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應用中藥板藍根於放射線黏膜炎之研究

Application of Ban-Lan-Gen (Radix of *Isatis indigotica*) in Radiation-Induced Mucositis

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摘要

本品為十字花科植物菘藍的根，是一種常用於涼血止血、清熱解毒、涼血利咽的常用中藥。根據現代藥學研究，板藍根主要成分含靛藍、靛玉紅等成分，具有抗病毒、清熱、解毒和抗發炎功效，並且毒性與副作用都很小。放射線口腔炎是頭頸部癌症病人接受放射治療期間最主要的急性副作用，目前主要的治療藥物其藥效有限，病人常需要暫停放射治療來緩和症狀。我們的目的是應用板藍根以降低放射性口腔炎並且評估其臨床效果，並探討可能的作用機轉，以期可在臨床廣泛的應用。

方法：

分為二部分：(1). 應用板藍根於降低放射線口腔炎的臨床試驗：針對頭頸部癌症病人接受放射治療時，將病人分成兩組，一組為口服板藍根組，另一組為對照組(口服生理食鹽水)，進行臨床觀察並評估放射性口腔炎的嚴重程度；(2). 小鼠在經放射線照射後投與板藍根，評估其抗發炎的效果：以 8 週 BALB/c 公鼠，經連續三天以放射線 9 MeV 照射全身共 5.4 Gy 的劑量，投與板藍根一周後犧牲，評估 BALB/c 公鼠體內的發炎指標。

結果：

在臨床試驗部分，一共有 20 位病人進行板藍根的臨床試驗，其中板藍根組 11 位，對照組 9 位。臨床試驗證明，板藍根組相對於對照組可有效降低放射線口腔炎(P=0.01)、厭食(P =0.002)和對於病人因放射線口腔炎而引起的吞嚥困難(P =0.02)能顯著改善。病人接受放射治療所需的休息天數也能下降，但未達到顯著意義(P =0.06)。板藍根組的病人血清中的炎性細胞素 IL-6 與對照組相比，在放射治療第 1、5、7 週有顯著下降。在動物實驗方面，投與板藍根能顯著降低放射線對於免疫器官的損傷，增加免疫器官的重量，並且使老鼠體內的血球數量如白血球、淋巴球等顯著上升，並且呈現劑量關係。發炎細胞素 IL-1β、IL-6 與腫瘤壞死因子 TNF-α則顯著下降，並且呈現劑量關係。

結論：

臨床試驗及動物實驗結果顯示，板藍根可有效降低因放射線所引起的細胞損傷，降低放射性黏膜炎傷害，對於細胞激素的調節有明顯的作用，本研究發現板藍根可成為放射治療之緩解患者因放射性黏膜炎之不適，其機轉應該是經由發炎細胞激素的調節。

關鍵字： 板藍根、放射性黏膜炎、臨床試驗、動物模式、發炎細胞激素。

Abstract

Radiotherapy plays major role in the treatment of malignancy. However, acute side effects such as radiation mucositis often cause oral pain and dysphagia of the patients, resulting in poor nutrition status. These disabilities usually influence the effect of radiotherapy. In this study, we evaluated the effect of Ban-Lan-Gen (BLG, radix of *Isatis indigotica* FORT) on acute mucositis and dermatitis induced by radiation.

Methods: In clinical trial there were total 20 head and neck cancer patients were randomized into two groups: 1. Control group with only normal saline, 2. BLG group: We prophylactic application of BLG consisted of gargling and then swallowing the BLG preparation on the irradiated oral mucosa as radiotherapy was being carried out. This was compared with control patients who received routine conventional analgesics and skin care. Therapeutic application was started on the first day of radiotherapy. We evaluated of acute radiation mucositis and dermatitis according to the gold standard scale proposed by RTOG. In animal study, total 57 BALB/c mice were divided into six groups: three BLG groups with low (BLG-L, 0.195 g/kg/day), moderate (BLG-M, 0.585 g/kg/day) and high dose (BLG-H, 1.170 g/kg/day), glutamine group (1.950 g/kg/day), control group (RO water 10 ml/kg/day) and naïve group. All mice except naïve group were irradiated with 5.4 Gy in three days and then treated according to each group's regimen for one week.

Results: The clinical trial showed BLG can reduce the severity of radiation mucositis (P=0.01), anorexia (P=0.002) and swallowing

(*P*=0.002). Although the result of resting days needed between groups without significant (*P*=0.06), but quiet difference still was noted (mean days 1.64±2.46 versus 5.89±6.7). Serum IL-6 was significant lower in BLG group in 1st, 5th and 7th weeks. In animal study, increased thymus and spleen weight were found in BLG groups and in dose-dependent relationship. Blood contents such as leukocyte, lymphocyte, granulocyte and monocyte showed the same result. Serum TNF-α, IL-1β and IL-6 were also significantly lower in BLG groups and in dose-dependent relationship. Histopathology assessments of intestine were done and villi number was increased in BLG-H group and glutamine group only.

Conclusion: BLG can improve radiation mucositis clinically. Animal study also showed its effects on immune organs preservations, increased cell subpopulation and down-regulated inflammatory cytokines expression in irradiated mice. We suggested that BLG has anti-inflammatory ability to reduce the mucosal damage caused by radiation.

Keywords: Ban-Lan-Gen, radiation mucositis, clinical trials, animal study, inflammatory cytokines.

Abbreviation

BLG	Ban-Lan-Gen (radix of *Isatis indigotica* FORT)
CDK	cyclin-dependent kinase
COX-2	cyclooxygenase-2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor-kappa B
PGE2	Prostaglandin E2
RT	Radiotherapy
RTOG	Radiation Therapy Oncology Group
ROS	Reactive oxygen species
SARS	Severe Acute Respiratory Syndrome
SWOG	Southwest Oncology Group
TNF-α	Tumor necrosis factor-alpha

Contents

Chapter 1: Introduction

Ionizing radiation is one of the most important modalities for the treatment of human malignancies. However, the acute and late effects of radiation on normal tissues often limit the total dose that can be delivered safely. Radiation mucositis is that condition wherein mucosa suffers toxic damage from direct or indirect action of radiation insult at the layer of mucosal epithelium, on the luminal surface of the mucosa and on the cells between lumen and basement membrane [1]. Radiation mucositis is the most common seen acute side effect in head and neck cancer patients receiving radiotherapy. Thus, radiation mucositis usually acts as a major dose-limiting side effect that influences optimal delivery of radiation [2]. To days, there is no satisfied strategy for preventing mucosal injury or lowering its severity. The current major drug for the treatment of radiation mucositis are steroid and non-steroid anti-inflammation drugs (NSAIDs). Unfortunately, due to the limited effectiveness of drugs, patients often need to rest radiotherapy to relief or reduce radiation mucositis symptoms. Additionally, radiation-induced mucosal damage influence other objective or subjective illness to qualify as mucositis such as pain, dysphagia and anorexia.

In order to improve the therapeutic effects of radiotherapy, clinical physicians often increase total radiation dose and size of daily fractions. However, acute and late effects of ionizing radiation on the vascular-connective tissue of bone and cartilage in the head and neck region still limited the total radiation dose. If it were possible to protect the oral and pharyngeal mucous membranes from the acute effects of irradiation such as radiation mucositis, patient morbidity would be substantially improved. Nonetheless, radioprotection of mucous membranes would reduce morbidity and make possible the incensement of irradiation dose. Besides, most

clinical trials showed great higher cure rate and local control rate in concomitants radiotherapy with chemotherapy. But these chemotherapeutics also cause acute desquamation of mucosal epithelium and exacerbate radiation mucositis [3]. Thus lowering of grade of mucositis caused from the acute effects of ionizing radiation and/or chemotherapeutic agents would reduce the dose-limiting toxicity of these two modalities and represent a therapeutic gain in the combined modality treatment of head and neck cancer.

Isatis indigotica FORT is a kind of the blue cruciferous plants. Its root is a commonly used Chinese herb to remove toxic heat, to reduce heat in blood, and to relieve convulsions. According to modern medical research, the major components of radix of *Isatis indigotica* FORT (BLG) including indirubin, indigotone and indigo pigment contents, with anti-virus, fever detoxification and anti-inflammatory efficacy. Its toxicity and side-effects are small. The purpose of this study was to prove the efficiency of BLG to reduce radiation mucositis in clinical trial, to discus the possible mechanism and to explore the possibility of widely clinical applications

Chapter 2: Literature Review

2-1 Radiotherapy

2-1-1 Introduction

Radiotherapy is a kind of treatment for cancer using radiation. Radiotherapy has experienced more than a century of history. Mr. Rontgen first discovered X-ray and his wife discovered radium respectively in 1895, then human soon used radiation in clinical treatment for skin cancer one year after. With advance in medical research and technique, non-invasive treatment became to be dominant in modern cancer treatment. Till now, approximately 70% of cancer patients in need of cancer treatment with radiotherapy and about 40% of the pain can undergo in curative purpose. The role and status of radiotherapy in the treatment of cancers had become increasingly prominent.

Radiotherapy was used in the treatment of malignancies which are radiosensitive such as lymphomas or Kaposi's sarcoma among others. Some tumors, such as the epithelial cancers of the upper aero-digestive tract, including the oral cavity, are moderately radiosensitive. Ionizing radiation to the head and neck regions is administered by means of external beams of X-ray, gamma rays or external beams of electrons directed to the tumor or by local implantation (brachytherapy) of radioactive needles of cesium, radium, gold, palladium or other metals which also emit x-rays or gamma rays. Most of the irradiative treatments for head and neck cancers consist of a total dose of 50 to 80 Grays (Gy) distributed in fractioned doses of 10 Gy per week during five weeks, at a rate of 2.0 Gy every 24 hours during a 5 day period (1.0 Gy = 100 rads).

2-1-2 Mechanism of radiation-induced cell damage

(1) Direct injury:

Major role in the direct rays resulting from organic elements pertaining to the DNA elements appear fractured and overlapping.

(2) Indirect damage:

Organization of ionizing water with energy-derived photons produced larger oxidative free radicals by using radiation on the human body. These large molecules interact with the DNA elements, usually correlate with irreversible damage.

2-2 Radiation mucositis

2-2-1 Structure of oral mucosa

The structure of oral mucosa is composed of stratified squamous epithelium that overlies the lamina propria and connective tissue, which consists of fibroblasts small blood vessels, inflammatory cells and extra-cellular matrix (ECM). The oral mucosa is a kind of constantly renewing tissue. Proliferating cells in the basal epithelial layer mitosis and produce daughter cells that then migrate to the mucosal surface [Figure 1].

2-2-2 Introduction of radiation mucositis

Radiation-induced damages are non-selective, and therefore may even trigger skin injury or mucositis, and even other fatal complications regardless of normal cells or malignant cells. Those side effects often limit the radiation dose to use in cancer treatment and therefore influence the therapeutic effects. Some side effects can be mitigated by medication methods.

One major complication of radiation therapy is the damage that occurs in the mucosal lining of the gastrointestinal tract, especially to the oral and oropharyngeal mucosa. This damage is called radiation mucositis. Severe oral mucositis is especially common among patients who receive radiation therapy as treatment for cancers of the oral cavity, oropharynx, nasopharynx and salivary glands [2].

Radiation mucositis is a painful inflammatory reaction of the oral mucosa to radiation therapy. The effect of radiation on the oral mucosa is one of cessation of the rapid proliferation of normal epithelial cells with

consequent cellular atrophy followed by necrosis, epithelial sloughing and ulceration. The exposed underlying connective tissue becomes an open door to infection which will be more marked in those patients which have undergone full body radiation and are in immune suppression patients.

The degree of radiation mucositis varies, depending on the dose and portal of the beam. Besides, age and general health of the patients affect the severity of radiation mucositis. The first reaction is usually noted during the second week of a 5 or 6 week treatment and consists of diffuse erythematic change, followed by desquamation and ulceration. Xerostomia adds to the discomfort and usually persists indefinitely to some degree. An alteration in taste often precedes the mucosal reaction and may persist, depending on the dose.

2-2-3 Mechanism of radiation mucositis

The mechanism of radiation mucositis was been postulated as occurring in four phases: initiation, cellular message generation and signal amplification, ulceration and healing.

2-2-3-1 Initiation phase

High energy photons can cause direct or indirect damage to cells. Mostly, indirect damages play major role in cell killing. Energy-derived photons interacted with water and generate reactive oxygen species (ROS), free radicals that can cause DNA strand breaks in the epithelium and submucosa. Cellular responses occur very soon, usually few milliseconds immediately after direct or indirect damages to DNA or other cellular

components by radiation insult. These responses initiate a cascade of other downstream biological events.

2-2-3-2 Cellular message generation and signal amplification

Cellular responses to radiation-induced DNA damage activate several transcription factors that affect a number of genes modulating protein synthesis and cell signaling. Among those numerous transcription factors which activated by radiation insult, one of the most important is nuclear factor-kappa B (NF-κB). This nuclear regulatory molecule coordinate nearly 200 genes involved with radiation mucositis. Some of those genes encode and regulate pro-inflammatory cytokines such as interleukin-6 (IL-6) and cell adhesion molecules [4, 5]. Elevated activity of synthesis of the cytokines interleukin-1β (IL-1β) and IL-6 were seen in the irradiated mucosa. Other enzymes activated by radiation, and sequential ROS include ceramide synthase and sphingomyelinases that can increase the rate of apoptosis. These transcription factors, cytokine and other substances together contribute to trigger a variety of destructive processes that can be lethal damage to epithelial cells and surrounding fibroblasts.

After initial cellular message generation, the next step involves feedback loops that further amplify the number and level of activating signals [Figure 2]. Following radiation damage, some pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), not only directly result in tissue injury but also further increase the activity of other signaling factors such as NF-κB and mitogen-activated protein kinase (MAPK) [4]. Then there is an ongoing cycle of amplification of cellular messages for radiation injury that persists well after the initial insult of radiation,

recruiting inflammation reaction cells. However, few symptoms are apparent in spite of all these cellular changes occurring during the initial stages of mucositis interestingly.

2-2-3-3 Mucosal ulceration

The advanced stage next to cycles of amplification of cellular message, mucosal ulceration occurs. The ulcer penetrates through the epithelium into the submucosa. The ulcerated mucosal surface often was infected by oral bacteria. The infection can produce toxins and recruit acute inflammatory cells such as macrophage, which release additional inflammatory cytokines and angiogenesis factors. This ulcerative phase takes great part of the main clinical symptoms of mucositis, such as oral pain, erymanthos inflammation, and dysfunction of swallowing.

2-2-3-4 Healing

In response to worse ulcer formation, mucosa starts healing process. Epithelial cells grow, migrate, and differentiate to form a wound under the stimulation of signals secreted by the extra-cellular matrix. These signals are then down-regulated to avoid hyperplasia. With the healing process under way, symptoms begin to abate.

2-2-4 Clinical course and severity grades of radiation of mucositis

During a course of fractionated external radiotherapy for head and neck cancer, the rapidly dividing mucosal epithelium is progressively

depleted with each succeeding radiation fraction. An acute radiation-induced mucositis usually begins during the third week of fractionated external irradiation, gradually increases in intensity until the end of irradiation, and then subsides over several weeks after.

Erythema characterizes the early mucosal reaction, erythema and patchy ulceration the intermediate, and erythema and confluent ulceration. The Radiation Therapy Oncology Group (RTOG) classified five grades to be a gold standard clinical guideline for physicians to objectively inspect and evaluate radiation-induced mucositis [Table 1].

2-3 Current treatment of radiation mucositis

Till now, there are no proven effective agents for prophylaxis of radiation mucositis, and thus no “gold standard” method exists. Many traditional forms of treatment, such as mouthwashes and chlorhexidine, have been largely ineffective. Medical intervention such as analgesics, steroids and antibiotics showed therapeutic benefits for symptoms improvement. But these methods have not demonstrated consistent efficacy in preventing and treating oral mucositis. Antimicrobial agents benefit but a few patients who develop a confluent exudative ulceration of the mucous membranes during irradiation. A far more common problem is the early appearance of a burning sensation and beefy red mucosa with or without the gray white plaques characteristic of candidiasis. Yeast can be documented by KOH smears or culture in roughly one quarter of patients undergoing irradiation for head and neck cancer and successfully treated with topical antifungal agents. Antimicrobial and antifungal agents successfully treat superimposed bacteria or fungal infection but do little to protect mucous membranes from the acute effects of irradiation. Although limited success was observed in pain moderation and improvements in inflammation with some of these procedures, to date, no agent has been granted a priori approval as a prevention or therapy for cytotoxic mucositis. Thus newer approaches or agents are needed to improve both prophylaxis and therapy in patients receiving radiation therapy.

2-4 Previous trials for radiation mucositis

Strategies to reduce the incidence and severity of acute radio mucositis include the use of antimicrobial and antifungal agents as well as thiol and prostaglandin radio-protectors. In the past, the use of chlorhexidine [6] sucralfate [7] and benzydamine hydrochloride [8] oral rinses for prevention of radiation induced oral and pharyngeal mucositis have been tried but only benzydamine hydrochloride has been shown to reduce the severity of mucositis when compared with placebo. To date, some ended clinical trials and agents are listed below.

2-4-1 benzydamine hydrochloride

Epstein reported a reduction in mucositis severity from a score of 3.2 ±0.51 for eighteen placebo patients to 2.20±0.56 for the 25 benzydamine hydrochloride patients (P=0.01) [8]. Benzydamine not only attenuates pro-inflammatory cytokines but also scavenges reactive oxygen species (ROS) [9].

2-4-2 aminothiols (WR-2721)

There are sulfhydryl compounds that protect from the effects of ionizing radiation mainly by scavenging free radicals. Charged with protecting military and civilian populations from radiation or chemical warfare the Defense Department undertook an extensive program to develop thiol radio-protectors based on the early work of Bacq and Herve [10]. The most effective radio-protector proved to be S-2-(3-amino-propyl amino)-ethyl phosphorothioic acid (WR-2721). WR-2721 has proven

radio-protective for a variety of normal tissues but its clinical usefulness has been limited by nausea, vomiting and hypotension [11].

2-4-3 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF has been postulated as one of the most promising agents for the prevention of radiation-induced mucositis on the basis of same preliminary reports [12-14]. GM-CSF is a glycoprotein with a molecular weight of 22 kD. It is a potent growth factor for the myeloid lineage of hematopoietic cells.

GM-CSF enhances colony formation of granulocytes, macrophages, and eosinophils and also regulates several functions of mature leukocytes, macrophages, and dendritic cells in the dermis and submucosa [15-17], was shown to have the ability of reduces the severity of radiation-induced mucositis.

2-4-4 glutamine

Glutamine, a nonessential amino acid, is the key fuel for rapidly dividing cells, such as enterocytes, renal tubular cells, lymphocytes, and malignant cells [18-19]. During critical illness, the balance of glutamine metabolism switches to favor enhanced use, leading to a state of total body glutamine depletion and a catabolic state [20-21]. Concentrations of glutamine are found to be decreased during stress, starvation, metabolic acidosis, postoperative stress, and advanced malignant states [22]. It has been shown previously to reduce the extent of intestinal injury from radiation and chemotherapy in laboratory rats [23]. Mechanism by which

glutamine may help decrease mucous membrane injury induced by radiation is by altering the inflammatory response. Glutamine has been shown to be a regulator of glutathione, a ubiquitous antioxidant [24]. Glutathione is an antagonist to prostaglandin E2 (PGE2) production, which is a strong inflammatory mediator.

