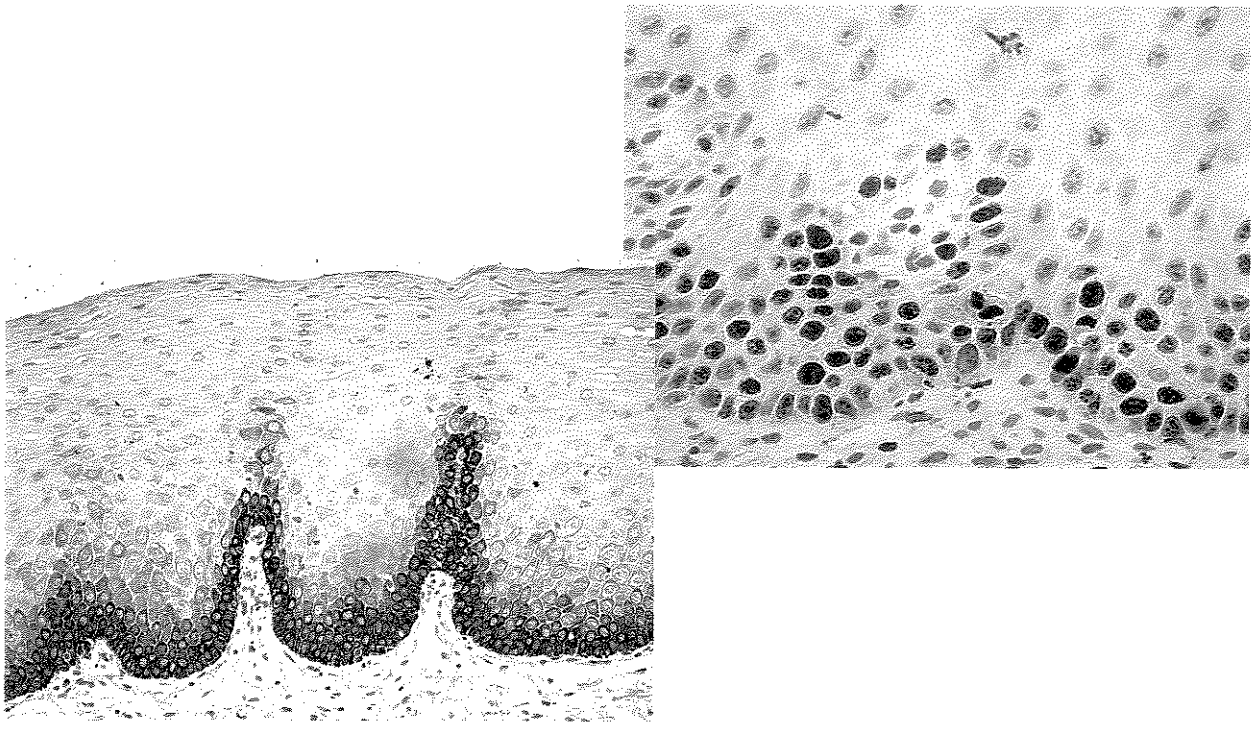
Paulo Rogério Ferreti Bonan

Radioterapia em Cabeça e Pescoço: Avaliação do Tratamento Odontológico e da Mucosite Oral



Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para a obtenção do Título de Doutor em Estomatopatologia, Área de Concentração-Estomatologia

PIRACICABA
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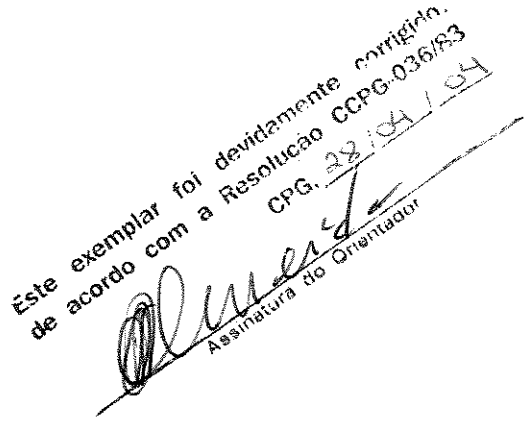

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Orientador:
Prof. Dr. Oslei Paes de Almeida

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 27 de Janeiro de 2004, considerou o candidato PAULO ROGÉRIO FERRETI BONAN aprovado.

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3. Prof. Dr. FÁBIO DE ABREU ALVES

4. Prof. Dr. MARCIO AJUDARTE LOPES

5. Prof. Dr. PABLO AGUSTIN VARGAS

Dedicatória

Ao meu grandioso Criador Jeová

"Pois estou convencido de que nem a morte, nem a vida, nem anjos, nem governos, nem coisas presentes, nem coisas por vir, nem poderes, nem altura, nem profundidade, nem qualquer outra criação será capaz de nos separar do amor de Deus, que está em Cristo Jesus, nosso Senhor"
Romanos 11:38-39, extraído da Tradução do Novo Mundo

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Sumário

Resumo

A radioterapia em cabeça e pescoço é usualmente empregada no tratamento de neoplasias malignas. Embora a telerradioterapia seja efetiva para esse propósito, efeitos colaterais resultantes da radiação tumoricida em campos cérvico-faciais, resultam em decréscimo na qualidade de vida e no aumento da morbidade. Os efeitos colaterais mais significativos da radioterapia em região orofacial são a mucosite oral, osteorradionecrose, xerostomia, candidose, cáries de radiação, disgeusia, trismo, halitose, radiodermatite e hiperpigmentação melânica. Os objetivos desse trabalho foram avaliar as condições dentárias; a importância e influência do tratamento odontológico prévio à radioterapia em 40 pacientes portadores de carcinomas espinocelulares em cabeça e pescoço; evidenciar as alterações histopatológicas dos tecidos epiteliais e conjuntivos em 10 casos de mucosite iniciais e investigar a expressão imunohistoquímica das citoqueratinas e Ki-67 na mucosite oral inicial. Exodontias antes da radioterapia foram freqüentemente realizadas devido à alta prevalência de doença periodontal e cáries avançadas. Embora apenas um caso de cárie de radiação tenha sido observado, ocorreram cinco casos de osteorradionecrose (12%), sendo um desses casos associado a exodontia prévia, um caso associado ao tratamento cirúrgico para a ressecção tumoral e 3 casos idiopáticos. Áreas de mucosite inicial apresentaram redução significativa de espessura, perímetro e área epiteliais comparadas com tecido normal. No tecido conjuntivo houve aumento significativo de células inflamatórias e vasos sanguíneos. As células CD68 positivas (macrófagos) foram mais freqüentes nos casos de mucosite do que na mucosa normal. As expressões das citoqueratinas Ck 1, 6, 10, 14, 16 estavam aumentadas na mucosite oral. A contagem de células positivas para Ki-67 foi similar nos casos de mucosite e displasia oral e estatisticamente superiores à mucosa normal. Em resumo, pacientes com neoplasias malignas de cavidade bucal apresentaram hábitos insatisfatórios de higienização bucal e condições dentárias precárias. O tratamento odontológico prévio à radioterapia foi importante na prevenção de episódios de cárie de radiação mas não impediram o surgimento de osteorradionecrose, que apresentou etiologia multifatorial. O tecido epitelial na mucosite oral inicial apresentou atrofia e diminuição das projeções epiteliais, com conjuntivo subjacente apresentando aumento de vasos e do infiltrado inflamatório mononuclear, formado por macrófagos mais numerosos do que o

tecido normal. O aumento da proliferação celular da camada basal do epitélio evidenciada pela expressão de Ki-67 e a modificação na expressão de citoqueratinas, sugerem resposta de defesa do epitélio nas fases iniciais da mucosite oral. Entretanto, para melhor compreensão das alterações morfológicas da mucosite oral, mais estudos são necessários.

Abstract

Radiotherapy is frequently employed in head and neck cancer treatment. Although teleradiotherapy is effective, side effects cause high morbidity decreasing quality of life. The most important side effect of orofacial radiotherapy are oral mucositis, osteoradionecrosis, xerostomia, candidosis, radiation caries, dysgeusia, trismus, halitosis, radiodermatitis and hyperpigmentation. The objectives of this work were to evaluate the dental conditions and necessity of dental treatment before radiotherapy in 40 patients with head and neck cancer, as well as the microscopical alterations of initial oral mucositis, considering the proliferative index using Ki-67 antibody, expression of cytokeratins in 11 cases of oral mucositis and connective tissue inflammatory infiltrate in 10 cases of oral mucositis . Dental extractions before radiotherapy were common due the high prevalence of advanced caries and periodontal disease. One case of radiation caries and five of osteoradionecrosis ocurred, 3 for idiopatic causes, one caused by mandibulectomy and one associated with previous dental extraction. Areas of initial mucositis presented significant reduction of epithelial thickness, perimeter and area and increased number of blood vessels and inflammatory cells, with macrophages (CD68 positive cells) increasing. Expressions of cytokeratins 1, 6, 10, 14 and 16 were increased in initial oral mucositis. In summary, Brazilian patients with head and neck cancer present poor dental health, and despite of preventive dental treatment, including several dental extractions, 5 out 40 patients, presented osteorradionecrosis. Alterations of the epithelial and connective tissues in the initial phases of oral mucositis, as increased proliferative index, expression of some citokeratins and increasing number of macrophages, suggest these mucosal alterations are related to defensive mechanisms against tissue injuries caused by radiation. There are few reports describing the microscopical and molecular effects of radiotherapy on oral mucosa, and for better understanding of the subject, futher studies are necessary.

Proposições

1) Avaliação do tratamento odontológico pré-radioterapia em 40 pacientes portadores de carcinomas de cabeça e pescoço
2) Estudar as características microscópicas da mucosite oral inicial radioterapia considerando:
   a- características morfométricas do epitélio
   b- expressão de citoqueratinas
   c- expressão de Ki-67 (índice de proliferação)
   d- expressão de CD68 (macrófagos)

Capítulo 1

Clinical, biological and histological features of oral mucositis induced by radiation therapy and its treatment modalities: a review.

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RUNNING TITLE

Oral mucositis induced by radiation: main features

KEYWORDS

Radiotherapy, mucositis, treatment .

ABSTRACT

Objectives: The purpose of this work was to review the main and more recent studies which discussed the clinical, biological and histopathological features of oral mucositis induced by radiation therapy and to describe the main approches recommended to treat or to prevent oral mucositis.

Conclusions: Although the clinical features of mucositis are intensily described in the literature, few studies commented about histopatological alterations in oral mucositis. The biological mechanisms involved in the radiation tissue damage have been only recently discussed and there are no consensus among the treatment modalities. The progressive knowledge in the histopathology and biological characteristics of oral mucositis probably will lead to more effective prevention and managment.

INTRODUCTION

The radiotherapic treatment, exclusively or associated with surgery, is effective to treat and to control head and neck carcinomas (Zarkrzewka, 1999, Scully and Porter, 2000; Scully and Porter, 2001). The modalities of radiotherapic treatment include two approachs: external beam radiotherapy and intersticial radiotherapy, few indicated on head and neck cancer management. The external beam radiotherapy includes telecolbaltherapy and linear acceleration, where traditional planning and intensity modulated (IMRT) are used (Chao *et* al, 2000; Sannomiya and Furukawa, 2000; Scully and Porter, 2000; Scully and Porter, 2001; Claus *et al*, 2002; Vineberg *et al*, 2002). Although the external beam radiotherapy is largely indicated, side effects derived from radiation cause systemic injuries and mainly, oral tissues alterations. (Pernot *et al*, 1997; Epstein *et al*, 1999; Sannomiya and Furukawa, 2000; Scully and Porter, 2000; Scully and Porter, 2001). Bones, oral mucosa, salivary glands, blood vessels, muscles, nerves and teeth are affected by radiation (Schubert and Izutsu, 1987; Maxymiw and Wood, 1989; Barrett *et al*, 1994; Garg and Malo, 1997; Pernot *et al*, 1997).

Head and neck irradiated patients, who answered questions about oral health and quality of life, referred that dry mouth, difficulties in speak, swallow and eat, and dysgeusia were very common after radiotherapy (Epstein *et al*, 1999). Xerostomia, trismus, radiation caries, candidosis, osteoradionecrosis, pigmentations and oral mucositis are the main side effects derivated from radiotherapy on head and neck (Morrish *et al*, 1983; Schubert and Izutsu, 1987; Maxymiw and Wood, 1989; Barrett *et al*, 1994; Garg and Malo, 1997; Scully and Porter, 2000; Sannomiya and Furukawa, 2000; Scully and Porter, 2001, Bonan *et al*, 2003).

The main acute side effect induced by radiation in head and neck is oral mucositis. (Spijkervet *et al*, 1989; Sur *et al*, 1994; Hlavaty *et al*, 1996; Rovirosa *et al*, 1998; Hejna *et al*, 1999; Plevová, 1999; Etiz *et al*, 2000; Sprinzla *et al*, 2001; Dörr *et al*, 2001). Oral mucositis induced by radiation results in serious pain and swallowing, eating and speech impairment and may be a portal for opportunistical infections with systemic relevance, decreasing the quality of life (Spijkervet *et al,* 1989; Leung *et al*, 2000; Köstler *et al*, 2001, Scully and Porter, 2001). Adding, oral mucositis may obligate partial or complete treatment

interruption, increasing the risk of tumor repopulation and reducing the treatment control (Dörr *et al*, 2001). The purpose of this work was to review the main and more recent studies which discussed the clinical, biological and histopathological features of oral mucositis induced by radiation therapy and to describe the main approches recommended to treat or to prevent oral mucositis.

EPIDEMIOLOGY AND CLINICAL FEATURES

Oral mucositis induced by radiation is a acute side effect which reaches almost all patients submitted to tumoricidal radiation doses on head and neck fields. (Spijkervet *et al*, 1989). Trotti *et al* (2003) reported that 97% out 2875 patients developed oral mucositis due to conventional radiotherapy, 89% out 1505 due to radiotherapy and chemotherapy association and 22% out 318 patients due to chemotherapy, exclusively. Handschel *et al* (2001b) accompained 13 head and neck irradiated patients and referred that all patients developed oral mucositis after two weeks of the treatment beggining.

The severity of oral mucositis are correlated with the type of radiation employed, radiation distribution, total doses, association with chemotherapy, individual response, tobacco and alcohol use and levels of EGF and PAF in saliva. (Hlavaty *et al*, 1996; Scully and Epstein, 1996; Denham *et al*, 1999; Dumbrigue *et al*, 2000, Epstein *et al*, 2000; Bentzen *et al*, 2001; Trotti *et al*, 2003). Oral mucositis is also more intense when patients are submitted to hyperfractionated doses than received conventional doses associated or not with chemotherapy. (Bentzen *et al*, 2001; Trotti *et al*, 2003). A study realized by Denham *et al* (1999) with 191 patients irradiated on head and neck, showed the importance of individual response to therapy and sinergical role of tobacco, alcohol, dental infections, desnutrition and poor oral care. High levels of PAF in saliva are associated with severe episodes of oral mucositis, due to inflammation induction (Hlavaty *et al*, 1996). Otherwise, low EGF levels are straightly relationed with intense mucositis due to difficulties in the mucosa healing (Epstein *et al*, 2000).

Concomintant infections associated with oral mucositis are another important aggravation factor. *Candida* colonization increases during radiotherapy causing, in some cases, infections in whole irradiated mucosa (Paula *et al*, 1990; Scully and Epstein, 1996, Ramirez-Amador *et al*, 1997). Although micoorganisms could cause secondary infections

associated with oral mucositis, two studies with anti-bacterial and anti-fungal drugs did not reduced the severity of oral mucositis when *Candida* and gram-negative bacterias were found in oral cavity (Wijers *et al*, 2001; Stokman *et al*, 2003).

