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ORIGINAL ARTICLE

A rat model against chemotherapy plus radiation-induced oral mucositis

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KEYWORDS

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Abstract *Objectives:* Present study was aimed at developing an experimental model of oral mucositis in rats using a combination of chemotherapeutic agent and radiation.

Study design: Female Wistar rats (150–200 g) were divided into 3 groups ($n = 6$). Rats in group 1 (normal control) and group 2 (mucositis control) were treated with vehicle. Rats in group 3 were treated with L-glutamine (1 g/kg, p.o.; 15 days) before and after mucositis induction. Oral mucositis was induced by busulfan (6 mg/kg, p.o.; 4 days) and the tongue exposed to infrared (IR) radiation of intensity 40 mV/cm^2 for 5 s on the 1st, 4th and 10th days of challenge using a tail flick apparatus. Parameters monitored were body weight, food intake, blood count and survival. Oral mucositis score (OMS) was recorded daily. Histological changes of the irradiated tongue were assessed by hematoxylin and eosin staining.

Results: Busulfan and IR radiation significantly reduced body weight and food intake of the mucositis control group as compared to normal control. Clear ulceration of the tongue reflected in the OMS. Histopathology of the tongue revealed intense lymphocytic infiltration, decreased thickness of squamous epithelial cell layer, decrease in number of blood vessels, and necrosis of cells along with pseudo-membrane formation in the mucositis control group. These findings suggested that oral mucositis was successfully induced and treatment with L-glutamine partially reversed these conditions.

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Conclusion: Oral mucositis was established successfully in rats by the combination of chemotherapeutic agent and IR radiation. This may be a useful model for screening drugs in the treatment of oral mucositis.

1. Introduction

Mucositis is the inflammation of the mucous membrane coating the digestive tract. When it involves the mucous membrane of the oral and oropharyngeal regions, it is termed as oral mucositis (OM). OM is a major problem for cancer patients receiving head and neck radiotherapy, stem cell transplantation and myelosuppressive chemotherapy for solid tumors (Raber-Durlacher et al., 2010). It is characterized by the atrophy and ulceration of squamous epithelial cells, vascular tissue damage and infiltration of inflammatory lymphocytes to the basement region (Sonis, 1998). This injury occurs as a consequence of chemotherapy (CT) and radiotherapy (RT), which are targeted to eliminate rapidly dividing cancer cells. While rapid cell division is essential for maintaining a healthy oral mucosal epithelium, it is this normal function that renders the oral epithelium an unintended target for CT and RT regimens, in cancer patients with hematologic malignancies undergoing hematopoietic stem cell transplantation (Pico et al., 1998).

Current treatments for oral mucositis in the clinical settings are local anesthetics, paliferin, glutamine, caphsol mouth rinse, amifostine and antimicrobial agents (Lionel et al., 2006; Yamamura et al., 1998). However, there are no established effective treatments for oral mucositis. Several animal models were developed for the induction of oral mucositis e.g., mouse lip (Parkins et al., 1983; Xu et al., 1984), mouse ventral tongue mucosa (Moses and Kummermehr, 1986) and hamster cheek pouch model (Sonis et al., 1990). Drawbacks of the aforementioned animal models were high mortality rate, un-intentional exposure of organs such as brain and disturbed mucous homeostasis (Bowen et al., 2011). In order to overcome the disadvantages, the present study was aimed at developing a simple and reliable model of oral mucositis in rats using chemotherapy and IR radiation.

2. Materials and methods

2.1. Animals

Eighteen female Wistar rats of 150–200 g, aged 14–16 weeks, were issued from Central Animal Research Facility, Manipal University, Manipal (License No. 94/1999 CPCSEA). The rats were housed in polycarbonate cages and were provided free access to standard rat food and filtered water from standard perspex drinking bottles. All rats used in this study were allowed to adapt to the housing conditions for one week prior to the commencement of the study. All procedures in this study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), Manipal University, Manipal, India (No. IAEC/KMC/70/2011–2012).

2.2. Chemicals

Busulfan was purchased from Sigma–Aldrich Co. LLC, St. Louis, MO, USA. The standard test drug, L-glutamine was supplied by Nirlife Healthcare, Ahmedabad, Gujarat, India. All chemicals and reagents used in the study were of laboratory grade and procured from Merck Specialities Private Limited, Mumbai, Maharashtra, India.