A trial conducted by Huang and colleagues [25] in 2000 examined whether oral glutamine could also inhibit radiation-induced mucositis. A total of 17 patients were randomized to receive oral glutamine suspension (2.0 g/30 ml) versus placebo. Objective responses were noted, with decreased duration of grade 3 and 4 mucositis as well as decreased severity in degree of mucositis (mean, 1 grade). These early results certainly warrant further investigation. But recently, a large randomized, double-blind, placebo-controlled study (SWOG-9908) in American conducted by Southwest Oncology Group (SWOG) showed that glutamine had no significant benefit to improve radiation mucositis.

2-5 Cytokines and blood contents effect related to radiation damage

Some cytokines such as TNF-α, IL-1β and IL-6 was found to be often elevated during radiotherapy. To date, a number of circulating cytokine levels have been measured in patient serum and appear to have clinical relevance: TNF-α [26-28], IL-1 [29-30], IL-6 [31-32] and IL-8 [33-35]. These cytokines are inflammatory or pro-inflammatory mediators, related to radiation-induced tissue damage, such as radiation mucositis [36]. Tumor necrosis factor-α could well provide a marker for both tissue damage and the induction of inflammatory processes. Circulating levels of these cytokines will be used as a measure of primary damage and activation.

Moreover, several studies showed these cytokines also take part of fatigue and thus influence the life quality of cancer patients. Bower and colleagues [37-40] compared breast cancer survivors with persistent fatigue to a control group of non-fatigued survivors. Fatigued survivors showed significant elevations in several markers of pro-inflammatory cytokine activity compared to non-fatigued controls. Fatigued survivors also reported behavioral changes consistent with pro-inflammatory cytokine activity, including depressed mood, sleep disturbance, decreased activity, and cognitive disturbance [37-40].

2-5-1 Tumor necrosis alpha (TNF-α)

TNF-α in particular has been shown to be of primary importance in pulmonary fibrosis following injury and has been demonstrated to coordinate and network secondary, down-stream cytokines and chemokines, recruiting and amplifying inflammatory cells and components [41, 42]. This

network ultimately leads to the expression of radiation-induced late effects.

2-5-2 Interleukin-1alpha and beta (IL-1α, IL-1β)

IL-1 procedure by monocytes and macrophages, activate CD4 helper T-cell. It can promote T-cell proliferation. IL-1 alpha (IL-1α) is a kind of cytokine expressed on cell plasma membrane. IL-1α that may secreted by astrocytes, fibroblasts, hepatocytes, keratinocytes, type II Greater alveolar cells, brown fat adipocytes, thymic myoid cells, T cells, eosinophils, monocytes, and dendritic cells, especially by macrophages, endothelial cells, dendritic cells and fibroblast. The cells which can express IL-1β are astrocytes, adrenal cortical cells, NK cells, macrophages, monocytes, endothelial cells, keratinocytes, megakaryocytes, platelets, neurons, neutrophils, oligodendroglia, osteoblasts, Schwann cells, trophoblasts, T cells and fibroblasts.

2-5-3 Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory cytokine secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation. Pons et al. reported that IL-6 gene expression was increased by irradiation [43].

2-5-4 Hemoglobin (Hgb)

The Radiation Therapy Oncology Group (RTOG) analyzed 521 patients treated as part of RTOG 85-27, showed that patients with anemia

(defined as hemoglobin <14.5 mg/dl for men and < 13.0 mg/dl for women) had significantly worse overall survival (*P*=0.0003). A trend was noticed with respect to local-regional control, with anemic patients having a worse outcome (*P*=0.065). Of note, there was also a trend toward fewer late complications in anemic patients (*P*=0.054); it is unclear whether this could reflect hypoxia in normal tissues as well as tumor or whether this finding was an artificial result of anemic patients having shorter survival and thus less time to develop late complications [44].

2-5-5 Neutrophils

Activated neutrophils can lead to microvascular injury resulting in increased permeability, hemorrhage, and thrombosis [36]. Activation of neutrophils was by cytokines such as IL-1 and TNF-α [45]. Mucosal damage observed with infection is thought to be caused by cytokine activated neutrophils [46].

2-6 Radix of *Isatis indigotica* FORT

2-6-1 Introduction

Radix of *Isatis indigotica*, also known as Ban-Lan-Gen (BLG), derived from the root of the isatis plant, which is a source of indigo dye. Its roots were widely used as a kind of traditional Chinese medicine herb. Indigo plant is used in China for infections associated with heat. The purified extracts of BLG have been utilized to make various preparations which can be used in clinical practice for treatment of influenza, epidemic hepatitis, epidemic encephalitis B, carbuncle, erysipelas [47, 48]. It's main purported uses are bronchitis, chest congestion and fever. It is used in combination with other herbs to treat the common cold, sore throat, mumps, respiratory aliments, other febrile diseases and malignant tumors [48-51]. In several studies, it appears that indigo plant root has immune stimulating and anti-inflammatory activity. Recent anecdotal reports indicate that BLG can be used in Severe Acute Respiratory Syndrome (SARS) [52], because BLG has antiviral activities. [53]

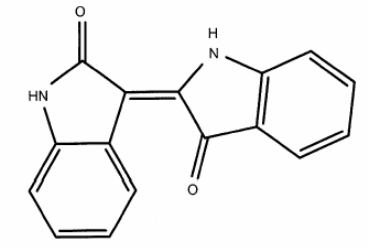
2-6-2 Constituents and possible mechanism of actions

Many chemical compounds have been isolated from BLG, including indigotin, indirubin, isatin, isatan A, isatan B, trytanthrin, purin, isaindigotidione, polysaccharides, organic acids and many amino acids [54].

These isolated components can be roughly divided to water-soluble and insoluble groups.

Water-soluble compounds: Mainly polysaccharides organic acids and amino acids take great part of the water-soluble compounds. Polysaccharide from indigowoad was shown to have immune-stimulatory effects by enhancing reticuloendothelial system function, especially lymphocyte, monocyte and NK cells. The organic acids in BLG had *in vitro* anti-endotoxic action and antiviral action [55].

Water-insoluble compounds: Indirubin, an active component of BLG, is a potent inhibitor of cyclin-dependent kinases (CDK) [56]. The chemical structure of indirubin was analyzed and shown below. Indirubin was proved to have cyclooxygenase-2 (COX-2) inhibitory-like effects [57]. Indirubin also had been proved an anti-cancer activity in the treatment of chronic granulocytic leukemia [56]. Recently, it was found that indigotin and indirubin were potent aryl hydrocarbon receptor (AhR) agonists [58, 59] and the alkaloid isaindigotone from BLG was reported to be a scavenger of superoxide generated [60].



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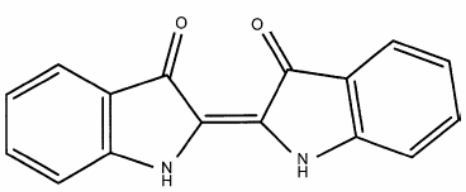

Indigotin

2-7. Clinical trial of BLG in radiation mucositis

Radiotherapy plays major role in cancer treatment. However, radiation mucositis is the most often acute side effect in head and neck cancer patients receiving radiotherapy. The current major drug for the treatment of radiation mucositis is steroid and non-steroid anti-inflammation drug (NSAIDs). Unfortunately, due to the limited effectiveness of drugs, patients often need to rest radiotherapy to relief reduce radiation mucositis symptoms. BLG is a commonly used Chinese medicine to remove toxic heat, to reduce heat in blood, and to relieve convulsions. According to modern medical research, the major components of BLG including indirubin, indigotone and indigo pigment contents, with anti-virus, fever detoxification and anti-inflammatory efficacy. Its toxicity and side-effects are small. Indirubin is the most important component. It is water-insoluable. The ethanol-extract part of BLG was proved to have major anti-inflammatory activity [61] However, there was no clinical trial to use BLG in radiation mucositis till now. The purpose of this study was to prove the efficiency of BLG to reduce radiation mucositis in clinical trial, to discus the possible mechanism and to explore the possibility of widely clinical applications.

Chapter 3: Materials and methods

Divided into two parts: (1) Application of BLG in radiation-induced mucositis clinical trials. (2) Assessment of BLG for the immune system after exposure to radiation effects.

3-1 Clinical trial: application of BLQ in Radiation-induced Mucositis

3-1-1 Inclusion criteria:

(1) Patients must have be victims of head and neck cancer (T1-T4, Any N, M0)

(2) Patients must be scheduled to receive (according to institutional standards) high dose radiation therapy (XRT) of at least 6,000 cGy with a daily dose of 180-200 cGy. Intensity Modulated Radiation Therapy (IMRT) may be substituted for standard XRT. XRT must be planned to begin 7 to 28 days after registration.

(3) Patients must have a Kornofsky performance score ≧ 70。

(4) No other malignancy is allowed except for the following: adequately treated basal cell or squamous cell skin cancer, in situ cervical cancer, adequately treated Stage I or II cancer from which the patient is currently in complete remission, or any other cancer from which the patient has been disease-free for five years.

(5) All patients must be informed of the investigational nature of this study and must sign and give written informed consent in accordance with institutional and federal guidelines.

3-1-2 Objectives

(1) Primary

Compare the efficacy of BLG versus placebo, in terms of maximum mucositis toxic effects and worst reported mouth pain during and after high-dose radiotherapy, in patients with head and neck cancer.

(2) Secondary

a. Compare the duration of severe mucositis in patients treated with BLG.

b. Compare the radiotherapy delay in patients treated with BLG.

c. Compare weight loss in patients treated with BLG.

d. Compare the toxic effects of BLG in these patients.

e. Compare patient-reported mouth pain success rate in patients treated with BLG.

f. Determine the compliance of patients treated with BLG.

3-1-3 Pretreatment evaluation

(1) Complete history and physical examination

(2) Document extent of tumor tissue biopsy (previous treatment)

(3) CBC with differential, platelet count, blood chemistries (SMA-12), liver profile.

(4) Chest X-ray.

(5) Pregnancy test for women of child-bearing potential.

3-1-4 Registration procedure

(1) Patients can be registered only after pretreatment evaluation is completed and eligibility criteria are met.

(2) The patient will be registered to the clinical trial and a case number will be assigned and confirmed.

(3) The Eligibility Checklist must be completed in its entirety prior to calling chairman.

(4) The completed, signed, and dated Checklist used at study entry must be retained in the patient's study file.

3-1-5 Radiation therapy

(1) Physical Factors

a. Equipment: Linear accelerators with appropriate photon and electron energies for supplemental boosting to the nodes.

b. Selection of appropriate photon energy should be based on optimizing the RT dose distribution within the target volume and minimizing the dose to the normal tissue.

c. Treatment distance must be S.A.D for isocentric techniques.

(2) Localization Requirements

a. Simulation: Simulation of all fields is mandatory. Patients must be reproducibly immobilized. The use of customized blocks to shape the treatment fields is recommended. Simulation films of each field, initial port films, and the calculation form will be sent to RTOG Headquarters in the first week of therapy, together with the treatment prescription for radiation therapy quality assurance review.

b. Verification: Beam verification (port) films must be obtained for each field. This should be done at least once in the first week of treatment and whenever any field adjustments are made. Port films of each field must be submitted to RTOG Headquarters.

(3) Target Volume Irradiation Portals

a. Standard three-field techniques using two parallel opposed lateral fields will be used for the primary tumor site at the discretion of the investigator for the case. A single anterior A-P field will be used to treat the neck below the fields for the primary tumor. When there is (are) positive node(s) in the lower neck, an additional posterior field may be necessary to deliver a supplemental dose to the positive node (s). All fields

must be treated on each treatment day. The lower neck and supraclavicular field should abut the primary field at the skin. For patients with short necks, right and left superior inferior oblique fields or any other standard techniques may be used. For oropharynx primaries, a midline block 2 cm wide and at least 2 cm in length on the skin surface will be placed in the anterior lower neck field to shield the larynx and the spinal cord in the junction region. For larynx and hypopharynx primaries, a lower lateral block, 2 cm in height, should be placed in the lateral upper neck fields to shield the areas from potential overlap of diverging beams over the spinal cord. Use of right and left superior – inferior oblique fields technique is also acceptable. Appropriate bolus around the stoma is to be used. The primary treatment fields should encompass the primary tumors with adequate margins along with sites of known and/or suspected lymph node disease in the upper neck. There should be a minimum 2-3 cm margin around the primary tumor and positive node(s) and should include upper neck nodes to be irradiated electively for the initial target volume. Appropriate field reductions are to be made at the discretion of the treating radiation oncologist. The maximum spinal cord dose should not be more than 45 Gy-46 Gy.

(4) Oropharynx:

a. The upper border of the field includes the nodes in the upper jugular region and should be placed at the level of the zygomatic arch, to include the parotids in the field.

b. The ipsilateral posterior cervical nodes must be irradiated if the

primary tumor is T3 or T4.

c. Both the ipsilateral and contralateral posterior cervical nodes must be irradiated if there are clinically positive cervical nodes in the anterior chain.

(5) Supraglottic larynx:

a. The upper border of the field includes the nodes in the upper jugular region and should be placed at the level of the zygomatic arch to include the parotids in the field.

b. The lower border of the field encompasses the larynx usually at or below the level of C5.

c. The ipsilateral posterior nodes should be treated for T3 and T4 lesions.

d. Both ipsilateral and contralateral posterior nodes should be treated if there are clinically positive nodes in the anterior chain.

(6) Hypopharynx:

a. The superior border is placed at the level of the zygomatic arch, to include the base of skull (above C1) and the retropharyngeal nodes. Nodes in the upper jugular region and posterior triangle are included.

b. The lower border of the field encompasses the lower border of the cricoid cartilage.

(7) Lower neck:

a. A single anterior lower neck field will be used to treat the neck and the supraclavicular fossa below the fields for the primary tumor. When there is (are) positive node (s) in the lower neck, an additional posterior field may be necessary to deliver a supplemental dose to the positive node (s).

b. The lower border of the field will be just below the clavicle or 1 cm below the clavicle when there are positive nodes in the supraclavicular fossa.

(8) Dose Calculation

a. Dose to the supraclavicular field is calculated at 3 cm depth or d Max depending on the clinical situation and at the discretion of the treating Radiation Oncologist. Cumulative isodose distributions at the level of the tumor center, a copy of the treatment record indicating cumulative doses, and boost field simulation and portal films must be submitted at the completion of radiotherapy.

b. Missing tissue equivalent compensators should be used to ensure homogeneity of dose distribution so that variation within the target volume does not exceed 10% of the target dose.

c. Boost doses will be specified at the actual site(s) of gross primary and nodal disease.

(9) Standard Fractionation

a. Treatment to the primary tumor and upper neck will be given at 1.8 Gy per fraction, once a day, five days a week to a total

dose of 54-70 Gy in 27-35 fractions in five and a half to seven weeks. Fields must be reduced to exclude the spinal cord at 38-44 Gy at the mid-plane. However, the entire neck must be irradiated to a dose of 54 Gy (even NO stage) as the anatomical levels of lymph node spread, usually 2-4 cm below the skin surface. Clinically positive neck nodes should receive a dose of 62-70 Gy in 31-35 fractions in 6-7 weeks. To supplement the dose to the posterior neck and clinically positive nodes, boost techniques may include additional electron beam (9 MeV) to the posterior neck. The anterior lower neck field will be treated at 2 Gy per fraction at 3 cm depth, once a day, to a total dose of 54 Gy in 22 fractions in 5.5 weeks. The total dose to the primary tumor and clinically positive nodes will be 62-70 Gy in 31-35 fractions in 6-7 weeks.

b. Radiation treatment is to be started within 4-6 weeks of surgery.

c. Wedge-pair techniques to boost the primary tumor can only be used if it does not interfere with the shielding of the transferred submandibular salivary gland in the submental space.

3-1-6 Radiation Therapy Toxicity Adjustments

(1) Treatment Interruptions: Interruptions in radiation therapy may be necessitated by skin reactions, mucositis, ulceration, edema, or other acute complications. Radiation therapy will be continued without interruption if at all possible. Any interruption of radiation therapy for whatever reason (pain, machine

malfunction, illness, lack of transportation or social obligation) must be clearly indicated in the treatment record.

(2) CBC is required to be done before radiation therapy or during the first week of radiation therapy.

Radiation mucositis judgments are according to RTOG classification of mucositis as Table 1.

3-1-7 Toxicity Reporting Guidelines

(1) For acute radiation effect, through day 90 of treatment, the NCI CTC Version 2.0 will be used.

(2) Late radiation effects will be evaluated and scored per the RTOG/EORTC Late Effects Scale.

(3) All fatal toxicities (grade 5) resulting from protocol treatment must be reported by telephone to the Group Chairman, to ACR Headquarters Data Management and to the Study Chairman within 24 hours of discovery.

(4) Required data forms, and, if requested, a written report, must be submitted to Headquarters within 10 working days of the telephone report.

3-1-8 Preparation the BLG

According to the textbook records of BLG, we prepared scientific Chinese medicine BLG powder manufactured by Sun-Ten Pharmaceutical

Co. Ltd. This BLG powder was composed of radix isatidis concentrated extracts and radix isatidis powder with absorption ratio to 2:1.

3-1-9 Clinical usage of BLG

We dissolve the concentrated powder of BLG in RO reverse water with daily dose 1.5 g in 90 ml solution. We educated our patients how to use it: mouthwash, gargle over throat and then swallow it, 30 ml per time and three times a day. (0.5 g BLG per time, 1.5 g per day)

During whole course of radiotherapy, we will give medication according to WHO three steps guideline (step 1: NSAIDs, step 2: partial narcotics, step 3: narcotics). BLG was supplied as adjuvant medication to lower radiation mucositis.

3-1-10 Statistical analysis

Parameters were documented at the beginning of radiotherapy until completion of radiotherapy: (1) grade of mucositis each fraction (from patient's subjective complaint and evaluation of physician. We used F-test to compare age, body weight, and treatment time between the two arms. Mean maximum grade of mucositis, mean maximum WHO step, mean body weight change, and mean fraction number of Grade 0-4 mucositis were compared by the Mann-Whitney U test. *P*-values less than 0.05 were considered to indicate statistical significance.

3-2 Assessment of BLG for the immune system after exposure to radiation effects.

3-2-1 Animals

Total 57 BALB/c mice strain aged 5-6 week and weighing between 20~25 g, , purchase from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, will housing in plastic cage with Ventilated Micro Isolator System (VMIS) and provide with steriled Purina rodent chow and water *ad libitum*. A 12-hour light/dark cycle was set. Animals were acclimated for a minimum of 48 hours prior to radiation to minimize the effects of stress due to shipping. All animals were weighed prior to radiation and monitored daily for survival. All protocols were approved by the Standing Committee on Animal Use of the China Medical University.