Clinically, two weeks after the radiotherapy beggining on head and neck, oral mucosa become whitish followed by erithema, and around 2500 cGy, epithelial atrophy leads to ulcerations recovered by fibrinous layer (Scully and Epstein, 1996) (**Fig.1** e **2**). Clinical distinguishement between intense mucositis and candidosis may be not easy due to be overlayed processes (Nicolatou-Galitis *et al*, 2003) (**Fig.3**). Pharyngeal mucosa may be more sensitive to radiation being the start point of the pain, leading to soft feeding (Blozis and Robinson, 1968; Sur *et al*, 1994). The pain resulted goes to a little discomfort to spontaneous bleeding which results in eating impairment leading to parenteral nutrition or nasogastric probes use (Spijkervet *et al,* 1989; Trotti *et al*, 2003). Aproximatelly 11% of cessation or radiotherapic regimen alteration occur due to oral mucositis development, being necessary, in some cases, to replan or to intern the patient (Trotti *et al*, 2003). For the majority of patients, 10 to 14 days are enough to heal the injured tissue but for patients with late repair and/or who received total doses above 6.500 cGy, this phase range to 14 to 21 days (Rothwell, 1987, Scully and Porter, 2000; Scully and Porter, 2001).

The evaluation of oral mucositis intensity may be realized trough the World Health Oraganization index (WHO), by the Hickey's, Van Der Schuren's; Spijkervet's, Stokman's methods and RTOG/EORTC classification (Spijkervet *et al*, 1989; Stokman *et al* 2002). The more recent studies used mainly the WHO classification, that grades the oral mucositis in five levels (0-no clinically noticeable changes; 1-enanthema, mild discomfort, 2-enanthema, small ulcers, solids; 3 –large and confluent ulcers, only liquid diet possible; 4-only parenteral nutrition possible) (Trotti *et al*, 2003). Mucositis grades 3 and 4 (WHO) are frequent after the middle of the treatment with tumoricidal doses (Handschel *et al*, 2001b, Trotti *et al*, 2003).

HISTOPATHOLOGICAL AND BIOLOGICAL FEATURES

Oral mucositis like a biological process have been only recently studied and mechanisms involving oral mucosa turnover, inflammatory cytokines, and local factors such as saliva and microbiota are present in mucositis pathogenesis. Oral mucositis induced

by radiation or chemotherapy may be classified in 4 distinctive phases: vascular, epithelial, ulcerative and healing (Pico *et al,* 1998; Sonis, 1998). Just after the beggining of radiotherapy, cytokines are released from epithelial tissue, such as TNF-α, IL-1 and maybe IL-6, starting the vascular phase. In the vascular phase, occurs increasing of subepithelial blood vessels and releasing of pro-inflammatory cytokines from connective tissue (Sonis, 1998). In sequence, damage in S phases and cellular death of basal cells by apoptosis occur. (Scully and Epstein, 1996; Sonis, 1997; Sonis, 1998). The cicle time of basal keratinocyte is about 4 days, and like the oral epithelium has 3 or 4 layers, the alterations caused by the radiation arise after 12 days of treatment beggining (Scully and Epstein, 1996). In the epithelial phase, occurs reduction, just on the first week of treatment, of the keratinocyte proliferation, leading to epithelial atrophy. In the second week after the radiotherapy beggining, partial recovering of epithelial proliferation occurs leading to transient epithelial repopulation (Dörr *et al*, 2001). Some experimental data have been showed the increasing of mucositis intensity when KGF, a very important epithelial proliferation promoter, is decreased, reforcing that biological mechanisms associated with epithelial repopulation are also alterated by the radiation (Dörr *et al*, 2002). In consequence of the decrease of cellular proliferation, occurs persistent atrophy and connective tissue exposure, recovered by fibrinous layer rich in neutrofiles (Pico *et al*, 1998). When the diary radiotherapic doses is 200 cGy, clinical manifestations of epithelial depletion emerge earlier than 180 cGy/day due to the umbalance between cellular death and epithelial repopulation caused by increased diary doses (Denham and Hauer-Jensen, 2002). After the ulcerative phase, due to proliferation events and differentiation, the epithelium is restored (Pico *et al*, 1998, Sonis, 1998). The healing process could be delayed if the cellular depletion was intense and deep, if a significative inflammatory infiltrate is present or the course of healing was alterated (Denham and Hauer-Jensen, 2002).

Histopathologically, in the connective tissue, mucositis induced by radiotherapy is not only an acute inflammatory response or an agressive form of chronical inflammation. Oral mucositis represents a process like a healing phase of inflammation (Handschel *et al*, 2001b). There are no significative differences in T lymphocyte and granulocytes counts, compared with non-irradiated tissue. Nevertheless, increased subpopulation of macrophages reactive for RM3/1 (macrophages found in late phases of inflammation) is

presented (Zwadlo *et al*, 1987; Handschel *et al*, 2001b). Blood vessels apear with large diameter and there are a remarkable evidence of vascular permeability and diapedesis (Handschel *et al*, 1999; Etiz *et al,* 2000) (**Fig. 4**). In the late consequences in oral mucosa found after radiotherapy, it was observed fewer blood vessels, increased diameter of the lumen of the residual blood vessels, and predominance of RM3/1 positive cells (Handschel *et al*, 2001a).

TREATMENT

The treatments usually employed to manage oral mucositis are genneraly palliative and very diversificate. Include profilatical approaches and pain relief (Köstler *et al*, 2001, Scully and Porter, 2001). Although many studies commented about mucositis managment, few of them were double-blind or used placebo(Köstler *et al*, 2001). The therapies include improvement of oral self care, to avoid tobacco and spice foods, growth factors (EGF, GM-CSF, KGF), coated salts (sucralfato e Maalox®), cytoprotectors and antioxidants, betametasone, glutamine, cytokines (interleucina-11), benzidamide, AAS, lidocaine, polimixine E, lozenges, tobramicine, low-energy lasers, criotherapy, and others (Wright *et al*, 1985; Sonis *et al*; 1992; Scully and Epstein, 1996; Cowen *et al*, 1997; Plevová, 1999; Biron *et al,* 2000; Huang *et al*, 2000; Sonis *et al*; 2000; Nicolatou-Galitis *et al*, 2001; Scully and Porter, 2001; Sutherland and Browman, 2001; Dörr *et al*, 2002). Recent papers commented about good outcomes with GM-CSF in the intense oral mucositis prevention maybe suggesting future therapeutic utilization, although this tendance is not unanimous (Hejna *et al*, 1999; Makkonen *et al*, 2000; Nicolatou-Galitis *et al*, 2001, Sprinzl *et al*, 2001). The trials of prevention and management of mucositis observing the different phases of oral mucositis could be interesting (Sutherland and Browman, 2001). For example, Biron *et al* (2000) encouraged the observation of different biological phases of oral mucositis to choose the apropriate therapy. In the vascular phases, criotherapy, vasoconstrictors, cytoprotectors, antioxidans, proliferation inibitors, such as TGF ß3, are recommended. In the epithelial phase, growth factor and epithelial renew drugs, such as sucralfate, GM-CSF e KGF, should be used. In the ulcerative phase, chlorexidine mouthwashes without ethanol and topical antibiotics should be employed. In the healing phase, low-energy lasers should be prescribed. Although this suggestion of therapy is very complete and interesting, there

are no consensus in therapeutic methods commonly suggested by Biron *et al* (2000), like showed antagonical studies about sucralfate efficacy to prevent oral mucositis (Scully and Epstein, 1996; Lievens *et al*, 1998; Etiz *et al,* 2000; Saarilahti *et al*, 2002) and the efficacy of antibiotics to reduce oral mucositis intensity (Adamietz *et al*, 1997; Epstein and Schubert, 1999; Wijers *et al*, 2001; Stokman *et al*, 2003).

CONCLUSION

Although the clinical features of mucositis are intensily described in the literature, few studies commented about histopatological alterations in oral mucositis. The biological mechanisms involved in the radiation tissue damage have been only recently discussed and there are no consensus among the treatment modalities. The progressive knowledge in the histopathology and biological characteristics of oral mucositis probably will lead to more effective prevention and managment.

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Figure 1. Confluent ulcerations in the tongue and comissure of patient submitted to radiotherapy in head and neck.

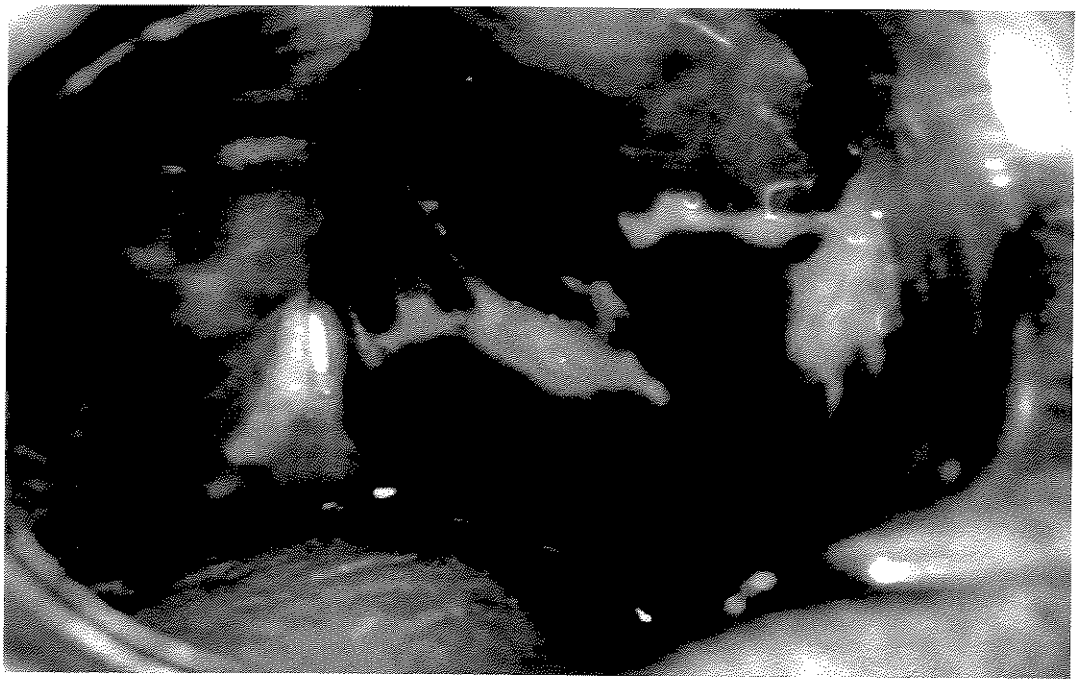

Figure 2. Erithematous mucosa covered by fibrinous layer in buccal mucosa of irradiated head and neck patient.

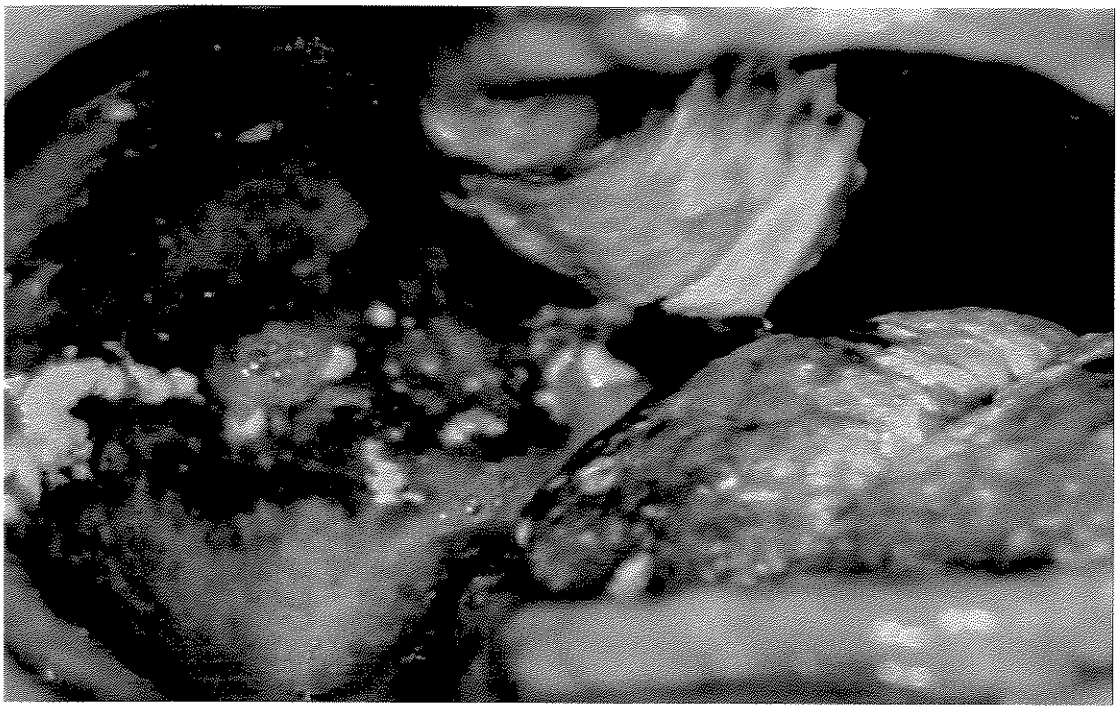

Figure 3. Pseudomembranous candidosis is overlying mucositis induced by radiotherapy.

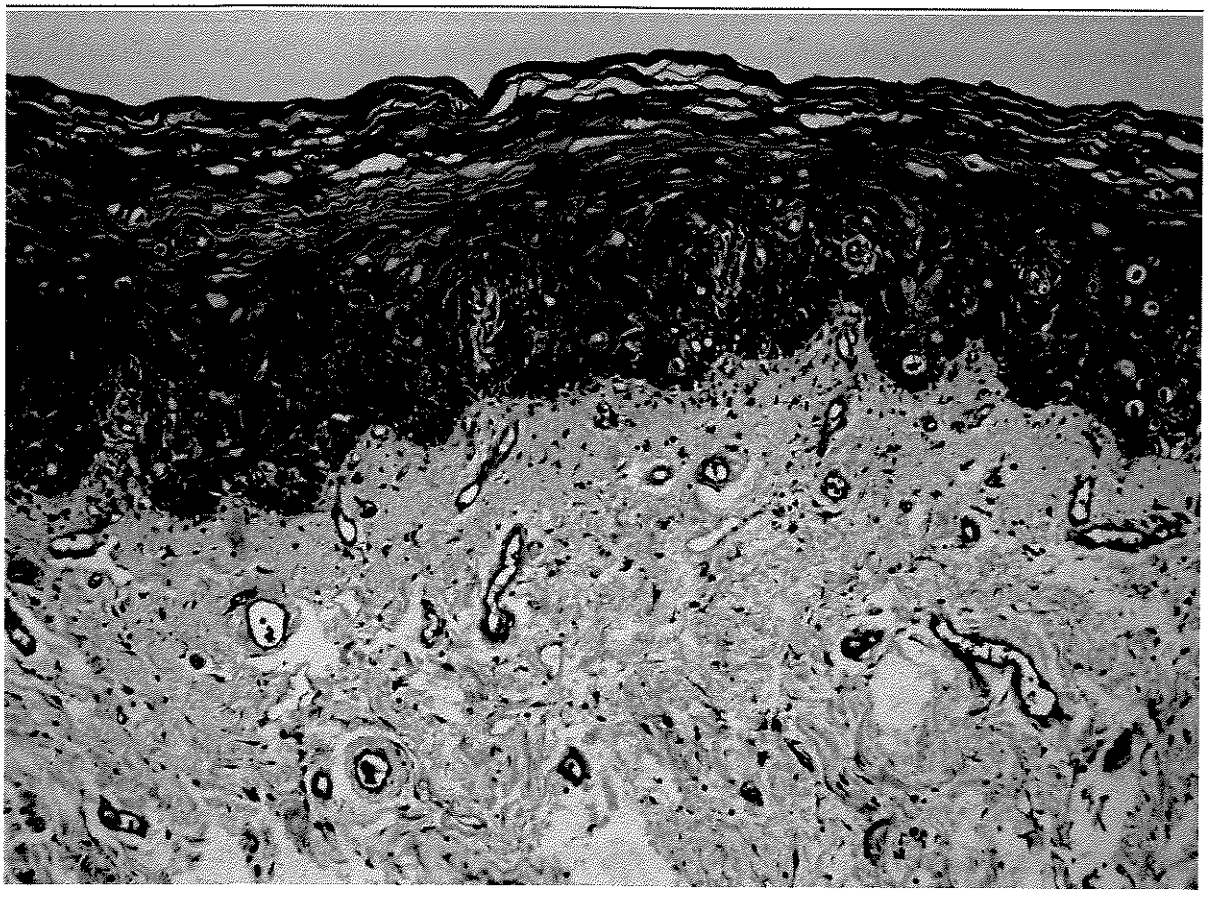

Figure 4. Hyperparakeratosis, hyperchromatic basal layer, mononuclear inflammatory infiltrate, dilated blood vessels in buccal mucosa of mucositis grade 1 (WHO). HE, X100.

heavy tobacco and alcohol use and presented poor nutritional and general status. ORN are not always associated with tooth extraction, and it can occur in edentulous regions, without previous extractions. Two reports showed that ORN developed in 3 out of 22 (13.5%) edentulous patients, and in 15 out of 21 (71.5%) irradiated patients, without relation to teeth extractions (23,28). Interval for development of ORN is variable, usually within one year after radiotherapy, ranging from 2 weeks to 34 months (23). In our study there was an average of 3.2 months between the end of radiotherapy and beggining of ORN, ranging from 1 to 10 months. Other risk factors considered for the development of ORN are radiation dose to bone, advanced stage of tumor and surgery (5,27). The mean total radiation dose of our 5 cases with ORN was 6900 cGy. Of these 5 cases, 3 patients presented clinical stage 3 or 4, with advanced tumors, and 2 patients presented stage 2. One case of ORN was associated with mandibulectomy performed after 7000 cGy in the jaws. Extractions before as well as after radiotherapy can cause ORN (23). According to EPSTEIN *ET AL* (29) the incidence of mandibular ORN is higher in patients having extractions just before RxT or immediately after. Extractions should be carried out at least 2-3 weeks before RxT starts (30). Unfortunattely, some of our patients needed to start radiotherapy 7 to 10 days after teeth extraction; even so, in only one case ORN was considered to be associated with extraction previous to RxT, with short time to alveolus healing. Conservative management for ORN (sequestrectomy and irrigation) were employed in four cases with complete healing in two cases. Hyperbaric oxygen therapy (HBO) was effective in one case. The efficacy of HBO is well accepted (31). One study demonstrated that of 18 patients who had undergone surgery and HBO for refractory ORN, 14 patients had complete healing (32).

