2 Gy cobalt-60 g-radiation. The animal experiment results showed that oral administration of RMDE increased feed intake in C57BL/6 mice. Serum proinflammatory cytokine levels (i.e. TNF-α, IL-1β and IL-6) and profibrotic cytokine (TGF-b) were significantly reduced in the RMDE group compared to control group ($p < 0.01$). For *in vitro* cell model, we evaluated the antiinflammative effects of AK, MS and CT in irradiated RAW 264.7 cells. Both AK and MS significantly reduced radiation-induced overexpression of proinflammatory cytokines and profibrotic cytokines. We have shown for the first time that *Monascus*-fermented products can attenuate ionizing radiation-induced inflammatory response. AK and MS are the main components of RMDE responsible for this effect.

Key words: *Monascus*, ankaflavin, monascin, citrinin, proinflammatory cytokine, profibrotic cytokine

INTRODUCTION

Radiotherapy (RT) is an important cancer treatment modality. Aside from cell-killing effect in tumor tissue, it also provokes normal tissue damage. Pro-inflammatory response as well as induction of cell death can occur as acute or subchronic side effects of radiation-based cancer therapy and impact the life quality of the patients.

Ionizing radiation breaks water molecules and produce reactive oxygen species (ROS) to increase intracellular oxidative damage[(1)]. ROS attack DNA molecules, causing double strand breaks. They can also attack and damage membranous protein and phospholipids, leading to cellular damage. Some study indicated that ionizing radiation has been shown to exaggerate inflammatory responses and to enhance the release of inflammatory mediators in experimental animal and human[(2,3)]. In addition, exposure to ionizing radiation rapidly induces expression of cytokines such as tumor necrosis factor-a (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) release. TNF-α is a member of systemic inflammation group that stimulate the acute phase reaction[(4)]. IL-1β is secreted mainly by macrophages. IL-1β is produced in response to various stimulants, such as bacteria, and cytokines[(5)]. IL-6 is a multifunctional protein. In innate immunity, it stimulates the synthesis of acute-phase proteins by hepatocytes and thus contributes to the systemic effects of inflammation[(6)]. Recent research indicates that inflammation promotes pathogenesis of cancer[(7)], of which there are three key mechanisms: 1) ROS molecules and free radicals lead to DNA damage and mutation[(8)]; 2) tumor cells can mutate to respond to proinflammatory factors[(9)]; 3) proinflammatory factors have a high degree of correlation to a tumor cell's ability to induce angiogenesis and metastasis[(10)]. On the other hand, radiation-induced overexpression of proinflammatory cytokines IL-1β and IL-6 can lead to activation of transforming growth factor-b (TGF-β). Once activated, TGF-β will suppress Smad pathway that lead to uncontrolled fibroblast proliferation, precipitation of extracellular matrix (ECM) and collagen, ultimately leading to fibrosis[(11,12)]. Consequently, minimizing the inflammatory side effects of

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radiotherapy has a great implication for treatment efficacy.

The *Monascus* species have been used as a traditional food fungus in Eastern Asia for several centuries. The secondary metabolites of *Monascus* in the contemporary research confirmed the suppression of cholesterol production[13], antiinflammation[14], anti-tumor[15] and tumor metastasis suppression[16]. *Monascus*-fermented dioscorea, known as red mold dioscorea (RMD), comprises a dioscorea root substance as well as several *Monascus*-fermented metabolites. Our previous study found that RMD had hight yellow pigment, monascin (MS) and ankaflavin (AK) production, greater hypolipidemic, antiatherosclerotic and antihypertensive effects than tranditional red mold rice (RMR) and unfermented dioscorea[17,18]. In addition, the dioscorea root is regarded as a functional food or a worthful herb because of the inclusion of many functional ingredients for the prevention of various diseases[19]. Polysaccharides, flavones, dioscorin, polyphenols, vitamin C, and sporamin of dioscorea are proven to exhibit great antioxidative ability[20], which should be of great benefit to blockade oxidation of nitric oxide[21].

In the current study, the ability of ethanol extract of red mold dioscorea (RMDE) to ameliorate radiation-induced inflammatory response in C57BL/6 mice and RAW 264.7 cells were investigated. In particular, we focused on their effects upon feed intake, proinflammatory cytokines (i.e. TNF-α, IL-1β and IL-6) and profibrotic TGF-β expression level after radiotherapy.