2.3. Experimental design

After the adaptation period, rats were divided into three groups containing 6 in each group. Rats in group 1 and 2 received vehicle and served as normal and mucositis control, respectively. Rats in group 3 received the standard drug L-glutamine (1 g/kg, p.o.). All the rats were pretreated with their respective dosage regimens for 3 days.

2.3.1. Induction of oral mucositis

After pretreatment, oral mucositis in control and standard group was induced by a combination of chemotherapy and radiation.

2.3.1.1. Chemotherapy. Busulfan was used as a chemotherapeutic agent and administered at the dose of 6 mg/kg by the oral route for 4 days.

2.3.1.2. Radiation. As a source of radiation, tail flick apparatus (model 37360, Ugo Basile Srl, Comerio, VA, Italy) was used to deliver IR radiation. The rats were anesthetized with light ether and the dorsal surface of the tongue was exposed to IR radiation of intensity 40 mV/cm^2 for 5 s on the 1st, 4th and 10th days of busulfan challenge.

The drug treatment was continued during the busulfan/IR radiation exposure and continued for 15 days. All the rats were carefully observed during the experiment. The change in body weight, feed intake, and oral mucositis score were recorded daily. The dorsal surface of the tongue of each rat for oral mucositis score was evaluated using a previously developed

Table 1 Oral mucositis scoring system.

Score	Description
0	Normal
0.5	Slight pink
1.0	Slight red
2.0	Severe reddening
3.0	Focal desquamation
4.0	Exudation covering less than one half of the irradiated mucosa
5.0	Virtually complete ulceration of mucosa

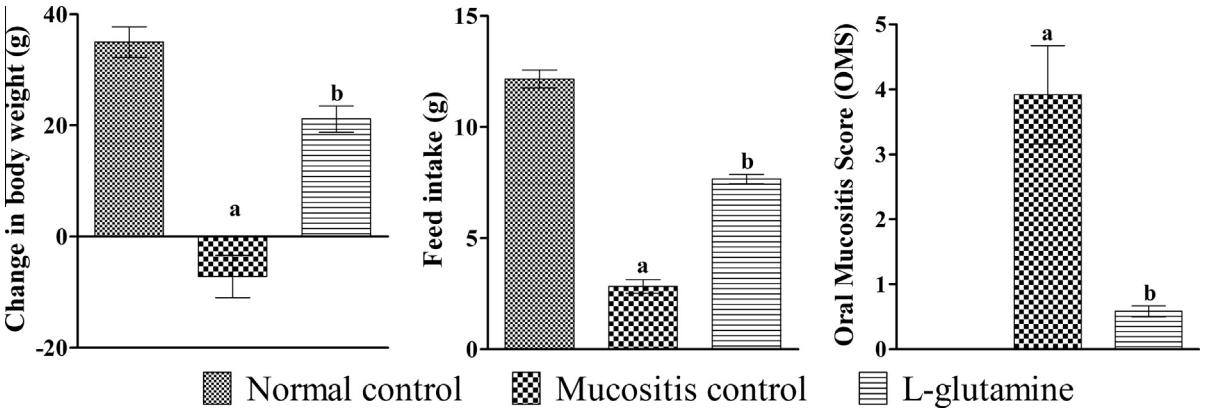

Figure 1 Effect of busulfan/IR radiation on change in body weight, feed intake and oral mucositis score (OMS) at the end of study period (15 days). Values are expressed as mean ± SEM. [a]$p < 0.01$ vs. normal control; [b]$p < 0.01$vs. mucositis control for body weight changes and feed intake. [a]$p < 0.01$ vs. normal control using Kruskal–Wallis test followed by Dunn's multiple comparison for OMS.

scoring system by Parkins et al. (1983), (Table 1) and the survival rate was calculated.

Blood was withdrawn from retro orbital plexus under light ether anesthesia and hematological parameters were measured by veterinary blood cell counter (model PCE-210VET, ERMA Inc., Tokyo, Japan) during the second week of experiment. Survival rate was estimated.

2.3.2. Histopathology

All rats of standard group and normal control group were sacrificed on the 15th day from the initiation of treatment by light ether anesthesia followed by carotid bleeding. Tongue specimens were collected, while those of mucositis group were collected when they died on the 13th and 14th days from the initiation of treatment. The specimens were stored in 10% neutralized buffered formalin, and processed for histopathological findings.