Although new techniques of radiotherapy with fewer side effects, as IMRT (modulated-intensity radiation therapy) and brachytherapy (33-34) are also available in Brazil (34-35), few patients have access to these treatment modalities. In summary, most of the low social economic HNSCC patients of Brazil with oral cancer need dental extraction before radiotherapy due to periodontal disease and caries. ORN, a multifactorial process, still is an important problem associated with high total doses, poor systemic and oral health and with tobacco smoking and alcohol use.

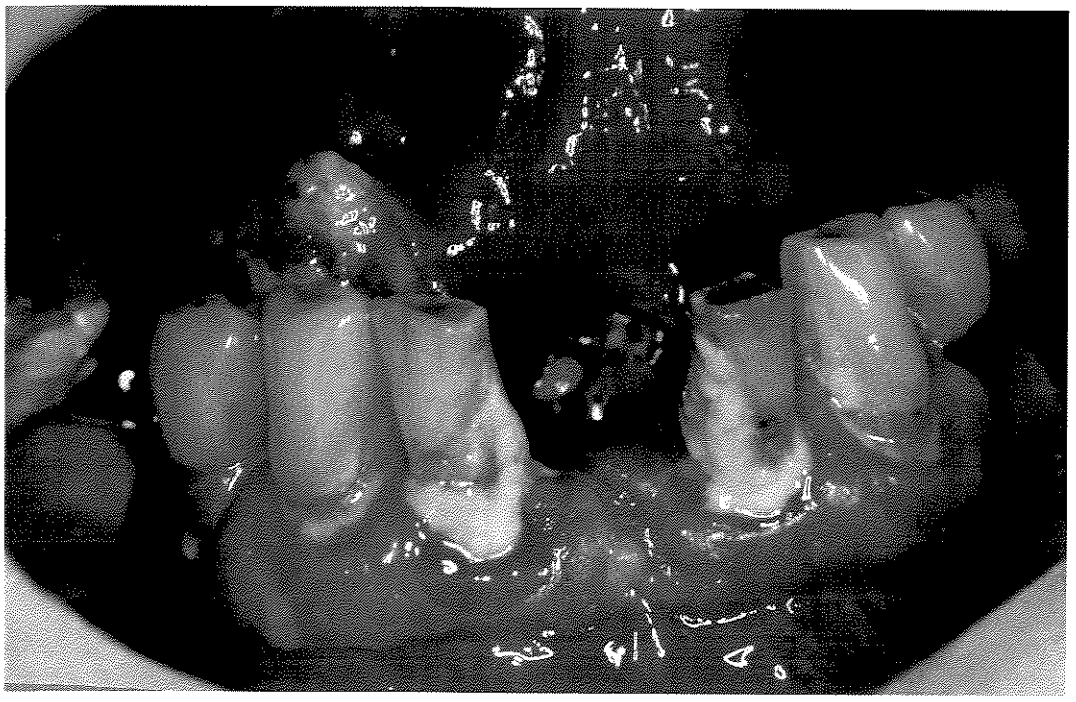

Figure 1. Abundant calculus, gingival recession, cavitations and tongue carcinoma. This patient received total dental extractions due moderate to severe periodontal disease and poor oral care.

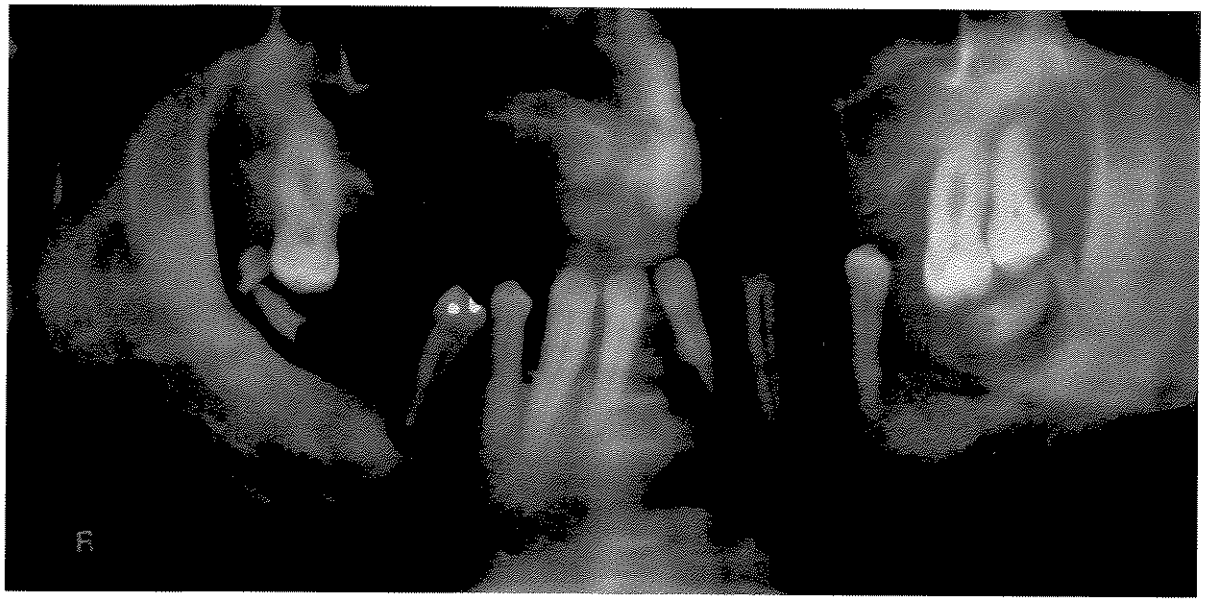

Figure 2. Severe periodontal disease, residual roots and periapical lesions of a patient with floor of the mouth carcinoma. It was necessary to remove all teeth before radiotherapy.

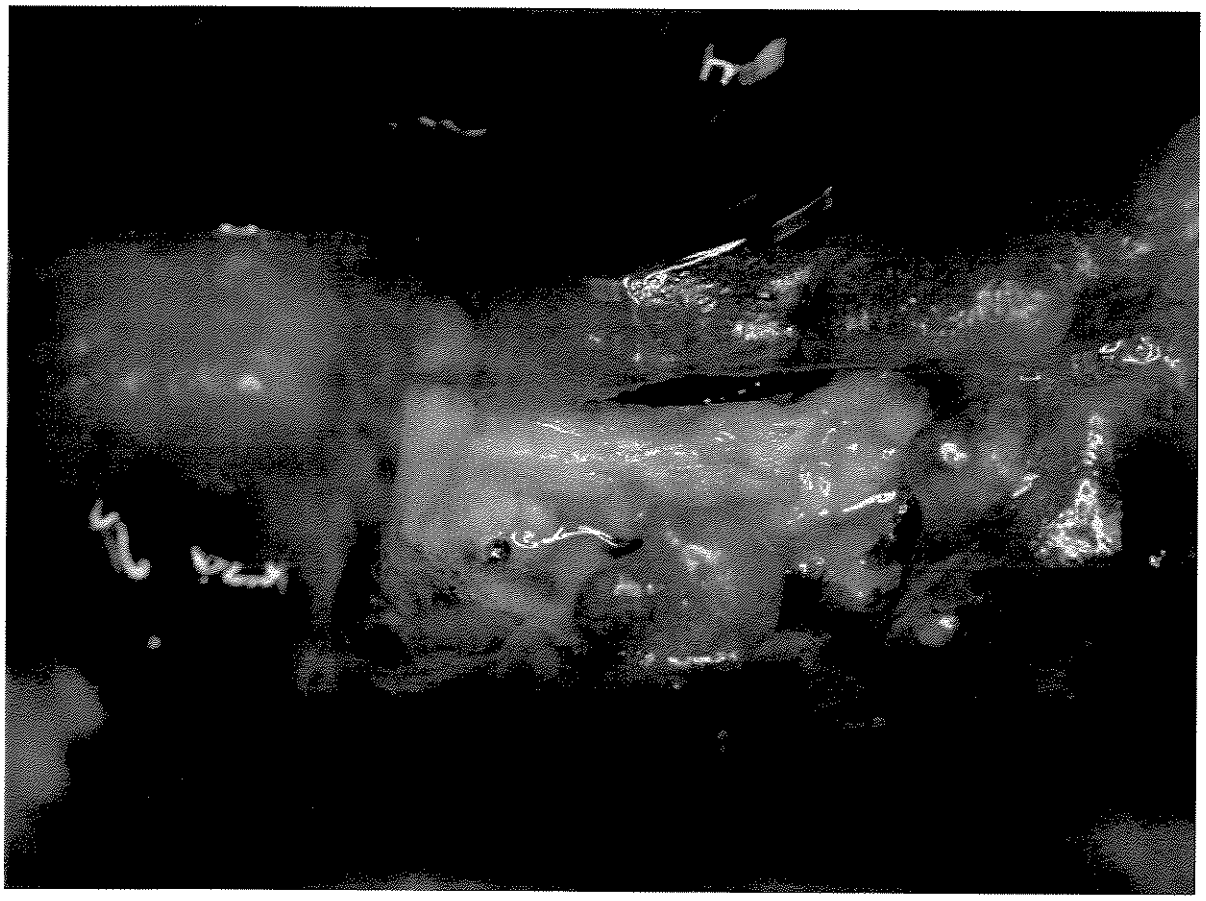

Figure 3. Osteoradionecrosis, on inferior alveolar rigde, arose 1 month after 6300 cGy.

Table 1. Age, gender, tobacco use, tumor site, clinical stage, total radiation doses (cGy) treatment modalities and follow-up of 40 patients with head and neck squamous cell carcinoma.

Patient	Age	Gender	Tobacco		Tumor site	Clinical stage (UICC)	Radiation total doses (cGy)	Treatment	Follow-up (months)
			Use	Period					
1	56	MALE	Yes	25 years	FLOOR OF MOUTH	4	7000	Radio	48
2	58	MALE	Yes	Over 30 years	FLOOR OF MOUTH	4	8100	Radio+Chem	48
3	61	MALE	Yes	Over 30 years	FLOOR OF MOUTH	4	7100	Radio	46
4	75	MALE	Yes	Over 30 years	TONGUE	3	7000	Radio	46
5	58	MALE	Yes	15 years	BUCCAL MUCOSA	4	7440	Radio	46
6	74	MALE	Ex	Over 30 years	FLOOR OF MOUTH	4	7000	Radio+Chem	45
7	83	MALE	Yes	Over 30 years	FLOOR OF MOUTH	3	7000	Radio	45
8	62	MALE	Ex	Over 30 years	TONGUE	4	7100	Surg+Radio+Chem	37
9	52	MALE	Yes	Over 30 years	TONGUE	2	7000	Surg+Radio	42
10	47	MALE	Ex	25 years	OROPHARYNX	4	7000	Radio	43
11	46	MALE	Yes	Over 30 years	TONGUE	4	5040	Radio	39
12	64	MALE	Yes	Over 30 years	OROPHARYNX	4	7000	Radio	40
13	69	MALE	Yes	Over 30 years	BUCCAL MUCOSA	4	6840	Radio	40
14	53	FEMALE	Yes	25 years	FLOOR OF MOUTH	2	7120	Radio	38
15	63	FEMALE	Yes	25 years	BUCCAL MUCOSA	4	7000	Radio+Chem	38
16	64	MALE	Ex	Over 30 years	OROPHARYNX	4	6000	Radio+Chem	37
17	48	MALE	Ex	Over 30 years	SOFT PALATE	3	6000	Surg+Radio	42
18	54	MALE	Ex	Over 30 years	LARYNX	4	7000	Surg+Radio	36
19	63	MALE	Yes	Over 30 years	OROPHARYNX	4	7000	Radio	31
20	41	MALE	Yes	Over 30 years	SOFT PALATE	1	4600	Radio	5
21	40	MALE	Yes	25 years	SOFT PALATE	2	7000	Surg+Radio	25
22	70	MALE	Ex	Over 30 years	TONGUE	4	7200	Surg+Radio	6
23	63	MALE	Yes	Over 30 years	TONGUE	2	6000	Surg+Radio	20
24	54	MALE	Yes	25 years	TONGUE	2	7200	Radio	21
25	73	MALE	Ex	25 years	TONGUE	3	7200	Radio	24
26	50	MALE	Ex	15 years	TONGUE	4	6300	Surg+Radio+Chem	22
27	58	MALE	Yes	Over 30 years	FLOOR OF MOUTH	4	9000	Radio+Chem	28
28	74	MALE	Yes	Over 30 years	RETROMOLAR	2	7200	Radio	32
29	36	MALE	Ex	15 years	RHINOPHARYNX	3	5040	Radio	32
30	46	MALE	Yes	25 years	FLOOR OF MOUTH	4	7040	Surg+Radio	28
31	65	MALE	Yes	Over 30 years	SOFT PALATE	4	7000	Surg + Radio	21
32	34	FEMALE	No	None	RETROMOLAR	4	6900	Surg+ Radio	41
33	62	MALE	Ex	25 years	TONGUE	3	6000	Surg+Radio	3
34	58	MALE	Yes	Over 30 years	FLOOR OF MOUTH	2	6600	Radio	2
35	76	MALE	Ex	Over 30 years	ALVEOLAR RIDGE	4	6000	Surg+ Radio	5
36	64	MALE	Yes	Over 30 years	MAXILLARY SINUS	3	7000	Radio+Chemo	15
37	52	MALE	Yes	Over 30 years	TONGUE	4	7200	Radio+ Chemo	22
38	78	MALE	Yes	Over 30 years	FLOOR OF MOUTH	4	7000	Radio+ Chemo	4
39	52	MALE	Ex	Over 30 years	LARYNX	4	4500	Surg+Radio	8
40	54	MALE	Yes	25 years	FLOOR OF MOUTH	4	7020	Surg+Radio	6

Surg-surgery, Radio-radiotherapy, Chemo-chemoterapy

Table 2. Number of present teeth, prosthesis use, radiographic findings and dental treatment before RxT of 40 head and neck squamous cell carcinoma patients.