MATERIALS AND METHODS

I. *Chemical and Reagents*

Lovastatin (LOV) was obtained from Standard Chem. & Pharm. Co. Ltd (Tainan, Taiwan). Fetal bovine serum (FBS) was purchased from Life Technologies (Auckland, New Zealand). Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from HyClone Laboratories (Logan, UT, USA). Ethanol (95%) was purchased from Taiwan Tobacco and Liquor Corp. (Taipei, Taiwan). The TNF-α, IL-1β, IL-6 ELISA kit was purchased from PeproTech Inc. (London, UK) and the TGF-β ELISA kit was purchased from R&D Systems Inc. (Minneapolis, MN, USA). The Trizol reagent and Super-Script™ III First-Strand Synthesis System for reverse transcription polymerase chain reaction (RT-PCR) kit were purchased from Invitrogen Corp. (New York, NY, USA).

II. *Preparation of Ethanol Extracts of Red Mold Dioscorea (RMDE)*

The dioscorea root (*Dioscorea batatas* Dence) was purchased from a local supermarket in Taiwan to be used for RMD production under solid-state cultivation. *Monascus purpureus* NTU 568 was used to prepare RMD via the method described in a previous study[18]. After fermentation, the crushed and dried RMD was further extracted by ethanol at 37°C for 1 day. The extracts were concentrated by a vacuum filtration device and dried by lyophilization. The methods used for analyzing the key components in RMDE have been previously described[22]. The standards of MS and CT were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and the purities were 98% and 100% respectively. The AK sample was kindly provided by Dr. Yao-Haur Kuo in National Research Institute of Chinese Medicine. The purity of AK was determined to be 95% by Dr. Kuo.

III. *Cell Culture*

Murine B16-F0 melanoma cell line and RAW 264.7 mouse macrophage cell line were obtained from the Bioresource Collection and Research Center (BCRC) in Taiwan and maintained in DMEM media supplemented with 10% FBS, 1% antibiotic and antimitotic solution, and 0.2% $NaHCO_3$. Cells were cultured in a 10 cm^2 dish and incubated in 5% CO_2, 95% humidified air at 37°C.

IV. *Animal Experiment*

Six-week-old, male C57BL/6 mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were randomly divided into six groups (n = 5) as follows: (a) normal control; (b) B16-bearing control; (c) 1× LOV (25 mg/kg/day); (d) 2× LOV (50 mg/kg/day); (e) 1× RMDE (100 mg/kg/day); and (f) 2× RMDE (200 mg/kg/day). LOV is an enzyme blocker (HMG-CoA reductase inhibitor), also known as a "statin". Statin is reported to promote the killing effects of tumor-therapeutic drugs and radiation on tumor cells[23]. In this study, LOV was used as a positive control and the dosage is consistent with previous reports[24]. All mice were pre-fed for one week. The normal control group was left untreated while other groups were injected with 1×10^6 B16-F0 melanoma cells into the right hind limb on the 8th day. Groups (b) through to (f) were then given treatment with 10 Gy Co-60 g-radiation /day (4700 Ci Picker C-9, Picker Co., Lynbrook, NY, USA) for five consecutive days, giving a total dose of 50 Gy. After radiotherapy (day 0), the mice were treated with oral gavage for a period of 28 days. Animals were sacrificed by carbon dioxide inhalation at the indicated time. Following this, serum was collected for analysis. These experiments were approved by the Council of Agriculture, Executive Yuan, R.O.C., as well as the Animal Center Committee of the College of Medicine of National Taiwan University in Taiwan.

V. *Quantification of TNF-a, IL-1β, IL-6 and TGF-β Protein by ELISA*

Serum levels of TNF-α, IL-1β and IL-6 were determined using the PeproTech Murine TNF-α, IL-1β and IL-6 ELISA Development Kit (PeproTech Inc.). Serum levels of TGF-β1 were determined using the DuoSet® ELISA Development System mouse TGF-β1 Kit (R&D Systems Inc.).

