Macroscopic and Microscopic Effects of GaAlAs Diode Laser and Dexamethasone Therapies on Oral Mucositis Induced by Fluorouracil in Rats

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Purpose: To present an animal model for mucositis induced by fluorouracil in rats, and test two therapeutic options, the GaAlAs laser and topical dexamethasone, analysing them with regard to the quality and quantity of tissue alterations and comparing them with the phases of mucositis.

Materials and Methods: Forty-five Wistar rats (250 g) were treated with fluorouracil (60 mg/kg) and, in order to mimic the clinical effect of chronic irritation, the palatal mucosa was irritated by superficial scratching with an 18-gauge needle. When all of the rats presented oral ulcers of mucositis, they were randomly allocated to one of three groups: group I was treated with laser (GaAlAs), group II was treated with topical dexamethasone, and group III was not treated. Excisional biopsies of the palatal mucosa were then performed, and the rats were killed. Tissue sections were stained with haematoxylin and eosin for morphological analyses, and with toluidine blue for mast-cell counts.

Results: Group I specimens showed higher prevalence of ulcers, bacterial biofilm, necrosis and vascularisation, while group II specimens showed higher prevalance of granulation tissue formation. There were no significant statistical differences in the numbers of mast cells and epithelial thickness between groups.

Conclusion: For the present model of mucositis, rats with palatal mucositis treated with laser (GaAlAs) showed characteristics compatible with the ulcerative phase of oral mucositis, and rats treated with topical dexamethasone showed characteristics compatible with the healing phase of mucositis. Topical dexamethasone was more efficient in the treatment of rats' oral mucositis than the laser.

Key words: chemotherapy, dexamethasone, low level laser, mucositis, rats

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Oral mucositis is a clinical condition that may affect patients undergoing chemo- and radio-therapy. The administration of fluorouracil (5-FU) was often associated with Grade 3–4 rates of oral mucositis in more than 15%. The addition of radiation therapy to 5-FU-based regimens may increase the risk of Grade 3–4 oral and gastrointestinal mucositis to more than 30%. The highest rates were observed when total body irradiation was used in patients who underwent stem cell transplantation, with the rate of Grade 3–4 mucositis exceeding 60% in most reports (Sonis et al, 2004).

The classic initial appearance of oral mucositis is an erythematous mucosa and lingual scalloping. Progression of mucositis intensity leads to a white scar or pseudomembrane, which may then progress to ulceration, followed by bleeding (Gabriel et al, 2003).

The damaged mucosal surface may become colonised and infected by a wide variety of microorganisms. Infected tissue provides a portal of entry,

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allowing microorganisms to translocate into the bloodstream and cause systemic infection. In addition, severe pain decreases nutritional intake and the willingness of patients to continue treatment; it may thus require costly hospitalisation, with parenteral nutrition and narcotics (Raber-Durlacher, 1999).

No therapy available today directly targets oral mucositis. Management of oral mucositis is currently directed primarily at palliation of the symptoms and prevention of infections (Bensadoun et al, 1999). It may consist of the administration of antioxidants, such as the aminofostina; of amino acids, such as the L-glutamine; of anti-inflammatories, such as benzidamine; of interleukins; as well as of growth factors [such as granulocyte-colony stimulating factor (GCSF), transforming growth factor-β3 (TGFβ3) and keratinocyte growth factor (KGF)], cryotherapy and low level laser (Peterson et al, 2004).

Recently, growth factors, such as palifermin, have proven to be effective in preventing oral mucositis. Palifermin is a recombinant human KGF with biological activity similar to that of the native protein, but with increased stability. Palifermin, used in a double-blind study with 212 patients with haematological cancers, in comparison with a placebo substance, consistently decreased the incidence and duration of severe oral mucositis and their clinical sequelae, regardless of the measure used, the participating centre, the type of underlying disease, and the number of radiation fractions used (Spielberger et al, 2004). However, the growth factors are rarely available at health service centres such as ours, as opposed to low level laser and topical corticosteroids.

Low level laser has been reported to be effective in reducing the intensity of oral mucositis lesions in a non-randomised trial, thus reducing the time of wound healing (Cowen et al, 1997). The effects of low level laser therapy are known based on clinical observations in humans. However, the laser's physics and its biomedical application have been observed and explained in many tissues experimentally, but not in tissues with mucositis.