PATIENT	NUMBER OF TEETH	RADIOGRAPHIC FINDINGS	PROSTHESIS USE	DENTAL TREATMENT
1	13	Periapical lesions (3)	None	Exodontia (13 teeth)
2	22	Periapical lesion (1)	CUD	Exodontia (22 teeth)
3	9	None	CUD	Exodontia (9 teeth)
4	0	None	None	Edentulous
5	0	Osteosclerosis	CUD+CLD	Edentulous
6	9	None	CUD	Exodontia (9 teeth)
7	1	None	None	Exodontia (1 teeth)
8	12	None	CUD	Exodontia (12 teeth)
9	28	None	None	Exodontia (5 teeth)+ periodontal scaling + fluortherapy and oral care
10	0	None	CUD	Edentulous
11	0	None	None	Edentulous
12	0	None	CUD+CLD	Edentulous
13	9	Periapical lesions (6)	None	Exodontia (9 teeth)
14	7	None	None	Exodontia (7 teeth)
15	19	Periapical lesion (1) + impacted tooth (1)	None	Exodontia (19 teeth)
16	0	None	CUD+CLD	Edentulous
17	22	Osteosclerosis	PUP	Fluortherapy and oral care
18	0	None	CUD+CLD	Edentulous
19	9	None	CUD	Exodontia (9 teeth)
20	17	None	None	Exodontia (6 teeth) + periodontal scaling+ fluortherapy and oral care
21	12	None	PUP	Exodontia (12 teeth)
22	2	Impacted tooth (1)	None	Exodontia (1 tooth)
23	13	Periapical lesions (3)	None	Exodontia (13 teeth)
24	14	None	None	Exodontia (14 teeth)
25	0	None	CUD+CLD	Edentulous
26	12	Tumor rarefation	PLP	Exodontia (12 teeth)
27	16	Tumor rarefation	None	Exodontia (16 teeth)
28	5	None	None	Exodontia (5 teeth)
29	23	None	PUP	Fluortherapy and oral care
30	19	None	PLP	Exodontia (19 teeth)
31	0	None	CUD	Edentulous
32	31	None	None	Fluortherapy and oral care
33	25	None	None	Exodontia (4 teeth) + fluortherapy and oral care
34	1	Impacted tooth (1)	PUP	Exodontia (1 tooth)
35	0	None	CLD	Edentulous
36	0	None	CUD	Edentulous
37	26	None	None	Fluortherapy and oral care
38	13	None	None	Exodontia (13 teeth)
39	15	None	PLP	Fluortherapy and oral care
40	17	None	PLP	Exodontia (9 teeth)+ periodontal scaling + fluortherapy and oral care

CUD- complete upper denture, CLD-complete lower denture, PUP- partial upper prosthesis; PLP- partial lower prosthesis

Table 3. Correlation of 5 osteoradionecrosis cases with age, localization, clinical stage, total and daily radiation doses (cGy), oncologic treatment, time of dental extraction before radiotherapy, etiology and time of manifestation after radiotherapy.

Patients	Age	Localization	Clinical Stage (UICC)	Total radiation doses (cGy)	Daily radiation doses (cGy)	Dental treatment	Oncologic treatment	Interval of extraction	Etiology	Time of manifestation
19	63	Posterior mandible	4	7000	200	Total extraction (9 teeth)	Radiotherapy	10 days	Idiopatic	10 months
21	40	Anterior mandible	2	7000	200	Total extraction (12 teeth)	Surgery + Radiotherapy	7 days	Dental extraction previous RxT	1 month
24	54	Posterior mandible	2	7200	180	Totat extraction (14 teeth)	Radiotherapy	10 days	Idiopatic	3 months
26	50	Anterior mandible	4	6300	180	Total extraction (12 teeth)	Surgery + radiotherapy + Chemotherapy	10 days	Idiopatic	1 month
33	62	Posterior mandible	3	7000	200	Extraction (4 teeth) +fluortherapy and oral care	Surgery+ Radiotheray	2 months	Mandibulectomy	1 month

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Capítulo 3

Histopathological features of initial oral mucositis after radiotherapy of the head and neck

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ABSTRACT

Objective: Many previous reports comment about clinical features and management but few articles deal with the histopathological features of radiation induced mucositis. The aim of this study was to describe the histopathological features of mucositis grade 1 (WHO).

Methods: Ten cases of oral mucositis were biopsied and the samples were submitted to morphometric and immunohistochemical analyzis.

Results: Oral epithelium presented distinctive hyperparakeratosis, nuclear picnosis and atypical keratinocytes. Epithelial thickness, area and perimeter were decreased in mucositis in comparison to normal epithelium. Cellular infiltrate and blood vessels were more numerous on mucositis than normal mucosa. CD68 positiveness was more evident in oral mucositis than in control group (p=0.0112) and CD68 positive cells were found close to epithelium.

Conclusions : Oral mucositis grade 1 caused by radiotherapy, presented distinctive epithelial alterations, increased number of blood vessels and infiltration of mononuclear cells with increased number of macrophages.

KEYWORDS

Radiotherapy; mucositis; oral cancer; CD68

ETHICAL FEATURES

This study was approved by the ethical committee of School of Dentistry of Piracicaba/UNICAMP (process 123/2001) and all patients consented with procedures.

INTRODUCTION

Head and neck carcinomas are usually managed by surgery and/or, radiotherapy (RxT). The criteria for treatment choice include size and tumor localization, bone and muscular involvement, cervical metastasis, total or partial resection possibilities and systemic status of the patients [1]. Although head and neck RxT is effective, chronic and

acute side-effects such as mucositis, xerostomia, hypogeusia, trismus, radiation caries, candidosis and osteoradionecrosis are frequently reported [2-7].

The most important acute head and neck RxT side-effect is oral mucositis [2, 8-10]. Oral mucositis can obligate partial or complete interruption of RxT, decreasing treatment efficacy [2]. Mucositis also causes serious pain and swallowing, eating and speech impairment [11]. Radiation induced-mucositis arises about two weeks after the beginning of RxT, and more than 50% of patients present severe mucositis after half of treatment is completed [12]. Severity of mucositis is dependent on type and intensity of RxT, individual response, epidermal growth factor (EGF) levels, tobacco and alcohol use. [4, 12-13].

The biology of RxT mucositis is not well understood. Interactions among mucosal cells, pro-inflammatory cytokines and local factors such as saliva and microorganisms probably play a key role [14-15]. The initial microscopic events seem to be increased submucosal vascularity and inflammatory cell infiltration mainly formed by macrophages [12,15]. Radiation causes cell lesion and liberation of cytokines from epithelium and connective tissue, resulting in cell death and reduction of basal cell proliferation [14-15]. Clinically, radiation mucositis is characterized by erithema, followed by ulceration covered by fibrinopurulent exsudate [4,11]. RxT mucositis has been studied experimentally [2] but few reports describe the microscopical aspects in humans [2,14-17]. A better understanding of the mechanisms involved in RxT mucositis can be helpful for its prevention and management [2, 17]. The aim of this study was to describe the microscopical aspects of oral mucositis grade I (enanthema, mild descomfort) (WHO) [12] caused by RxT used for treatment of head and neck cancer.

MATERIAL AND METHODS

The present study was composed by 10 patients with head and neck carcinoma (HNC). Patients received RxT treatment (teletherapy) exclusively or associated with surgery. All patients received oral care orientation and drugs such as pilocarpine, artificial saliva, sucralfate and fluortherapy on dentate patients. Refering to xerostomia the patients were classified in absent, (no xerostomia complaints); moderate, (xerostomia ameliorated with pilocarpine uses) and severe, (pilocarpine was not effective to alleviate xerostomia). Biopsies with 5 mm punch, under local anesthesia, were taken from erythematous or

whitish areas of the buccal mucosa, but without ulcerations, at least two weeks after the beggining of RxT when patients presented mucositis grade 1 (WHO) (**Figs 1** and **2**). Nine normal mucosa specimens from the buccal mucosa were taken, at least 3 cm far from the tumor, from patients with HNC before RxT and used as control.

Nine patients received teleradiotherapic treatment from Linear Accelerator (6 MeV) and one patient received Co ^{60} therapy. Eight patients were male and two female. Nine patients referred past or present tobacco use and 8, past or present chronical alcohol use. Eight patients were treated by surgery and RxT, and 2 received RxT exclusively. Total doses were, on average, 6466 cGy, ranging from 4600 to 8000 cGy. Fractionated doses were, on average, 192 cGy, ranging from 180 to 200 cGy. When biopsy was taken from mucositis grade 1 areas, the average dose received by each patient was 3320 cGy ranging from 2700 to 5000 cGy. Association of pilocarpine and sucralfate was recommend for 8 patients, and in one case, artificial saliva was indicated. The majority of patients complained of mild xerostomia.. Data about gender, age, tumor localization, TNM, doses, palliative protocols and xerostomia complaint are shown on **Table 1**.

Microscopical Analyzis

All specimens were fixed in 10% formaline, and embedded in parafin. Five μm sections, stained with H&E were studied by light microscopy, and histomorphometry of the epithelium, blood vessels and inflammatory infiltrate was performed with a KS 400 software (Kontrol KE 2.00). All measurements were done on triplicate. Measurement of epithelial thickness, perimeter and area were performed on X40 magnification, inside a square of 16606610.95 μm^2. Vessels and cellular quantification were done at X400 magnification. Data were analyzed by t two-tailed test.

Immunohistochemistry

Immunostaining was performed using 3μm sections of paraffin-embedded tissue of oral mucositis and control cases, fixed in 10% buffered formalin. All reactions followed standart protocols. The sections were deparaffinized and rinsed for 5 min under running water. Endogenous peroxidase activity was blocked by incubating the slides in 3% H_20_2 . Antigen retrieval was obtained by 10 mM citric acid digestion, pH 6.0, using 2 cycles of 12

min in a microwave. After cooling for 15 min, the slides were transferred to phosphate buffered saline, incubated overnight with primary antibody for CD68 (PG-M1, Dako, Carpenteria, CA, USA, 1:400), followed by streptavidin-biotin peroxidase complex (StrepABC Complex/HRP Duet kit, Dako, Denmark). Reactions were developed with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma) containing 0.01% H_2O_2 and counterstained with hematoxylin. Cytoplasmic and membranous labeling were considered as positive. For quantification, both the positively stained and all negatively stained cells were counted in five sampled high-power fields (magnification X400), immediately below the epithelial basal stratum, using the KS 400 software (Kontrol KE 2.00). The percentual of positive cells was calculated based in total number of counted cells [12]. The Mann-Whitney test was performed for CD68 positive cells analyzis.

RESULTS

Microscopically most of the cases of mucositis grade 1 showed epithelial hyperparakeratosis (8 cases), ectasic and numerous blood vessels (all cases) and mild inflammatory infiltrate (7 cases). Seven cases presented evident epithelial atypia characterized by pleomorfism, hypercromasia, eosinophilia, increased nuclear-cytoplasmic ratio and enlarged nuclei and six cases showed evident vacuolization of epithelial cells. Histopathological findings are shown on **Table 2** and on **Fig. 3** to **5**. Epithelial thickness in mucositis was significantly smaller than normal tissue ($p=0.002$), as well as epithelial area and perimeter ($p=0.02$ and $p=0.007$, respectively). Increased vascularity and inflammatory infiltrate were also significantly higher is mucositis than in normal tissue ($p=0.0001$ and $p=0.019$, respectively) (**Table 3**). CD68 positive cells in oral mucositis (29.78 %, ± 6.889) were found just below the basal stratum. Sporadic CD68 positive cells were found in the normal mucosa (18.557 %, ± 5,333). CD68 positive cells were more common in the oral mucositis cases than in the normal mucosa ($p=0.0112$). The CD68 positive cells pattern in both groups is illustrated in **Fig 6**.

DISCUSSION

Mucositis is a limitating acute side-effect of radiotherapy that can even cause interruption of RxT treatment [2, 9-11]. Mucositis usually arises after two weeks of RxT

treatment, and after half of radiation course almost all patients present severe ulcerative mucositis [11-12]. In our study, patients were biopsied when received, 3370 cGy on average. All patients presented clinically mucositis Grade 1 (WHO), although they were submitted to pilocarpine and sucralfate therapies. Pilocarpine is helpful to minimize dry mouth sensation [18-19] and sucralfate is an useful adjunctive therapy for mucositis [20-23]. Although some studies showed that sucralfate does not prevent or treat mucositis [21-22], a histopathological study demonstrated a reduction of submucosal inflammatory infiltrate when it was used [20].

Epithelial hyperparakeratosis was found on eight cases and this probably results in whitening of the oral mucosa after two weeks of radiotherapic treatment [4]. A study with samples taken from 22 patients before and during irradiation for squamous cell carcinomas in the head and neck region showed microscopically keratosis patterns on irradiated mucosa [2]. Mature and well differentiated cells, which promote epithelial keratinization, were also detected on desquamation of irradiated epithelium after two weeks of RxT [24].

Nevertheless, thinning of the epithelium and denuded areas were also observed on the end of the second week of the RxT [2]. In our study, epithelial perimeter and area of irradiated mucosa were significantly lower than normal mucosa. There was also a reduction on epithelium thickness and rete pegs were less pronounced. This is explained by the reduction of proliferative cells after the first week of treatment and, even with a recover in the second week, proliferation level remains lower than non-irradiated tissue [2]. The same study reported a progressive reduction of germinal and functional cell layers during RxT [2]. This explains why the mucosa turns erythematous and then ulcerate [4]. Adding, one study evaluated two different mice strains submitted to RxT and showed reduced ulcer duration in a strain with higher proliferative rate of oral mucosal cells [25]. It was also reported that administration of KGF (Keratinocyte Growth Factor) reduced the incidence of oral mucosal ulceration [26].

Epithelial atypia is evident on repair-like cells affected by radiotherapy with some being misinterpreted as malignancies [27]. Cellular pleomorfism, hypercromasia, eosinophilia, vacuolization, enlarged nuclei, increased nuclear/cytoplasmic ratio were observed on atypical cells in our study. One study evaluating esophageal cancer cells after chemoradiotherapy revealed 7.5% of cases of dysplasia-like epithelial atypia, with similar

histomorphology as our atypical cells [28]. In this report, dysplasia-like cases were less positive for p53 and MIB 1 than true dysplasia. In several instances, the dysplasia-like changes were misinterpreted as neoplastic, which, in retrospect, led to unnecessary treatment. Similar diagnosis difficulties can be found between dysplasia-like induced by radiation and true dysplasia in the esophagus [28].

Inflammatory mononuclear infiltrate found on our cases were similar to described by Etiz *et al* [20] and Handschel *et al* [12]. The latter study showed that the infiltrate was composed by CD4+ and 8+ lymphocytes and macrophages, mainly RM3/1 subtype macrophages. Granulocytes were rarely found. De Oliveira Rodini and Lara [29], studyng periapical cysts, revealed that the highest CD68 positive cells concentrations was close to cystic epithelium and in areas of active inflammation. CD68 positive cells in mucositis cases were intensely found close to the epithelium maybe downregulating T lymphocytes, may be involved in suppressor functions [12]. Nevertheless futher investigations are necessary to stablish the macrophage role in the oral mucositis. In short, during radiotherapy, RM3/1 macrophage seems to be the most prevalent inflammatory cell in oral mucositis [17]. Etiz *et al* [20] compared sucralfate versus placebo use in head and neck radiotherapy and described statistical differences of the inflammatory infiltrate between irradiated tissues with and without previous sucralfate use. We used sucralfate in nine patients, therefore we can not evaluated its interference on the inflammatory infiltrate. Nevertheless, the infiltrate was more intense than found in normal tissue.

Our study showed significant increased number of blood vessels compared with non irradiated mucosa. This corroborates the findings of Etiz *et al* [20] and Handschel *et al* [17], who reported increased number of blood vessels during RxT and increased vascular permeability. A previous paper commented that ICAM-1 and E-selectin molecules and beta2-integrin expression were increased in irradiated tissue and VCAM-1 expression was lower, indicating increased mononuclear leukocyte transendothelial migration and vascular preservation during RxT [30]. Although vascular alterations are better known on chemotherapy [15], vascular proliferation possibly is associated with inflammation, but this awaits further evidences.

In conclusion, on radiation-induced mucositis Grade 1 (WHO) the epithelium showed hyperparakeratosis and cell atypia. Epithelial thickness, area and perimeter were

significantly reduced comparing to normal mucosa and increased vascularity, inflammatory infiltrate and macrophages counts were evident.

