ABSTRACT

BACKGROUND: Mucositis is a common side-effect of high-dose chemotherapy regimens. The black seed Nigella sativa L (NS) represents a rich source of thymoquinone (TQ) with the potential to exert anti-inflammatory and anti-neoplastic effects. We evaluated NS for its capacity to decrease the severity of chemotherapy-induced mucositis in a rat model.

METHOD: twenty five male albino rats were randomly divided into control group, untreated group and treated group. A model of chemotherapy induced mucositis was established by intraperitoneal injection of 5-flouro-uracil (FU) [150 mg/kg] and the right cheek pouch was scratched with a wire brush to induce mucositis. rats in the untreated group received physiologic saline orally. The rats in treated group were gavaged with 1 ml NS seed extract (400 mg/kg) daily (day 5-13). Rats were sacrificed at day 14, the cheek pouch areas were excised and prepared for histological and immunohistochemical analysis using Bcl-2 and PCNA immunolabelling. RESULTS: Compared with controls and 5-FU untreated rats, NS decreased the histologically observed damage of the cheek mucosa in the mucositis model, reduced Bcl-2 immunoreactivity and increased expression of PCNA. Based on these observations NS seemed to attenuate the 5-FU-induced reduction of mucosal thickness and ameliorate oral mucosal damage induced by 5-FU in rats. NS may represent a promising prophylactic adjunct to conventional chemotherapy for reducing the severity of oral mucositis.

INTRODUCTION

Chemotherapy- and radiotherapy-induced oral mucositis represents a therapeutic challenge frequently encountered in cancer patients. This side effect causes significant morbidity and may delay the treatment plan, as well as increase therapeutic expenses. The pathogenesis of this debilitating side effect can be attributed to the direct mucosal toxicity of cytotoxic agents and ionizing radiation and to indirect mucosal damage caused by a concomitant inflammatory reaction exacerbated in the presence of neutropenia, and the emergence of bacterial, mycotic, and viral infections.

Nigella sativa L. (Ranunculaceae family) seeds, commonly known as black seed or black cumin, have been employed for thousands of years as a spice and food preservative, as well as a protective and curative remedy for numerous disorders (1,2).
Many studies have been conducted, particularly during the last two decades, on the effect of (NS) L. seed extracts on various body systems in vitro or in vivo. The pharmacological investigations of the seed extracts reveal a broad spectrum of activities including immunopotentiation (3) and antihistaminic (4), antidiabetic (5), anti-hypertensive (6), anti-inflammatory (7), and antimicrobial activities (8). Many of these activities have been attributed to the quinone constituents of the seed (9, 10). Furthermore, black seed preparations may have a cancer chemopreventive potential and may reduce the toxicity of standard antineoplastic drugs (11). In fact, topical application of a black seed extract inhibited the two-stage initiation-promotion of skin carcinogenesis in mice by dimethylbenz[a]anthracene (croton oil) (11). In addition, others have reported an antitumor activity of some crude and purified components of N. sativa.

At present the basic strategies dealing with oral mucositis aim at pain relief and prevention of infectious complications. The current approaches to management include frequent oral bicarbonate rinses, topical anesthetics and analgesics. Other approaches include the use of medications to reduce exposure of the oral mucosa to chemotherapeutic drugs that are secreted in saliva. Antimicrobial approaches have met with conflicting results, little effect being seen with systemic as well as topical antimicrobials in the prevention of mucositis although there may be reduction in oral colonization by Candida.

The efficacy of the aforementioned conventional treatments is considerable; however the failure rate is still often. Many researchers are now seeking alternatives to conventional medicine such as the use of herbal medicines or phytomedicinal products as mouth gargle have been clinically tested and proved to be useful methods against oral mucositis prevention and sharp pain alleviation from the chemotherapy.

This approach of employing botanical and herbal therapies needs additional study and investigation to determine its effectiveness with respect to the prevention of chemotherapy – induced oral mucositis, symptom management as well as to determine appropriate doses and frequencies of intervention. This study was designed to determine the efficacy of NS L seed extract as an adjuvant treatment of chemotherapy induced oral mucositis developed in albino rats model

**MATERIAL AND METHODS**

Twenty five adult Albino rats weighing 250-300 g are used in this study: Animals were randomly divided into three groups. Group A: 5 rats served as rats control. Two experimental groups each of 10 rats, the two experimental group received intraperitoneal injection of 5 flourouracil 5-FU (150 mg/kg) for 5 days and the right cheek pouch was scratched with a wire brush to induce mucositis. Group B: Untreated group received physiologic saline orally. Group C: treated group were gavaged with 1 ml NS seed extract (400 mg/kg) daily (day 3-11). Rats were sacrificed at day 12. The cheek pouch areas were excised and prepared for histological examination and immunohistochemical analysis using Bcl-2 and PCNA immunolabelling.

**Histological Method**

The cheeks of the rats dissected and the lesions in the experimental groups were located. The lesions were sectioned and fixed in 10% formalin at 4°C, and then the samples were routinely processed for embedding in paraffin, and cut into 5micron thick and stained with hematoxylin and eosin and observed under a microscope.

