Therapeutic effect of compound of White Peony Root Oral Liquids on radiation-induced esophageal toxicity via the expression of EGF and TGF-β1

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Abstract. The predominant pathological processes of radiation-induced esophageal toxicity include inflammatory reactions in the early stage and the fibrotic process in the late stage. An increased expression of the epidermal growth factor (EGF) and transforming growth factor β1 (TGF-β1) is capable of reducing inflammatory reactions and TGF-β1 is considered responsible for the initiation, development and persistence of fibrosis. In the present study, we investigated *in vivo* the therapeutic effect of the compound of white peony root oral liquids (cWPROL) on reducing the toxicity via modulating the expression levels of EGF and TGF-β1. Adult male Wistar rats were treated and tissue sections were obtained. The tissue sections were stained using histological, Masson and immunohistochemical staining. The results revealed that cWPROL had a higher rate of repairing damaged structures compared with the control group. In addition, immunohistochemistry showed that although cWPROL and the mixture of lidocaine, dexamethasone and gentamycin (mLDG) induced levels of EGF and TGF-β1 expression, there were differences between the two types of intervention. These results are significant for understanding that the mechanism of therapeutic effect of cWPROL varied to some extent from that of mLDG.

Introduction

A large proportion of patients with thoracic carcinomas receive thoracic radiotherapy (TRT) as part of their treatment. Some of these patients are likely to have esophageal toxicity such as acute radiation-induced esophagitis (ARIE) and radiation-induced fibrosis (RIF). The occurrence of these toxicities results in unplanned treatment delays or interruption of treatment. In addition, tumor control and survival rates as well as patient quality of life may also be reduced.

ARIE, which is the primary dose-dependent complication for radiotherapy, is fairly common. ARIE has been reported in 5-50% of the patients treated with TRT at different volumes of thoracic irradiation, and this rate was further increased by concurrent chemotherapy (1,2). Dysphagia, odynophagia and substernal burning sensation are the major clinical features of ARIE. Inflammatory cell infiltration in esophageal tissues is a prominent histopathological change that occurs in ARIE. These inflammatory cells including mast cells, macrophages and lymphocytes may secrete pro-inflammatory cytokines and growth factors that are important in the inflammatory processes (3,4). Of those factors, epidermal growth factor (EGF) is crucial in the growth and proliferation ability of various cells including the epithelium, endothelium and fibroblasts (5,6). Similarly, transforming growth factor β1 (TGF-β1) is a type of substantial growth factor involved in the start and termination of tissue repair. Furthermore, TGF-β1 downregulates the peroxides and nitric oxide generated by inflammatory cells to reduce the extent of inflammation (7).

Following the inflammatory response induced by irradiation RIF, a late sequela of radiation therapy, is mainly characterized by an increased production of extracellular matrix (ECM) components and mesenchymal cell proliferation, migration and accumulation. RIF is an occasional irreversible damage that is unavoidable and may continue for years after TRT (8). In the fibrotic process, a number of cytokines and growth factors have been shown to participate in this process. TGF-β1, via the Smad proteins, is considered responsible for the initiation, development and persistence of fibrosis, and to be the main cytokine involved in the process of RIF *in vivo*. TGF-β1 is also important in the synthesis and deposition of collagen (8-10).

At present, treatments including adrenocorticotropic hormone and certain antibiotics, such as mixture of lidocaine,

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dexamethasone and gentamycin (mLDG), constitute the main drugs used. However, these drugs have not proven to be efficacious in a wide range of patients (4,11). Moreover, adverse effects of these drugs such as the increased risk of osteoporosis and resistance to antibiotics negatively impact the therapeutic ratio. Drugs of herbal origin with few side-effects are of great interest as alternatives and the traditional Chinese herbal medicine (tChm) may provide a novel therapy that may relieve clinical symptoms and improve general functions such as eating, sleeping and immune function (12,13).

Compound of White Peony Root Oral Liquid (cWPROL) is a prescription formula independently developed by our investigators. Previous experimental studies revealed a certain therapeutic effect of cWPROL on ARIE (14). The present study was designed to evaluate the therapeutic effect of cWPROL in an animal model of radiation-induced toxicity as well as to elucidate the molecular mechanisms underlying this therapeutic effect.