VI. *Cell Inflammation Model and RT-PCR*

RAW 264.7 cells were pretreated with 0, 2, 4 and 8 mg/mL pure forms of the components (AK, MS, and citrinin; CT) of RMDE for 30 min; then, the cells were irradiated with 2 Gy g-radiation. Total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and analyzed by polymerase chain reaction (PCR). All PCR products of an expected size were run on agarose gel and identical levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were clearly detected in all samples. For reverse transcription-PCR analysis, cDNA was synthesized from 1 μg of total RNA using reverse transcriptase and oligo-dT primers in a volume of 20 μL. PCR was performed with a cDNA reaction mixture using Super-Script™ III First-Strand Synthesis System for RT-PCR kit (Invitrogen Corp.) and appropriate primers in a volume of 50 μL. Primers were designed using the NCBI Primer-BLAST. The specific primers used in this study are listed in Table 1. Amplification was performed in an automated thermal cycler at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 min for 35 cycles, followed by an additional 10-min extension period at 72°C. To account for variations in the amount of RT-RNA between samples, all data were normalized to GAPDH, which was measured by the same technique. For identification of PCR products, aliquots from each PCR were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining. The gels were analyzed by laser scanning densitometry using a Molecular Dynamics Densitometer and Image Quant Software (Sunnyvale, CA, USA).

Table 1. The primers used in this study

	Primer	Size (bps)
GAPDH	f: TGTGCAGTGCCAGCCTCGTC r: CGGCCTTGACTGTGCCGTTGA	212
TNF-α	f: ACTCCAGGCGGTGCCTATGTC r: CCTCCACTTGGTGGTTTGCTACGA	231
IL-1β	f: GCCAAGCTTCCTTGTGCAAGTGTC r: GTAGCTGCCACAGCTTCTCCACA	264
IL-6	f: TCTGCAAGAGACTTCCATCCAGTTGC r: GACAGGTCTGTTGGGAGTGGTATCCT	242

Table 2. The concentration of monacolin K, ankaflavin, monascin and citrinin in ethanol extracts of red mold dioscorea

Compound	Concentration (mg/g RMDE)
Monacolin K (MK)	N/D*
Ankaflavin (AK)	3.1 ± 1.0
Monascin (MS)	9.8 ± 0.9
Citrinin (CT)	0.06 ± 0.00

* N/D = not detected, n = 3.

VII. *Statistical Analysis*

Data were expressed as mean ± standard deviation. Statistical comparison was performed using one-way ANOVA with Duncan's test. Statistical significance was determined at $p < 0.05$.

RESULTS

I. *Composition Analysis of RMDE*

In this experiment, we extracted the RMD by ethanol. There are many secondary metabolites in the RMDE. Several yellow pigments such as MS and AK obtained from *Monascus* species have been reported to have antiinflammatory effects and possess cytotoxic activity against cancer cells[23,25]. RMDE contained 9.8 ± 0.9 mg/g of MS, 3.1 ± 1.0 mg/g of AK, and 0.06 ± 0.00 mg/g of CT (Table 2). Monacolin K was not detected.

II. *Effects of RMDE on Feed Intake after Radiotherapy in B16-Bearing Mice*

During radiotherapy, patients often experience pain and discomfort due to inflammation, which adversely affects their appetite and impedes food digestion and absorption; this in turn causes malnutrition, muscle and fat tissue wasting, and ultimately cachexia. Therefore, in the first set of experiments, we investigated the effect of RMDE supplementation on post-radiotherapy feed intake of B16-bearing mice. The feed intake levels of the 1st week of each experimental group remained comparable with those of the control group, indicating that appetite was not affected during or immediately after radiotherapy (Table 3). However, from the 2nd week, the differences in feed intake between the experimental groups and the control group became more obvious. In particular, the average intake of the B16-bearing control group on the 2nd week, which was 10.0 g, decreased to 8.4 g by 4th week (Table 3). The average intake of the 2× LOV group was 8.8 g on the 3rd week, and decreased to 7.1 g by the 4th week; anorexia is a known side effect of LOV. In contrast, the average intake of the 1× LOV, 1× RMDE, and 2× RMDE groups compared to B16-bearing control group were increased by 2.4 g, 2.9 g, and 2.1 g, respectively, on the 4th week. These findings suggested that RMDE as a feed supplement increases the post-radiotherapy feed intake in mouse models.