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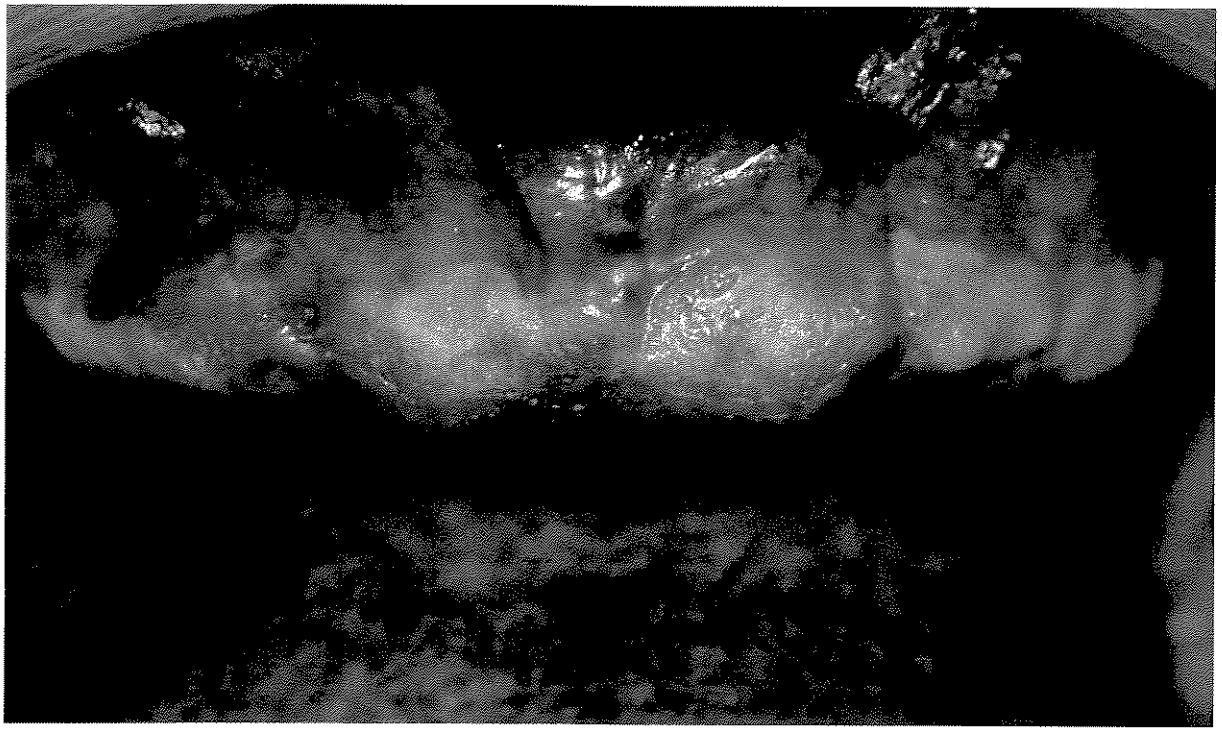

Figure 1. Whitish upper alveolar mucosa of mucositis grade 1(WHO) after received 4600 cGy.

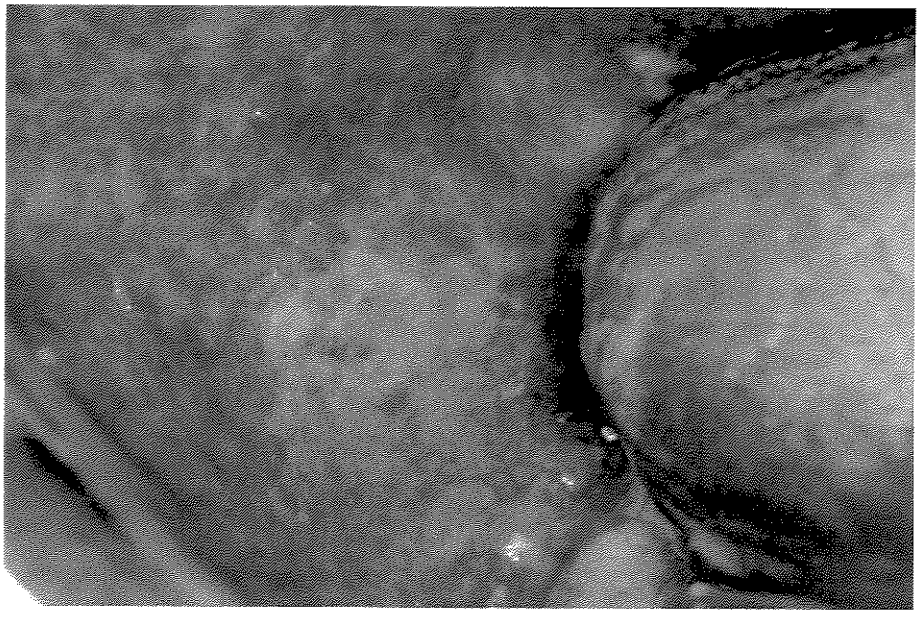

Figure 2. Buccal mucosa of a patient with mucositis grade 1 (WHO). Focal areas of keratosis are observed after two weeks of radiotherapic treatment.

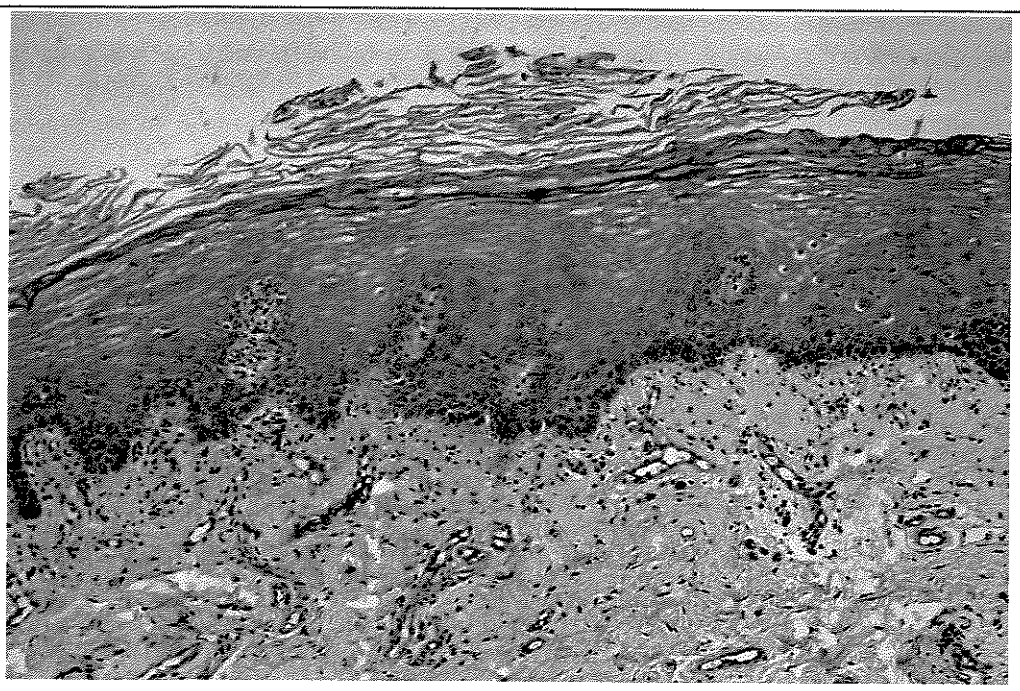

Figure 3. Hyperparakeratosis and mononuclear cell infiltrate associated with increased number of blood vessels in mucositis Grade I (WHO). HE (X100).

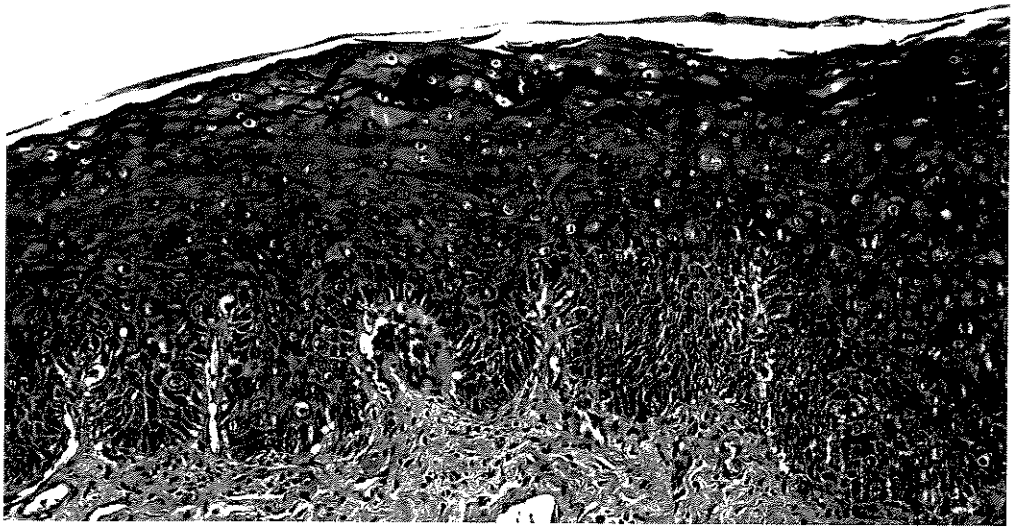

Figure 4. Epithelial cells showing hypercromatic and pleomorphic nuclei and cytoplasmatic eosinophilia in mucositis Grade 1 (WHO). HE (X100).

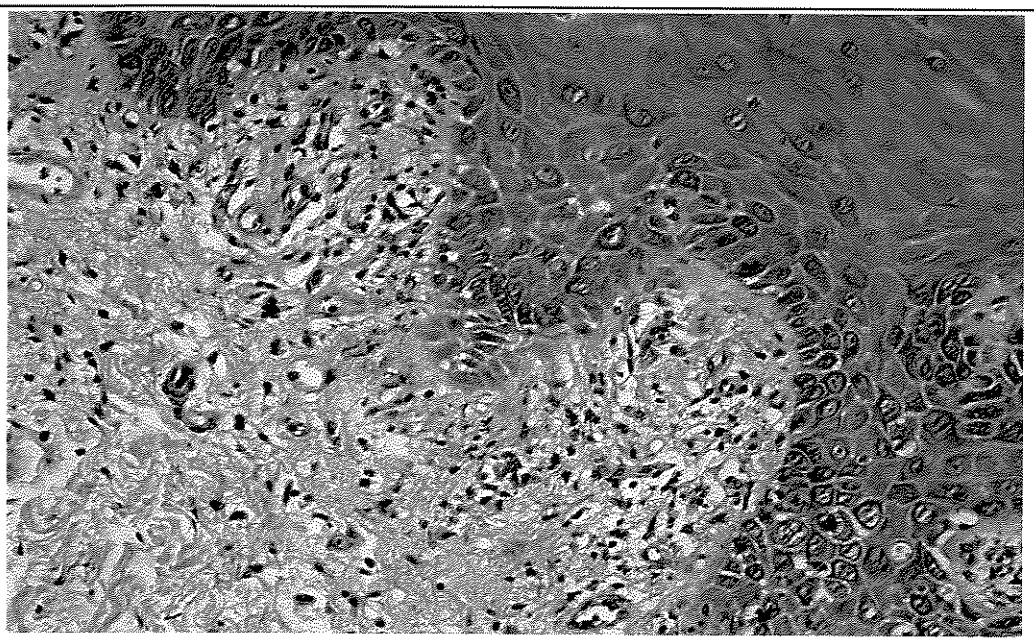

Figure 5. Mild mononuclear infiltrate and blood vessels subjacent to the epithelial basal layer in mucositis Grade 1 (WHO). HE (X200).

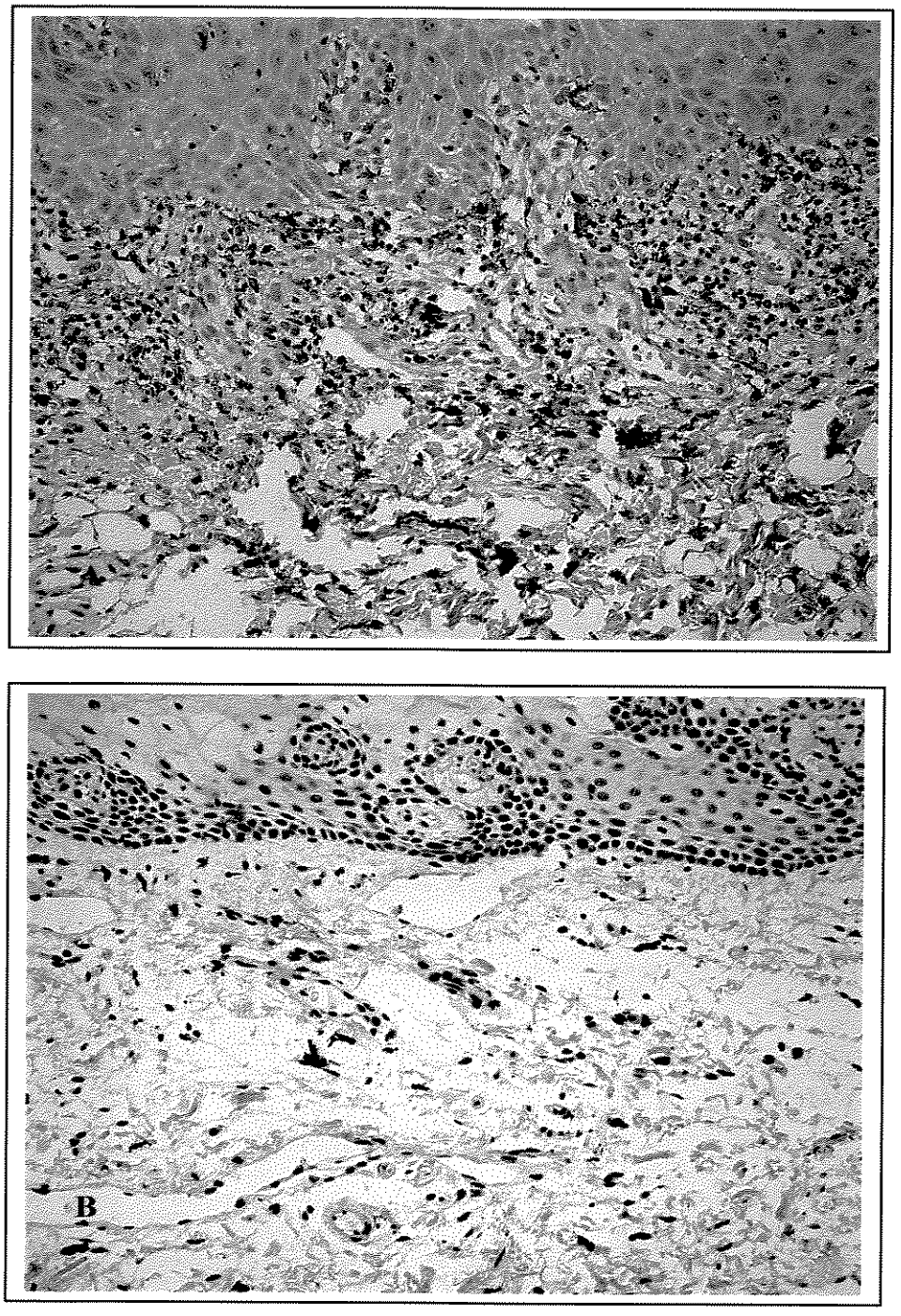

Figure 6. CD68 positive cells (X200). **A**. Oral mucositis showing many CD68 positive cells close to epithelium.

B. Normal mucosa showing sporadic CD68 positive cells.

Table 1. Distribution of 10 irradiated patient according to gender, age, tumor localization, TNM, daily doses, at the moment of biopsies, and total doses on facial fields, drugs used and xerostomia complaints.

Patient	Gender	Age	Site of Tumor	TNM	RxT(cGy)			Drugs	Xerostomia
					Daily	Biopsy	Total		
1	M	48	Conjunctive	IV	200	3000	6000	Pilocarpine, sucralfate	Absent
2	F	54	Tonsil	IV	200	3000	4600	Pilocarpine, sucralfate	Severe
3	M	55	Larynx	III	180	3200	6900	Pilocarpine, sucralfate	Moderate
4	M	64	Cervical metastasis	IV	180	3000	6480	Artificial saliva, sucralfate	Absent
5	M	60	Pyriform sinus	III	180	2700	5040	Pilocarpine, sucralfate	Moderate
6	M	50	Retromolar Area	IV	200	3400	8000	Pilocarpine, sucralfate	Moderate
7	M	54	Floor of the mouth	IV	180	3400	7040	Pilocarpine, sucralfate	Moderate
8	F	43	Palate	IV	200	4600	6000	Pilocarpine, sucralfate	Severe
9	M	42	Tonsil rigde	I	200	5000	7000	Pilocarpine	Absent
10	M	58	Floor of the mouth	IV	200	2400	7600	Pilocarpine, sucralfate	Moderate

Table 2. Histopathological features of 10 initial oral mucositis.