Materials and methods

Experimental animals. A total of 64 adult male Wistar rats with an average weight of 180-220 g were used in the present study. Animals were housed with 12-h light/dark cycle and had access to food and water *ad libitum*. The experimental animal techniques and animal handling procedures were approved by the Institutional Animal Care and Use Committee of the Hebei Medical University, and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (certification no. DK0512053).

Grouping and irradiation. Animals were divided into four groups: the cWPROL treatment group (ctg) where rats were administered cWPROL at a dose of 0.475 g/ml by intra-esophageal perfusion after irradiation; the mLDG treatment group (mtg) in which rats were treated with mLDG using the same route of administration after irradiation; the radiation group (rg) in which rats were not administered any treatment following irradiation; and the non-intervention group (nig) where rats did not receive irradiation or administration of drugs. Animals received administrations at a volume of 2 ml each time, three times a day starting on the seventh day following irradiation and continuing for 7 days. Rats were deprived of food and water for 30 min following drug administration.

Following arousal of rats, single irradiation on chest with a total dose of 43 Gy was performed with a ^{60}Co therapy apparatus at a dose rate of 0.111 Gy/min. The irradiation field was 3x30 cm with a centre dose point on the back of rats 1 cm under the body surface and an irradiation range of 3 cm on the upper esophagus, while other parts were covered. Rats in nig were not irradiated, but otherwise treated as the irradiated ones.

Staining. Rats were anesthetized with 2% pentobarbital sodium administered by intraperitoneal injection (45 mg/kg). Esophageal samples were fixed with 4% paraformaldehyde for 24 h, embedded in paraffin and sectioned at 4 μm.

Histological staining. Paraffin sections were stained with hematoxylin and eosin as usual following deparaffinization and rehydration. Light and electron microscopes were used to observe the histopathologic and ultrastructural changes of esophageal tissue. The extent of pathological changes comprised tissue damage and infiltration of phagocytes.

Masson staining. Tissue sections were deparaffinized and hydrated, stained in hematoxylin for 3 min and differentiated in 1% hydrochloric acid alcohol for 3-5 sec. The sections were then treated with ponceau for 3 min, differentiated in 1% phosphoric acid molybdenum for 1 min, counterstained in aniline blue for 1 min and dehydrated rapidly through 95% alcohol, followed by two changes of 100% alcohol, until the collagen was green.

Immunohistochemistry. Tissue sections were deparaffinized with xylene, and rehydrated through an ethanol series and Tris-buffered saline (TBS), and then immersed in 3% formaldehyde hydrogen peroxide liquid to block endogenous peroxidase. Antigen retrieval was performed by microwave treatment in the presence of antigen retrieval solution. The sections were incubated with primary antibody at 4˚C overnight and treated with biotin-labeled secondary antibody at 37˚C for 20 min, followed by the addition of streptavidin peroxidase-conjugated antibody at 37˚C for 20 min. Antibodies for EGF (1:50) and TGF-β1 (1:100) served as the primary antibodies. The sections were counterstained with hematoxylin, dehydrated, transparentized and then sealed with neutral gum. Black control and replacing control were treated with phosphate-buffered saline (PBS) and normal rabbit serum. The appearance of brown particles in the stained sections was regarded as the positive judgment standards. Five successive visual fields centering on the lesion area of each section under the microscope (magnification, x400) were obtained, and the average of their integral optical density was regarded as the representative value.

Statistical analysis. Experimental results were analyzed for statistical significance using the SPSS13.0 software package. Groups were compared by one-way ANOVA. The Student-Newman-Keuls test was used when the variance was equal, while the Kruskal-Wallis H test was used when the variance was unequal. Results were presented as the mean ± standard deviation (SD). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Light and electron microscope analysis. Inflammation caused by direct exposure to radiation results in epithelium apoptosis or necrosis, and mast cells and leukocytes are then recruited to the site of the damage. The pathological criteria of injury centers around two main aspects: the extent of damaged mucous epithelium, and the extent and depth of infiltrating inflammatory cells.