3-2-2 Regimen dosing

In animal study, total 57 BALB/c mice were divided into six groups: three BLG groups with low (BLG-L, 0.195 g/kg/day), moderate (BLG-M, 0.585 g/kg/day) and high dose (BLG-H, 1.170 g/kg/day), glutamine group (1.950 g/kg/day), control group (RO water 10 ml/kg/day) and naïve group. Each group was assigned the different test regimens (feed immediately after irradiation for seven days before sacrificed) as following:

Group	Sample size	Radiation modeling	Treatment
No treatment	10	+	— (RO, 10 ml/kg/day)
BLG-L	10	+	BLG, 0.195 g/kg/day (1×)
BLG-M	10	+	BLG, 0.585 g/kg/day (3×)
BLG-H	10	+	BLG, 1.170 g/kg/day (5×)
Glutamine	9	+	Glutamine, 0.520 g/kg/day (1×)
Control	8	−	—

3-2-2 Irradiation

Each groups except naïve group received whole body irradiation with 1.8 Gy/day for consecutive three days, total 5.4 Gy. We used Elekta Precision Linac to perform irradiation. High energy electron beam 9 MeV was applied with SSD technique (SSD, 110 cm).

3-2-3 Immune organs and blood cells and differentiation measurement

All 57 mice were sacrificed after consecutive feeding with testing regimens. The weight of immune organs, such as spleen, thymus and pancreas were measured.

Red blood cells were removed by BD FACS Lysing Solution. Wash cells one time in cold wash buffer (DPBS/0.1% NaN_3); and centrifuge at 350 ×g for 5 minutes. And then re-suspend cell pellet and adjust the cell concentration to 2×10^7 cells/ml. Dilute primary fluorochrome-conjugated

mAbs to predetermined optimal concentrations in wash buffer. Deliver 1×10^6 cells in 50 μl to each well already containing 50 μl of mAb. Mix by gently vortexing or tapping. Then Incubate at 4°C for 20-40 minutes in the dark. Wash two times with 3.0 ml of wash buffer. After each centrifugation, 350 ×g for 5 minutes, aspirate tube to remove supernatant. Vortex gently or tap plate to loosen pellet prior to adding next wash or diluted secondary reagent. Use 500 μl of analysis buffer (DPBS/0.1% NaN_3/1.0% fetal bovine serum) to resuspend pellet in FACS tubes appropriate for flow cytometer. Acquire sample data on flow cytometer as soon as possible after staining. Live gate in 5000 cells for differentiate lymphocytes, monocytes and granulocytes in the dot plot of forward scatter (FSC) and side scatter (SSC).

3-2-4 IL-1β and IL-6 by ELISA protocol

The "Mouse cytokine ELISA Ready-SET-Go!" and "Human cytokine ELISA Ready-SET-Go!" "eBioscience" reagent set contains the necessary reagents, buffers and diluents for performing quantitative enzyme linked immunosorbent assays (ELISA).

(1). Capture Antibodies and Detection Antibodies (biotin-conjugate) were pre-titrated, purified antibodies. The clones list below in table:

Cytokines	Capture antibody	Detection antibody	Sensitivity	Detection range
mTNF-α	1F3F3D4	XT3/XT22	8 pg/ml	8-1000 pg/ml
mIL-1β	B122	Rabbit polyclone	8 pg/ml	8-1000 pg/ml
mIL-6	MP5-20F3	MP5-32C11	4 pg/ml	4-500 pg/ml
hIL-1β	CRM56	CRM57	8 pg/ml	8-1000 pg/ml
hIL-6	MQ2-13A5	MQ2-39C3	2 pg/ml	2-200 pg/ml

(2). Standard: Recombinant cytokine for generating standard curve and calibrating samples

(3). Coating Buffer: 10× concentrated

(4). Assay Diluent: 5× concentrated

(5). Detection enzyme: pre-titrated Avidin-HRP

(6). Substrate Solution: Tetramethylbenzidine (TMB) Substrate Solution

(7). 96 Well Plate: Corning Costar 9018 or NUNC Maxisorp flat-bottom

(8). Coat 96 well ELISA plate with 100 µl/well of capture antibody in Coating Buffer (dilute as noted on Certificate of Analysis, which is included with the reagent set). Seal the plate and incubate overnight at 4°C.

(9). Aspirate wells and wash 3 times with >300 µl/well Wash Buffer. Invert the plate and blot on absorbent paper to remove any residual buffer.

(10). Dilute 1 part 5× concentrated Assay Diluent with 4 parts DI water.

Do NOT add sodium azide. Sodium azide inhibits HRP activity. Block wells with 200 µl/well of 1× Assay Diluent. Incubate at room temperature (RT) for 1 hour.

(11). Aspirate/wash as in step 9.

(12). Add 100 µl/well of standard (dilute as noted on Certificate of Analysis) to the appropriate wells. Perform 2-fold serial dilutions of the top standards to make the standard curve. Add 100 µl/well of your samples to the appropriate wells. Seal the plate and incubate at RT for 2 hours.

(13). Aspirate/wash as in step 9. Repeat for a total of 5 washes.

(14). Add 100 µl/well of detection antibody diluted in 1× Assay Diluent (dilute as noted on Certificate of Analysis). Seal the plate and incubate at RT for 1 hour.

(15). Aspirate/wash as in step 9. Repeat for a total of 5 washes.

(16). Add 100 µl/well of Avidin-HRP diluted in 1× Assay Diluent (dilute as noted on Certificate of Analysis). Seal the plate and incubate at RT for 30 minutes.

(17). Aspirate and wash as in step 9. In this wash step, soak wells in Wash Buffer for 1 to 2 minutes prior to aspiration. Repeat for a total of 7 washes.

(18). Add 100 µl/well of Substrate Solution to each well. Incubate plate at room temperature for 15 minutes.

(19). Add 50 µl/well of Stop Solution to each well.

(20). Read plate at 450 nm by ELISA reader (Multiskan Spectrum, Thermo Electron Corporation, San Jose, CA, USA), and subtract the values of 570 nm from those of 450 nm and analyze data.

3-2-5 Haematoxylin and Eosin Staining (HE stain)

(1). Rinse sections to water.

(2). Place sections in Mayers haematoxylin solution for 5 minutes.

(3). Wash in tap water.

(4). Place sections in 1% acid alcohol for a few seconds.

(5). Wash in tap water.

(6). Place sections in eosin (1%) for 5 minutes.

(7). Wash in tap water.

(8). Dehydration by serial concentrated alcohol (70%, 80%, 90%, 100%).

(9). Mount sections and photograph record.

Chapter 4: Results

4-1 Clinical results correlate to BLG

Between October 2005 and May 2006, 20 patients were randomized in clinical trial. Of these 20 patients, 11 were enrolled in the BLG group and 9 were enrolled in the placebo group. The distributions of patients according to age, body weight, gender, radiation dose, chemotherapy and diagnosis were similar between the two treatment groups [Table 4]. The clinical results of between two groups were listed in table 5.

Our clinical trial showed that BLG improved objective symptom-radiation mucositis (P=0.01). Lower the severity of clinical subjective symptoms such as anorexia (P=0.002) and swallowing difficulty (P=0.002). Most patients need to take rest during radiotherapy course because of severe radiation-induced mucositis and dermatitis. In our clinical trial, although patients' resting day didn't showed significance (P=0.06), we still can found that less rest were needed in BLG group and higher complete radiotherapy rate without rest (4/11 versus 2/9).

The result of blood contents analysis in clinical trial was shown in Table 6. Less WBC counts was found in BLG group, especially in 5^{th} weeks after start of radiotherapy (4972.4 ± 2196.1 vs. 6502.2 ± 4365.0 /mm^3, P=0.046). Differential count of WBC disclosed that less eosinophils and lymphocytes. These results may explain why less WBC was found in 5^{th} weeks. In contrary, absolute monocyte was higher in control group and showed significance in 7^{th} week (P=0.0022). Hemoglobin showed no significant difference between BLG and control group.

Serum cytokines analysis was analyzed by ELISA assay. The results

were shown in Table 7. Pro-inflammatory cytokines IL-1β was lower in BLG group but didn't showed significance except at 3rd weeks. Serum IL-6 level was much lower in BLG group at 1st, 5th and 7th week after radiotherapy and showed significantly difference when compared with control group.

4-2 Influence of BLG to immune organs in irradiated mice

As listed in Table 8, immune organs such as spleen, thymus and pancreas were found to be significantly lower in irradiated control group compared with naïve group (0.0029±0.0008, 0.0256±0.0032 and 0.0093± 0.0011 vs. 0.1131 ± 0.0194, 0.0498±0.0076 and 0.0285±0.0106 g, respectively, $P<0.001$). That means this animal model was effective. Among BLG groups, BLG-M and BLG-H group showed higher immune organs preservation ability of thymus and spleen and in dose-dependent relationship. In contrary to spleen and thymus, BLG-M showed best preservation ability for pancreas ($P<0.05$).

4-3 Influence of BLG to blood counts in irradiated mice

The result of mice blood contents analysis was listed in Table 9. Blood contents and its differentiation count such as granulocyte, monocyte and lymphocyte were found to be significantly lower in irradiated control group compared with naïve group that means this animal model was effective. BLG-treated groups had significantly higher lymphocyte, monocyte and granulocyte when compared to control group and in dose-dependent relationship. These results were corresponding to immune

organs preservation effect of BLG. Glutamine also has similar effects but not as well as BLG groups.

4-5 Influence of BLG to cytokines in animal model

Serum cytokines was quantitatively analysis its amount by ELISA assay. TNF-α, IL-1β and IL-6 quantitative amount were made and these results were shown in Table 10. BLG groups takes great part in lowering serum TNF-α, IL-1β and IL-6, that were correlate with BLG dose. Higher BLG dose was correlated with lower serum pro-inflammatory cytokines in linear relationship. Glutamine has effect in lower TNF-α but plays no role in IL-1β and IL-6 (P=0.008, 0.331 and 0.352, respectively).

4-6 Histopathologic assessment in animal model

The results of histopathologic assessment in animal model were listed in Table 11. Ileal villous number and height were found to be significantly lower in irradiated control group compared with naïve group (9.17±0.98 and 0.32±0.07 /mm vs. 12.67±0.52 and 0.49±0.09 mm, respectively, P <0.01 and P<0.001). High dose BLG significantly showed a increase in villi number but not villi height when compared with control group. Glutamine pretreatment before irradiation significantly prevented a decrease in villous number and height (10.83±0.75 vs. 9.17±0.98 mm, P=0.008).

Chapter 5: Discussion

Acute radiation mucositis is a result of mitotic cell death in the mucosa, disruption of the epithelial barrier, and mucosal inflammation. Pathogenesis of radiation mucositis is presumed to be an inflammatory process in which various mediators take place. The activation of inflammatory cells leads to the synthesis and release of certain cytokines, inflammatory mediators, and reactive oxygen metabolites. Among these cytokines, TNF-α and IL-6 are key pro-inflammatory mediators, which are often over-expression in cancer patients. TNF-α is produced after stimulation of mainly macrophages and monocytes. In addition, TNF-α binds to receptors on endothelial cells to initiate angiogenesis then produces vascular damage and the expression of IL-6 [62]. IL-6 is released by stimulation of T and B lymphocytes, macrophages, fibroblasts and endothelial cells. Radiation is found to induce TNF-α which may exacerbate the cytotoxic effects of radiation [63, 64]. Recent clinical studies have shown that the circulating serum level of IL-6 correlates with the disease progression and prognosis of cancer patients [65, 66].

Haveman et al. reported no increase in TNF-α level was observed while a significant radiation-induced rise in circulating IL-6 levels in experimental study [67]. However, Tang et al. evaluated the effect of pelvic irradiation on IL-6 and CRP levels in patients with cervical carcinoma and found no significant difference in patients treated with external beam RT [68]. The significance failure might have been a result of an inadequate sample size. In a recent clinical study, Akmansu et al. showed a significant rise in TNF-α level with radiotherapy in all patients and also in IL-6 levels in patients treated with postoperative adjuvant radiotherapy [69]. Yuhchyau et al. analyzed 24 lung cancer patients who received radiotherapy and

evaluated the correlation between serum cytokines and radiation pneumonitis, IL-lα and IL-6 were the only 2 cytokines that correlated significantly with radiation pneumonitis [70].

BLG is one of the plants in Cruciferae family. BLG was known to have antiviral effects to against influenza, hepatitis virus and Japanese encephalitis virus. Many chemical compounds were found in BLG, including indigotin, indirubin, isatin, isaindigotidione, organic acids and amino acids. In previous studies, water-insoluble component extracted by ethanol and chloroform was proved to have anti-endotoxic effect [61]. The indirubin and alkaloid isaindigotone are part of water-insoluble component from BLG. Alkaloid isaindigotone was first isolated in 1997 by Xiaoyun Wu and Guowei Qin et al. [71] and later found to be a scavenger of superoxide [72]. Indirubin was widely studied and reported to be one of most effective compounds in BLG, have anti-inflammatory and cyclin-dependent kinases (CDK) inhibitory reaction. However, the functional mechanisms of indirubin are still not very clear. According to Liu et al. study in 1997, meisoindigo was found to be a second-generation derivative of indirubin, could down-regulate the expression of c-myb mRNA, which is one of the transcriptional regulators for expression of interferon-γ mRNA [73]. Hoessel et al. studies indirubin in 1999 and reported that indirubin suppresses cyclin-dependent kinase (CDK) activities [56]. Animal study indicated that indirubin inhibit interferon-γ production from a low concentration at which indirubin did not affect cell growth and confirmed its anti-inflammatory activity [74]. Indirubin was also found to have inhibitory reaction on RANTES mRNA expression in influenza-infected bronchial epithelial cells [75].

In our clinical trial, BLG can effectively reduce the severity of

maximal mucositis (P=0.01), improved patients' life qualities such as anorexia (P=0.002) and swallowing ability (P=0.002). Although less significance was found, BLG can lower the rest days (P=0.06), which most comment needed in illness patients who receive radiotherapy. Serum IL-6 level was significantly lower in BLG group when compared with control group (P<0.001). According to the animal study of Liu et al, they used LPS to induce elevation of TNF-α and IL-6 then measured by ELISA method. Chloroform and butanol extracted fraction of BLG could lower endotoxin-induced TNF-α and IL-6 [76]. Later Lin et al. used similar mice model and further reported that chloroform and butanol extracted BLG can inhibit mitogen-activating-protein-kinases (MAPKs, p38) thus reduced LPS-induced TNF-α and IL-6 elevation [77]. In our animal study, the similar result was found. Serum IL-1β and IL-6 are much lower in BLG groups. It confirmed the anti-inflammatory effect of BLG. Furthermore, as higher dose of BLG and lower serum IL-1β and IL-6 was found to be linear-dose significance (P=0.012 and 0.001 respectively).

In contrary to clinical study, our animal study disclosed BLG can increase immune organs weight; elevate blood leukocyte, granulocyte, and lymphocyte. But the possible mechanism is not clear. Several studies in China Mainland proposed that polysaccharides of BLG have immune-stimulating effect but the evidence was weak. This phenomenon remains further study in the future.

Chapter 6: Conclusion

BLG was used for thousands years in China and was recognized by our ancients to have anti-infection activity. We used BLG to reduce radiation mucositis clinically and in mice. These data suggested the anti-inflammatory effect and enhanced the immune cell proliferation. But the exact active compounds, mechanisms and pathways still need further analysis and evaluation.

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Table 1. The RTOG describes five grades of acute mucositis.

Grade	Mucous membrane
0	No change over base line
1	Injection; mild pain not requiring analgesics
2	Patchy mucositis which may produce an inflammatory serosanguinous discharge; may experience moderate pain requiring analgesics
3	Confluent fibrinous mucositis; may include severe pain requiring narcotics
4	Ulceration, haemorrhage or necrosis

Table 2. The RTOG describes five grades of anorexia.

Grade	Mucous membrane
0	No change over base line
1	Loss of appetite without alteration in eating habits
2	Oral intake altered without significant weight loss or malnutrition; oral nutritional supplements indicated
3	Associated with significant weight loss or malnutrition (e.g., inadequate oral caloric and/or fluid intake); IV fluids, tube feedings or TPN indicated
4	Life-threatening consequences

Table 3. The RTOG describes five grades of dyshpagia.

Grade	Mucous membrane
0	No change over base line
1	Symptomatic, able to eat regular diet
2	Symptomatic and altered eating/swallowing (e.g., altered dietary habits, oral supplements); IV fluids indicated <24 hrs
3	Symptomatic and severely altered eating/swallowing (e.g., inadequate oral caloric or fluid intake); IV fluids, tube feedings, or TPN indicated ≥24 hrs
4	Life-threatening consequences (e.g., obstruction, perforation)

Table 4. Patient characteristics

Baseline factors	BLG group N=11	Control Group N=9	*P* value
Mean age (years)	56.5±10.6	57.9±13.1	*P*=0.789[a]
Body weight	62.3±12.8	58.9±11.6	*P*=0.548 [a]
Gender			
Male	9	9	
Female	2	0	
Radiation Dose	6787.3±754.7	6820.0±651.8	*P*=0.920 [a]
Chemotherapy	5	5	
Diagnosis			
Nasopharyngeal cancer	2	2	
Oropharyngeal cancer	7	6	
Salivary gland cancer	2	1	

[a] Data was analyzed with independent F-test and present here as mean ± SD. The BLG group was no significant difference when compare with control group.

Table 5. Distribution of clinical parameters between the two groups

Clinical parameter	BLG group N=11	Control Group N=9	*P* value
Maximum mucositis grade			*P*=0.01*[a]
Grade 0	0	0	
Grade 1	3	0	
Grade 2	7	3	
Grade 3	1	6	
Grade 4	0	0	
Maximum anorexia grade			*P*=0.002**[a]
Grade 0	0	0	
Grade 1	4	0	
Grade 2	7	3	
Grade 3	0	6	
Grade 4	0	0	
Maximum swallowing grade			*P*=0.002**[a]
Grade 0	0	0	
Grade 1	4	0	
Grade 2	7	3	
Grade 3	0	6	
Grade 4	0	0	
Resting condition			
Without rest	4/11	2/9	
Rest days	1.64±2.46	5.89±6.7	*P*=0.06 [b]
Body weight change (kg)	-3.9±3.9	-4.7±4.4	*P*=0.66 [b]

* $P < 0.05$, ** $P < 0.01$ indicated the BLG group was significantly difference that compare with control group. [a] Data was analyzed with Mann-Whitney U test, [b] data was analyzed with independent F-test and present here as mean ± SD.