Patient	Histopathological features
1	Hyperparakeratosis, picnosis and atypia, increased number of blood vessels, pigmentar incontinentia , mild mononuclear infiltrate
2	Hyperparakeratosis, epithelial atrophy and atypia, increased number of blood vessels, mild mononuclear infiltrate
3	Hyperparakeratosis, picnosis and epithelial vacuolization, increased number of blood vessels, mild mononuclear infiltrate
4	Hyperparakeratosis, picnosis, atypia and epithelial vacuolization and increased number of blood vessels
5	Hyperparakeratosis, picnosis and epithelial atypia, increased number of blood vessels, mild mononuclear infiltrate
6	Hyperparakeratosis, epithelial vacuolization, increased number of blood vessels
7	Hyperparakeratosis, epithelial vacuolization, increased number of blood vessels, mild mononuclear infiltrate
8	Parakeratosis, apoptotic bodies, vacuolization and epithelial atypia, mixed infiltrate, increased number of blood vessels
9	Parakeratosis, atrophy and epithelial atypia, apoptotic bodies, increased number of blood vessels, vascular dysplasia
10	Hyperparakeratosis, vacuolization and epithelial atypia, increased number of blood vessels, mononuclear infiltrate

Table 3: Epithelial characteristics, blood vessels and inflammatory infiltrate of normal mucosa and mucositis

Patient groups	Epithelial thickness (μm)	Epithelial Perimeter (μm)	Epithelial area (μm ^{2}/per field)	Blood vessels (vessels /per field)	Inflammatory infiltrate (cells /per field)
Mucositis (n=10)	353.7 ±161.01	4536,09 ±763.03	476279.5 ±155754.87	10,85 ±3.66	28.85 ±11,86
Control (n=9)	427.18 ±203.35	5084,57 ±724.48	592922.5 ±212552.59	7,22 ±2.87	14.63 ±29.56
p =	0.002	0.007	0.02	0.0001	0.019

Capítulo 4

Cytokeratin expression in initial oral mucositis of head and neck irradiated patients

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RUNNING TITLE
Cytokeratins in initial oral mucositis

ABSTRACT

Backgroud: The aim of this study was to analyze citokeratin (Ck) expression in initial radiation induced oral mucositis.

Methods: Eleven cases of initial radiomucositis of the buccal mucosa and nine normal specimens were immunostained for Cks 1, 5, 6, 7, 8, 10, 14, 16, 18, and 19 by immunoperoxidase method.

Results: Expression of Cks 1, 6, 10 and 16 was stronger in mucositis than in normal mucosa. Cks 7, 8 and 18 were negative for both control and study groups. Cks 5, 13 and 14 were strongly positive for both groups, nevertheless suprabasal staining for Ck 14 was more evident in mucositis than in controls. Sporadic staining for Ck 19 was observed in one case of mucositis and in 2 controls.

Conclusions: These Cks can be associated with the reactive proliferation of the epithelium and increasing resistance of the oral mucosa during the initial phases of radiotherapy.

KEYWORDS

Cytokeratin, mucositis, oral cancer.

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INTRODUCTION

The most important acute head and neck radiotherapy (RxT) side-effect is oral mucositis (1-3), causing pain, swallowing, eating and speech impairment (4). The biology of RxT induced mucositis is not well understood. Interactions among epithelial cells, pro-inflammatory cytokines and local factors such as saliva and microorganisms are involved. The connective tissue shows increased vascularity and inflammatory infiltrate (5,6). Radiation causes lesions on epithelium, with liberation of cytokines resulting in cell death and reduction of basal cell layer proliferation (5,7). Epithelial renovation decreases on the first week of radiotherapy, followed by restoration of compensative epithelial repopulation on the second week (7,8). These epithelial changes possibly lead to differential expressions of Cks (9-10). Keratins are the predominant cytoskeletal component of stratified keratinized epithelial cells and are the most sensitive indicators of epithelial differentiation and proliferation (11-12). However, no previous studies focused cytokeratin (Ck) expression in oral mucositis induced by radiotherapy, and this is the aim of this study.

MATERIAL AND METHODS

The present study was composed by 11 patients with head and neck carcinomas (HNC) (**Table 1**). Patients received head and neck radiotherapic treatment (teletherapy) exclusively or associated with surgery. All patients received oral care orientation and drugs such as pilocarpine, artificial saliva, sucralfate and, on dentate patients, fluoride. Biopsies with 5mm punch, under local anesthesia, were taken from erythematous or whitish buccal mucosa, when patients presented mucositis Grade 1 (WHO), when oral mucosa became whitish or erithematous, about three weeks after the beggining of radiotherapy. Eight patients were treated by surgery before RxT, and 3 received RxT exclusively. Total doses were, on average, 6532.7 cGy, ranging from 4600 cGy to 8000 cGy. Fractionated daily doses were, on average, 190.9 cGy, ranging from 180 to 200 cGy. On average patients had received 3570.9 cGy when biopsied presenting mucositis grade I (WHO). Association of pilocarpine and sucralfate was recommended for 8 patients, and in two cases, artificial saliva was used for medical reasons. Nine normal buccal mucosa specimens were taken from patients with HNC before RxT and used as control.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed using 3μm sections of paraffin-embedded tissue, fixed in 10% buffered formalin. All reactions followed standart protocols. The sections were deparaffinized and rinsed for 5 min under running water. Endogenous peroxidase activity was blocked by incubating the slides in 3% H_2O_2. Antigen retrieval was performed in 10 mM citric acid digest, pH 6.0, using 2 cycles of 12 min in a microwave. After cooling for 15 min, the slides were transferred to phosphate buffered saline; incubated overnight with primary antibodies for Ck 1, 5, 6, 7, 8, 10, 13, 14, 16, 18 and 19 (**Table 2**), followed by streptavidin-biotin peroxidase complex (StrepABC Complex/HRP Duet kit, Dako). Reactions were developed with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma) containing 0.01% H_2O_2 and counterstained with hematoxylin. Positive and negative controls were included in all reactions. Reactions were considered as negative, when was negative or few positive, moderate or intense, the latter when strong labeling was found in one or more epithelial layers.

ETHICAL FEATURES

The methodology of this study was approved by the ethical committee of Dental School of Piracicaba / UNICAMP (process 123/2001).

RESULTS

All cases of radiation mucositis presented parakeratosis, intense atrophy was evident in two cases and 7 out 11 cases showed epithelial dysplasia-like features (**Fig.1**). Normal buccal mucosa presented parakeratosis. Immunohistochemistry for Cks showed stronger positivity of Cks 1 and 10 in mucositis than in normal tissue in the suprabasal layers and very strong positive staining was found in atrophic regions of mucositis (**Fig.2**). Cks 6 and 16 also were also expressed in the suprabasal layer of normal mucosa and mucositis, but stronger in the latter. Cks 7, 8 and 18 were negative for both groups while Cks 5 and 14 were strongly positive in both. Nevertheless Ck 14 in normal mucosa was expressed only in the basal layer, while in mucositis suprabasal cells were also positive. Ck 13 suprabasal expression was strong in both groups, while slight positiveness for Ck 19 was observed in 3 cases (one case of mucositis and 2 controls). No differences in the Ck pattern were

observed between dysplastic-like and non-dysplastic-like mucosa in mucositis. The patterns of Ck expression are shown on **Figures 3**, **4** and **5** and data from Ck expression are shown on **Table 3**.

DISCUSSION

Our 11 patients with head and neck carcinomas were irradiated with tumoricidal doses and presented mucositis grade 1 (WHO) about three weeks after the beginning of radiotherapy, when accumulated doses were on average 3570.9 cGy. DÖRR *et al* (8) observed rapid suppression in epithelial cell proliferation in the first week of treatment, followed by restoration of compensative epithelial repopulation on second week. It was hypothesized that cytokines such as IL-1, IL-6 and TNF α released from epithelium and adjacent connective tissue are relevant for mucositis development (5). Keratosis and epithelial damage are evident after two weeks of radiotherapy and are followed by epithelial atrophy and ulcerations (1,7).

Cytokeratins are structural proteins which are either constitutively present or induced after tissue injury (11). Cks 1 and 10 are expressed in keratinized oral epithelium and it is suggested that these proteins protect epithelium from trauma or damage. Increased expression in the buccal mucosa leads to epithelial differentiation and keratinization (13-15). BLOOR *et al* (16) using 6 biopsies from normal buccal mucosal epithelium showed that only few cells were positive for Cks 1/10 and another study showed that Ck 10 was absent on normal buccal mucosa (12). Our study showed that Cks 1 and 10 expression are increased on initial mucositis, contrasting with the sporadic positivity on normal mucosa. It can be suggested that it is a response to protect the epithelium from radiation injury (15). In hyperkeratotic lesions of the oral mucosa, Ck 10 is expressed homogeneously in the suprabasal layers, differing from normal epithelium which is either negative or lightly positive (14). The presence of substantial amounts of suprabasal Cks 1 and 10 could impare rigitidy properties to the keratinocyte and this is in concern with the increased positiveness found in atrophic areas in oral mucositis compared with normal thickness in the same cases (17).

Cks 6 and 16 are related with wound repair and repopulation of keratinocytes (14, 18,19). Probably these Cks may be induced by a hyperproliferative keratinocyte response, under the control of cytokines, maybe interferon-gamma (20,21). Due radiotherapy, cytokines are probably released from epithelial and connective tissue (5,21). In our study, epithelium from areas of mucositis expressed more Cks 6 and 16 than normal buccal mucosa, emphasizing the epithelial repopulation and healing attempt after the first week of radiotherapy (8). Epidermal injury, as caused by radiation, induces elevated expressions of Ck 6 and 16, which is accompanied by a dramatic reorganization of intermediary filamentous network and enhanced motility of keratinocytes during the re-epithelization process (19). Interestingly, inactivation of Ck 6a and Ck 6b genes, which promote Ck 6 synthesis, resulted in fragility of the oral mucosa in neonates (11).

All our control and mucositis cases were similarly positive for Cks 5 and 14, in the mytotic basal cell layer of the stratified epithelium. The main characteristic feature of inherited Ck 5 and Ck 14 alterations is the cytolysis of epithelial cells expressing these mutated keratins, resulting in blistering and fragility of the epithelium (17,22). Although these Cks are expressed mainly in the basal cell layer of the buccal mucosa, they were also found in the suprabasal layers (17). Ck 5 staining in the basal cells of the buccal mucosa can be stronger than epidemis, including suprabasal positiveness (17,23). A previous study referred that Ck 14 is detected almost exclusively in the basal cell layer of non-cornified epithelium but it can be found in the upper layers (excluding stratum corneum), when the epithelium is submitted to frequent friction, trauma and stress (24). Five out of mucositis cases presented expression of Ck 14 also in the spinous layer. Mucositis epithelium suffers trauma and stress from radiation and possibly answers with additional protection maintaining this structural protein in the upper layers (14,17,24). Cks 5 and 14 form a filament network that is particularely resilient or tough and elastic resulting in the epithelial protection, yet retaining some plasticity to accomodate new cells as a result of cell divisions, or changes in shape of the tissue (17), as observed in oral mucositis. Indeed, in actinic cheilitis, Ck 14 was described in the suprabasal layers (24) eventually in response to inflammatory cells cytokines. Similarly to actinic cheilitis, radiation mucositis also shows inflammatory infiltrate and dysplasia.

Expression of Ck 13, a suprabasal keratin, was equally found in both groups. This is in agreement with previous studies, which showed Ck 13 staining on parakeratotic lesions of the buccal mucosa (14-15). Ck 19 was positive only in 3 cases, 2 controls and 1 mucositis, staining only basal and parabasal cells. According with Su *et al* (25), studying this Ck in oral epithelium, Ck 19 expression is practically absent in dysplasias. All cases of both groups were negative for Cks 7, 8 and 18, usually expressed in simple and glandular epithelium (13).

Epithelial dysplasia-like mucosa had the same Ck pattern than non-dysplastic mucosa in mucositis. Epithelial dysplasia-like is not yet well described and understood in head and neck mucosa and only few reports commment about dysplasia-like changes induced by radiation in salivary glands and gastric mucosa describing nuclear and cytosplasmatic pleomorphism (26-27).

In summary, Ck immunohistochemistry showed increased suprabasal expression of Ck 14 and increased expression of Cks 1, 6, 10 and 16 in the epithelium of radiation-induced mucositis comparing. These Ck patterns can be related to proliferation and protection of epithelium during initial phases of mucositis. The molecular mechanisms involved in radiation-induced mucositis and Ck expression need further studies.

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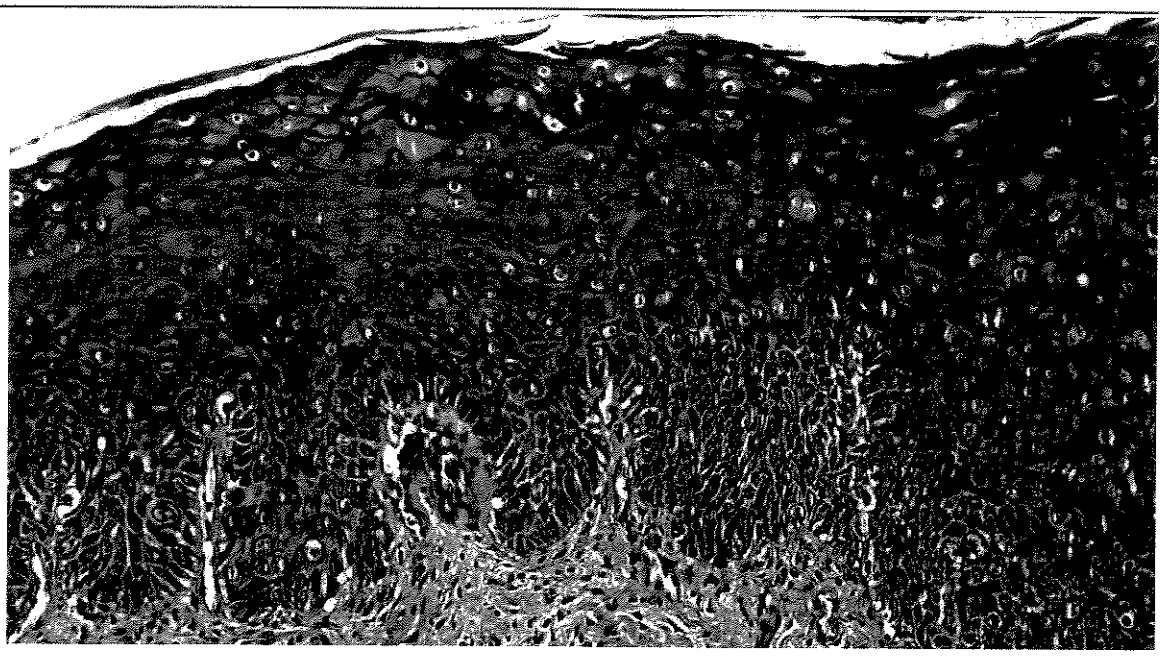

Figure 1. Oral mucositis Grade I (WHO) showing parakeratosis, and dysplasia-like features showing hypercromasia, pleomorphic nuclei and cytoplasmatic eosinophilia. HE (X100).