The normal structure of a Wistar rat esophagus consists of a horny layer, a mucous membrane, a muscular layer and the tunica adventitia. The mucous membrane is intact and contiguous, and there are no inflammatory cells infiltrating under the mucous membrane (Fig. 1A). Following radiation, mucous erosion, telangiectasias, defluxion of the epithelium and recruitment of inflammatory cells in the lamina propia,

Table I. Pathology score of rats in each group.

	Pathology injury of esophagus				Infiltration of inflammatory cells			
Group	0	I	II	III	0	I	II	III
nig	16	0	0	0	16	0	0	0
rg	0	2	6	8	0	2	4	10
ctg	13	1	2	0	3	8	3	2
mtg	11	1	4	0	3	5	6	2

nig, non-intervention group; rg, radiation group; ctg, cWPROL treatment group; mtg, mLDG treatment group.

Table II. RTOG/EORTC late esophagitis morbidity grading criteria.

Grade	Description
0	No change over baseline
1	Mild fibrosis, slight difficulty in swallowing solids, no pain on swallowing
2	Unable to take solid food normally, swallowing semisolid food, dilatation may be indicated
3	Severe fibrosis, able to swallow only liquids, may have pain on swallowing, dilatation required
4	Necrosis/perforation, fistula

RTOG/EORTC, Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer.

the submucosal and muscular layers and the tunica adventitia were observed through pathological examination (Fig. 1B).

The radiation-induced alterations of the subcellular level organizations and functions play a significant role in the development of acute radiation injury. Damage at this level in cell organelles has been manifested. Structural changes of some organelles were also observed in this study. The organelle structure of capillary endothelium was normal in the nig group (Fig. 1C). After radiation, elongation and branching of the mitochondria as well as an increase of their size and the development of giant forms, degranulation of endoplasmic reticulum membranes as well as their dilatation and vesicularization, and an increased number and volume fraction of lysosomes were observed (Fig. 1D).

Rate of repair in each group. The determination of tissue repair was based on the pathology score, according to two aspects: the histopathological injury and inflammatory cell infiltration. The scores were divided into different grades and amount of rats according to grade in each group (Table I). The data showed the repair rate to be 81% (13/16) in the ctg group, which was higher compared with 69% (11/16) in the mtg group. However, inflammatory cell infiltration showed a marked decrease in the ctg compared with the mtg group.

The variation of EGF and TGF-β1 expression. Figs. 3 and 4 show the variations of EGF and TGF-β1 expression by immunohistochemistry, respectively. In the normal epithelium of esophageal mucosa, a weak expression of EGF and TGF-β1 was observed, as well as a slight EGF expression in basilar membrane cells and a slight TGF-β1 expression in the fibroblasts of the lamina propria (Figs. 2A and 3A). The level of EGF expression was significantly upregulated following radiation compared with the nig group, which was mainly distributed in the epithelium surrounding ulcers, fibroblasts and vascular endothelium in inflammatory tissues (Fig. 2B). No significant difference in EGF expression was detected between the ctg and rg groups (P=0.071), although a significant difference was observed compared with the nig group (P=0.027) (Fig. 2C). In the mtg group, EGF expression was higher compared with that in the rg group (P=0.001) and significantly higher compared with that in the nig group (P<0.001) (Fig. 2D). The comparison of EGF between the ctg and mtg groups elucidated a difference between the two groups, although this difference was not statistically significant (P=0.927) (Fig. 4).