2.4. Statistical analysis

All values were expressed as mean ± SEM for 6 animals in each group. The data were analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA). Score for oral mucositis was analyzed by Kruskal–Wallis test followed by Dunn's multiple comparison tests. The level of significance was set at $p < 0.05$.

3. Results

3.1. Body weight

Following treatment with busulfan and exposure to IR a significant body weight reduction was observed in rats compared to control. Treatment with standard drug, L-glutamine showed a progressive increase in the body weight during the 15 days of study period, and was significant compared to mucositis control (Fig. 1).

3.2. Feed intake

The average food intake of normal control rats was found to be 12.16 ± 0.40 g during the study period. Mucositis control group showed a progressive decrease in food intake which was significant when compared to normal control from day 5 till the death of the animal. The decrease in feed intake may be attributed to difficulty in chewing and swallowing because of ulceration of the tongue. Treatment with L-glutamine showed improvement in feed intake which was significant when compared to mucositis control (Fig. 1).

3.3. Oral mucositis score (OMS)

In normal rat group, no oral mucositis was observed and the score was zero. In mucositis control the presence of clear ulceration in 4 out of 6 rats was observed and a maximum score of 5.0 was recorded. However, in 2 rats there was redness of the mucosa though no ulcers were observed. The scoring was done as shown in Table 1. Treatment with L-glutamine showed reduction in OMS however it is not significant compared to mucositis control (Fig. 1).

3.4. Blood components

A significant decrease in the leukocyte and platelet counts was observed in mucositis control group compared to control, while RBC remained unchanged. Treatment with L-glutamine did not significantly improve the parameters when compared with mucositis control (Table 2).

3.5. Mortality rate

The mortality rate was determined during 15 days of study period in each group. In mucositis control group, 3 rats died on the 12th day and the remaining 3 rats died on the 13th day of study period so the percentage of mortality was found to be 100% at the end of study period. In L-glutamine group, none of the rats died during 15 days of experiment period, the percentage of mortality was found to be 0% at the end of experiment. Treatment with L-glutamine showed a protective effect against toxicity of busulfan by decreasing mortality proportion and increasing survival proportion during 15 days of experiment period compared to mucositis control (Fig. 2).

Table 2 Effect of busulfan/IR radiation on blood components.

Groups	WBC ($\times 10^3$ cells/m^3)	LY ($\times 10^3$ cells/m^3)	MO ($\times 10^3$ cells/m^3)	GR ($\times 10^3$ cells/m^3)	RBC ($\times 10^6$ cells/m^3)	PLT ($\times 10^3$ cells/m^3)
Normal control	13.66 ± 0.46	11.43 ± 0.433	1.30 ± 0.05	1.11 ± 0.04	8.06 ± 0.07	612.50 ± 32.01
Mucositis control	3.80 ± 1.34[a]	3.28 ± 1.21[a]	0.21 ± 0.08[a]	0.35 ± 0.06[a]	6.37 ± 0.25	47.50 ± 13.95[a]
L-Glutamine (1 g/kg)	4.90 ± 1.39[a]	4.35 ± 1.27[a]	0.20 ± 0.07[a]	0.31 ± 0.04[a]	5.94 ± 0.42[a]	33.00 ± 5.00[a]

WBC—white blood cell; LY—lymphocyte; MO—monocyte; GR—granulocyte; RBC—red blood cell; PLT—platelets.
[a] Values are expressed as mean ± SEM. Level of significance was set as $p < 0.05$ vs. normal control.

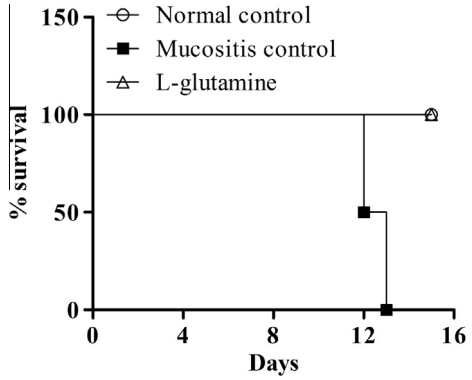

Figure 2 Percentage survival of different groups after busulfan/infrared irradiation.

