Oral mucositis induced by 5-fluorouracil in hamsters: clinical and histopathological evaluation of experimental methods

Mucosite bucal induzida por 5-fluoruracila em hamsters: avaliação clínica e histopatológica de modelos experimentais

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Abstract

Objective – To evaluate three methods to induce mucositis in hamsters. **Methods** – Intraperitoneal injection of variable doses (60, 90 or 100 mg/kg) of 5-fluorouracil (5-FU) was performed in 45 hamsters followed by mechanical irritation of the oral mucosa at different days. Mucositis was scored daily. Cheek pouches biopsies obtained on days 0, 4, 8, 12 and 15 were processed for microscopic examination. **Results** – The group that received 100 mg/kg of 5-FU developed severe mucositis and this dose caused a high mortality rate. When the animals received injections of 60 and 90 mg/kg associated with oral mucosa scratching on days 1 and 3, a very discrete mucositis was observed. **Conclusion** – Doses of 90 and 60 mg/kg of 5-FU associated with oral mucosa irritation on days 3 and 4 were more effective in producing mucositis in hamsters, representing a good model for future treatment agents.

Descriptors: Mouth mucosa; Fluorouracil; Hamsters; Neoplasms

Resumo

Objetivo – Comparar três modelos experimentais para induzir mucosite em hamsters. **Métodos** – Mucosite foi induzida por meio de injeção intraperitoneal de diferentes doses do quimioterápico 5-fluoruracila (60, 90 or 100 mg/kg) associada à escarificação da mucosa jugal dos animais em dias diferentes (1 e 3 ou 3 e 4). Foram utilizados 45 animais divididos em três grupos. As alterações orais foram classificadas diariamente e exame histopatológico foi realizado a partir de biópsias obtidas nos dias 0,4,8,12 e 15. **Resultados** – O grupo que recebeu 5-FU na dose de 100 mg/kg apresentou mucosite severa e alta taxa de mortalidade. O grupo que recebeu doses alternadas de 60 e 90 mg/kg e foi escarificado nos dias 1 e 3 apresentou apenas leve alteração na mucosa jugal. **Conclusão** – Concluiu-se que doses de 60 e 90 mg/kg associadas à escarificação nos dias 3 e 4 foram mais efetivas para induzir mucosite oral em hamsters, representando um bom modelo para teste de agentes que possam tratar e previnir a mucosite.

Descritores: Mucosa bucal; Fluoruracila; Hamsters; Neoplasias

Introduction

Toxic and dose-limiting effects of antineoplastic agents in oral mucosa are frequently observed during cancer therapy. One of the most debilitating side-effect caused by chemotherapy is the oral mucositis, for which there is no established treatment[1-5]. This oral complication restricts intake of food and liquids, causes discomfort and severe pain and may represents a portal of entry for pathogenic microorganisms[6-7].

The oral mucosa presents a high rate of DNA synthesis and fast turn over time[8]. For this reason, the oral tissue is especially susceptible to the chemotherapeutic agents as a result of their nonselective inhibitory effect on mitosis[9-10]. The inhibition of cell division results in atrophy followed by ulceration of the mucosal barrier[8]. In addition, the drugs used for chemotherapy are able to stimulate the release of inflammatory cytokines from the epithelium and connective tissue. Consequently, local damage of the oral mucosa associated with inflammatory response may give rise to the oral mucositis[10].

Considering the clinical significance of chemotherapy-induced mucositis, it seems reasonable to determine how to prevent and treat this debilitating condition. The current available treatment is palliative and includes administration of analgesic, oral hygiene, nutritional support and prevention of secondary infections[11]. New concepts of treatment and preventive strategies such as laser therapy[7,12-17], cytokines and inflammatory modifiers[18-21] have been evaluated.

In an attempt to mimic the clinical conditions observed in human beings, Sonis *et al.*[22] have proposed animal models to assess the oral mucositis induced by cancer chemotherapy. However, in the current literature different protocols concerning the administration of chemotherapeutic dose and the adequate period to perform the mechanical irritation of the oral mucosa can be found[2-3,22-23]. In this way, the aim of this in vivo study was to evaluate three experimental methods for chemotherapy-induced mucositis in hamsters by clinical and histological evaluation of the alterations found in the oral mucosa.