With regard to the levels of TGF-β1, the results were similar to those of EGF. The expression of TGF-β1 in normal esophageal tissues was weak positive, and was mainly distributed in the cytoplasm of the epithelial cells of the esophageal mucosa and cells in the muscular layer (Fig. 3A). Following radiation, the level increased but without any significance as compared to the nig group (P=0.101). The expression of TGF-β1 presented in the sections with ulcers, mainly located in the cytoplasm of the epithelial cells of the esophageal mucosa and the fibroblast around the inflammation, as well as vascular endothelial cells in the ulcer and cells in the muscular layer (Fig. 3B). The expression of TGF-β1 in the ctg and mtg groups was significantly induced compared with that in the nig group (P=0.013 and 0.016, respectively), while the difference was not statistically significant compared with that in the rg group (P=0.082 and 0.184, respectively) (Fig. 3C and D). The level of TGF-β1 in the mtg group was

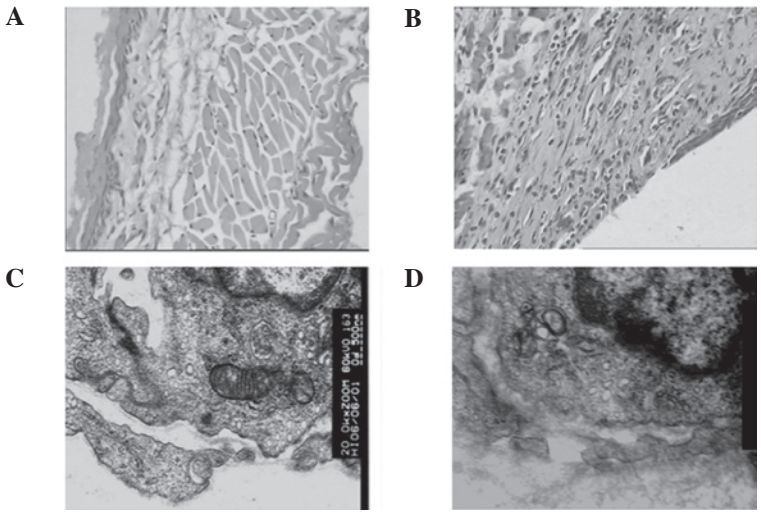

Figure 1. The tissue damage and organelle injuries under light and electron microscopy. (A) Tissue structure of normal esophagus (magnification, x400). (B) Damaged esophageal tissue following radiation (magnification, x400). (C) Structure of organelles in basal cell of normal esophageal tissue (magnification, x20,000). (D) Structure of organelles in the endothelium of radiated esophageal tissue (magnification, x20,000).

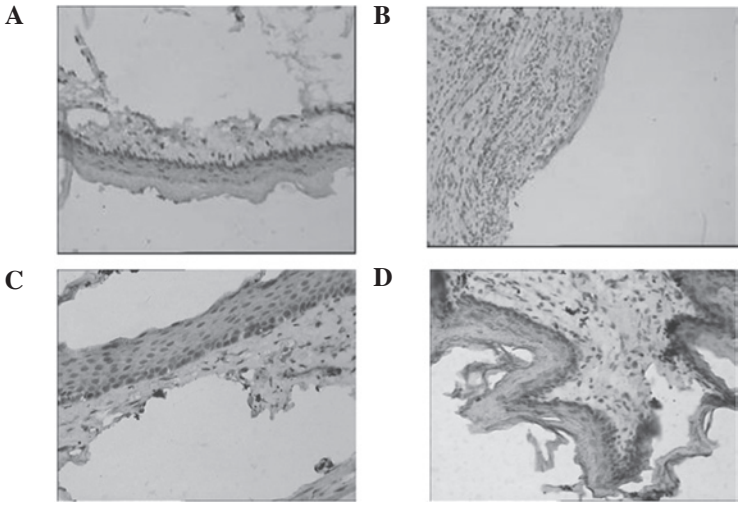

Figure 2. Immunohistochemistry results of the expression of epidermal growth factor (EGF) in esophageal tissues of rats in each group. (A) Esophageal tissue of rat in the non-intervention group (magnification, x400). (B) Esophageal tissue of rat in radiation group (magnification, x100). (C) Esophageal tissue of rat in the cWPROL treatment group (magnification, x400). (D) Esophageal tissue of rat in the mLDG treatment group (magnification, x400).