III. *RMDE Decreases Serum Proinflammatory Cytokine Levels in C57BL/6 Mice*

In order to investigate whether RMDE can reduce radiotherapy-induced proinflammatory reaction, proinflammatory

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Table 3. The body weight and feed intake after radiotherapy

	Normal control	B16-bearing control	1× LOV	2× LOV	1× RMDE	2× RMDE
Initial BW (g)	22.1 ± 2.1	23.5 ± 1.6	21.1 ± 1.4	20.8 ± 1.8	22.6 ± 0.9	22.1 ± 1.3
Final BW (g)	26.3 ± 1.2	29.1 ± 1.9	30.3 ± 2.5	24.4 ± 3.0	26.0 ± 2.2	27.0 ± 1.6
After IR						
Feed intake (g/mouse/1st week)	5.3	5.5	5.5	5.4	6.1	6.0
Feed intake (g/mouse/2nd week)	9.2	10.0	8.8	8.3	10.0	9.3
Feed intake (g/mouse/3rd week)	9.4	9.3	9.7	8.8	11.2	10.4
Feed intake (g/mouse/4th week)	9.5	8.4	10.8	7.1	11.3	10.5

1X LOV: 25 mg/kg/day; 2X LOV: 50 mg/kg/day; 1X RMDE: 100 mg/kg/day; 2X RMDE: 200 mg/kg/day

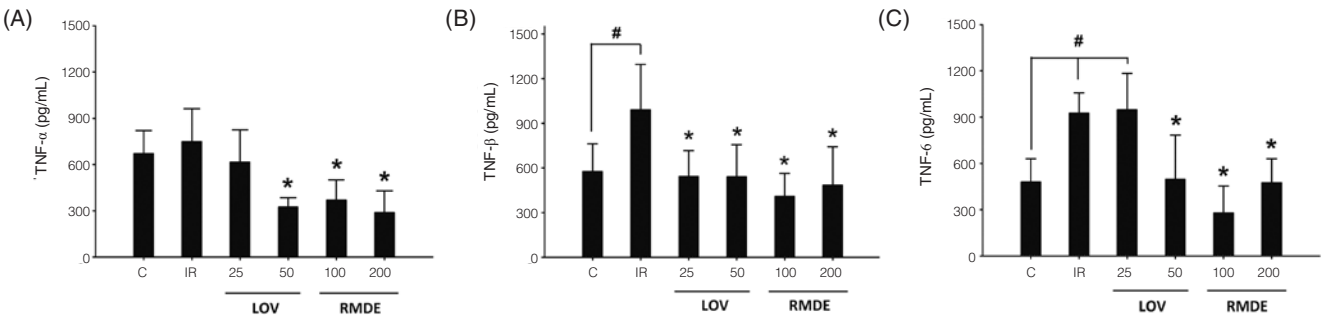

Figure 1. Serum proinflammatory cytokines levels in C57BL/6 mice after radiotherapy. A: TNF-α; B: IL-1β; C: IL-6. Results are expressed as the mean ± SD, $^{\#}p < 0.01$ vs. control, $^{*}p < 0.01$ vs. irradiation only (IR), n = 5.

cytokine (TNF-α, IL-1β, and IL-6) levels in mouse sera were analyzed after sacrifice. The results indicated that the serum TNF-α level of post-radiotherapy in the B16-bearing only group did not differ from the normal control group. However, serum TNF-α level in the B16-bearing only control group compared to the post-radiotherapy, these levels in 2× LOV, 1× RMDE, and 2× RMDE groups decreased by 57%, 51%, and 61%, respectively ($p < 0.01$) (Figure 1A). An increase of 42% was noted in the post-radiotherapy serum IL-1β level of the B16-bearing control group as compared to that of the normal control groups ($p < 0.01$), indicating that serum IL-1β levels increase after radiotherapy. However, the following statistically significant ($p < 0.01$) decreases in the post-radiotherapy IL-1β levels were noted in the LOV- and RMDE-fed groups: 45% in the 1× LOV group and 2× LOV group, 59% in the 1× RMDE group, and 51% in the 2× RMDE group (Figure 1B). The serum IL-6 level increased by 48% in the B-16 bearing control group as compared to that of the normal control group ($p < 0.01$). Although no significant differences in IL-6 levels were detected in the 1× LOV and B16-bearing control groups, decreases of 46%, 70%, and 49% were noted in these levels in the 2× LOV, 1× RMDE, and 2× RMDE groups, respectively ($p < 0.01$) (Figure 1C). Although radiotherapy may cause an increase in the IL-1β and IL-6 levels, this increase does not seem to directly affect the TNF-α level. RMDE incorporated as a feed supplement can, however, effectively decrease the serum levels of proinflammatory cytokines, thereby reducing the degree of inflammation.