Methods

Forty five Golden Syrian hamsters, aged 6-8 weeks, were assigned into 3 experimental groups. Another five hamsters represented the control group. Animals were individually numbered, caged in small groups and fed a standard laboratory diet and water *ad libitum*. The research protocol was approved by the Animal Ethics Committee of the Araraquara School of Dentistry (Proc. CEEA nr. 14/2007), and the procedures were conducted in accordance with the Brazilian College of Animal Experimentation (COBEA).

The animals were anesthetized with intraperitoneal injection of the general anesthesic ketamine hydrochloride (Ketamina Agener, União Química Farmacêutica Nacional S/A, Embu-Guaçu, Brasil) containing 0,08 mL per 100g body weight associated with the sedative, muscle relaxant and analgesic Xylazine 10% (Virbaxyl 2%, Virbac do Brasil Ind. Com. Ltda, São Paulo, Brasil) containing 0,04mL per 100g body weight.

To induce oral mucositis, intraperitoneal injection of different doses of 5-fluorouracil (5-FU – ICN Farmacêutica Ltda, Campinas, Brasil) was carried out. In addition, the animals had both cheek pouches mechanically irritated on different days. For this purpose, an orthodontic wire device was used (Figure 1).

Figure 1. Irritation of the oral mucosa using a device made with orthodontic wire

According to the treatment, the animals were randomly divided into the following groups: **Group 1**: administration of 5-FU containing 90 or 60 mg/Kg on days 0 and 2, respectively. Cheek pouches were scratched on days 3 and 4 for five consecutive times until slight erythema was observed; **Group 2**: administration of 5-FU containing 90 or 60 mg/Kg on days 0 and 2, respectively. The oral mucosa was scratched on days 1 and 3 as described above; **Group 3**: administration of 5-FU containing 100 mg/Kg on days 0 and 2. The oral mucosa was scratched on days 3 and 4 as described previously; **Group 4 (control group)**: five animals received no intervention.

Mucositis severity was daily assessed from day 4 up to day 15. For this purpose, the cheek pouches were photographed with a digital camera (Canon Power Shot G3, Canon USA, Inc., United States). At the end of the experiment (day 15), all photographs were randomly coded by an independent person and scored by a blinded and calibrated examiner. The characteristics of the oral mucositis were classified by using a six-point grading system[3] (Table 1). The statistical analysis of Kruskal-Wallis was used to determine if the scores obtained from every specimen were different at the 95% of confidence level.

Table 1. Mucositis scores for clinical evaluation[3]

Escore	Mucositis severity
0	Cheek pouch completely healthy
1	Mild erythema
2	Severre erythema and superficial erosion
3	Formation of ulcers in one or more places
4	Cumulative ulcer formation about 50% of cheek pouch surface area
5	Complete ulceration of the cheek pouch mucosa

Three animals of each group were randomly selected for sacrifice on days 0, 4, 8, 12, and 15 when biopsies of both cheek pouches were performed and processed for microscopic evaluation. The histologic events were described and scored according to a five-points scale as shown in Table 2.

Table 2. Histophatologic scores

Escore	Histopathologic event
0	No inflamation
1	Mild inflamation
2	Moderate inflamation
3	Severe inflamation
4	Abscess formation

Results

Clinical evaluation

Oral mucositis was observed in all experimental groups. However, the statistical analysis of Kruskal-Wallis showed significant difference among the groups (*P = 0.0000*) concerning the intensity of these oral lesions.

Based upon the clinical scores determined for all specimens, a daily mean value of mucositis was determined (Graph 1). Mucositis was more severe in group 3, followed by groups 1 and 2. In control group, no changes in the oral mucosa were observed. The intensity of oral mucositis clinically observed varied from erithematous area (Figure 2), ulcerative lesions and complete ulceration of the cheek pouch mucosa (Figure 3).

No statistical difference (*P>0.05*) between groups 2 and 4 (control) was determined, which characterized the unsatisfactory mucositis induction method employed in group 2. Mucositis observed in group 1 was statistically different (P<0.05) from group 2 e 4. In group 3, in which the animals received a high dose of 5-FU (100mg/Kg), more severe mucositis was observed on day 7. It was also demonstrated that the oral mucositis occurred earlier in this experimental group when compared to the groups 1 and 2 (Graph 1).