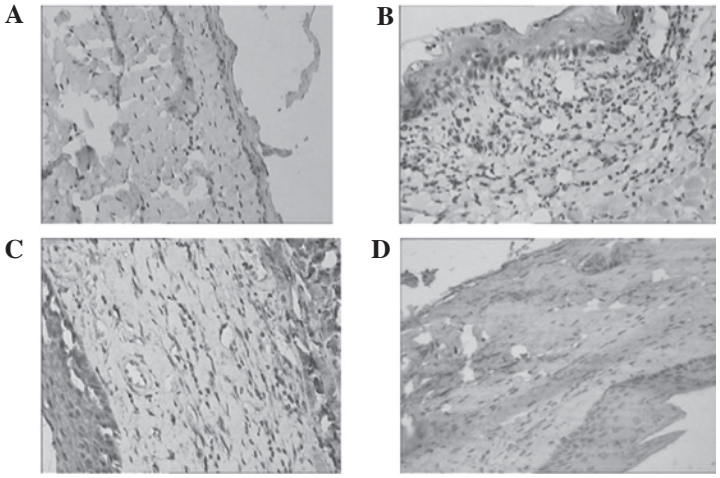

Figure 3. Immunohistochemistry results of the expression of transforming growth factor β1 (TGF-β1) in esophageal tissues of rats in each group (magnification, x400). (A) Esophageal tissue of rat in the non-intervention group. (B) Esophageal tissue of rat in the radiation group. (C) Esophageal tissue of rat in the cWPROL treatment group. (D) Esophageal tissue of rat in the mLDG treatment group.

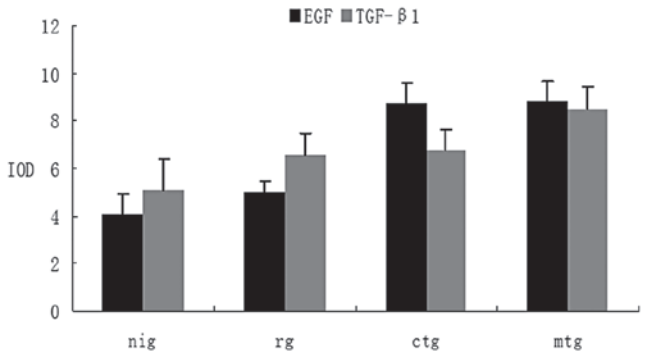

Figure 4. Expression level of epidermal growth factor (EGF) and transforming growth factor β1 (TGF-β1).

higher compared with that in the ctg group, although the difference was not statistically significant (P=0.246) (Fig. 4).

Comparison of collagen fibers in each group. There were a few sparse collagen fibers in the lamina propria of the esophagus of the rat in the nig group (Fig. 5A). A number of exudate and inflammatory cell infiltrations were present in the inflammatory regions in the rg group, and the proliferation of collagen fibers was distributed in several inflammatory cells (Fig. 5B). In the ctg group, mature granulation tissues were evident in the lamina propria and the collagen fibers exhibited a slight increase compared with those in the nig group (Fig. 5C) In the mtg group, the collagen fibers in the lamina propria of the esophagus exhibited mild proliferation (Fig. 5D).

Discussion

Radiotherapy aims to deliver an effective dose to the tumor, while maintaining an acceptable dose for the neighboring normal tissues in order to maximize the therapeutic ratio. However, the radiotherapy of thoracic neoplasms often exposes the esophagus to high levels of ionizing radiation. Radiation-induced esophageal toxicity triggered by various molecular responses induces acute and chronic effects in the normal tissues following radiation therapy. In the early stage, patients often complain of non-specific symptoms such as dysphagia, odynophagia and substernal burning sensation following radiotherapy. In the late stage, patients may experience a serious degree of dysphagia and require endoscopic dilation, caused by the fibroatropic process of the esophagus to radiation (1). Table II defines the Radiation Therapy Oncology Group (RTOG)/European Organization for Research and Treatment of Cancer (EORTC) late esophageal toxicity grading. Table II data indicate that the grading criteria are mainly based on the clinical symptoms instead of on histopathological evidences.