IV. *Effects of RMDE on Profibrotic Cytokine TGF-β1 Expression*

Some studies have shown that increased levels of IL-1β and IL-6 due to radiotherapy can induce post-treatment fibrosis[11, 12]. Mice sera were analyzed to determine whether administration of RMDE decreases serum levels of TGF-β1. Results showed that B16-bearing control showed a 29% increase in TGF-β1 compared to normal control ($p < 0.05$). The 1× LOV, 2× LOV, 1× RMDE and 2× RMDE groups showed a respective decline in TGF-β1 of 23%, 27%, 33% and 26%, respectively (Figure 2). In particular, the 2× LOV, 1× RMDE, and 2× RMDE groups showed a statistically significant decline compared with B16-bearing controls ($p < 0.05$) (Figure 2). This indicates that RMDE can effectively reduce the expression of TGF-β1 levels.

V. *Effects of AK, MS, and CT on the mRNA Expression Levels of Proinflammatory Cytokines RAW 264.7 Cells*

On the basis of animal results, we further investigated which of the active ingredients of RMDE were effective against radiation-induced inflammation. According to recent studies, solid tumor comprises not only tumor cells, but also tissue matrix and many stromal cells such as fibroblasts and macrophages[26, 27]. Hence, we tested the effects of concentrations (0, 2, 4, and 8 mg/mL) of the main components of RMDE, i.e., MS, AK, and CT, on mouse

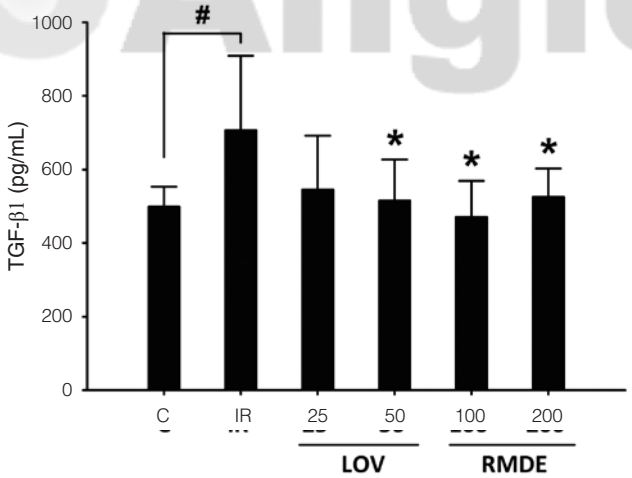

Figure 2. Serum TGF-b1 levels in C57BL/6 mice after radiotherapy. Results are expressed as the mean ± SD, $^{\#}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. irradiation only (IR), n = 5.

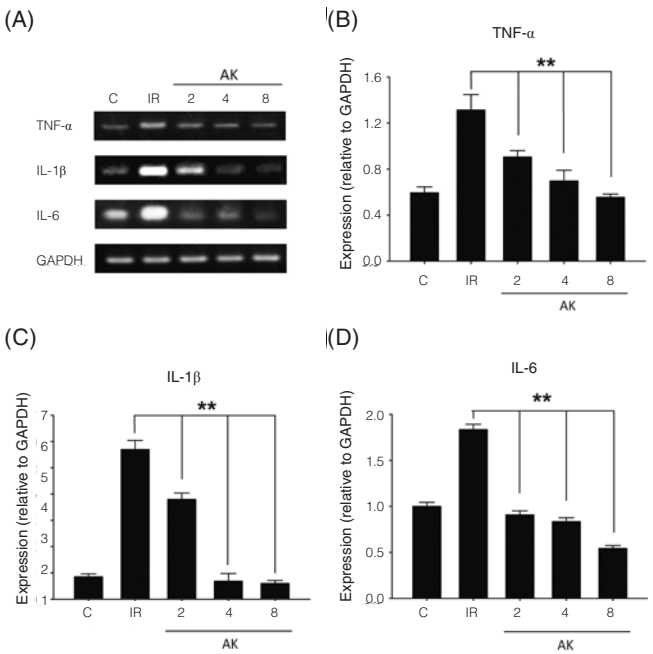

Figure 3. Effects of ankaflavin on the mRNA expression levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) in RAW 264.7 cells. (A) Agarose gel; (B) TNF-α; (C) IL-1β; (D) IL-6. Significance: $p < 0.01$.