3.6. Histological findings

Histological findings revealed that normal control showed intact epithelium, no lymphocytic infiltration and normal number of blood vessels (Fig. 3A). In mucositis control, the thickness of epithelial layer was altered along with lymphocyte infiltration and reduction in the number of blood vessel which indicates the presence of oral mucositis (Fig. 3B). Treatment with L-glutamine (1 g/kg, p.o.) showed normal thickness of the epithelial layer, normal number of blood vessels and absence of lymphocyte infiltration which indicates protection against oral mucositis induced by busulfan and infrared radiation (Fig. 3C).

4. Discussion

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 (Raber-Durlacher et al., 2010). Oral mucosa comprises membranes with high mitotic index (rapid epithelial turnover and maturation rates). This renders the mucosa vulnerable to the adverse effects of chemotherapy and radiotherapy (Sonis, 1998).

A complex mechanism is involved in the pathophysiology of mucositis induced by chemotherapy and radiotherapy. Both chemotherapy and irradiation generate reactive oxygen species (ROS) which are deleterious to the DNA of epithelial cells. ROS may induce a cascade of biological events such as activation of transcription factors like nuclear factor-kappa B (NF-κB), which in turn result in the synthesis of various pro-inflammatory cytokines. These cytokines target epithelium, endothelium and connective tissue, thereby causing tissue injury. Chemotherapy and radiation also activate the apoptotic pathway, leading to mucosal disintegration. This exposes the nerve ends causing severe pain and bacterial infections (Sonis, 2004).

Some of the limiting factors associated with the currently available animal models of oral mucositis are high mortality rate, difficulty in attaining homogenous exposure and the need for sophisticated instruments. In addition, owing to the highly keratinized nature of the rat tongue, it is difficult to induce ulceration in rats (Bowen et al., 2011). The present animal model involved the combination of busulfan and IR radiation. Busulfan, a chemotherapeutic agent, known for its use in different types of cancer and in bone marrow transplantation, is reported to induce mucositis (Zerbe et al., 1992).

IR radiation, a non-ionizing form of radiation, is employed frequently in the treatment of sports injuries, muscle aches and a few chronic diseases including the treatment of cancer (ICNIRP, 2006). In pre-clinical studies IR is used to induce pain in tail flick apparatus. Acute and chronic exposure to IR may cause damage to the skin and eyes. Histopathological analysis of the IR exposed skin showed vasodilatation and perivascular accumulation of degranulated mast cells. Exposure

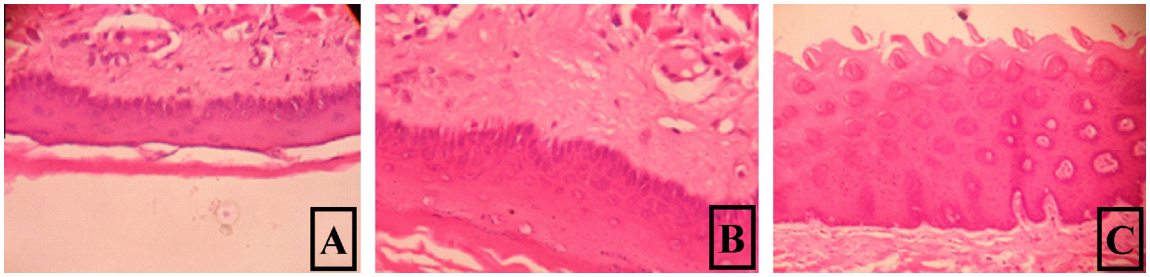

Figure 3 Histology of tongue sections of different groups after busulfan/infrared irradiation. (A) Normal control; (B) mucositis control; (C) glutamine (1 g/kg, p.o.).

to IR also induces the expression of prostaglandins (Juhlin et al., 1983).

In the present context, rats treated with busulfan and exposed to IR radiation showed clear mucositis and ulceration. Histological study of the tongue revealed decreased thickness of epithelium, lymphocyte infiltration and a decrease in the number of blood vessels indicating induction of mucositis. These findings are at par with the previous reports on animal models of oral mucositis (Chen et al., 2007).

5. Conclusion

From the above findings, it can be concluded that an experimental model of OM in rats was successfully established. The method was simple, effective and easily reproducible. This model can be used to assess the prophylactic and therapeutic interventions used for oral mucositis.

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