Table 6. Blood contents expression between BLG and control group

Serum parameter	BLG group	Control Group	*P* value [a]
WBC (/mm^3)			
Pre-treatment	5830.0±1280.1	6183.3±2143.3	*P*=0.1295
1st week	7045.6±1959.0	8471.1±7484.3	***P*=0.0003*****
3rd week	5564.6±2006.2	5087.8±2452.7	*P*=0.5416
5th week	4972.4±2196.1	6502.2±4365.0	***P*=0.0462***
7th week	4894.6±1910.7	5448.8±1946.8	*P*=0.9253
Neutrophil (/mm^3)			
1st week	4813.0±1681	5771.1±4526.9	***P*=0.0052****
3rd week	4012.6±1669.2	4143.4±1555.9	*P*=0.8587
5th week	3622.0±1776.5	4825.0±3289.6	*P*=0.0721
7th week	3597.6±1552.7	4029.1±158.2	*P*=0.7673
Monocyte (/mm^3)			
1st week	564.0±191.6	731.5±592.0	***P*=0.0017****
3rd week	497.8±207.3	609.9±379.4	*P*=0.0776
5th week	414.2±243.5	487.8±360.8	*P*=0.2432
7th week	260.2±141.7	512.4±433.0	***P*=0.0022****
Eosinophil (/mm^3)			
1st week	257.7±74.8	126.1±117.7	***P*=0.0054****
3rd week	178.9±124.2	115.4±82.2	*P*=0.2556
5th week	288.8±290.3	188.5±122.6	***P*=0.0227***
7th week	282.4±288.7	182.1±433.0	***P*=0.0019****
Lymphocyte (/mm^3)			
1st week	1437.5±595.3	1274.6±967.4	*P*=0.1524
3rd week	901.4±366.2	915.5±697.5	*P*=0.0606
5th week	615.0±275.2	948.2±663.6	***P*=0.0121***
7th week	650.4±291.1	680.2±158.2	*P*=0.1189
Hgb (mg/dl)			
1st week	13.1±2.0	13.1±1.9	*P*=0.8745
3rd week	12.9±2.0	12.3±1.3	*P*=0.2420
5th week	12.1±1.8	12.3±2.0	*P*=0.8514
7th week	11.9±1.7	11.7±2.0	*P*=0.5833

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicated the BLG group was significantly difference that compare with control group. [a] Data was analyzed with independent F-test and present here as mean ± SD.

Table 7. Serum cytokines expressions between BLG and control groups

Serum parameter	BLG group	Control Group	*P* value [a]
Blood contents			
IL-1β (pg/ml)			
1st week	9.4±10.6	5.0±7.5	*P*=0.3293
3rd week	10.0±11.8	3.5±3.8	***P*=0.0039****
5th week	11.3±19.2	9.9±12.0	*P*=0.1974
7th week	8.5±7.8	6.3±6.1	*P*=0.5347
IL-6 (pg/ml)			
1st week	13.7±4.3	62.5±60.5	***P*<0.001*****
3rd week	19.1±9.9	32.4±18.7	*P*=0.065
5th week	16.9±5.3	51.1±58.9	***P*<0.001*****
7th week	11.6±5.1	35.4±25.6	***P*<0.001*****

** $P < 0.01$, *** $P < 0.001$ indicated the BLG group was significantly difference that compare with control group. [a] Data was analyzed with independent F-test and present here as mean ± SD.

Table 8. Immune organs of irradiated mice (mg)

Group	Thymus	Spleen	Pancreas
Control	**49.8±7.6$^{###}$**	**113.1±19.4$^{###}$**	**28.5±10.6$^{###}$**
Radiation condition			
Water (R.O. 10 ml/kg/day)	25.6±3.2	29.0±0.8	9.3±1.1
BLG-L (0.195 g/kg/day, 1×)	28.4±3.8	31.0±3.6	**13.0±3.9***
BLG-M (0.585 g/kg/day, 3×)	**30.1±3.0***	**31.5±1.8***	**15.6±4.1****
BLG-H (1.170 g/kg/day, 5×)	**30.0±2.8***	**33.7±3.0****	11.0±3.1
Glutamine (0.520 g/kg/day, 1×)	**28.5±2.0***	**30.4±1.6***	11.0±3.5

$^{###}$ P<0.001 indicated the radiation condition in water group was very significantly decrease when compare with control group. * $P < 0.05$, ** $P < 0.01$ indicated the medication treatments were significantly difference that compare with the water group in radiation condition. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Table 9 . Absolute blood contents of irradiated mice (/mm^3)

Group	Leukocyte	Granulocyte	Monocyte	Lymphocyte
Control	**24643±3443$^{###}$**	**1283±266$^{###}$**	**1292±195$^{###}$**	**5676±560$^{###}$**
Radiation group				
Water (R.O. 10 ml/kg/day)	3917±826	342±47	418±138	1298±361
BLG-L (0.195 g/kg/day, 1×)	**9303±3240****	**565±87****	693±413	**2775±361***
BLG-M (0.585 g/kg/day, 3×)	**10897±2997****	**752±326***	**1090±679***	**3631±1076****
BLG-H (1.170 g/kg/day, 5×)	**8383±1584****	**735±166****	**1083±347****	**3979±688****
Glutamine (0.520 g/kg/day, 1×)	**7264±2788***	294±61	**267±85***	**1646±161***

$^{###}$ P<0.001 indicated the radiation condition in water group was very significantly decrease when compare with control group. * P < 0.05, ** P < 0.01 indicated the medication treatments were significantly difference that compare with the water group in radiation condition. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Table 10. Serum cytokines of irradiated mice (pg/ml)

Group	TNF-α	IL-1β	IL-6
Control	**7.10±1.74$^{###}$**	**4.82±2.52$^{##}$**	**215.53±58.33$^{#}$**
Radiation group			
Water (R.O. 10 ml/kg/day)	27.36±17.64	10.37±5.04	490.59±233.15
BLG-L (0.195 g/kg/day, 1×)	**13.41±8.40***	7.47±3.49	354.13±332.29
BLG-M (0.585 g/kg/day, 3×)	**7.51±1.91***	**6.06±2.00***	**279.56±114.61***
BLG-H (1.170 g/kg/day, 5×)	**7.31±1.13***	**5.43±2.47***	**211.12±71.93***
Glutamine (0.520 g/kg/day, 1×)	**9.49±3.04***	7.79±6.19	369.41±324.72

$^{#}$ $P<0.05$, $^{##}$ $P<0.01$, $^{###}$ $P<0.001$ indicated the radiation condition in water group was significantly difference when compare with control group. * $P < 0.05$ indicated the medication treatments were significantly difference that compare with the water group in radiation condition. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Table 11. Histopathologic assessment of irradiated mice.

Group	Villi number (/mm)	Villi height (mm)
Control	**12.67±0.52**[#]	**0.49±0.09**[#]
Radiation group		
Water (R.O. 10 ml/kg/day)	9.17±0.98	0.32±0.07
BLG-L (0.195 g/kg/day, 1×)	10.17±0.75	0.36±0.08
BLG-M (0.585 g/kg/day, 3×)	10.00±0.89	0.32±0.08
BLG-H (1.170 g/kg/day, 5×)	**10.83±0.75***	0.39±0.07
Glutamine (0.520 g/kg/day, 1×)	**11.00±1.26***	0.35±0.09

[#] $P<0.05$ indicated the radiation condition in water group was significantly difference when compare with control group. * $P < 0.05$ indicated the medication treatments were significantly difference that compare with the water group in radiation condition. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Figure 1. Structure of mucosa

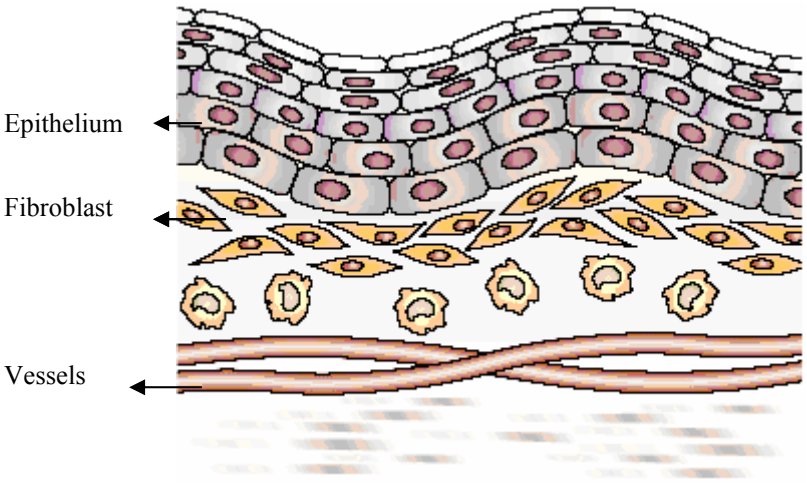


Figure 2. Possible cellular signals pathway in radiation mucositis

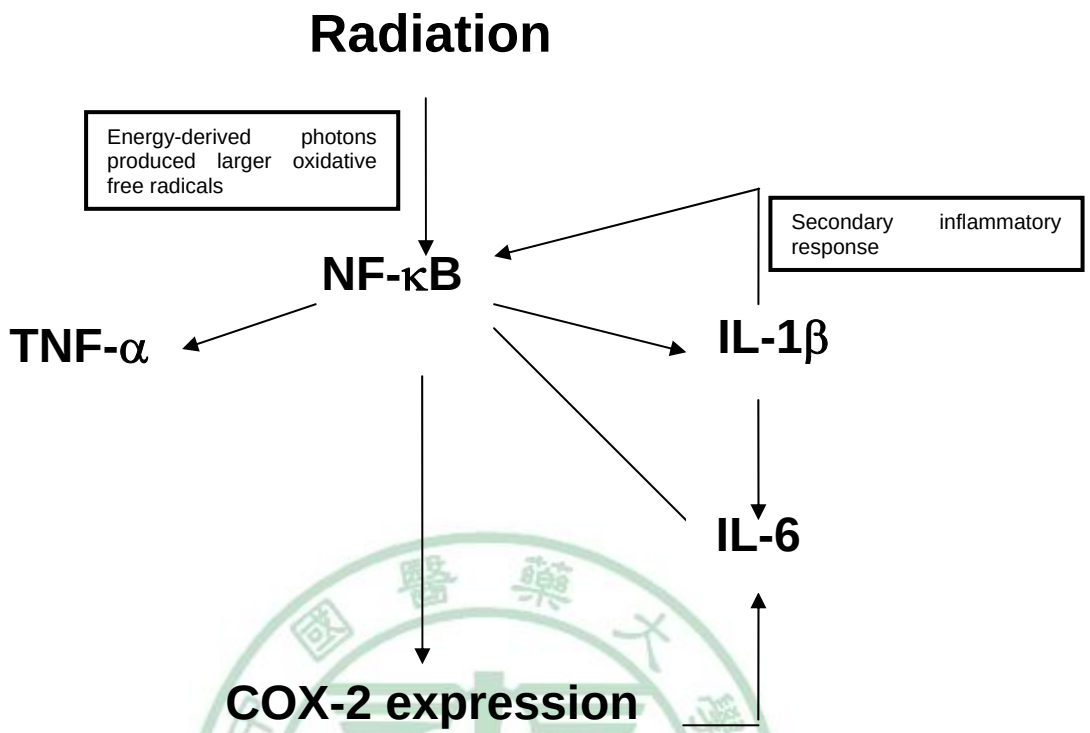
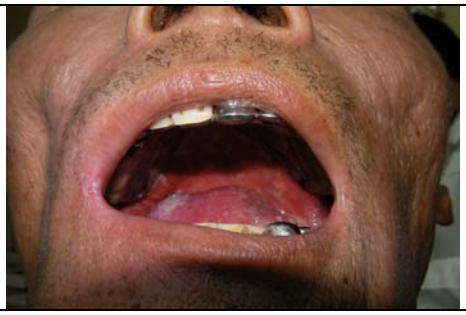
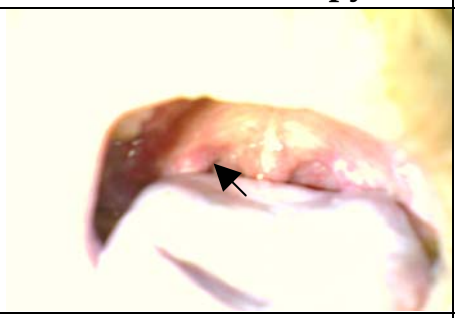
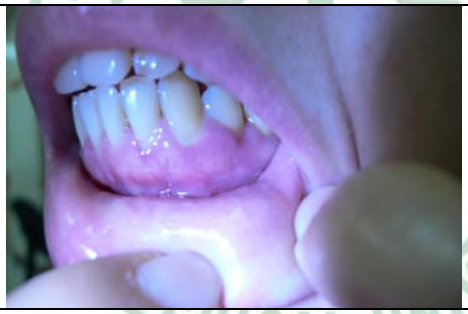
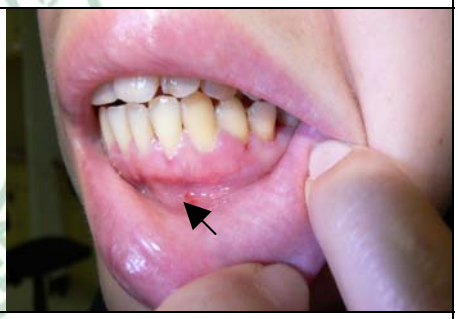
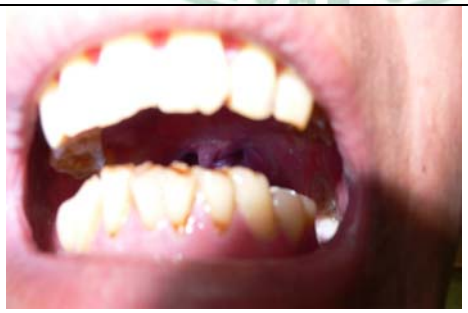
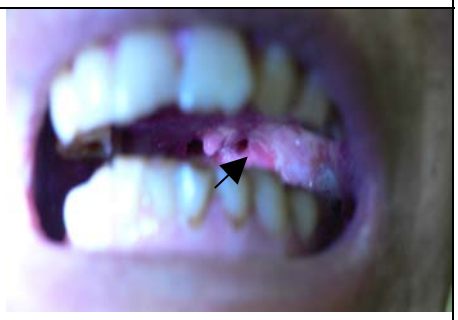
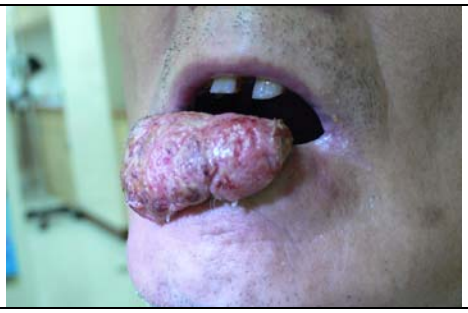
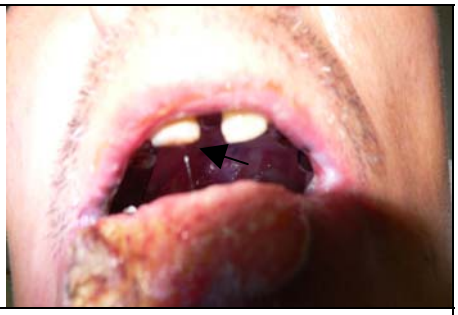
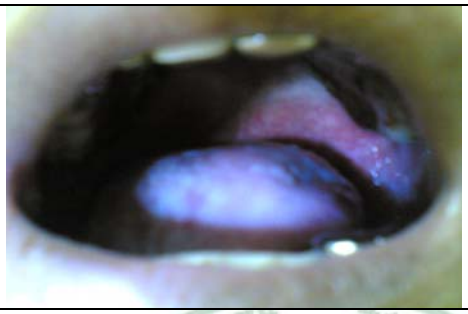
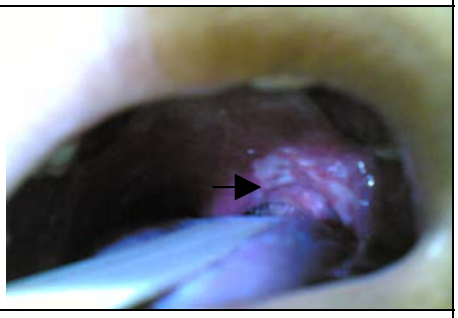
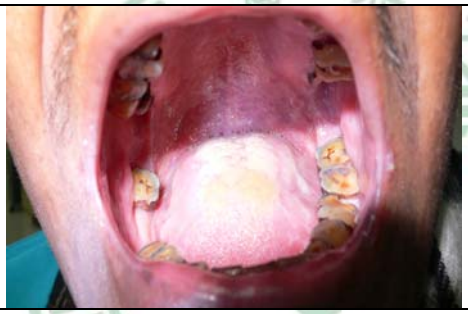
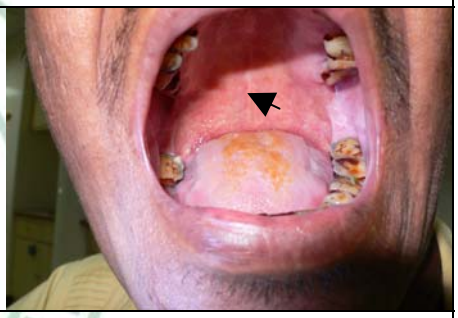
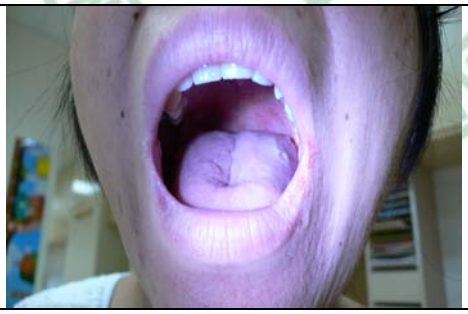
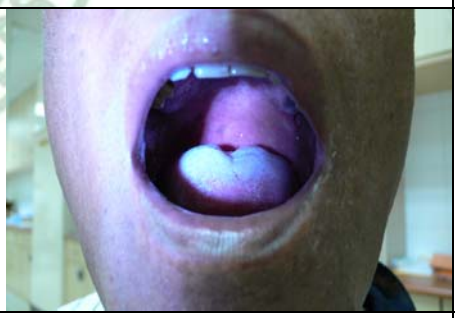
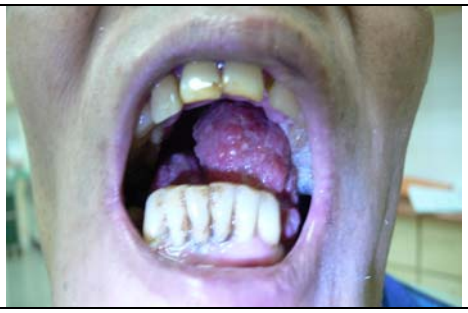
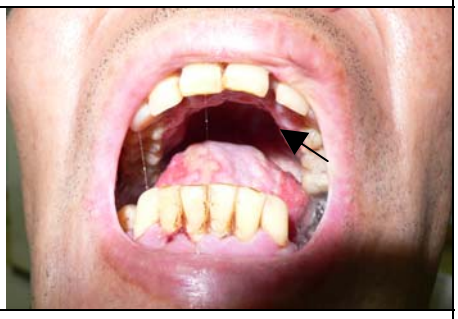


Figure 3. Clinical photography of patient oral mucosa

Group	Pre-radiotherapy	Post-radiotherapy
Control 1		
	Grade o mucositis	**Grade 2 mucositis**
Control 2		
	Grade o mucositis	**Grade 3 mucositis**
Control 3		
	Grade o mucositis	**Grade 3 mucositis**
Control 4		
	Grade o mucositis	**Grade 3 mucositis**