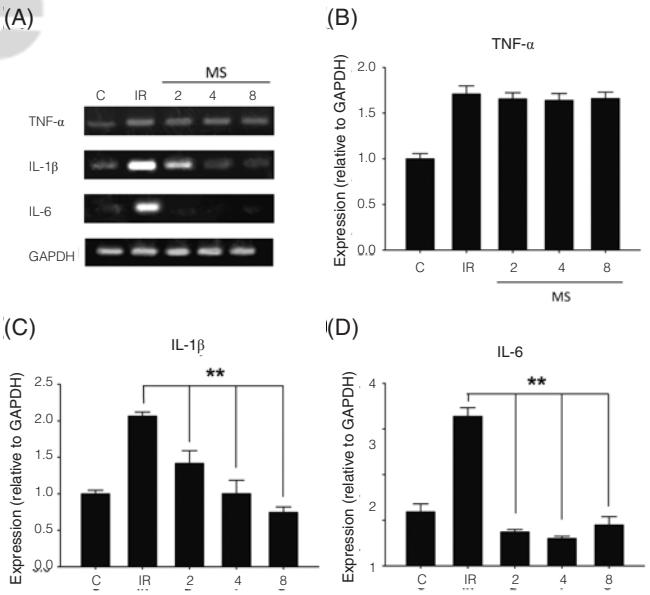

Figure 4. Effects of monascin on the mRNA expression levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) in RAW 264.7 cells. (A) Agarose gel; (B) TNF-α; (C) IL-1β; (D) IL-6. Significance: $p < 0.01$.

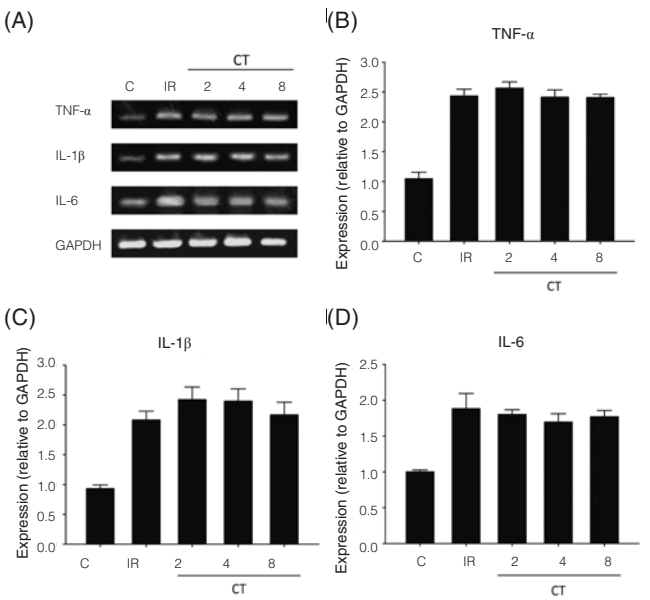

Figure 5. Effects of citrinin on the mRNA expression levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) in RAW 264.7 cells. (A) Agarose gel; (B) TNF-α; (C) IL-1β; (D) IL-6.

macrophage cell line RAW 264.7. The results showed that 2 Gy g-radiation could cause proinflammatory cytokines (TNF-α, IL-1β, and IL-6) overexpression in RAW 264.7 cells (Figure 3, 4, and 5). AK was found to significantly suppress the mRNA expression of all 3 proinflammatory cytokines ($p < 0.01$) (Figure 3B, 3C, and 3D). MS was found to significantly suppress IL-1β and IL-6 mRNA expression ($p < 0.01$) (Figure 4C and D), but it did not affect TNF-α mRNA expression (Figure 4B). This result suggests that MS specifically inhibits IL-1β and IL-6 mRNA expression. Our results showed that CT, which is a mycotoxin, had no effect on the mRNA expression of any of the 3 proinflammatory cytokines (Figure 5B, 5C, and 5D). Based on these results, CT has no antiinflammation ability. In summary, AK and MS were found to suppress the radiation-induced overexpression of proinflammatory cytokine (TNF-α, IL-1β, and IL-6) mRNAs in RAW 264.7 cells.