**Immunohistochemical staining**

The sections were deparaffinized and rehydrated routinely. Antigens were retrieved by heating the sections in a microwave oven at 700 W in 10 mmol/L citrate buffer (pH 6.0) for 10 min. After blocked with 3 mL/L H2O2 and goat serum, specimens were then incubated with the primary antibodies, directed against bcl-2 (Maxim Biotech Co., China) and PCNA (Senta Cruz). The staining was performed by streptavidin peroxidase enzyme conjugate method using a S-P kit (Zymed).
Reaction products were visualized by DAB. Brown-yellow granules in cytoplasm were recognized as positive staining. After immunostaining, the sections were lightly counterstained with Mayer’s hematoxylin.

**RESULTS**

Light microscopic investigations of H & E stained specimens in control group A revealed that the buccal mucosa of the control group demonstrated structural integrity of rat stratified squamous keratinized buccal mucosa with regular arrangement of underlying lamina propria. The basement membrane showed mild folding. The granulation tissues showed progress of healing. The number of blood vessel is less than normal.

In the mucositis untreated model (group B), the specimens of the oral lesions revealed loss of continuity of surface epithelium (ulcerated mucosa). In mucositis areas the exposed superficial connective tissue showed a marked infiltrate of inflammatory cells.

The present epithelium showed damaged keratinocytes with extensive cytoplasmic vacuolization, and hyperchromatic nuclei. Prominent subepithelial inflammatory cell infiltration in the lamina propria and submucosa was observed (Fig. 2).

In the mucositis model treated with NS extract, (group C): the epithelium restore its integrity and are intact but are thin than in group A. inflammatory features were still remarkable in the lamina propria, numerous blood capillaries were detected in the lamina propria (Fig. 3).

**Immunohistochemical results**

In group A, strong positive nuclear PCNA immunostaining was mainly observed in the basal and prickle cell layers (stratum germinativum) of the buccal mucosa. Some cells showed negative immunoreaction. (Fig. 4).
Meanwhile, in group B, the immunoreactivity of PCNA was negative and only confined to few basal cells. The lamina propria showed positive immunostaining in some cells. (Fig. 5)

In group C, it was noted that PCNA immunostaining was strong involving the whole thickness of the epithelium. While connective tissue cells showed positive and negative PCNA immunoreaction (Fig. 6).

On the other hand, the immunolabeling of Bcl-2 protein in normal rat buccal mucosa (group A) showed negative immunoreaction in the whole epithelial thickness with presence of only few positive cells. (Fig. 7)

The lamina propria cells showed negative Bcl-2 immunostaining (Fig. 7)

Immunoreactivity of Bcl-2 of group B revealed that positive immunoreactivity of Bcl-2 was greatly restricted to granular and cornified cell layers. However, the lamina propria revealed negative Bcl-2 immunolabelling (Fig. 8).

In group C: the immunoreactivity of Bcl-2 is upregulated more than in group B, as positive immunoreactivity is shown in some basal cells and prickle cell layer. (Fig. 9)
DISCUSSION

Intensive combination chemotherapy protocols, high dose chemotherapy regimens and allogeneic bone marrow transplantation are being increasingly used in the treatment of both lymphoproliferative malignancies and solid tumors. Ideally, a chemotherapeutic agent should only destroy malignant cells. Unfortunately, anticancer drugs with such a sparing effect on normal tissues are not yet available and therefore, some damage to normal tissues is inevitable, particularly those in which rapid cell division normally occurs (i.e., hair, skin, mucous membranes and the hematopoietic system).

Chemotherapeutic agents that have a high potential for precipitating oral mucosal damage are alkylating agents such as busulfan, cyclophosphamide, procarbarzine, and thiopete; anthracyclines such as daunorubicin, doxorubicin, and epirubicin; antimetabolites such as cytosine arabinoside, hydroxyurea, 5-FU. (13,14)

The direct inhibitory effects of chemotherapy on DNA replication and mucosal cellular proliferation result in a reduction in the renewal capacity of the basal epithelium and therefore, the direct stomatotoxicity of the chemotherapy occurs. These events are believed to result in mucosal atrophy, collagen breakdown, and eventual ulceration. (15,16)

Mucositis is a common dose-limiting complication in patients receiving systemic anticancer chemotherapy, bone marrow transplantation, and local irradiation for tumors in the head and neck area. It appears clinically as erythematous or diffuse ulcerative lesions. (15,16)

Mucositis develops in four phases; an initial inflammatory/vascular phase; an epithelial phase; an ulcerative/bacteriological phase and a healing phase. Depending on the chemotherapeutic regimen used, erythematous mucositis develops in 3 to 5 days after the initiation of therapy and ulcerative mucositis in approximately 7 days. (17-19)

Oral mucosa is comprised of membranes of a high mitotic index with rapid epithelial turnover and maturation rates. This causes the mucosa to be vulnerable to the adverse effects of chemotherapy. (20-22)

The chemotherapy alters the integrity of mucosa, the microbial flora which normally inhabit the oral cavity, salivary quantity and composition, as well as the epithelial maturation. (22,23)

There has been growing interest in naturally occurring compounds with anti-cancer potential. Black seed is one of the most extensively studied plants. This annual herb grows in countries bordering the Mediterranean Sea and India. Thymoquinone (TQ) is the bioactive constituent of the volatile oil of black seed. It has been shown to exert anti-neoplastic and anti-inflammatory effects. The molec-
ular pathways of TQ action are not clear. Nevertheless, TQ is known to induce apoptosis by p53-dependent and p53-independent pathways in cancer cell lines. Growth inhibition is associated with induction of cell cycle arrest. TQ also acts on the immune system by modulating the levels of inflammatory mediators.