Also, elixir forms of corticosteroids, such as dexamethasone, can be used as an oral rinse for patients with multiple ulcerations (Eisen and Lynch, 2001), such as apthous ulcers (McBride, 2000). This could be an alternative therapy to oral mucositis, which has yet to be experimentally or clinically tested.

The objectives of this study were to present an animal model for mucositis induced by 5-FU in rats, and to test the following therapeutic options: GaAlAs (gallium-aluminum-arsenium) diode laser and dexamethasone elixir, analysing them with regard to the quality and quantity of tissue alterations and comparing them with the phases of mucositis.

MATERIALS AND METHODS

All experiments were performed in accordance with regulations for animal research. The protocol for animal use was approved by the Animal Ethics Committee of the University of Brasilia Biology Department.

A total of forty-five 12-week-old male Wistar rats (*Rattus norvegicus*, Bioagri, Planaltina, Brazil), weighing around 250 g, were used. The animals were fed a standard laboratory diet and water ad libitum. Illumination was in a 12-hour light. The rats were kept in groups of five, observed for 7 days before the commencement of the experiment.

All the animals received two intraperitoneal injections of 5-FU (50 mg/ml; ICN Laboratory), on days 0 and 5, at a dose of 60 mg/kg. A pilot study demonstrated that this dose and schedule were optimal for producing mucositis with minimal systemic morbidity or mortality.

To mimic the clinical effect of chronic irritation, the hard palate mucosa was irritated by superficial scratching with the tip of an 18-gauge needle, until erythematous changes were noted. For the purposes of observations, rats were anaesthetised with halotane daily. By day 7, all the rats showed palatal mucosal ulcerations, clinically diagnosed as mucositis (Fig 1).

On day 7, the rats were randomly divided into three groups and the wounds treated three times every 2 hours, under halothane anaesthesia, as follows. Group I: diode-laser GaAlAs (MMOptics, São Carlos, Brazil), 780 nm, 70 mW, power density 4 J/cm^2. Laser was irradiated by manual scan, linear velocity 1 cm/sec, beam perpendicular to the wound at a distance of 1 mm, covering all ulcers. Group II: dexamethasone elixir (1 mg/ml), 0.1 ml dose, administered with a non-absorbent brush over the entire wound. Group III: control, without ulcer treatment.

Two hours after the third and last treatment, all the animals were anaesthetised with an intramuscular injection of 0.4 ml of ketamine (Ketamin-S (+), 50 mg/ml, Cristália) and 0.2 ml of xylazine (Coopazine 2%, Coopers). The palatal mucosa was carefully dissected out, delimited by the superior incisors, superior lip and palatal folds. Standard samples (about 2 cm^2) were excised, fixed in 10% buffered formalin, and embedded in paraffin. Preparations (5 μm) were made and stained with haematoxylin-eosin (for morphological analyses) and with 0.1% of toluidine blue (for mast cell counts).

Table 1 Qualitative microscopic analysis guide

Epithelium	Size	☐ Not Hyperplasic ☐ Hyperplasic
	Ulceration	☐ Absent ☐ Present
	Exocitosis	☐ Absent ☐ Present
	Biofilm	☐ Absent ☐ Present
	Hyperkeratosis	☐ Absent ☐ Present
	Duplication of the basal layer	☐ Not duplicated ☐ Duplicated
	Droplet-shaped rete processes	☐ Absent ☐ Present
	Mitosis	☐ Absent ☐ Few ☐ Many
Conjunctive - inflammatory alterations	Intensity	☐ Discrete ☐ Moderate ☐ Intense
	Composition	☐ PMN ☐ Neutrophils ☐ Eosinophils
		☐ MN ☐ Lymphocytes ☐ Plasmocytes ☐ Giant cell
	Distribution	☐ Focal ☐ Diffuse
	Necrosis	☐ Absent ☐ Present
	Blood vessels	☐ Present (+) ☐ Present (++) ☐ Congested ☐ Not Congested
	Granulation tissue	☐ Absent ☐ Present

Qualitative analysis

The analysis of qualitative data, shown in Table 1, was carried out simultaneously by three examiners, using a blind method. In the analysis, the epithelium along the edges of the ulcers or next to the granulation tissue was considered.

Quantitative analysis

The quantitative data were the thickness of epithelium and the mast cell counts.

The thickness of the epithelium was obtained in a 2.5x objective, using morphometric software (Image Pro-Plus 4,0®, Measured Cybernetics, Silver Spring, MD, USA). The contour of the epithelium of all portions of the mucosa samples was carried out by the tracing of two lines, and using a program tool, a maximum, average and a minimum measure of distance between these lines were obtained.