Control 5		
	Grade o mucositis	**Grade 3 mucositis**
Control 6		
	Grade o mucositis	**Grade 3 mucositis**
Control 7		
	Grade o mucositis	**Grade 2 mucositis**
BLG 1		
	Grade o mucositis	**Grade 1 mucositis**
BLG 2		
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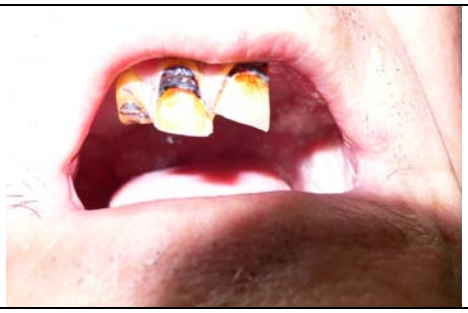
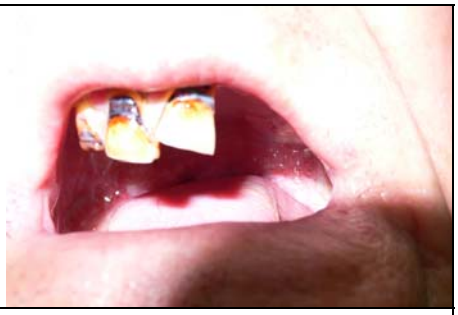
BLG 3		
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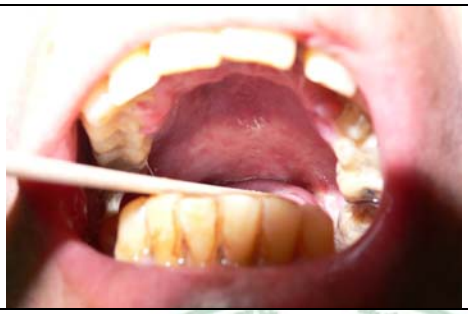
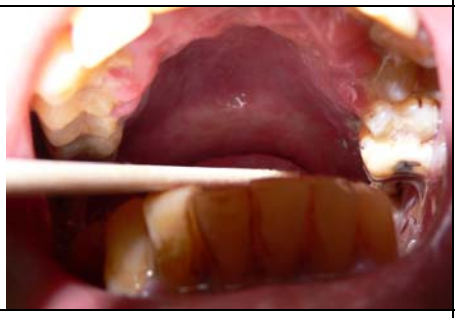
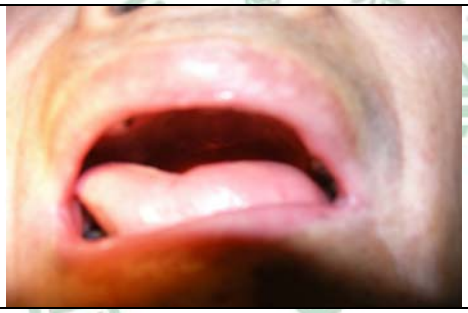
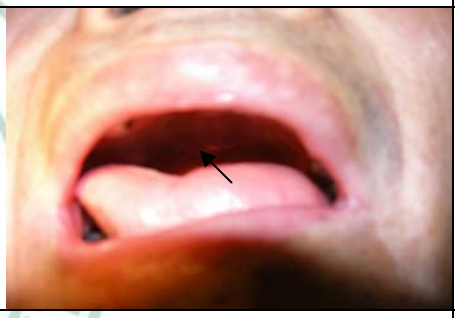
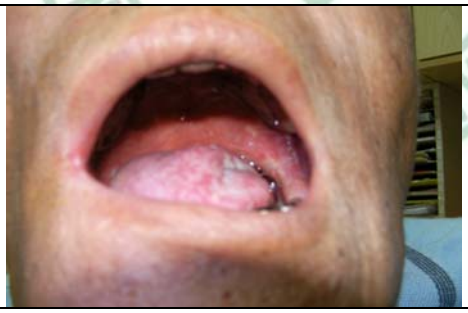
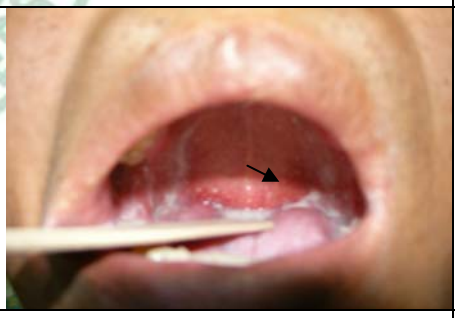
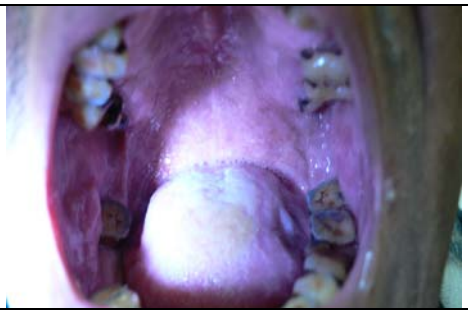
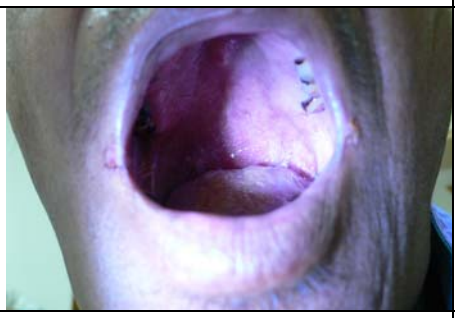
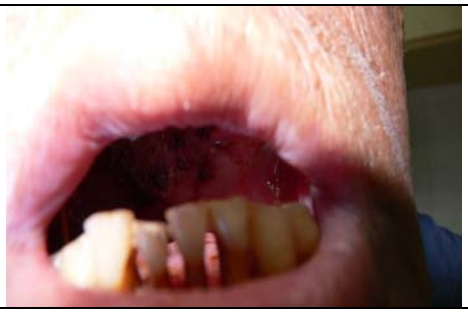
BLG 4		
	Grade o mucositis	**Grade 1 mucositis**
BLG 5		
	Grade o mucositis	**Grade 2 mucositis**
BLG 6		
	Grade o mucositis	**Grade 2 mucositis**
BLG 7		
	Grade o mucositis	**Grade 1 mucositis**

BLG 8		
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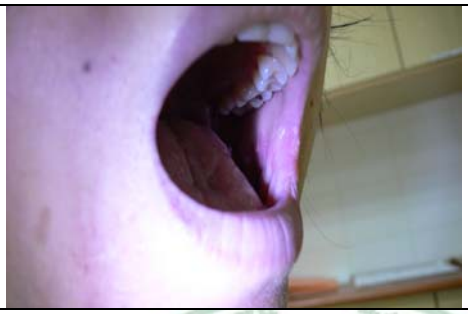
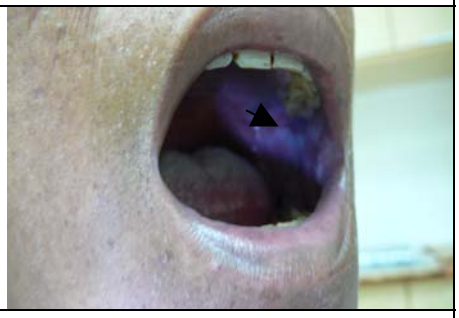
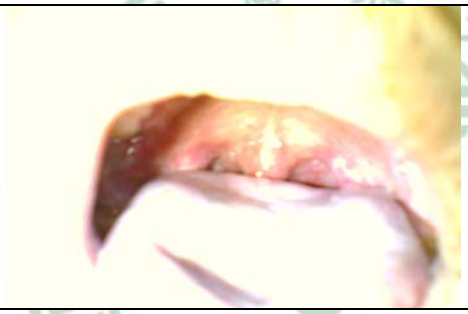
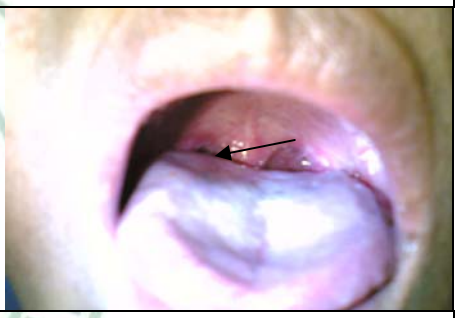
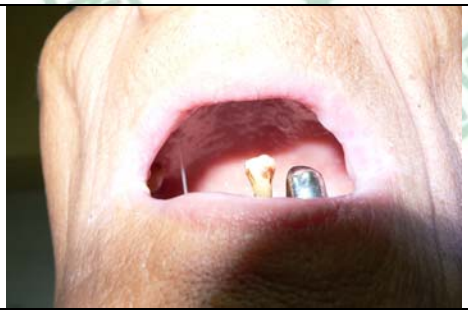
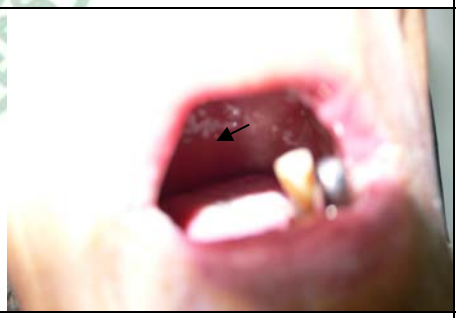
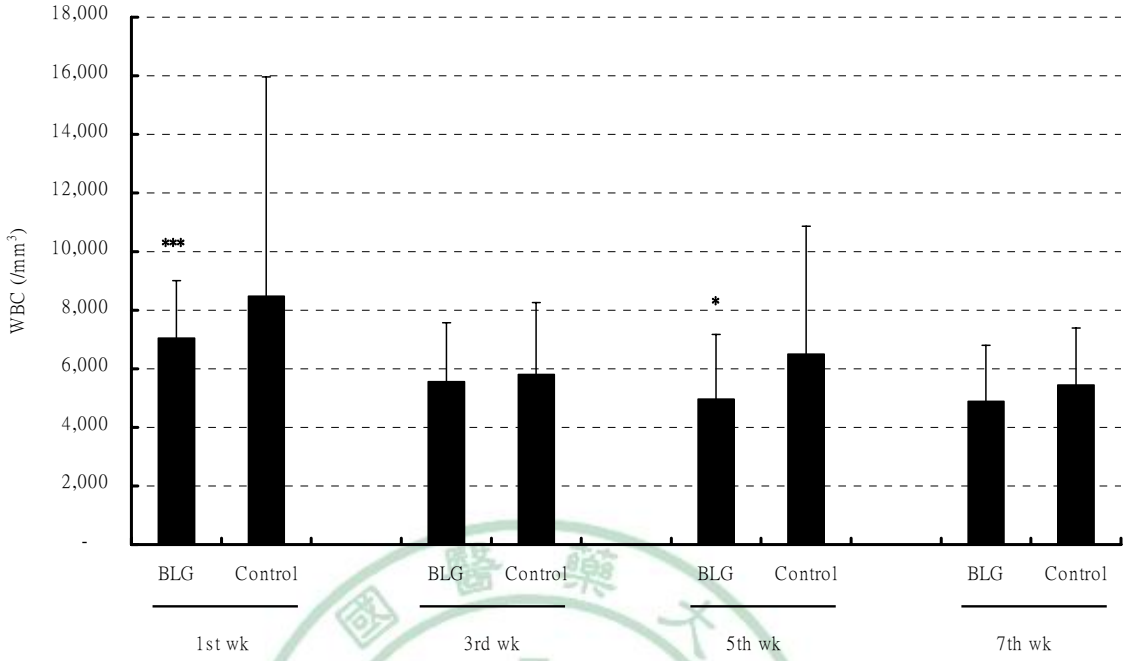
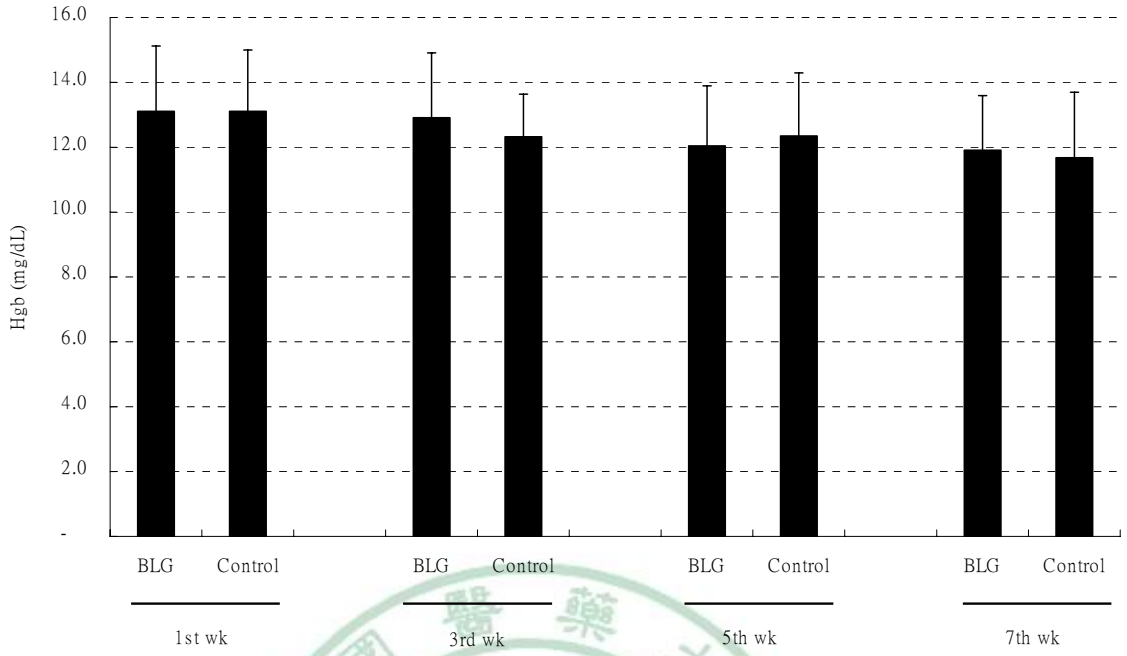
BLG 9		
	Grade o mucositis	**Grade 3 mucositis**
BLG 10		
	Grade o mucositis	**Grade 2 mucositis**
BLG 11		
	Grade o mucositis	**Grade 2 mucositis**

Figure 4. WBC count expression of human during radiotherapy



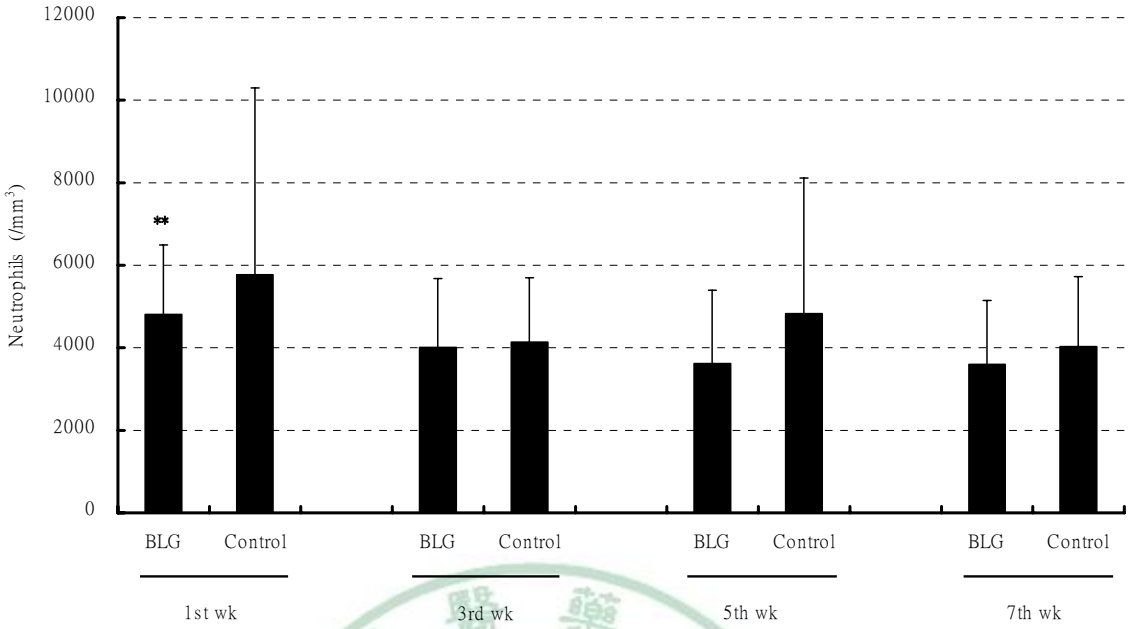
* $P < 0.05$, *** $P<0.001$ indicated the BLG treatments were significantly difference that compare with the control group. Data were analyzed with one-way ANOVA and present here as mean ± SD. This data showed that less WBC counts was found in BLG group, especially in 5th weeks after start of radiotherapy (4972.4±2196.1 vs. 6502.2±4365.0 /mm^3, P=0.046). Differential count of WBC disclosed that less eosinophils and lymphocytes. These results may explain why less WBC was found in 5th weeks.

Figure 5. Hgb expression of human during radiotherapy



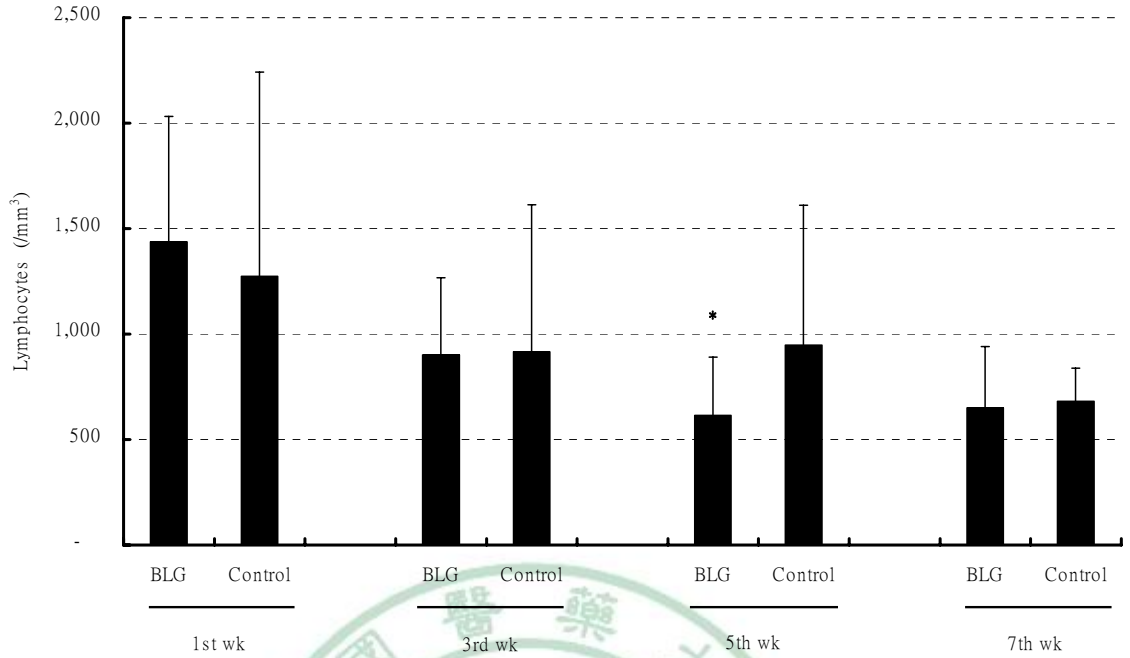
Data were analyzed with one-way ANOVA and present here as mean ± SD. This data showed no difference in hemoglobin (Hgb) level between BLG and control group. This result implicated that BLG take no effect in myeloproliferated pathway.