The pathological progression of radiation-induced toxicity in normal esophageal tissues is apparently a consequence of an early inflammatory phase followed by late stromal alterations. Acute or early reactions are primarily characterized by rapidly occurring changes, such as cell death as well as inflammatory cell adhesion and infiltration (1,15). Cell death caused by ionizing irradiation has been categorized into two main classes, manifested as apoptosis and necrosis (16). The mitochondria, endoplasmic reticule, Golgi-complex and the lysosome system have long been considered to be direct intracellular targets of irradiation. Consistent with our results, the necrosis process that ends in the irreversible swelling and lysis of cells has the morphologic hallmarks of mitochondrial swelling, dilatation and degranulation of endoplasmic reticule and lysosomal rupture (17) (Fig. 1D). Apoptosis is suggested to be the main form of ionizing radiation-induced cell death in several cell lines. However, the dose of irradiation may also be important in determining the type of cell death (18). The death and defluxion of the mucous epithelium were observed in our study outcomes (Fig. 1B). Following treatment with cWPROL or mLDG, the

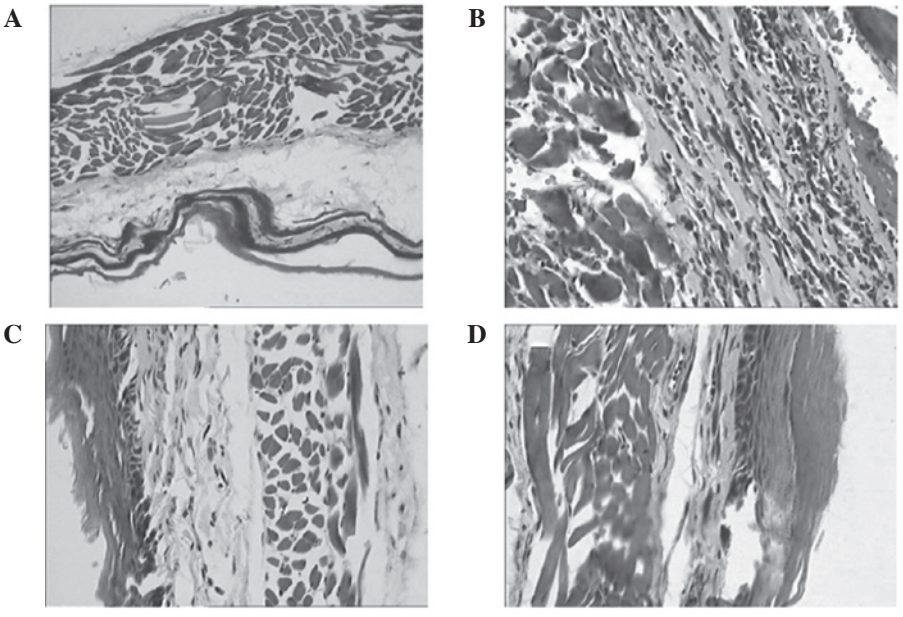

Figure 5. The collagen deposition in each group (red, muscle fiber; green, collagen fiber; brown, nucleus). (A) Non-intervention group (magnification, x200). (B) Radiation group (magnification, x200). (C) cWPROL treatment group (magnification, x400). (D) mLDG treatment group (magnification, x400).

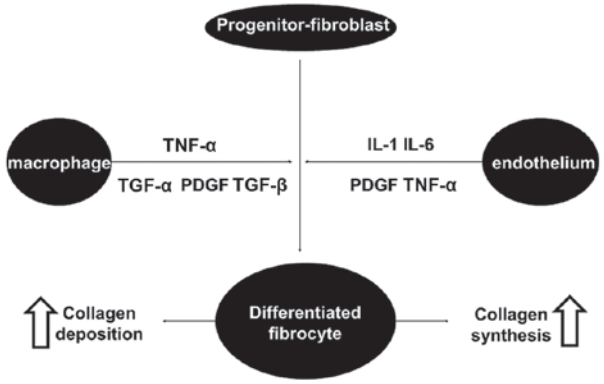

Figure 6. Cytokine-mediated multicellular interactions in fibrotic process.

injured esophageal tissues were repaired via various biological activities were associated with cell proliferation. In the present study, we identified that cWPROL and mLDG promoted mucous epithelium proliferation by increasing the expression of EGF. EGF is crucial in cell proliferation, migration and locomotion. It is a monomeric peptide that promotes mitogenesis in tissues of endodermal, mesodermal and ectodermal origin (19,20). Following the binding of EGF to its receptors, the modulatory effects exerted by EGF were associated with the differentiation retardation and proliferation enhancement via the cell cycle regulating genes (21).