The same program was used for mast cell counts. Microscopic fields of all the regions of the sections was digitalised by means of capture, using a colour video camera CCD (Sony, Montvale, NJ, USA), connected to the top of a light microscope (Axioskop, Zeiss). The cells were counted in each field, at a magnification of 100x, in a semiquantitative form. The total number of cells was expressed in relation to the area of each field, calculated from the contour of the image, since the samples were not identical in size.

Statistical analysis

The results of the qualitative data were analysed descriptively, using multiple contrasts and the comparison of averages among the three groups. The results of the quantitative data were evaluated by ANOVA and confirmed by Tukey's Test.

RESULTS

On day 0 of the experiment, the animals received the first dose of 5-FU and presented normal mucosa. Between day 1 and day 3, the formation of erythema in the palatal mucosa of the animals was observed. On

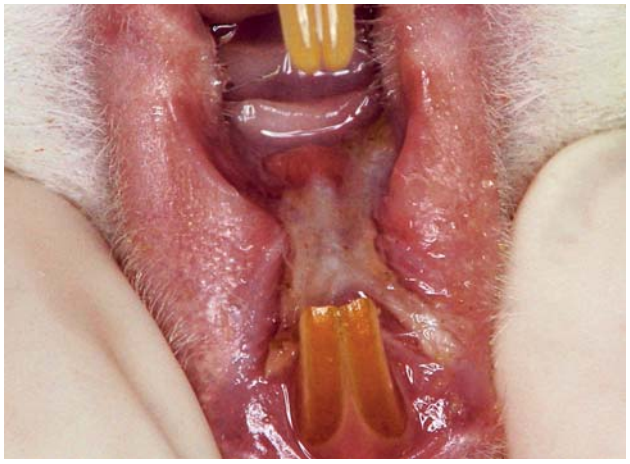

Fig 1 Oral mucositis. Extensive areas of ulcers recovered by yellowish pseudomembrane and surrounded by erythema in the mucosa of the palate extending laterally.

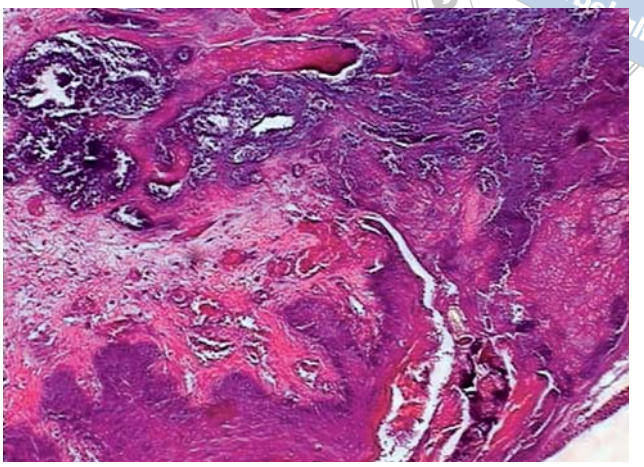

Fig 2 Sample of group I, showing the presence of an area without epithelial covering. In the conjunctive tissue displayed, bacterial presence of biofilm and tissue disarrangement was observed, characteristics observed in greater percentages in this group (haematoxylin and eosin, objective 10x).

Table 2 Classification of the presence of ulcers per group (%)

Group	Absent	Present
I - Laser	33.33	66.67
II - Dexamethasone	66.67	33.33
III - Control	46.15	53.85

Table 3 Classification of the presence of necrosis per group (%)

Group	Absent	Present
I - Laser	33.33	66.67
II - Dexamethasone	53.33	46.67
III - Control	46.15	53.85

Table 4 Classification of the presence of biofilm per group (%)

Group	Absent	Present
I - Laser	33.33	66.67
II - Dexamethasone	60.00	40.00
III - Control	46.15	53.85

day 4, all animals received the second dose of 5-FU. Lastly, on day 7, all the rats presented well-established ulcers in the palatal mucosa, covered by a pseudomembrane, and surrounded by erythema, characteristic of oral mucositis (Fig 1).

The same ulcers were visualised under light microscopy. An area without epithelial covering was easily identified and, on the displayed conjunctive tissue, the presence of a bacterial biofilm was observed. The biofilm was especially related to the areas of tissue disarrangement, evidenced as areas of necrosis under greater magnification, and to the conjunctive tissue itself, suggesting greater extension of contamination (Fig 2).