Figure 6. Absolute neutrophils expression of human during radiotherapy



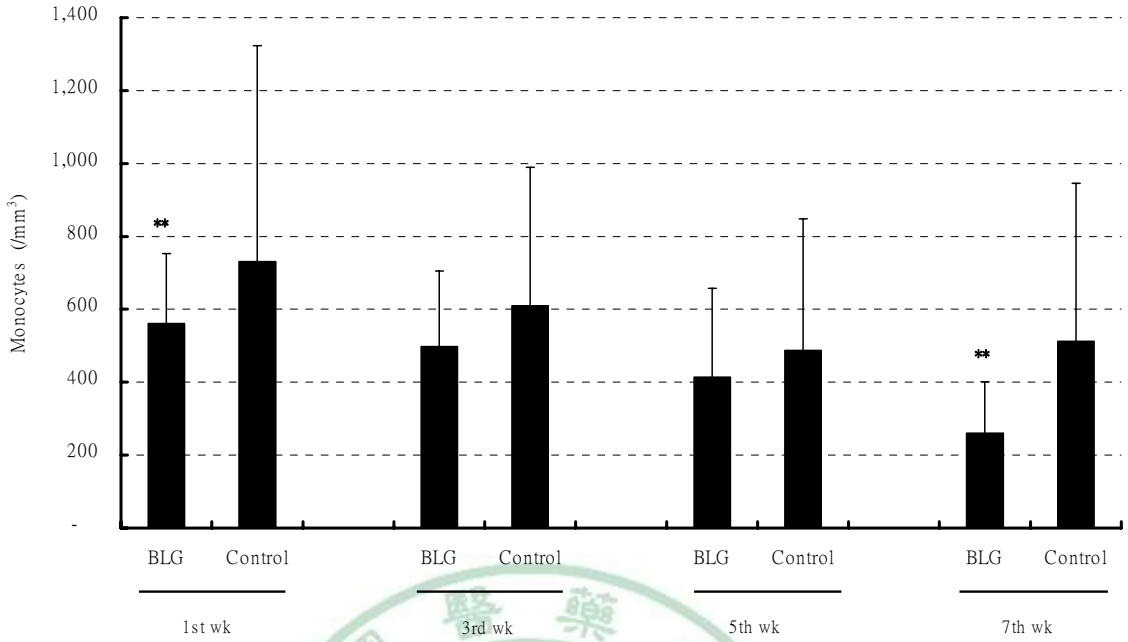
* $P < 0.05$ indicated the BLG treatments were significantly difference that compare with the control group. Data were analyzed with one-way ANOVA and present here as mean ± SD. This data disclosed lower neutrophils was observed in BLG group in 1st week when compared with control group.

Figure 7. Absolute lymphocyte expression of human during radiotherapy



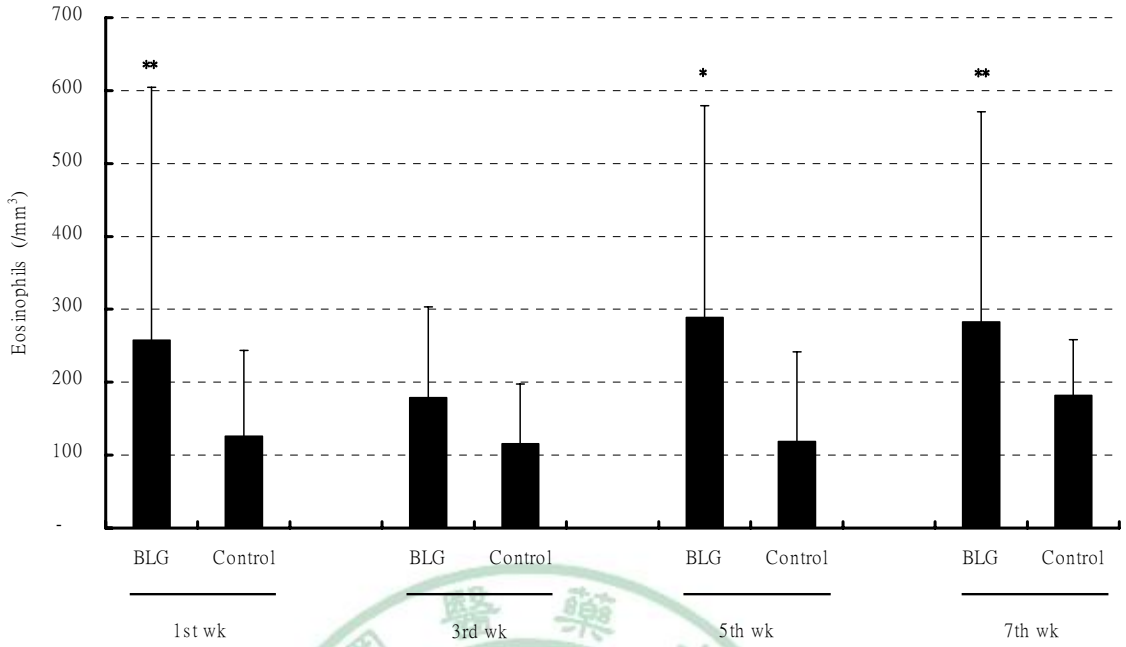
* $P < 0.05$ indicated the BLG treatments were significantly difference that compare with the control group. Data were analyzed with one-way ANOVA and present here as mean ± SD. This data showed no difference in lymphocyte expression except in 5th week, higher lymphocytes were found in control group.

Figure 8. Absolute monocyte expression of human during radiotherapy



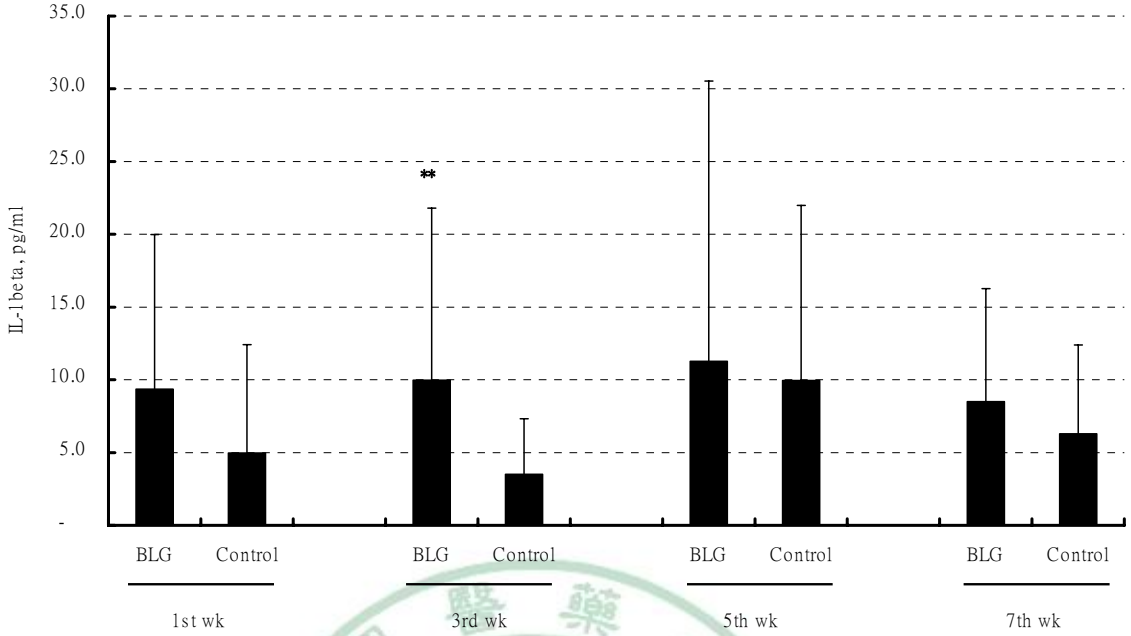
** $P<0.01$ indicated the BLG treatments were very significantly difference that compare with the control group. Data were analyzed with one-way ANOVA and present here as mean ± SD. This data disclosed that higher monocyte level was found in 1st and 7th week.

Figure 9. Absolute eosinophils expression of human during radiotherapy



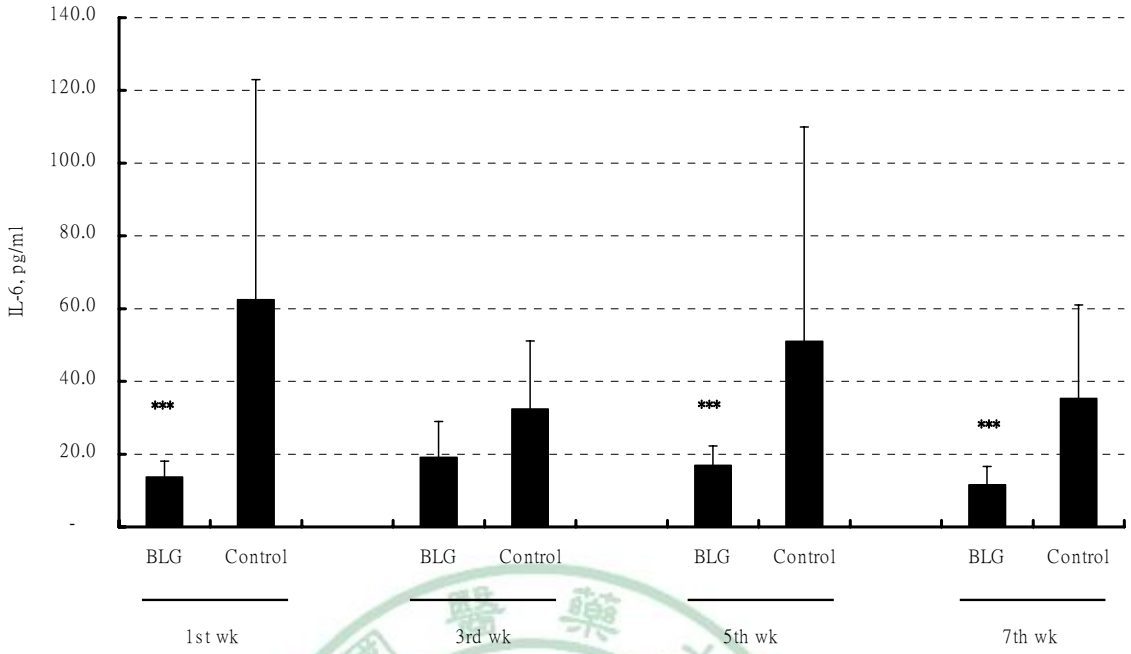
* $P < 0.05$, ** $P<0.01$ indicated the BLG treatments were significantly difference that compare with the control group. Data were analyzed with one-way ANOVA and present here as mean ± SD. This data showed that higher eosinophils were observed in BLG group in 1st, 5th and 7th week in significantly.

Figure 10. Serum IL-1βexpression of human during radiotherapy



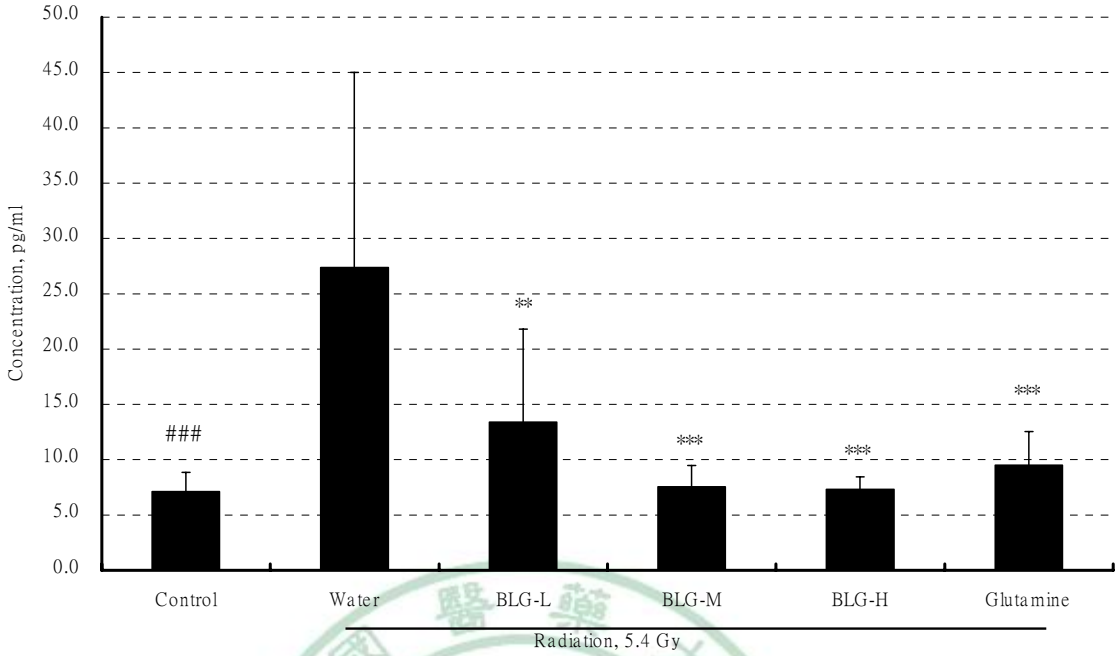
** *P*<0.01 indicated the BLG treatments were significantly difference that compare with the control group. Data were analyzed with one-way ANOVA and present here as mean ± SD. This data showed higher IL-1β was found in BLG group in 3rd week in significantly. These results disclosed the complicated cytokines expression and implicate the potential radio-protective role of BLG.

Figure 11. Serum IL-6 expression of during radiotherapy



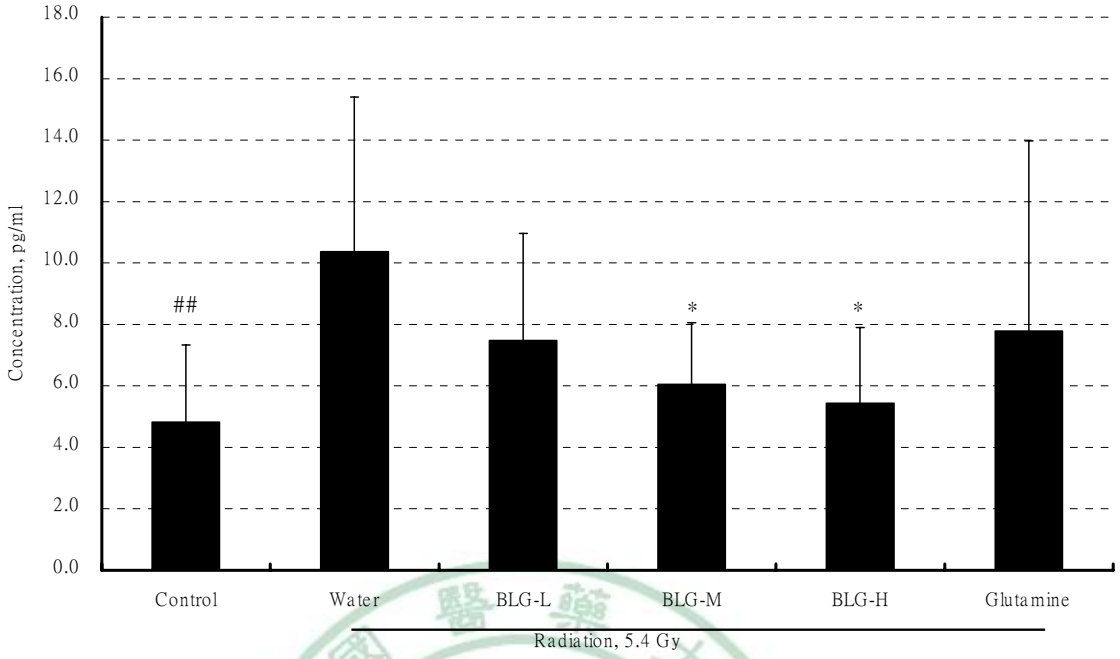
*** $P<0.001$ indicated the BLG treatments were significantly difference that compare with the control group. Data were analyzed with one-way ANOVA and present here as mean ± SD. This data showed that BLG group has lower IL-6 expression in 1st, 5th and 7th week. These results confirm the anti-inflammatory role of BLG.

Figure 12. Serum TNF-alpha (α) expression of irradiated mice



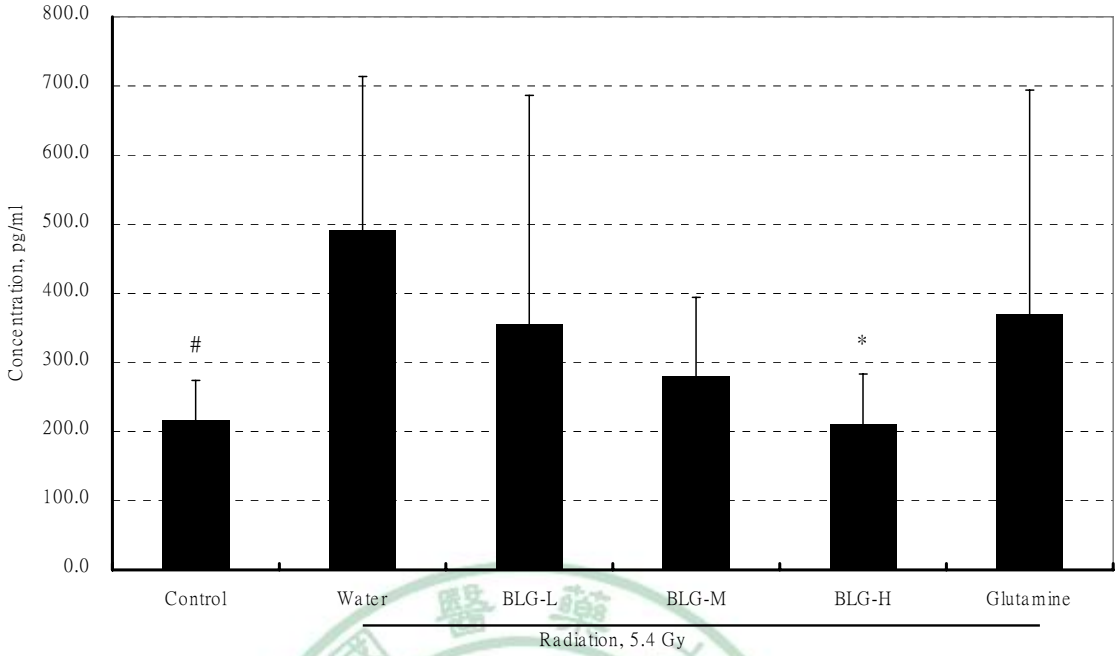
$P<0.001$ indicated the radiation condition in water group was significantly difference when compare with control group. ** $P<0.01$, *** $P<0.001$ indicated the medication treatments were significantly difference that compare with the water group in radiation condition. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Figure 13. Serum IL-1beta (β) expression of irradiated mice