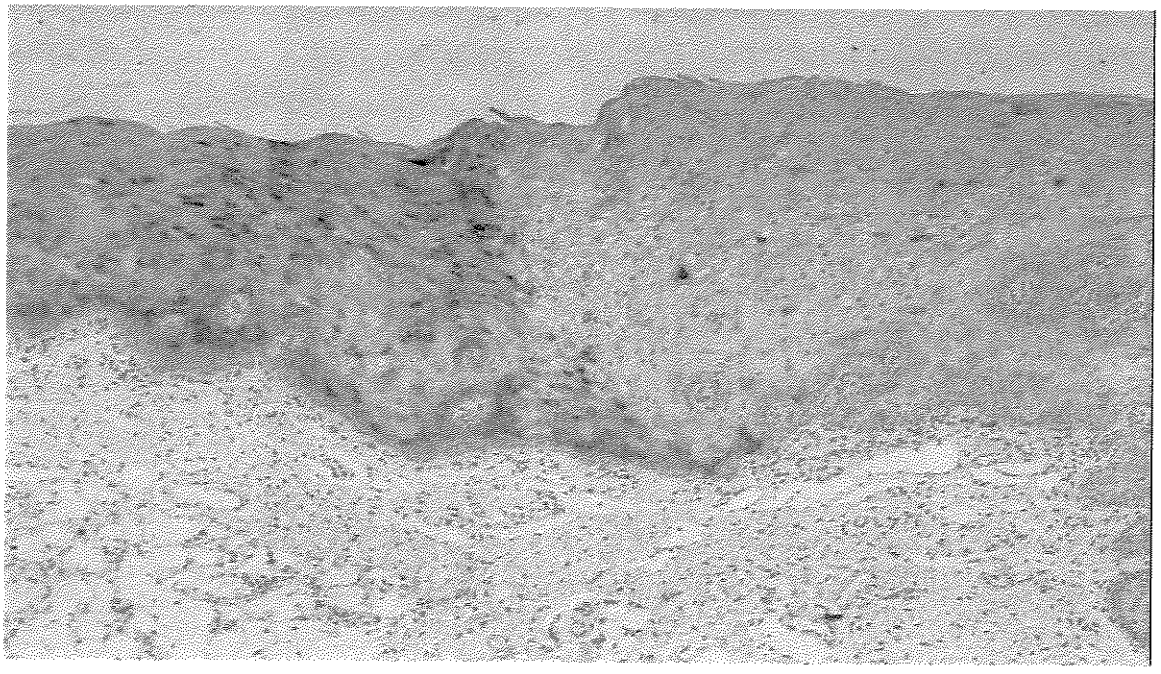

Figure 2. Expression of anti CK1 antibody in atrophic epithelial area of mucositis. Adjacent non-atrophic epithelium shows only few positive cells (X40).

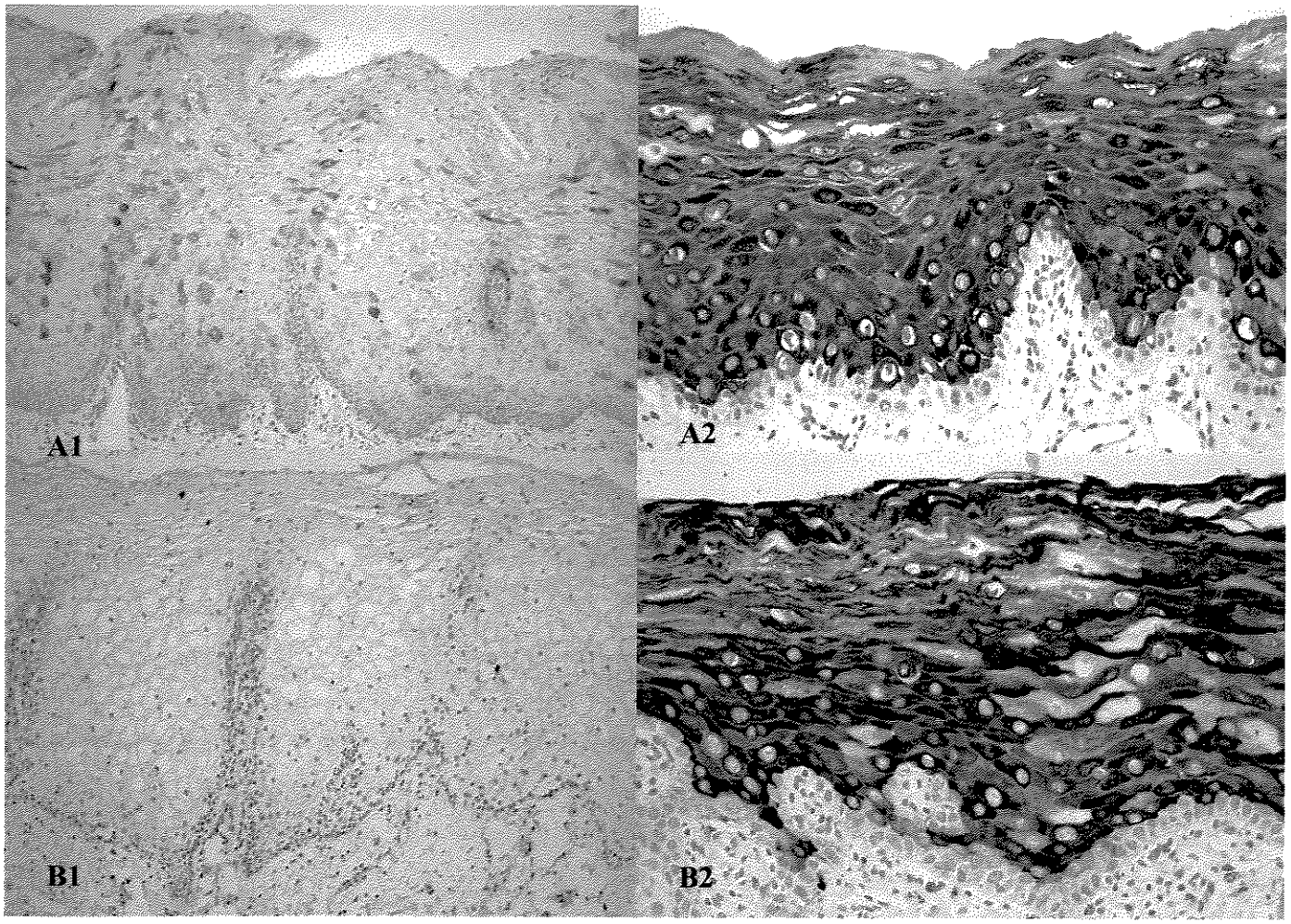

Figure 3. A. Ck1, buccal mucosa X200. Ck1 expression is strongly positive in oral mucositis (A2), while normal mucosa (A1) is positive in only few cells.

B.Ck10, buccal mucosa X200. Suprabasal strong positiveness in oral mucositis (B2) and negative staining of the normal mucosa (B1).

B.Ck14, buccal mucosa. X200. Ck 14 positiveness was observed in all cases of oral mucositis (B2) and normal mucosa (B1), but in mucositis both basal and suprabasal layers were stained.

C. Ck 13, buccal mucosa. X200. Similar strong positiveness was observed in normal mucosa (C1) and mucositis (C2).

Table 1. Distribution of 11 irradiated patients with HNC according to gender, age, tumor site, TNM, daily, at the act of biopsy and total doses, drugs used and xerostomia complaints.

Patient	Gender	Age	Site of Tumor	TNM	RxT(cGy)			Drugs	Xerostomia
					Daily	Biopsy	Total		
1	M	48	Conjunctive	IV	200	3000	6000	Pilocarpine, sucralfate	Absent
2	F	54	Tonsil	IV	200	3000	4600	Pilocarpine, sucralfate	Severe
3	M	55	Larynx	III	180	3200	6900	Pilocarpine, sucralfate	Moderate
4	M	64	Cervical metastasis	IV	180	3000	6480	Artificial saliva, sucralfate	Absent
5	M	60	Pyriform sinus	III	180	2700	5040	Pilocarpine, sucralfate	Moderate
6	M	50	Retromolar Area	IV	200	3400	8000	Pilocarpine, sucralfate	Moderate
7	M	54	Floor of the mouth	IV	180	3400	7040	Pilocarpine, sucralfate	Moderate
8	F	43	Palate	IV	200	4600	6000	Pilocarpine, sucralfate	Severe
9	M	42	Tonsil rigde	I	200	5000	7000	Pilocarpine	Absent
10	M	58	Floor of the mouth	IV	200	2400	7600	Pilocarpine, sucralfate	Moderate
11	M	54	Tongue	IV	180	5500	7200	Artificial saliva, sucralfate	Absent

Table 2. Characteristics of antibodies used

Anti-Ck	Diluition	Clone	Source
1	1:200	34ßB4	Novocastra Lab. Ltda, Newcastle,UK
5	1:400	XM 26	Novocastra Lab. Ltda, Newcastle, UK
6	1:200	LHK6B	Novocastra Lab. Ltda, Newcastle, UK
7	1:400	OV-TL 12/30	Dako Corp. Carpenteria, CA, USA
8	1:200	35ßH11	Dako Corp. Carpenteria, CA, USA
10	1:200	DE-K10	Dako Corp. Carpenteria, CA, USA
13	1:400	KS-1AE	Novocastra Lab. Ltda, Newcastle, UK
14	1:200	LL002	Novocastra Lab. Ltda, Newcastle, UK
16	1:200	LL025	Novocastra Lab. Ltda, Newcastle, UK
18	1:400	DC10	Dako Corp. Carpenteria, CA, USA
19	1:400	RCK108	Dako Corp. Carpenteria, CA, USA

Table 3. Expression of cytokeratins in 11 cases of oral mucositis and 9 cases of normal mucosa.

CK	Control			Mucositis		
	negative	moderate	intense	negative	moderate	intense
Ck 1 *	4 (44.4%)	3 (33.3%)	2 (22.2%)	1 (9.0%)	5 (45.4%)	5 (45.4%)
Ck 10*	6 (66.6%)	3 (33.3%)	0 (0%)	3 (27.2%)	3 (27.2%)	5 (45.4%)
Ck 5	0 (0%)	0 (0%)	9 (100%)	0 (0%)	0 (0%)	11 (100%)
Ck 14	0(0%)	0 (0%)	9 (100%)	0(0%)	0 (0%)	11 (100%)
Ck 7	9(100%)	0 (0%)	0 (0%)	11 (100%)	0 (0%)	0 (0%)
Ck 8	9(100%)	0 (0%)	0 (0%)	11 (100%)	0 (0%)	0 (0%)
Ck 16*	5(55.55%)	3 (33.3%)	1 (11.1%)	4 (36.3%)	2 (18.1%)	5 (45.4%)
Ck 13	0(0%)	2 (22.2%)	7 (77.7%)	0 (0%)	0 (0%)	11 (100%)
Ck 6*	7(77.77%)	2 (22.2%)	0 (0%)	2 (18.1%)	6 (54.5%)	3 (27.2%)
Ck 18	9 [100%)	0 (0%)	0 (0%)	11 (100%)	0 (0%)	0 (0%)
Ck 19	7(77.77%)	2 (22.22%)	0 (0%)	10 (90.9%)	1 (9.0%)	0 (0%)

*Expression more intense in mucositis than control

Obs: Suprabasal Ck 14 expression was found in 5 cases of mucositis and in none of the control

Capítulo 5

Ki-67 expression in oral radiation-induced mucositis: comparative analyzes with oral dysplasia, buccal mucosa of head and neck cancer patients and normal mucosa

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ABSTRACT

Objective: The aim of our study was to evaluate Ki-67 staining in initial oral mucositis induced by radiotherapy.

Methods: Ki-67 immunostaining was performed in 11 cases of oral mucositis and in 13 samples of oral dysplasia, 9 samples of buccal mucosa of head and neck cancer patient (NMCP) and 7 samples of normal buccal mucosa (NM).

Results. Seven cases (63.6%) of oral mucositis presented dysplastic-like features. No statistical differences were observed when oral mucositis and NMCP samples were compared (p=0.0742) but differences were observed in oral mucositis and NM (p=0.0018). Oral dysplasia showed significant increased counts of Ki-67 positive cells (p=0.0005 and p=0.0003, respectively) comparing to NMCP and NM samples, and no statistical differences were observed in oral dysplasia and oral mucositis (p=0.1558).

Conclusion: Oral mucositis presented reactive epithelial proliferation even with radiation damage and dysplastic-like finding presented no different proliferation indexes compared with oral dysplasia.

INTRODUCTION

Oral mucositis is the most important acute side-effect of radiotherapy on head and neck [1-3]. Morbidity of oral mucositis is clearly identified in speeking, swallowing and eating impairments [4]. Initially, epithelial alterations result in a whitish oral mucosa followed by erithema [5] and epithelial cell death and reduction of cell proliferation leads to painful ulcerative lesions [5-6]. Radiotherapy causes changes in the epithelium that mimic dysplasia as described by Brien *et al* [7] in gastric mucosa. Similar alterations were also described in head and neck irradiated epithelium in salivary glands [8]. These epithelium alterations can be misinterpreted as neoplastic, leading to unnecessary treatment [7]. The microscopical features of early mucositis are yet not well known, and the proliferative capacity of the epithelium remains few studied [6]. It is well established that Ki-67, found only in dividing cells, is a reliable marker of cell proliferation [9-11]. The aim of this study was to evaluate Ki-67 expression in oral mucositis, and compare the results with those of oral dysplasia, buccal mucosa of head and neck cancer patients and normal mucosa.

MATERIAL AND METHODS

Eleven head and neck cancer patients who received head and neck teleradiotherapy and presented oral mucositis participated in this study. Characteristics of the patients and treatment received are shown on **Table 1**. Seven patients were treated by surgery plus RxT, one treated by RxT plus surgery and three received RxT exclusively. Total doses had a mean of 6532.7 cGy and the patients had received 3570.9 cGy, in average, when biopsied. All patients received oral care orientation and drugs such as pilocarpine, artificial saliva, sucralfate and fluortherapy on dentate patients. Biopsies with 5 mm punch, under local anesthesia, were taken from erythematous or whitish areas of the buccal mucositis, but without ulceration, about two weeks after the start of radiotherapy when patients presented mucositis Grade 1 (WHO). Thirteen specimens of mild to moderate oral dysplasia, according with pathologist criteria, all clinically diagnosed as leucoplakia, were used for comparative studies (**Table 2**). Nine buccal mucosa specimens obtained from head and

heck cancer patients before radiotherapy (NMCP) at least 3 cm far from the tumor. Eight of them referred previous and present tobacco and alcohol use. Seven normal buccal mucosa (NM) specimens obtained from young dentistry students were used as controls. All of them referred no deleterious chronical habits. The mean ages of MMCP and NM patients were 54.4 years, ± 15.2 years and 19.5 years, ± 0.5 year, respectively.

5μm sections stained with hematoxylin and eosin were used for the histological observations. Ki-67 immunostaining was performed in 3μm sections using standard avidin-biotin complex technique. In short, sections were deparaffinized and rinsed for 5 min under running water. Endogenous peroxidase activity was blocked by incubating the slides in H_2O_2 (3%) for 5 min. The slides were then heated in microwave oven for 24 min in 10 mM citrate buffer at pH 6.0. and after cooling for 15 min, the slides were transferred to phosphate buffered saline. After overnight incubation with primary antibody anti-Ki-67 (Clone MIB1, Dako, Carpenteria, CA, USA, 1:200), the samples were incubated with streptavidin-biotin peroxidase complex (StrepABC Complex/HRP Duet kit, Dako). Reactions were developed with 3,3- diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Company, St. Louis, Missouri, USA) containing 0.01% H_2O_2 and counter stained with hematoxylin. Ki- 67 counts were performed using the software KS 400 (Kontrol), using X400 magnification, considering, on average, 1000 cells per slide (positive/negative), sampled in different fields. Statistical analyzes were performed using Mann-Whitney test .

RESULTS

Seven out 11 (63.6%) oral mucositis specimens presented parakeratosis, epithelial atrophy, reduced rete-pegs and epithelial dysplastic-like cells from the basal layer to half of the epithelium classified as mild to moderate. Atypical-like cells on light microscopic showed nuclear pleomorfism, proeminent nucleoli, mitosis, increased nuclear-cytoplasmic ratio and hyperchromasia, cytoplasmic eosinophilia and vacuolization (**Fig.1**). Areas indicative of hyperemia and mononuclear infiltrate were frequent findings in the subjacent connective tissue. The 4 cases without dysplasia presented hyperparakeratosis and atrophy in one case.

All cases of true oral dysplasia were classified as mild to moderate, with basal and parabasal cells presenting nuclear pleomorphism, loss of nuclear polarity and hyperchromasia.