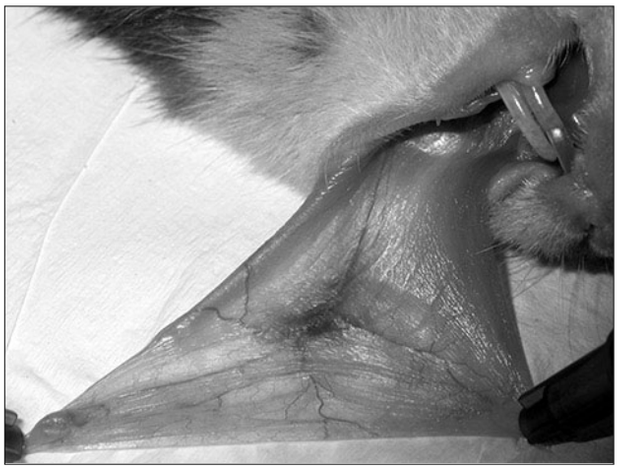


Figure 2. Group 3 (4-day period). Cheek pouch showing severe erythema (score 2)

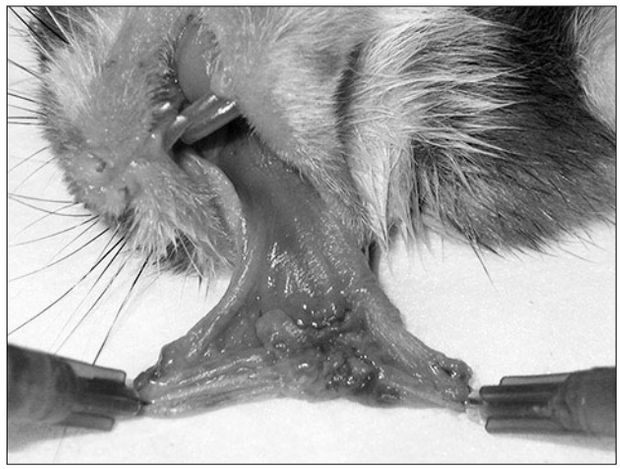
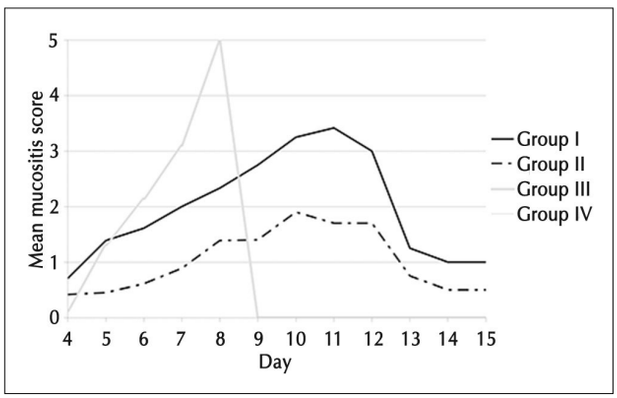


Figure 3. Group 1 (12-day period). Complete ulceration of the cheek pouch associated with clinical characteristics of necrosis (escore 5)



Graph 1. Mean mucositis score by day: comparison among groups

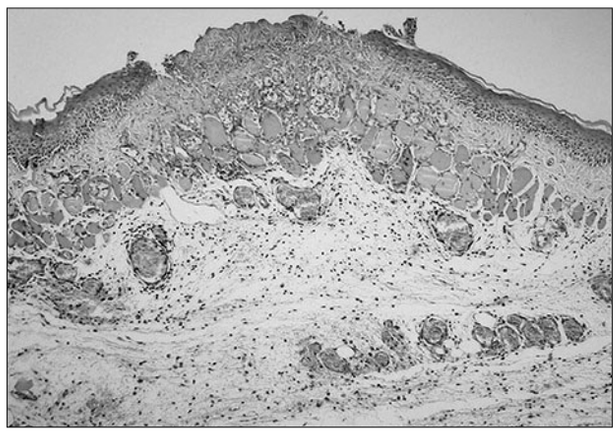


Figure 4. Group 1 (4-day period). Disruption of the ephitelium layer. The subjacent connective tissue exhibits discrete inflammatory reaction associated with notable local edema (score 1). H/E, ± 100x

Histopathological evaluation

The scores determined for inflammatory reaction is demonstrated in Table 3.