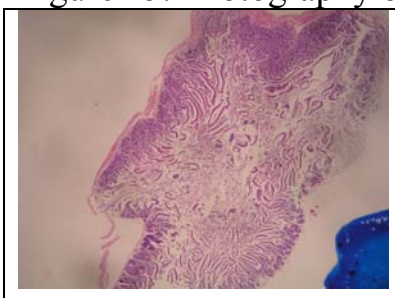
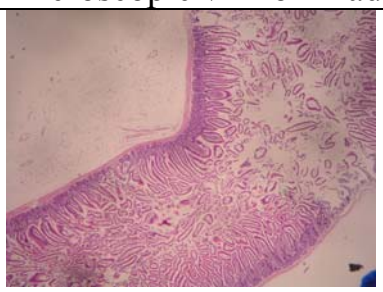
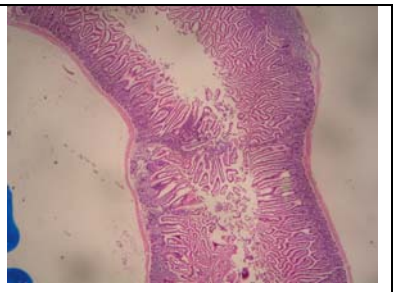
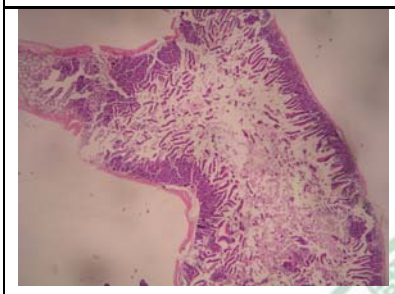
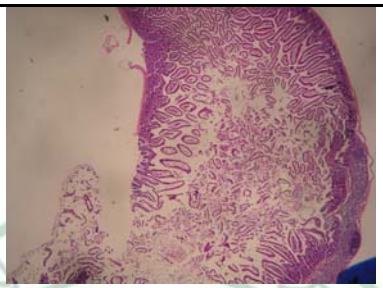
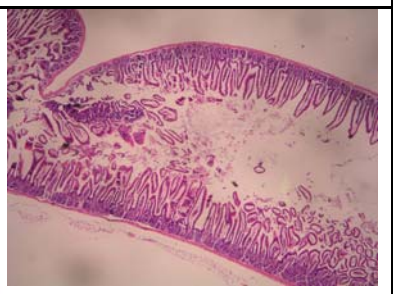
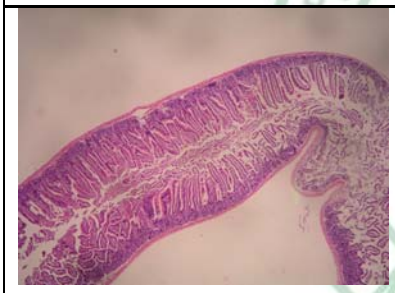
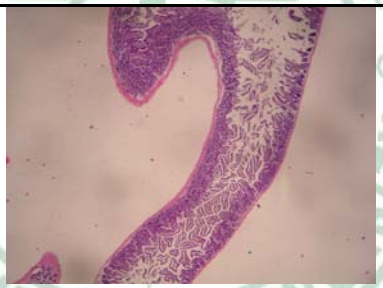
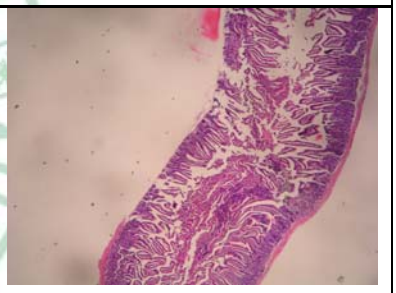
$P<0.01$ indicated the radiation condition in water group was significantly difference when compare with control group. * $P < 0.05$ indicated the medication treatments were significantly difference that compare with the water group in radiation condition. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Figure 14. Serum IL-6 expression of irradiated mice



[#] $P<0.05$ indicated the radiation condition in water group was significantly difference when compare with control group. * $P < 0.05$ indicated the medication treatments were significantly difference that compare with the water group in radiation condition. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Figure 15. Photography of microscopic villi of irradiated mouses

		
Control group Number 1	**Control group Number 2**	**Control group Number 3**
		
Control group Number 4	**Control group Number 5**	**Control group Number 6**
		
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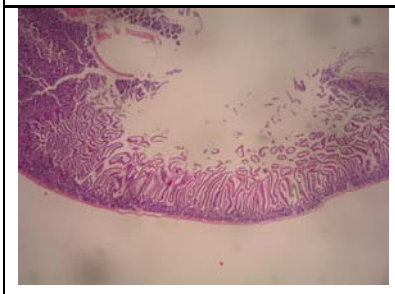	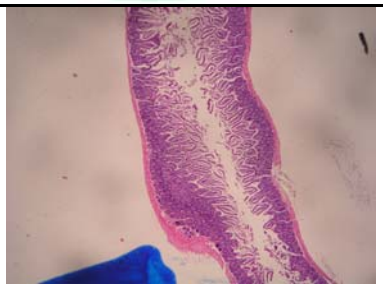	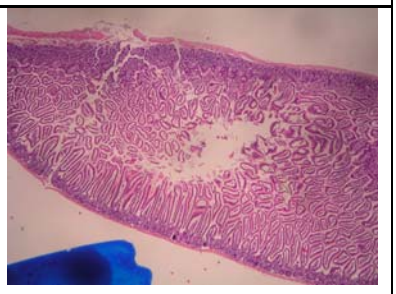
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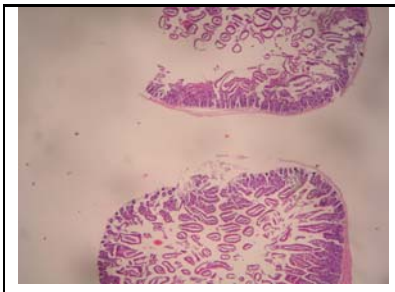	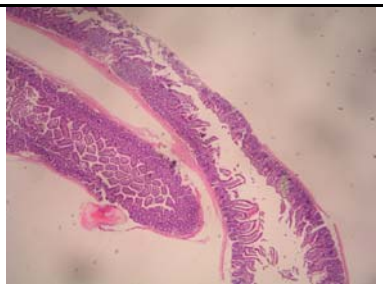	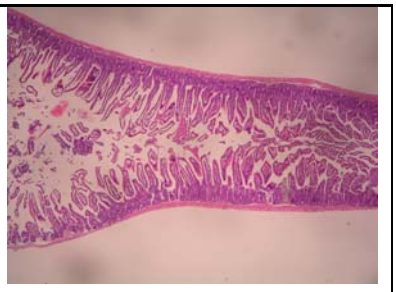
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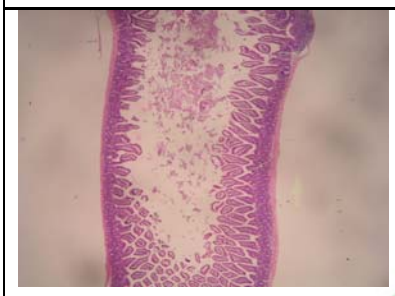	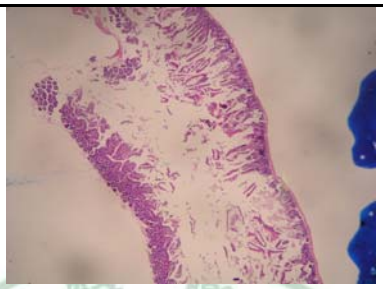	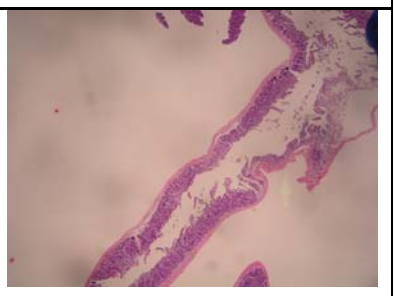
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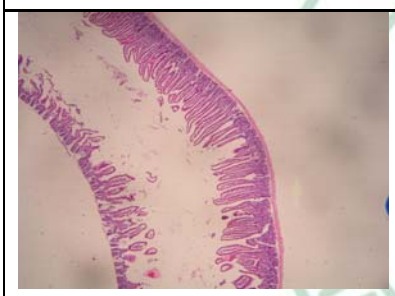	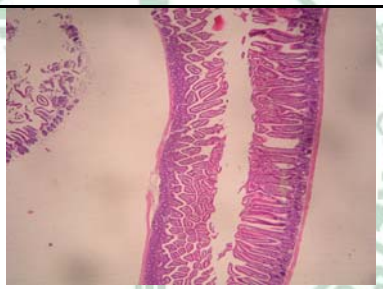	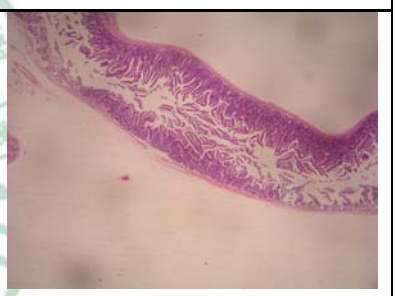
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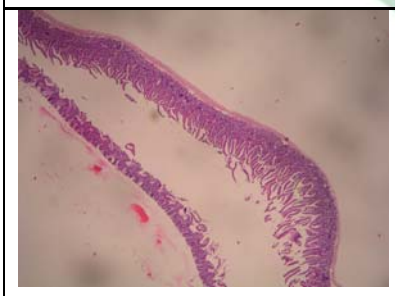	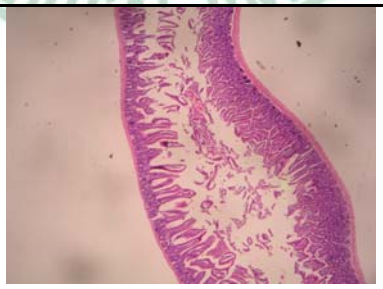	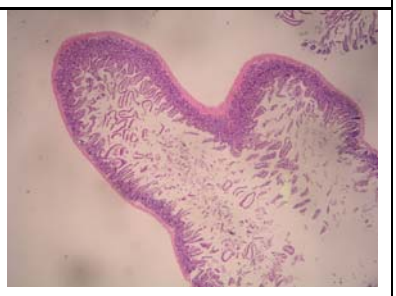
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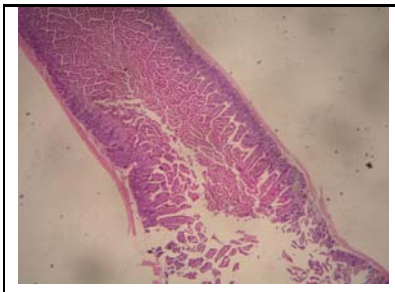	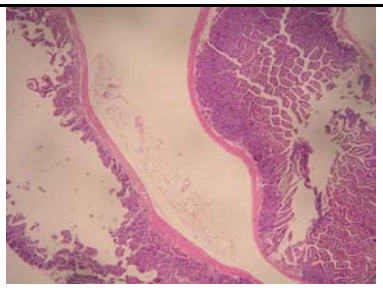	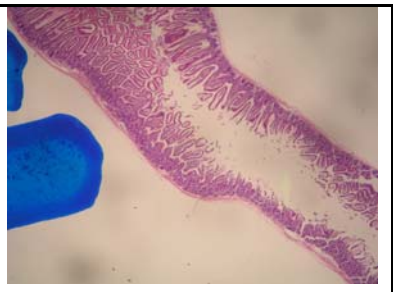
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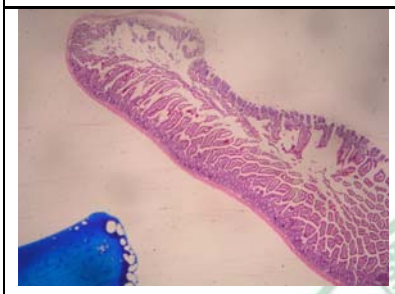	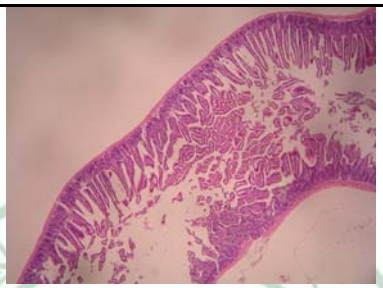	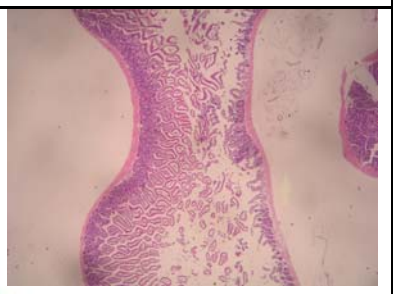
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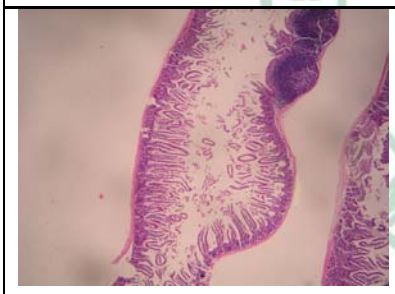
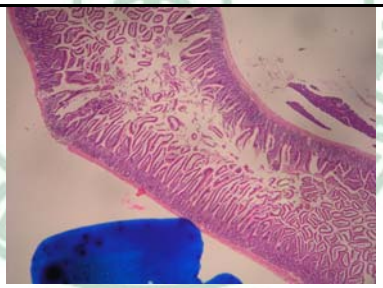
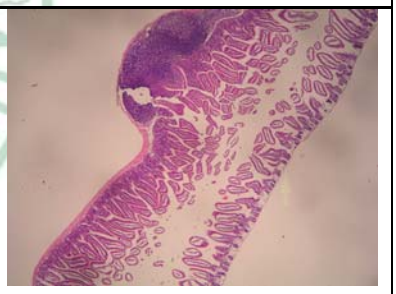
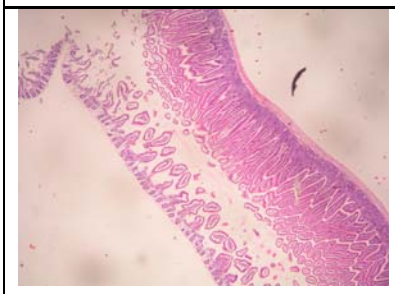
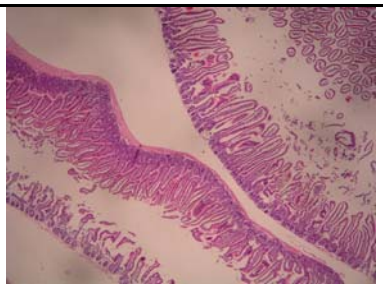
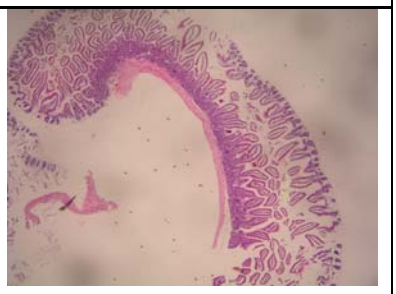
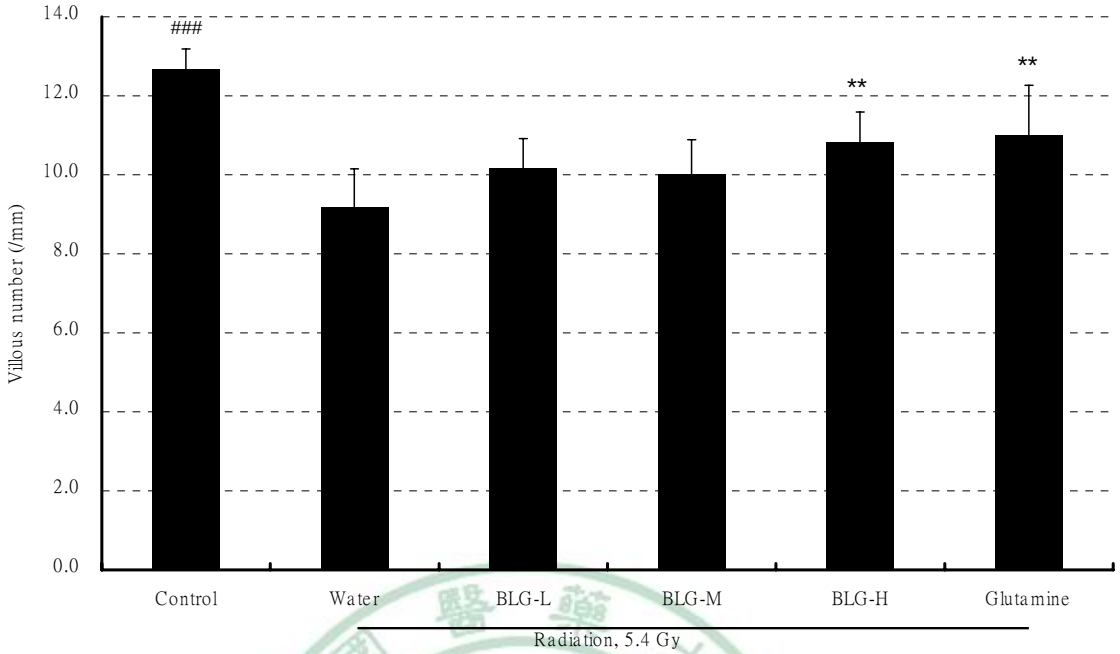
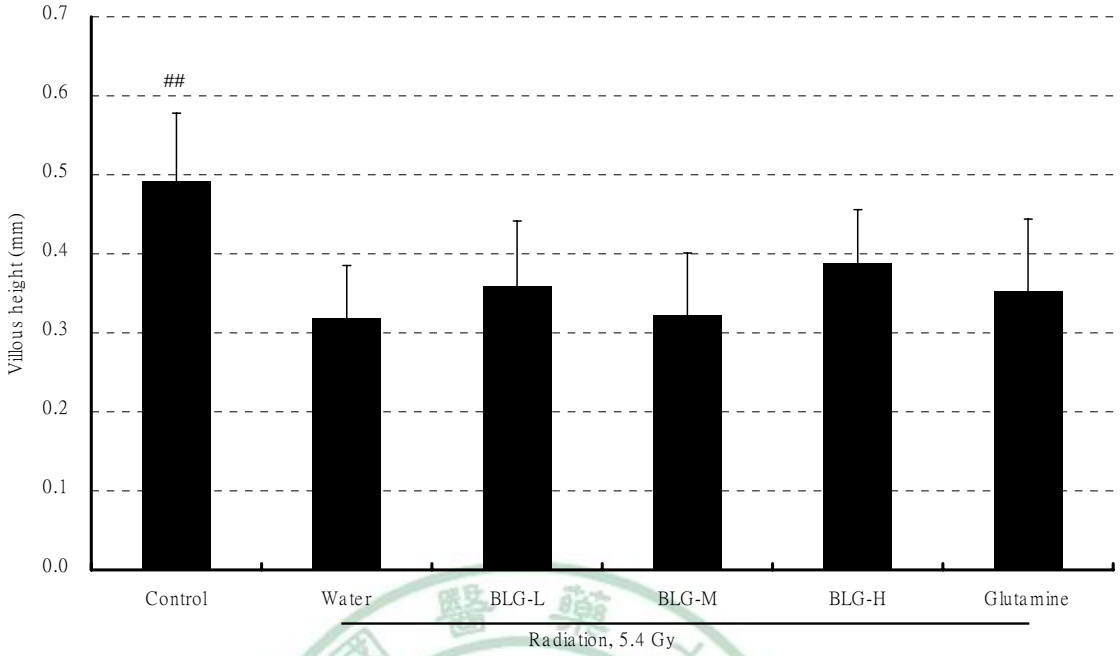
		
Naïve group Number 1	**Naïve group Number 2**	**Naïve group Number 3**
		
Naïve group Number 4	**Naïve group Number 5**	**Naïve group Number 6**

Figure 16. Microscopic villi number (/mm) of irradiated mice



$P<0.001$ indicated the radiation condition in water group was significantly difference when compare with control group. ** $P<0.01$ indicated the medication treatments were significantly difference that compare with the water group in radiation condition. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Figure 17. Microscopic villi height (mm) of irradiated mice



$P<0.01$ indicated the radiation condition in water group was significantly difference when compare with control group. There are no significantly differences in each medication treatment after radiation modeling. Data were analyzed with one-way ANOVA and present here as mean ± SD.