Preventive measures and treatment of established oral mucositis. Several clinical observations showed the potential beneficial role of betacarotene and some hematopoietic cytokines in the maintenance of mucosal integrity following high dose chemotherapy. Betacarotene is an antiproliferative agent who produces regression of leukoplakia, and in patients, who received supplemental beta-carotene, the grade of mucositis was lower. At present time no agent has been shown to be uniformly efficacious and can be accepted as standard therapy, but those agents with beneficial effects may be recommended for prevention and management of oral mucositis in patients receiving chemotherapy. The results of ongoing trials and future cooperative clinical oncology group protocols will be of benefit to develop more efficient strategies.

The combination of TQ with clinically used anti-cancer drugs has led to improvements in their therapeutic index and prevents non-tumor tissues from sustaining chemotherapy-induced damage.

PCNA is a highly conserved 36 kDa nuclear polypeptide identified as the auxiliary protein of DNA polymerase delta. PCNA is expressed throughout the cell cycle and its concentration is increased further in the S-phase. Szabo and Vincze, 2000 have demonstrated that the capacity to accelerate the ulcer healing process depends on many factors, such as platelet-derived growth factor, fibroblast growth factor, and vascular-endothelial growth factor stimulation of angiogenesis and cell proliferation.

In the present study, it was noted that in the NS extract treatment of the chemotherapy induced ulcer (group C) NS promoted the proliferation of the epithelial cells as immunoexpression of PCNA is greatly increased in group C in comparison to Group B, this results are coincide with Kitajima et al. (1993) which demonstrated the proliferative activity during the healing of gastric ulcers using the PCNA method.

On the other hand, the Bcl-2 immunoexpression in the present work was restricted to only few sporadic cells within the epithelium which may suggest that these cells are the keratinocyte stem cells. The expression of Bcl-2 in these cells suppresses their apoptosis, thus keeps them from growth and terminal differentiation. The absence of Bcl-2 expression in terminally differentiated tissues suggests that apoptosis keeps pace with cell differentiation. However, the high level of Bcl-2 immunoreaction in the epithelium of group B rats might be an indication of impaired differentiation of the epithelium as it was claimed that Bcl-2 interferes with differentiation of epithelial cells. It was also reported that Bcl-2 protein plays a role in maintaining a stem cell population suggesting that Bcl-2 proto-oncogene plays an important role in opposing the commitment of keratinocytes to differentiation and preserving stem cells.

Until recently, cancer therapy-induced oral mucositis was thought to be a process involving the epithelium only. Chemotherapy and radiation directly damage the basal cells of the mucosal epithelium, compromising the capacity of this tissue to regenerate itself. This results in epithelial thinning as no new cells are being developed at the basal layer and existing cells migrate to the surface and are exfoliated as seen in group B in the present study. As more layers of cells are lost, the epithelium will become thinner and thinner, resulting in erythema initially and eventually ulceration. Chemotherapy caused basal cell damage when the drugs permeate to these cells from the blood vessels of the submucosal connective tissue.

The results of the present study revealed that administration of NS could partly reduce the severity of chemotherapy induced oral mucositis and promote healing of the oral which is reflected in healing oral lesions of group C by the increased expression of bcl2 within the renewing epithelium. These findings are in good agreement with a recent study by El-Denshary et al. The anti-ulcerogenic effects of NS can be
attributed to the improvement of the antioxidant status of animals due to the presence of free radicals scavenging substances such as TQ which is the main active constituent of Nigella sativa. Therefore, NS could protect the mucosa by increasing the bioavailability of anti inflammatory cytokines, resulting in biosynthesis of the cytoprotective prostaglandins as has been reported previously. NS has also been reported to produce a marked inhibition on the release of leukotrienes, which cause mucosal tissue injury and hypoxemia. Therefore, it may alter the delicate balance between prostaglandins and leukotrienes in the oral mucosa favoring cytoprotection. Thymoquinone, derived from N. sativa, has also been demonstrated to induce apoptosis of human colon cancer cells and this might enhance progression of cell cycle which in turn enhances the healing process of mucosal lesions.

The above results clearly illustrate the massive therapeutic potential of N. sativa, of which the anti-carcinogenic effects of N. sativa hold the maximum therapeutic potential. Given the significant benefits associated with its administration, broad-spectrum studies are clearly and urgently needed to further assess and elaborate its therapeutic benefits in treating chemoradiotherapy induced oral mucositis.

REFERENCES


31) Feld R. The role of surveillance cultures in patients likely to develop chemotherapy-induced mucositis. Support Care Cancer 1997;5:371-5