The group I specimens, treated with laser, were the ones that presented the highest rates of ulcer presence, necrosis and biofilm, with an occurrence of 66.67% for all three characteristics (Tables 2, 3 and 4).

Blood vessels were diffusely distributed, with discrete predilection for the areas below the ulcers. Vascular congestion was also a widely observed to be characteristic in these regions.

Vascularisation was evaluated qualitatively, by consensus among the three examiners, according to the greater or lesser number of vessels, excluding those with granulation tissue, if present. In group I, large numbers of intensely vascularised conjunctive tissue were observed (Table 5).

The analysis of the specimens was conducted simultaneously by the three examiners, attributing a quantitative score depending on the number of inflammatory cells. Thus, it was stipulated, arbitrarily, that the infiltrate would be considered as absent, discrete, moderate or intense, by comparison among specimens.

There was a predominance of discrete inflammatory infiltrate, diffuse and rich in lymphocytes, observed in the three experimental groups. However, the group treated with dexamethasone presented the lowest percentage of intense infiltrate (13.33%), as well as a small percentage of absence of inflammatory infiltrate (6.67%), which was not observed in the other groups (0.00%) (Table 6).

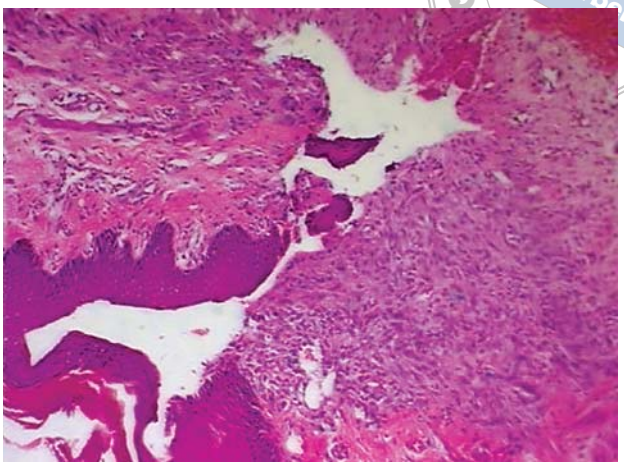

Fig 3 Sample of group II, showing granulation tissue in association with the closing ulcer region, a characteristic which was more present in this group (haematoxylin and eosin, objective 10x).

The granulation tissue, when present, was intensely vascularised, cellularised and permeated by staple collagen fibres and young fibroblasts, evidencing, under greater magnification, marks of angiogenesis, cellular proliferation and deposition of an extracellular matrix (Fig 3).

Contrary to what was observed in group I, which received laser therapy, group II, treated with dexamethasone, presented the largest percentage of granulation tissue (Table 7).

In the superficial and deep regions, there were no significant statistical differences in the numbers of

Table 5 Classification of the vascularisation per group (%)

Group	Absent	Present
I - Laser	40.00	60.00
II - Dexamethasone	66.67	33.33
III - Control	61.54	38.46

Table 6 Classification for the intensity of the inflammatory infiltrate per group (%)

Group	Absent	Discrete	Moderate	Intense
I - Laser	0.00	46.67	33.33	20.00
II - Dexamethasone	6.67	46.67	33.33	13.33
III - Control	0.00	38.46	23.08	38.46

Table 7 Classification for the presence of granulation tissue per group (%)

Group	Absent	Present
I - Laser	73.33	26.67
II - Dexamethasone	26.67	73.33
III - Control	46.15	53.85

mast cells among the three groups ($p > 0.05$) (Table 8). The same occurred with the specimens' epithelial thickness in all groups (Table 9).

In all groups, there was a predominance of hyperorthokeratotic hyperplasic epitheliums, which maintained their architecture, and presented well-marked epithelial retes.

In all groups, with a discrete predominance in the control group, the epithelial retes assumed the form droplet-shaped rete processes, with apical expansions, instead of uniform expansions with acantosis.

In the basal layer, there was evidence of epithelial activity, exemplified by the presence of more than one layer of basal cells, and by typical and atypical mitosis.

DISCUSSION

The animal model of oral mucositis, established in the pilot study and described in the present study, resulted from the association of two previously established models: one for the rat, an animal that develops gastrointestinal mucositis (Howarth et al, 1998), reacts similarly to humans with regard to gastrointestinal disturbances, and is easy to handle and low cost; the second is the chemotherapy protocol and physiological trauma simulation of the animal model of mucositis in hamsters (Sonis et al, 1990).