In control group, a continuous keratinized epithelium was observed. The subjacent connective tissue exhibited normal extracellular matrix associated with fibroblasts, a few blood vessels and notable muscle bundle zone. Similar histological features were also observed for all experimental groups at the 0-day period. Discrete to moderate inflammatory reaction mediated by mononuclear cells occurred in most of the specimens in group 1 and 3 at the 4-day period (Figure 4). In these specimens, disruption of the epithelial layer occurred.

Due to the high mortality rate occurred in group 3, only two samples were evaluated at the 8-day period. In these samples, deep zone of tissue necrosis associated with abscess was observed (Figure 5). In group 3, at the 12 and 15-day periods all animals were dead. In group 1, most of the specimens showed signs of healing related to a mild inflammatory reaction. Only one specimen exhibited a large ulcer with abscess, tissue degeneration and noticeable inflammatory reaction mediated by polymorphonuclear neutrophils in these experimental periods.

Table 3. Scores of inflammatory reaction according to the periods and experimental groups

Inflammatory reaction	G1 (days)					G2 (days)					G3 (days)				
	0	**4**	**8**	**12**	**15**	**0**	**4**	**8**	**12**	**15**	**0**	**4**	**8**	**12**	**15**
No	6	2	1	0	3	6	6	4	4	2	4	1	0	0	0
Mild	0	2	2	3	1	0	0	2	1	2	2	3	0	0	0
Moderate	0	2	1	0	0	0	0	0	0	0	0	2	0	0	0
Severe	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Abscess	0	0	1	1	0	0	0	0	1	0	0	0	2	0	0
Total	6	6	6	4	4	6	6	6	6	4	6	6	2	0	0

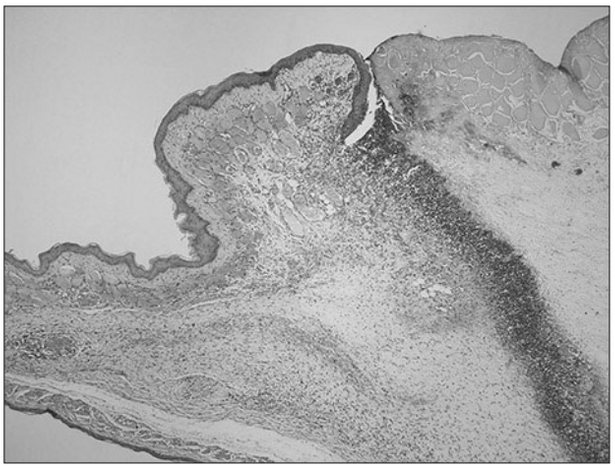
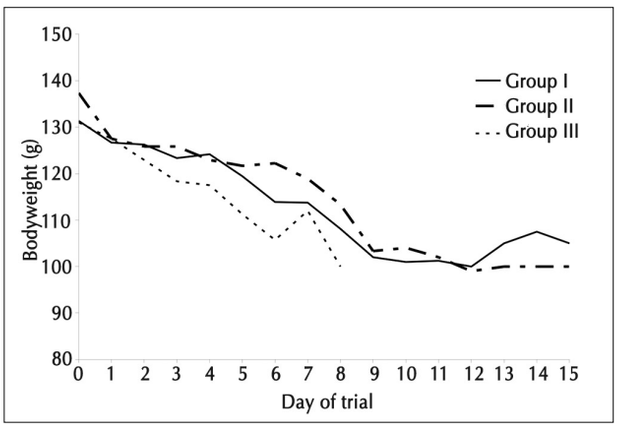


Figure 5. Group 3 (8-day period). Presence of a large ulcer. Between the necrotic zone associated with abscess and the connective tissue recovered by continuous epithelium there is a layer of intense inflammatory reaction (escore 4). H/E, ± 40x

Mortality rate and bodyweight loss

In groups 1, 2, and 3 the mucositis induction protocol caused a mortality rate of 13,3%, 6,6%, and 60%, respectively. This data was associated with the bodyweight loss (Graphy 2).