Another feature of acute toxicity is inflammatory cell infiltration. Inflammation caused by exposure to irritants triggers a cascade of cytokines released that results in an inflammatory response and tissue damage. Activated inflammatory and immune cells, such as neutrophils, macrophages, monocytes and natural killer cells, are recruited to the site of inflammation and generate reactive oxygen species in the inflamed tissue, leading to tissue injury (15,22). The present study demonstrated that inflammatory cells mainly comprising neutrophils in the lamina propia, the submucosal and muscular layer and the tunica adventitia were observed in the rg group, while the amount of inflammatory cells was reduced following administration in particular with cWPROL (image not shown).

Late reactions following radiation exposure include fibrosis, organ dysfunction and tissue necrosis (23). Of these reactions, fibrosis is a fundamental pathological process (8,10,23,24). The fibroblast cell system plays a predominant role on the fibrotic process due to its secretary function, which produces the components of ECM and ensures its renewal in a balance between synthesis and degradation. Similar to other fibrotic responses, RIF is a multi-cell process driven by intercellular communications via cytokines and growth factors (22,23,25) (Fig. 6). TGF-β1 stimulates proliferation of early progenitor fibroblast and myofibroblasts, which may be an initial step in the onset of fibrosis. With regard to the process of tissue remodeling, TGF-β1 stimulates, through TGF-β1 receptors and Smad signaling, the synthesis of most matrix proteins, decreases the production of matrix degrading enzymes and increases the production of the inhibitors of these enzymes (26,27). Thus, TGF-β1 has a key role in the development of fibrotic tissue alterations. Findings of the present study have demonstrated that RIF may be reversed by administration with cWPROL via a decrease in the expression of TGF-β1 following repair of the injured tissue, which corresponds to collagen depositions in the ctg and mtg groups.

For the preventive strategies of radiation-induced esophageal toxicity, minimizing the amount of esophagus irradiated is an effective means, however, reducing this amount is likely to reduce the control of thoracic malignances. Investigators previously studied the utility of sucralfate in preventing ARIE. However, 58% of patients dropped out of that study due to nausea and vomiting (28). Amifostine, considered the best radioprotective compound screened by the U.S. Army, was not shown to effectively reduce ARIE in a large clinical trial (RTOG 9801) (29). With regard to preventing and treating RIF, several drugs have also been studied, including D-penicillamine, angiotensin II blocker, interferon γ and antioxidant (29-32), however, no clinical evidence has been found that supports the hypothesis that these drugs may reverse RIF. Therefore, other strategies to minimize radiation-induced esophageal toxicity need to be investigated.

The present study focused on the use of cWPROL in treating an animal model of ARIE and demonstrated that this prescription formula was able to repair the damaged esophageal tissues. Although it has been proven that tChm has the exact function of improving clinical symptoms for the dysphagia in particular, no report is currently available on the underlying mechanisms of the effect. According to the findings of our study, histopathological analysis allowed for the detection of the decrease of collagen deposition in the ctg group, combined with a significant reduction of TGF-β1 expression; cWPROL decreased the TGF-β1 expression level following complete repair of the damaged esophageal tissue. However, this change was not observed in the mtg group. As mentioned above, TGF-β1 plays a critical role in the fibrotic process in late stromal alterations, therefore, we conclude that cWPROL likely promotes the repair of ARIE via an increase in the expression of EGF and TGF-β1, and prevention of RIF via the reduction of TGF-β1. Future studies are required to confirm our conclusion in the RIF animal model via monitoring of the level of TGF-β1 locally and in the blood circulation.

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