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DISCUSSION

In this study, we investigated the putative beneficial effects of RMDE on the radiation-induced inflammatory response in C57BL/6 mice. Anorexia accompanied by nausea and vomiting is an important manifestation preceding radiation syndrome[28]. According to the statistics, 80% cancer patients exhibit signs of cachexia[29]. The presence of cachexia complicates therapeutic intervention and is an important cause of death in cancer patients. Some evidence from animal models suggests that various proinflammatory cytokines (i.e., TNF-α, IL-1β, and IL-6) play a role in the genesis of cancer-associated cachexia[30,31]. Our data showed that compared to LOV, RMDE as a feed supplement could effectively increase appetite (Table 3) and decrease the serum TNF-α, IL-1β, and IL-6 levels in mice after radiotherapy (Figure 1). Several yellow pigments such as MS and AK from *Monascus* have been reported to have anti-inflammatory potential and cancer cell cytotoxic activities. Therefore, we also deduce that RMDE increases food intake by reducing radiation-induced inflammatory reactions, and thus, prevents any discomfort; this in turn prevents anorexia and reduces the possibility of malnutrition and cachexia.

Inflammation is the most common side effect of radiotherapy. Some studies indicated that radiotherapy-induced IL-6 production can cause tumor cells to increase vascular endothelial growth factor (VEGF) secretion, leading to neoangiogenesis within the tumor, and this reduces the sensitivity of tumor cells to radiotherapy[32]. Furthermore, recent studies have indicated that radiation-induced overexpression of proinflammatory cytokines IL-1 and IL-6 naturally occurs in many patients, and they go on to develop pulmonary fibrosis[11]. Pro-fibrosis occurs via the activation of TGF-β. Ionizing radiation directly/indirectly activates TGF-β through the dissociation of the latency associated peptide (LAP) from the active mature form of TGF-β. Once activated, TGF-β by suppresses early Smad pathways and causes uncontrolled fibroblast proliferation, increased extracellular matrix (ECM) and collagen deposition, and ultimately fibrosis[12]. Our results demonstrated that RMDE effectively lowers the post-radiotherapy increase in serum TGF-β1 ($p < 0.05$) (Figure 3). According to the result, we deduce that RMDE treatment can inhibit TGF-β1 by blocking IL-1β and IL-6 production. However, the role of RMDE on radiotherapy induced fibrosis effect would be investigated in the future.

In Figure 1, RMDE could reduce the TNF-α level in IR-treated mice, which IR alone did not affect the TNF-α level. About this issue, TNF-alpha secretion is an acute inflammatory response after irradiation. In our experiment, the mice serum was analyzed in day 28 after irradiation. This stage belongs to chronic inflammatory phase, so we did not observe the increase TNF-α level in mice serum. Overall, RMDE treatment can inhibit ionizing radiation induced inflammatory response.

It is interesting to note that the predicted HMG-CoA reductase inhibitor, MK, was not isolated and detected in RMDE. AK and MS are the major components of RMDE in this study (Table 2). Earlier studies used lipopolysaccharide (LPS) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to induce inflammatory reactions and it was found these two active ingredients had antiinflammatory effects[14,33]. To the best of our knowledge, radiation-induced inflammatory responses have not yet been studied. Further, tumors contain varying numbers of macrophages. During radiotherapy, radiation can activate macrophages, leading to secrete proinflammatory cytokines, thereby causing an inflammatory response[26,27]. Therefore, our study was based on these reactions, and we investigated which of the bioactive ingredients of RMDE (AK, MS, or CT) possessed activity against these reactions. The results showed that AK effectively inhibited TNF-α, IL-1β, and IL-6 mRNA expression (Figure 3) while MS specifically inhibited IL-1β and IL-6 mRNA expression (Figure 4). On the basis of these results, we confirmed that AK and MS were the main RMDE ingredients that suppressed the radiation-induced overexpression of proinflammatory cytokines. Therefore, AK and MS may serve as nontoxic natural antiinflammatory agents in the development of clinical radiotherapy.

In future studies, we will continue to investigate the effects of RMDE or its pure components (AK and MS) on mechanisms of fibrosis and angiogenesis after irradiation. Thus, RMDE may be considered as a functional food supplement for cancer patients upon completion of the radiotherapy regime.

ACKNOWLEDGMENTS

This work was supported by a research grant (NSC 97-2313-B-002-032-MY3) from the National Science Council of Taiwan, Republic of China. We thank Professor Chia-Hsien Cheng (Department of Oncology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan) for providing the Co-60 radiation machine.

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