Graph 2. Mean mucositis bodyweight loss: comparison among groups

Discussion

Oral mucositis is a common toxic side-effect observed during antineoplasic treatment[16-17]. The chemotherapy causes damage to the oral mucosa including the development of ulcerative lesions which cause discomfort and intense pain[8-10]. Despite of the clinical significance of this oral complication, only a few investigations have been performed in an attempt to replicate mucositis in animals with the purpose of evaluating different therapies or procedures to prevent this oral complication[1,3,11,18-20,22-25].

In the present in vivo study, three methods of mucositis induction were investigated. Hamsters have been selected due to the large volume of the cheek pouches and this investigation was based on the experimental mucositis model of Sonis *et al.*[22]. 5-FU is one of the most widely used chemotherapeutic agents for the treatment of oral cancer and other malignant diseases[26]. This drug presents high toxicity, facilitates mucositis onset and induces the occurrence of myelosuppression since it is able to cause damage to the bone marrow cells[27].

Several in vivo studies have induced mucositis in hamsters by traumatizing the oral mucosa immediately after administration of the chemotherapeutic agent (day 0) or at the 48 hour-period (day 2)[11,23]. On the contrary, in this study no significant mucositis was observed when the animals had their oral mucosa scratched at earlier periods (group 2).

This fact could be explained by the hypothesis of mucositis development described by Sonis[10]. According to the author, the chemotherapeutic agent causes reduction in the epithelial renewal about 4 to 5 days after drug administration. Once the epithelium becomes atrophic and its renewal is inhibited, functional trauma leads to ulceration. This hypothesis was confirmed in the present investigation by the histological evaluation which demonstrated that the association between 5-FU injection and mechanical trauma at the 3 to 4-day period causes tissue ulceration (group 1 and 3).

Variations in the technique to produce the mechanical irritation of the oral mucosa have been reported in the literature, including the use of needles and burrs at low speed[2,22]. However, in the present study the scarification was performed with an adapted metallic device made with orthodontic wire which was adequate to standardize the clinical procedure.

The protocol described for group 1 consistently produced moderate to severe mucositis and can be considered an effective method to induce mucositis in hamsters. The development of oral mucositis was faster and more severe in group 3, in which intraperitoneal injection of a high dose of 5-FU was performed. In this group, a mortality rate of 60% was observed which did not allow the complete period evaluation. These data clearly demonstrated that the mucositis induction protocol used in group 3 was not appropriate.

On the other hand, the low mortality rate observed in groups 1 and 2 is desirable and characterizes an acceptable percentage for this study. These data were corroborated by Clarke *et al.*[11], who found a 3,1% mortality rate using 90 or 60 mg/Kg of 5-FU.

Although the mortality rate is related to the 5-FU dose, the anesthesia procedures carried out every day to take the photographs of the oral mucosa seem to play an important role in the lost of animals during the experiment. The anesthesia protocol used in the current study has been described by Sonis *et al.*[28] (2004) and is recommended by the CCAC (Canadian Council on Animal Care)[29] which indicates the association of the dissociative anesthetic ketamine hydrochloride with the tranquillizer xylazina. However, many workers have chosen inhalant anesthetics such as ether-based volatile agents[1,3,11,22-23].

The histologic features observed in this study corroborate with histological characteristics of the oral mucositis demonstrated by Morvan *et al.*[2] (2004) and Sonis *et al.*[22] (1990). Large areas of epithelial breakdown and

frank ulceration (Figure 5) were associated with the presence of lesions clinically observed. Extensive areas of necrosis and abscess were noted and microorganisms were evidenced in the connective tissue underlying ulcerated mucosa by the Brown and Brenn technique. The disruption of epithelial integrity provides a portal of entry for microorganisms[1,22,30]. It has been demonstrated that the presence of bacteria exacerbates the lesions induced by the chemotherapy and facilitates the development of higher mucositis scores[1].

Conclusions

In the present investigation, the intraperitoneal injection of 5-FU associated with irritation of the oral mucosa produced breakdown of the epithelial integrity.

It was concluded that the experimental method described in group I (90 and 60 mg/Kg of 5-FU administrated on days 0 and 2 and mechanical irritation of the oral mucosa on days 3 and 4) was more effective to produce mucositis in hamsters.

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