5-FU, in contrast with metotrexate, proved to be cytotoxic, but did not compromise the individuals systemically, allowing the visualisation of the ulcers, without major morbidity. Despite the induction of apoptosis by 5-FU, the treatment with this drug induced alterations in the keratinocytes of rats, which were compatible with autophagic degeneration (von Bültzingslöwen et al, 2001).

This animal model of mucositis induced by 5-FU in rats allowed the visualisation of the phases of mucositis (Sonis, 2004a; 2004b). Erythema was observed, followed by the formation of a pseudomembrane and ulcers in the rats' palate mucosa. However, the timescale was different from the one established in the first model developed by Sonis et al (1990), in hamsters, in which 6 days were needed for the development of the ulcers, another 6 days for the closing of the wounds, and there were a further 4 days until the end of the healing process. However, ulcers in three of the seven animals in the Sonis et al (1990) study closed 1 day after the formation of these wounds, coinciding with the results of our pilot study. This timescale is nevertheless in accordance with that observed by Cornelissen et al (1999), in which the mechanisms of palate mucoperiosteum repair in immature rats were studied. It suggests that, whenever the rat oral chemotherapy-induced mucositis model is adopted, tests should be conducted on the same day of the ulcers' appearance, since these wounds tend to close on the following day.

Studies on mucoperiosteal repair have been conducted by light microscopy; however, quantitative data are still scarce. Therefore, the quantification of mast cells, which are cells that seem to participate in the healing process, and that seem to be influenced by laser and dexamethasone, becomes necessary. How-

Table 8 Total number of mast cells per area (mm^2) per group and p-value in accordance with ANOVA

Group I		Group II		Group III		p-value
Rat	Total/area	Rat	Total/area	Rat	Total/area	
1	384/7.58	16	182/3.99	31	307/8.28	0.0946
2	261/5.90	17	831/16.04	32	510/5.63	
3	324/5.40	18	315/5.37	33	740/9.50	
4	389/10.09	19	428/7.30	34	496/8.13	
5	332/5.03	20	359/2.41	35	241/5.87	
6	394/6.51	21	318/4.26	36	499/8.87	
7	178/4.42	22	325/3.52	37	491/7.23	
8	334/5.43	23	649/7.28	38	778/11.41	
9	453/4.74	24	1099/19.3	39	283/5.08	
10	277/5.46	25	419/9.06	40	371/6.58	
11	252/3.82	26	213/2.37	41	414/7.30	
12	369/5.21	27	790/8.44	42	488/5.73	
13	468/6.22	28	174/2.56	43	500/7.38	
14	140/1.86	29	677/8.97			
15	326/6.10	30	575/7.07			

Table 9 Average thickness of the epithelium per group and the p-value in accordance with ANOVA

Group I		Group II		Group III		p-value
Rat	Thickness (µm)	Rat	Thickness (µm)	Rat	Thickness (µm)	
1	169.03	16	150.85	31	267.18	0.0698
2	120.27	17	212.12	32	218.13	
3	247.58	18	268.52	33	187.65	
4	254.97	19	223.96	34	173.01	
5	110.52	20	113.39	35	136.03	
6	196.01	21	127.70	36	192.77	
7	172.21	22	126.68	37	298.45	
8	229.62	23	97.85	38	173.17	
9	244.43	24	233.22	39	147.22	
10	177.29	25	209.42	40	258.42	
11	195.45	26	65.62	41	368.48	
12	137.35	27	234.34	42	298.07	
13	189.57	28	245.74	43	257.85	
14	275.29	29	221.18			
15	129.07	30	167.71			

ever, in the present study, there were no significant statistical differences in the numbers of mast cells among the three groups, allowing us to state that neither laser nor dexamethasone influenced the amount of mast cells in the oral palate mucositis process. However, nothing may be inferred regarding the effect of the therapies on the action of mast cells, qualitatively, with regard to inflammation and repair.

Qualitative data showed a difference among the treatments used, allowing us to distinguish the group treated with laser in the ulcerative phase of mucositis from the animals from the group treated with dexamethasone in the proliferative phase or healing phase of mucositis.

The group treated with laser presented results that were more representative of the ulcerative phase of mucositis because the majority of tissues were ulcerated (66.67%). Ulcers were filled with necrotic material in 66.67% of the cases, and the same percentage was colonised by bacterial biofilm. These numbers were higher than those observed in tissues treated with dexamethasone and the controls for these three characteristics. However, the group treated with dexamethasone presented even lower percentages of these characteristics compared with the control group.