Appendix I 人體試驗委員會 臨床試驗審核同意書

安泰醫院人體試驗委員會審查意見表

計劃主持人：	安泰醫院放射腫瘤科 游惟強醫師	
計劃名稱：	應用 L-glutamine 以及中藥板藍根於放射治療頭頸部癌症病人放射性口腔炎之評估(Application of L-glutamine and Isatic indigoaca Fort. In radiation-induced mucositis of head and neck patient.)	編號：94006

總評： 推　　薦 V　　修正後推薦＿＿＿

修正後再審＿＿＿　不　推　薦＿＿＿

綜合委員之建議

相關 六、試驗對象 (一)對象之資格第(參)點及受試同意書 3. 試驗之主要納入與排除條件第參點中提及

病人在開始接受放射治療不能接受任何化學治療，如果須接受化學治療，必須在放射治療結束3週後。

建議修正為『病人若需接受化學治療則退出本實驗。』

審查意見：為使研究計畫順利執行得到寶貴之成果，並保障志願受試者之權益，敬請審查委員務必表達意見。篇幅不足，敬請另紙繕附，並簽名。

94 年 12 月 30 日　主任委員簽名：

交付受審單位

安泰醫院(TIAN-SHENG MEMORIAL HOSPITAL)

Appendix II 動物試驗 審核同意書

中國醫藥大學動物實驗管理小組審查同意書

Affidavit of Approval of Animal Use Protocol
China Medical University

動物實驗申請表暨同意書：95-76-D

計畫申請人：謝長奇　職稱：助理教授
單位：中西醫結合研究所　飼養/應用地點：動物中心 / 動物中心
計畫名稱：中草藥對放射線黏膜炎之保護作用

本計劃之「動物實驗計畫書」業經動物實驗管理小組 ☑實質 ☐形式審查通過。本計畫預定飼養應用之動物如下：

動物種類	動物數量	飼養及應用期間
BALB/c	90 隻	95 年 2 月 1 日至 95 年 7 月 31 日

The animal use protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC)

Protocol Title：Study the effects of Chinese medicines on repair and prevention of radiation oral mucositis of mice
Protocol No：CMUATH-95-01 95-76-D
Period of Protocol：Valid From：02/01/2006 To：07/31/2006 (mm/dd/yyyy)
Principle Investigator (PI)：Hsieh, Chang-Chi

動物實驗管理小組召集人：　日期：

IACUC Chairman：　Date：July. 31. 2006

Appendix III 板藍根科學中藥 成品檢驗報告

成品檢驗報告表

判定標對者

品名：板藍根 (GS193)　　劑型：G　　批號：265648

檢驗成績

項　　目	規　　格	結　　果	檢驗者
(一)、性狀			
性狀	本品為淺褐色顆粒，具淡香，味淡。	合格	
(二)、一般檢查			
重量差異	內容物重量與平均重量之差異不得超過平均重量之± 5%	合格	
平均重量	不得低於標誌重量。	合格	
崩散度	三十分鐘內須崩散完畢或呈軟泥狀。	合格	
(三)、雜質檢查			
重金屬試驗	檢液之顏色不得比對照液之顏色深，即 50ppm 以下。	合格	
乾燥減重	10.0% 以下	4.9%	
總灰分	5.0% 以下	3.1%	
酸不溶性灰分	1.0% 以下	0.9%	
(四)、含量測定			
水抽提物	4.0% 以上	37.2%	
稀醇抽提物	4.0% 以上	37.3%	
(五)、鑑別 板藍根	對照藥材溶液與檢液於 Rf 值約 0.42 處有淺褐色螢光斑點。	陽性	

判定：合格　　主管

論文刊於九十四年度委辦研究計畫成果發表會暨第二十一屆天然藥物研討會

中藥板藍根對放射線口腔黏膜炎之保護作用

游帷強 謝長奇

中國醫藥大學 中西醫結合研究所

在癌症治療方面，放射治療和藥物治療是二種主要之治療方式，臨床上為治癒癌症，常必須提高藥物或游離輻射的劑量，如此常會對身體的正常組織或器官造成極大的傷害。放射線黏膜炎是放射線治療癌症的組織傷害因素，黏膜炎嚴重的時候，常使放射線治療中斷，為了研究中藥板藍根是否有效抑制放射線口腔黏膜炎，本研究利用頭頸部癌患者進行放射線治療後，以板藍根沖劑 (Ban-Lan-Gen, BLG, 每次0.5 g，每天1.5 g)的劑量連續投與七週，為照組給予生理食鹽水進行對照處置，於藥物投與後於一、三、五及七週分別採血，並於第七週評估患者口腔黏膜炎之程度，並評估週邊血中發炎細胞激素之含量，結果顯示，對照組與板藍根組在年齡、體重、放射線劑量之投與無顯著差異下，板藍根組在口腔黏膜炎之程度(P=0.01)、厭食(P=0.002)與吞嚥(P=0.002)之評估均達到與對照組差異之顯著性；週邊血中發炎細胞激素之含量顯示，前發炎細胞激素IL-1表現板藍根組在個週均高於對照組，在第三週達到顯著性差異(9.9811.83 vs. 3.513.83 pg/ml, P=0.0039)，但發炎細胞激素IL-6表現方面，板藍根組在個週均低於對照組，並且分別在第一、五及七週極顯著的低於對照組(P<0.001)，在最後的口腔黏膜炎的觀察中，整體患者的口腔黏膜炎的評估中，板藍根組的處理是有效的，雖然前發炎細胞激素(IL-1)之表現均略高於對照組，但最後發炎細胞激素(IL-6)之表現是有明顯的降低，顯示整體發炎反應所受到的調控非常複雜，IL-6的表現與最後口腔黏膜炎的病理評估有相關性，值得進行進一步動物試驗或細胞培養的模式來觀察其相關分子調控機轉。

Radiotherapy plays major role in the treatment of malignancy. However, acute side effects such as radiation mucositis often cause oral pain and dysphagia of the patients, resulting in poor nutrition status. These disabilities usually influence the effect of radiotherapy. In this study, we evaluated the effect of Ban-Lan-Gen (BLG, radix of *Isatis indigotica* FORT) on acute mucositis and dermatitis induced by radiation. In clinical trial there were total 20 head and neck cancer patients were randomized into two groups: 1. Control group with only normal saline, 2. BLG group: We prophylactic application of BLG consisted of gargling and then swallowing the BLG preparation on the irradiated oral mucosa as radiotherapy was being carried out. This was compared with control patients who received routine conventional analgesics and skin care. Therapeutic application was started on the first day of radiotherapy. We evaluated of acute radiation mucositis and dermatitis according to the gold standard scale proposed by RTOG. The clinical trial showed BLG can reduce the severity of radiation mucositis (P=0.031), functional mucositis (P=0.002), anorexia (P=0.002), swallowing (P=0.002). Serum IL-6 was significantly reduced at 1st, 5th , and 7th weeks peripheral blood harvest. BLG can improved radiation mucositis clinically. We postulated that BLG has ability to reduce the mucosal damage from radiation. But IL-1 expression in BLG group was higher than the control group in each peripheral blood harvest and significantly higher in 3rd weeks. The modulation of the inflammatory response in complicated in vivo, there should be further animal or in vitro culture system to evaluated the anti-inflammatory effect of BLG.

Materials and methods

Chemicals, kits and herb medicine preparation

Tris, EDTA, Triton X-100, and other chemicals were purchased from Sigma-Aldrich, Inc. IL-1, IL-6 and TNF- levels were measured by using the ELISA kit purchased from eBioscience (ELISA Ready-SET-Go, eBioscience, SanDiago, USA) Radix of *Isatis indigotica* FORT (Ban-Lan-Gen, BLG) was purchased from Sun-Ten Pharmaceutical Co. Ltd. in Taiwan that was obtained as brown dry powder power. This BLG powder was composed of radix isatidis concentrated extracts and radix isatidis powder with absorption ratio to 2:1.

Patients and treatment

Patients with head and neck cancer receiving radiotherapy were entered into this trial. At least one-half of the oral cavity mucosa needed to be included in the fields of irradiation. Patients had to tolerate solid food at study entry. The radiation schedule was 1.8 Gy/fraction, 5 fractions per week and 25 fractions for initial fields. Patients were ineligible for this study if they had mouth sores, diabetes, trismus, underwent chemotherapy, used other prophylactic drugs or mouthwashes, or had Karnofsky's Performance Status higher than 70. Patients were sequentially divided to two treatment arms: (I) BLG group: 0.5 g BLG in 30 ml normal saline; (II) placebo: 30 ml normal saline. BLG 8.0 g in 480 ml normal saline was prepared for suspension in a plastic bottle. The solution was stored in the refrigerator; shaking the bottle before administration was mandatory. Patients mouth-washed 30 ml test solution for 3 minutes and swallowed it before meals daily.

Mucositis evaluation

Radiotherapy was delivered with a 10 MV linear accelerator with intensity-modulated radiotherapy technique and a cervical anterior field initially. Patients received radiotherapy at 1.8 Gy per fraction daily, 5 days a week, in the initial large fields. Weight was checked once a week. Patients were to be examined by radiation oncologists each day of treatment. The subjective severity of mucositis was graded as follows: Grade 0: no pain; Grade 1: painful mucositis not necessitating a change in oral intake; Grade 2: painful mucositis restricting intake to soft foods; Grade 3: painful mucositis restricting oral intake to liquids; Grade 4: mucositis preventing oral intake.

The objective severity of mucositis was graded as follows according to the RTOG/EORTC criteria: Grade 0: no mucositis; Grade 1: injected mucosa; Grade 2: patchy mucositis; Grade 3: confluent fibrinous mucositis; Grade 4: ulceration, hemorrhage, or necrosis. Medication was prescribed when the patient became symptomatic. Parameters were documented at the beginning of radiotherapy: (I) grade of mucositis each fraction (from patient's subjective complaint and evaluation of physician); (II) body weight change. Distributions of gender, age, and diagnosis and radiation dose between the two arms were calculated by Fisher's exact test. Mean maximum grade of mucositis, mean body weight change, and mean fraction number of grade 14 mucositis, anorexia and difficulty in swallowing were compared by the Mann-Whitney U test. *P*-values less than 0.05 were considered to indicate statistical significance.

Cytokines determination

Cytokines determination was followed by standard protocol from data sheet of kits. Briefly, after coat and block in 96 well ELISA plate with capture antibody in and washed out the non-binding antibody with wash buffer. Serum (100 μl) from patients was add to each well and incubated for four hours and wash out the non-specific binding. Enzyme (HRP) linked ideotypic antibody was used to detected the specific cytokine and colorized subtracts (TMB) was indicated and compared with standard curve for determination.

Result

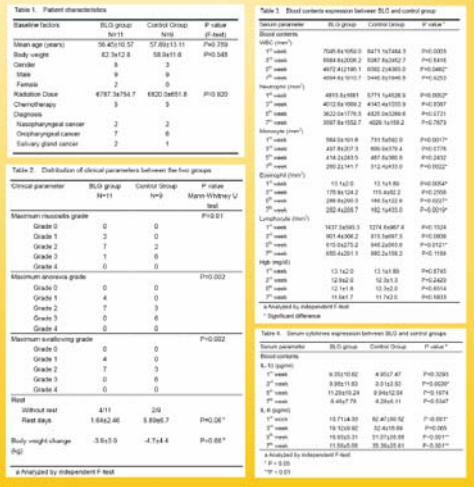


Table 1. Patient characteristics

Baseline factors	BLG group N=11	Control Group N=9	P value (F-test)
Mean age (years)	56.45±10.57	57.89±13.11	P=0.759
Body weight	62.3±12.8	58.9±11.6	P=0.548
Gender			
Male	8	9	
Female	3	0	
Radiation Dose	4787.3±754.7	4820.0±651.8	P=0.920
Chemotherapy	3	3	
Diagnosis			
Nasopharyngeal cancer	3	3	
Oropharyngeal cancer	7	6	
Salivary gland cancer	3	1	

Table 2. Distribution of clinical parameters between the two groups

Clinical parameter	BLG group N=11	Control Group N=9	P value Mann-Whitney U test
Maximum mucositis grade			P=0.01
Grade 0	0	0	
Grade 1	3	0	
Grade 2	7	2	
Grade 3	1	6	
Grade 4	0	0	
Maximum anorexia grade			P=0.002
Grade 0	0	0	
Grade 1	4	0	
Grade 2	7	3	
Grade 3	0	6	
Grade 4	0	0	
Maximum swallowing grade			P=0.002
Grade 0	0	0	
Grade 1	4	0	
Grade 2	7	3	
Grade 3	0	6	
Grade 4	0	0	
Rest			
Without rest	4/11	2/9	
Rest days	1.64±2.46	5.89±6.7	P=0.06*
Body weight change (kg)	-3.6±3.9	-4.7±4.4	P=0.66*

a Analyzed by independent F-test

Table 3. Blood contents expression between BLG and control group

Serum parameter	BLG group	Control Group	P value*
Blood contents			
WBC (mm³)			
1st week	7585.8±1050.0	8471.1±7484.5	P=0.0005
3rd week	6984.6±3208.2	9387.8±2452.7	P=0.5416
5th week	4972.4±2195.1	8392.2±4360.0	P=0.0462*
7th week	4994.4±1910.7	9446.8±1946.9	P=0.4253
Neutrophil (mm³)			
1st week	4893.8±1681	5771.1±4236.5	P=0.0002*
3rd week	4012.6±1656.2	4340.4±1333.9	P=0.8367
5th week	3622.0±1776.5	4826.0±3269.6	P=0.5721
7th week	3097.8±1552.7	4826.1±1558.2	P=0.7573
Monocyte (mm³)			
1st week	564.0±101.8	731.5±592.9	P=0.0017*
3rd week	497.8±257.3	899.0±570.4	P=0.0776
5th week	474.2±243.5	487.8±390.9	P=0.2432
7th week	290.2±141.7	512.4±433.0	P=0.0022*
Eosinophil (mm³)			
1st week	13.1±2.0	13.1±1.89	P=0.0054*
3rd week	178.9±124.2	115.4±82.2	P=0.2709
5th week	286.8±290.3	146.5±132.9	P=0.0227*
7th week	282.4±268.7	182.1±433.0	P=0.0019*
Lymphocyte (mm³)			
1st week	1637.3±995.3	1274.6±967.4	P=0.1524
3rd week	901.4±306.2	815.5±997.5	P=0.0808
5th week	815.0±275.2	946.2±950.8	P=0.0121*
7th week	650.4±201.1	880.2±158.2	P=0.1159
Hgb (mg/dl)			
1st week	13.1±2.0	13.1±1.89	P=0.8745
3rd week	12.8±2.0	12.3±1.3	P=0.2409
5th week	12.1±1.8	12.3±2.0	P=0.8514
7th week	11.5±1.7	11.7±2.0	P=0.5833

a Analyzed by independent F-test

* Significant difference

Table 4. Serum cytokines expression between BLG and control groups

Serum parameter	BLG group	Control Group	P value*
Blood contents			
IL-1β (pg/ml)			
1st week	9.35±10.62	4.95±7.47	P=0.3293
3rd week	9.89±11.83	3.01±3.82	P=0.0029*
5th week	11.28±19.29	9.94±12.04	P=0.1974
7th week	8.46±7.79	4.28±6.11	P=0.0347
IL-6 (pg/ml)			
1st week	15.71±4.39	62.4±100.52	P=0.001*
3rd week	19.12±9.92	52.4±18.89	P=0.065
5th week	16.93±5.31	31.37±16.68	P=0.001**
7th week	11.59±5.58	35.36±31.61	P=0.001**

a Analyzed by independent F-test

* P < 0.05

** P < 0.01

Discussion

BLG is one of the plants in Cruciferae family. BLG was known to have antiviral effects to against influenza, hepatitis virus and Japanese encephalitis virus. Many chemical compounds were found in BLG, including indigotin, indirubin, isatin, isaindigotidone, organic acids and amino acids. In previous studies, water-insoluble component extracted by ethanol and chloroform was proved to have anti-endotoxic effect. The indirubin and alkaloid isaindigotone are part of water-insoluble component from BLG. Alkaloid isaindigotone was first isolated in 1997 by Xiaoyun Wu and Guowei Qin et al. and later found to be a scavenger of superoxide. Indirubin was widely studied and reported to be one of most effective compounds in BLG, have anti-inflammatory and cyclin-dependent kinases (CDK) inhibitory reaction. However, the functional mechanisms of indirubin are still not very clear. According to Liu et al. study in 1997, meisoindigo was found to be a second-generation derivative of indirubin, could down-regulate the expression of c-myb mRNA, which is one of the transcriptional regulators for expression of interferon-γ[16]. Hoessel et al. studies indirubin in 1999 and reported that indirubin suppresses cyclin-dependent kinase (CDK) Activities. Animal study indicated that indirubin inhibit interferon- production from a low concentration at which indirubin did not affect cell growth and confirmed its anti-inflammatory activity [18]. Indirubin was also found to have inhibitory reaction on RANTES mRNA expression in influenza-infected bronchial epithelial cells [19].

In our clinical trial, BLG can effectively reduce the severity of maximal mucositis (P=0.01), improved patients' life qualities such as anorexia (P=0.002) and swallowing ability (P=0.002). Although less significance was found, BLG can lower the rest days (P=0.06), which most comment needed in illness patients who receive radiotherapy. Serum IL-6 level was significantly lower in BLG group when compared with control group (P<0.001). According to the animal study of Liu et al, they used LPS to induce elevation of TNF-α and IL-6 then measured by ELISA method. Chloroform and butanol extracted fraction of BLG could lower endotoxin-induced TNF-α and IL-6 [20]. Later Lin et al. used similar mice model and further reported that chloroform and butanol extracted BLG can inhibit mitogen-activating-protein-kinases (MAPKs, p38) thus reduced LPS-induced TNF-α and IL-6 elevatio. In this study, serum IL-6 was significantly reduced at 1st, 5th , and 7th weeks peripheral blood harvest. BLG can improve radiation mucositis clinically. We postulated that BLG has ability to reduce the mucosal damage from radiation. But IL-1 expression in BLG group was higher than the control group in each peripheral blood harvest and significantly higher in 3rd weeks. The modulation of the inflammatory response in complicated in vivo, there should be further animal or in vitro culture system to evaluate the anti-inflammatory effect of BLG.

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