These results presented by the group treated with dexamethasone are in accordance with the results of Shirasaki et al (2004), which suggest that the low amount of mononuclear and polymorphonuclear cells found could be explained by a suppression of the inflammation promoted by dexamethasone, probably by the inhibition of ICAM-1 (intercellular adhesion molecule-1) on inflammatory cells to the area of the injury.

A study by Bayat et al (2005) is also in agreement with these findings. They evaluated the effect of low level laser HeNe therapy on the healing of second-degree burns in Wistar rats. Two groups of 15 rats each were treated with two different doses of laser daily, in order to increase their healing activities. A control group was not treated. The number of macrophages, neutrophils, fibroblasts and blood vessels, as well as bacterial species, were measured and compared, concluding that the laser did not have significant effect on the number of cells present in the repair, and that laser therapy did not diminish the incidence of bacteria in these wounds. The rats treated with laser presented less inflammatory cells, fibroblasts and bacterial species, although the vascularisation was more intense.

The presence of greater vascularisation on the tissue samples of the group treated with laser in the present study also ratifies the classification of this group in the ulcerative phase of the mucositis. Respectively, 60% of the animals treated with laser, compared with 33.33% and 38.46% of the animals of the group treated with dexamethasone and the control group, presented more vascularisation. The ulcerative phase of mucositis is also characterised by the presence of blood vessels of greater number and size (Sonis, 1998; 2004a; 2004b).

Repair ends with the formation of scar tissue, covered superficially by epithelial cells, are compatible with the final phase of mucositis (Sonis, 2004a; 2004b). The presence of granulation tissue in 73.33% of the animals from the group treated with dexamethasone, more than the other groups, associated with the low rates of ulceration, necrosis and biofilm, evidenced that the majority of the specimens from this group was in the proliferative phase of mucositis.

Although there was a predominance of discrete inflammatory infiltrate, diffuse and rich in lymphocytes in the three groups of this experiment, the presence of slightly higher amounts of neutrophils and eosinophils in tissues treated with laser and in the control group could be related to the ulcerative phase of mucositis in which these specimens were found. Therefore polymorphonuclear cells predominate in the first hours after the injury, and are then substituted by mononuclear cells. However, the role of the inflammatory cellular infiltrate that follows radiation or chemotherapy is still undefined. Neither an acute infiltrate nor a mononuclear one is distinctive of the initial phases of mucositis. In animal models of chemo- and radio-therapy-induced mucositis, an inflammatory infiltrate made up of mononuclear cells and neutrophils was observed only just before the formation of the ulcers (Sonis, 1998).

The objective of oral mucositis treatment is to diminish healing time or to facilitate repair, since the induced ulcers can heal spontaneously. All this proliferative activity could be observed in the three groups of this experiment, with slightly lesser indices in group I, when considering the epithelium of the ulcers' edges or next to the granulation tissue, compared with more distant regions. The control group presented slightly increased amounts of epithelial hyperplasia and droplet-shaped rete process formation, which evidences the organism's capacity of regeneration.

The presence of a hyperplasic epithelium, with droplet-shaped rete process formation, the presence of duplicated basal layer and mitoses in these cells are characteristics of ulcer plugging and re-establishment. All these characteristics showed high prevalence in all groups, since they are also observed in the repair of the normal palatal mucosa that does not receive chemotherapy (Cornelissen et al, 1999).

The similarity of results for epithelial data suggests that the treatments used were not capable of modify-

ing them in vivo, contrary to the results of the study of Koutná et al (2003), which showed greater proliferation of HeLa cells in vitro after irradiation with low level laser, compared with the non-irradiated controls.

We conclude that the animal model of mucositis induced by 5-FU-based chemotherapy in rats may be used to study oral mucositis, especially the prevention and treatment of the sequelae, unless the objective is to study particularities of the different healing phases. Moreover, we may affirm that the rats with palatal mucositis treated with low level laser (GaAlAs) presented morphological characteristics, observed in light microscopy, compatible with the ulcerative phase of the oral mucositis, and that the rats treated with topical dexamethasone presented compatible morphological characteristics with the proliferative phase or healing phase of mucositis. Therefore, the present study showed that the application of dexamethasone elixir produced results that were more significant in the treatment of the experimental mucositis in rats.

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