The percentual of Ki-67 positive cells was 17.5 % for NM samples, 29.8 % for NMCP, 41.0 % for oral mucositis and 51.7 % for true oral dysplasia. These data are shown on **Figs. 2** and **3**. NMCP, oral mucositis and oral dysplasia showed significantly increased numbers of Ki-67 positive cells (p=0.005, p=0.0018 and p= 0.0003, respectively) than NM. Oral dysplasia showed increased number of Ki-67 positive cells than NMCP (p=0.0005) and no statistical differences were shown when oral dysplasia and oral mucositis were compared (p=0.1558). No differences were observed between oral mucositis and NMCP (p=0.0742). Suprabasal staining (over two layers up the basal) were evident in oral dysplasia but also presented in oral mucositis. Basal and parabasal (up to two layers up the basal) staining were found in NM, NMCP and oral mucositis being basal labeling more evident in the last. Ki-67 labeling pattern and intensity are illustrated on **Fig. 4**.

DISCUSSION

Radiation induced mucositis results in decreased quality of life of head and neck cancer patients. Histopathologic features of oral mucositis induced by radiotherapy are not well known and a scarcity of studies dealt with their pathological alterations. Handschel *et al* [9] described vascular and inflammatory infiltrate increasing during radiotherapy and Dõrr *et al* [6] observed thinning of epithelium and denuded areas after two weeks of radiotherapic treatment. Epithelial keratosis is also expected in initial phases of mucositis [5]. Dysplastic-like epithelial cells were observed by Brien *et al* [7] who gave a detailed description in gastric mucosa after chemoradiotherapy. Pleomorphism and nuclear hyperchromasia were evident in irradiated epithelium of salivary glands according to Busuttil [8]. Thinning of epithelium and areas of parakeratosis were observed in our cases. Dysplastic-like cells were found in 7 out 11 cases (63%) and hyperemia and mononuclear infiltrate were frequent findings in the subjacent connective tissue, in accordance with the literature.

Clinical managment of oral mucositis is extensely discussed in the literature, but few studies focused the proliferative capacity of irradiated epithelium. Dõrr *et al* [6] studied

22 mucosal biopsies taken before and during radiotherapy submitting the samples to in vitro incubation with thymidine for autoradiographic identification of DNA-synthesizing cells. It was observed that at the end of the first week of treatment the proliferative activity of epithelial cells decreased. Epithelial celular turnover was partially restored after the end of first week and the continuing rate of declining cellular counts during the remainder of therapy reduced substantially. These proliferative reactions can occur due to breaking the normal restriction to asyimmetric stem cell division [12] and in addition, cells normally destined to exfoliate without division, regain their capacity to divide [13].

Ki-67 is widely used as a marker of cell proliferation [11,14]. Cells in G1, S, G2 and mitosis are positive for Ki-67, but cells in G0 and early G1 are Ki-67 negative [15]. Differences between NMCP and oral mucositis counts were not observed although statistical differences were shown when NM samples were included. This is probably explained by the reduction of basal proliferation of epithelial cells during radiotherapy, and even with a little recover after the second week of treatment, the S phase positive cells were decreased comparing with NMCP [6]. However, 10 of 11 oral mucositis patients used sucralfate, a coated salt which binds with EGF, promoting epithelial proliferation probably increasing epithelial renew [16]. Even with epithelial atrophy, the remaining epithelium reacts promoting cellular repopulation [6]. Probably the increased Ki-67 positive cells in oral mucositis and epithelial atypia found in oral mucositis could also contributed for mucositis counts [17].

NMCP samples showed statistical higher Ki-67 positive cells than NM. This probably happened due to chronical use of tobacco and alcohol in NMCP that contributed for mytogenesis and proliferation. Increased Ki-67 expression after cessation of smoking could indicate permanent epithelial alteration [18]. Adding, Kotelnikov *et al* [19] studied normal oral mucosa without dysplasia near to malignant tumors and observed high counts of positive cells for IdUrd and BrdUrd proliferation markers probably resulting from cancerization field. Although our samples were taken at least 3 cm far from the tumor, they were also exposed to carcinogens [20].

Oral dysplasia presented the higher Ki-67 positive counts, significantly higher than NMCP and NM. In a study with rats, Sato *et al* [21] referred that Ki-67 labeling was significantly higher in dysplasia and early cancer induced by 4 nitroquinoline 1-oxide than

normal tissue. In accordance, Macluskey *et al* [22] using two methods for quantification of Ki-67 and comparing normal mucosa, dysplasia and squamous cell carcinoma, found proliferative indexes significantly higher in dysplastic lesions than in the normal oral mucosa. These authors concluded that epithelial proliferation accompanies the transition from normal to dysplastic tissue, and Ki-67 increasing is an early marker of disease progression in the oral mucosa. Significant changes were observed in the labeling index of p53 and Ki-67 in leukoplakia, as epithelial dysplasia progressed from mild to moderate or severe [23]. Liu *et al* [24] described Ki-67 labeling and showed a very significant change, with a 9-fold increase in the basal layer, when dysplastic leukoplakias were compared with normal tissue. In agreement, Piatelli *et al* [25] described statistical differences between normal mucosa and mild dysplasia. Futhermore, Sittel *et al* [26] reported more elevated Ki-67 indexes in oropharynx and oral cavity carcinomas from patients suffering from treatment failure than non-failures, indicating the possiblity of using Ki-67 labeling as prognostic marker.

Oliver *et al* [27], studying cases of oral epithelial dysplastic lesions, described intense suprabasal Ki-67 labeling. Indeed, Girod *et al* [28] observed Ki-67 positive cells in true dysplastic lesions, located not only in basal layer but also in suprabasal layers. It was also confirmed by Gonzalez-Moles *et al* [10], who described suprabasal positive Ki-67 cells and association between suprabasal labeling with the severity of dysplasia.

Although dysplasia and oral mucositis presented higher counts of Ki-67 positive cells, no statistical differences were observed when both were compared. This probably happened due to the dysplastic alterations observed in mucositis which presented similar dysplastic features, in majority of cases, than oral true oral dysplasia [10]. Interestingly, basal proliferation was evident in mucositis. The same finding was observed by Kotelnikov *et al*[19] and it can be explaned due to the alteration of the balanced replacement of proliferative parabasal cells and the break of normal restriction to asymmetric basal cell division [12,19].

Different sites of dysplastic lesions were analyzed in our cases including lower lip, palate and buccal mucosa. Experimentally, seven samples from normal lower lip, seven from normal palate and seven from normal buccal mucosa stained with Ki-67 were compared and no statistical differences were observed (unpublished data). In addition,

statistical differences in Ki-67 counts of different sites of oral dysplasia were not observed by Liu and Klein-Szanto [17].

In summary, oral mucositis presented epithelial atrophy, parakeratosis and epithelial dysplasia-like features. Significative Ki-67 positive cells increasing was observed in oral dysplasia and oral mucositis comparing to scarce labeled cells in NM. NMCP samples presented more Ki-67 positive cells than NM. No difference among oral mucositis and oral dysplasia was shown. Basal labeling in oral mucositis and suprabasal positiveness in oral dysplasia were evident. Futher investigations are necessary to stablish histopathological and cellular differences between oral dysplasia and dysplasia induced by radiotherapy.

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ETHICAL FEATURES

The patients formally consented with the procedures and the study methodology was approved by ethical committee of Dental School of Piracicaba/Brazil (Process nº:123/2001).

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Table 1. Distribution of 11 irradiated patients with HNC according to gender, age, tumor site, TNM, daily, at the act of biopsy and total doses and drugs used.

Patient	Gender	Age	Site of Tumor	TNM	RxT(cGy)			Drugs
					Daily	Biopsy	Total	
1	M	48	Conjunctive	IV	200	3000	6000	Pilocarpine, sucralfate
2	F	54	Tonsil	IV	200	3000	4600	Pilocarpine, sucralfate
3	M	55	Larynx	III	180	3200	6900	Pilocarpine, sucralfate
4	M	64	Cervical metastasis	IV	180	3000	6480	Artificial saliva, sucralfate
5	M	60	Pyriform sinus	III	180	2700	5040	Pilocarpine, sucralfate
6	M	50	Retromolar Area	IV	200	3400	8000	Pilocarpine, sucralfate
7	M	54	Floor of the mouth	IV	180	3400	7040	Pilocarpine, sucralfate
8	F	43	Palate	IV	200	4600	6000	Pilocarpine, sucralfate
9	M	42	Tonsil rigde	I	200	5000	7000	Pilocarpine
10	M	58	Floor of the mouth	IV	200	2400	7600	Pilocarpine, sucralfate
11	M	54	Tongue	IV	180	5500	7200	Artificial saliva, sucralfate

Table 2 . Clinical data about 13 cases of oral epithelial dysplasia

Patient	Gender	Age (years)	Site
1	F	56	Buccal mucosa
2	M	72	lower lip
3	M	43	palate
4	F	60	palate
5	F	71	Buccal mucosa
6	M	38	Buccal mucosa
7	M	79	Lower lip
8	M	60	Lower lip
9	M	33	Buccal mucosa
10	M	39	Buccal mucosa
11	M	41	Buccal mucosa
12	M	69	Buccal mucosa
13	M	47	Buccal mucosa

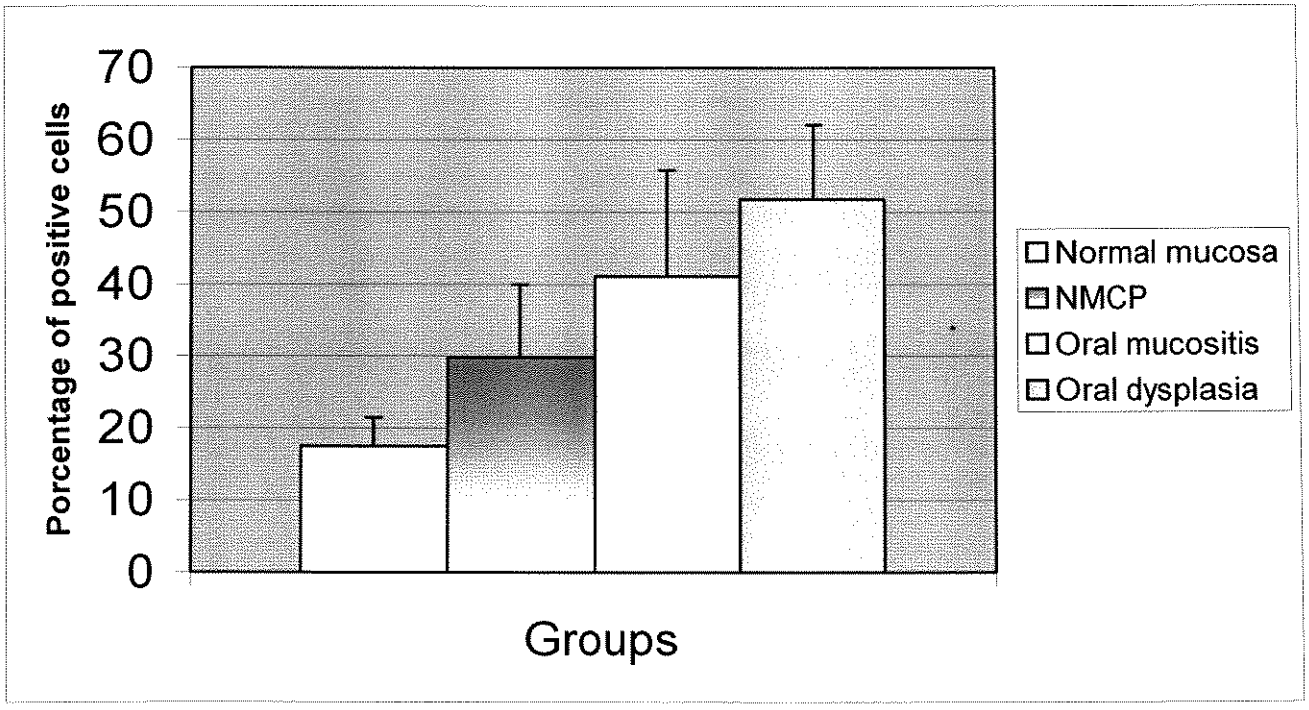

Figure 3. Porcentage of Ki-67 positive cells in the four groups. The means are the bars and standard deviations are shown.

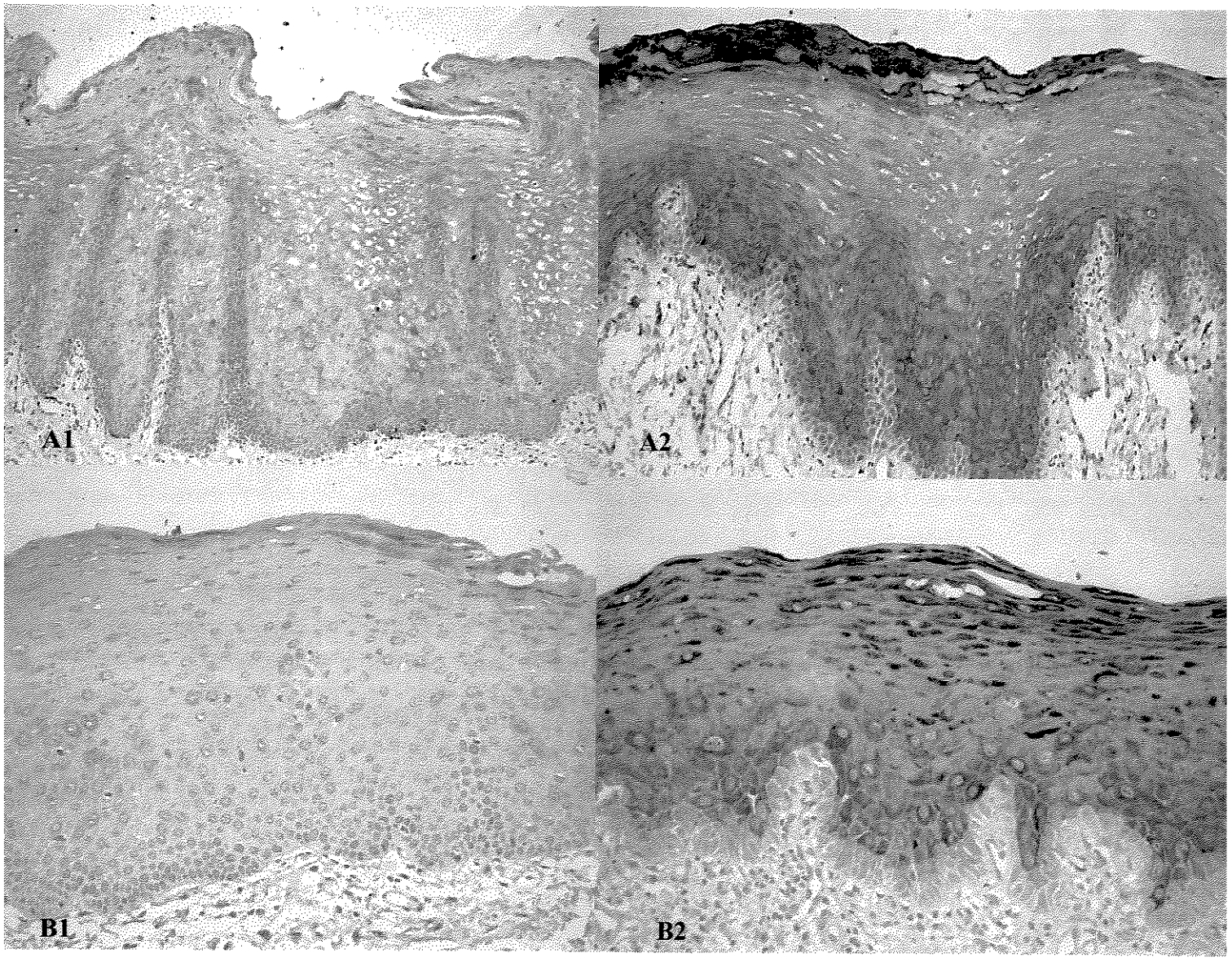

Figure 4. A. Ck 6, buccal mucosa. X200. Oral mucositis (A2) was positive for Ck 6 and normal mucosa (A1) was negative.

B. Ck 16, buccal mucosa. X200. Suprabasal strong positiveness in oral mucositis (B2) while the normal mucosa (B1) was negative.

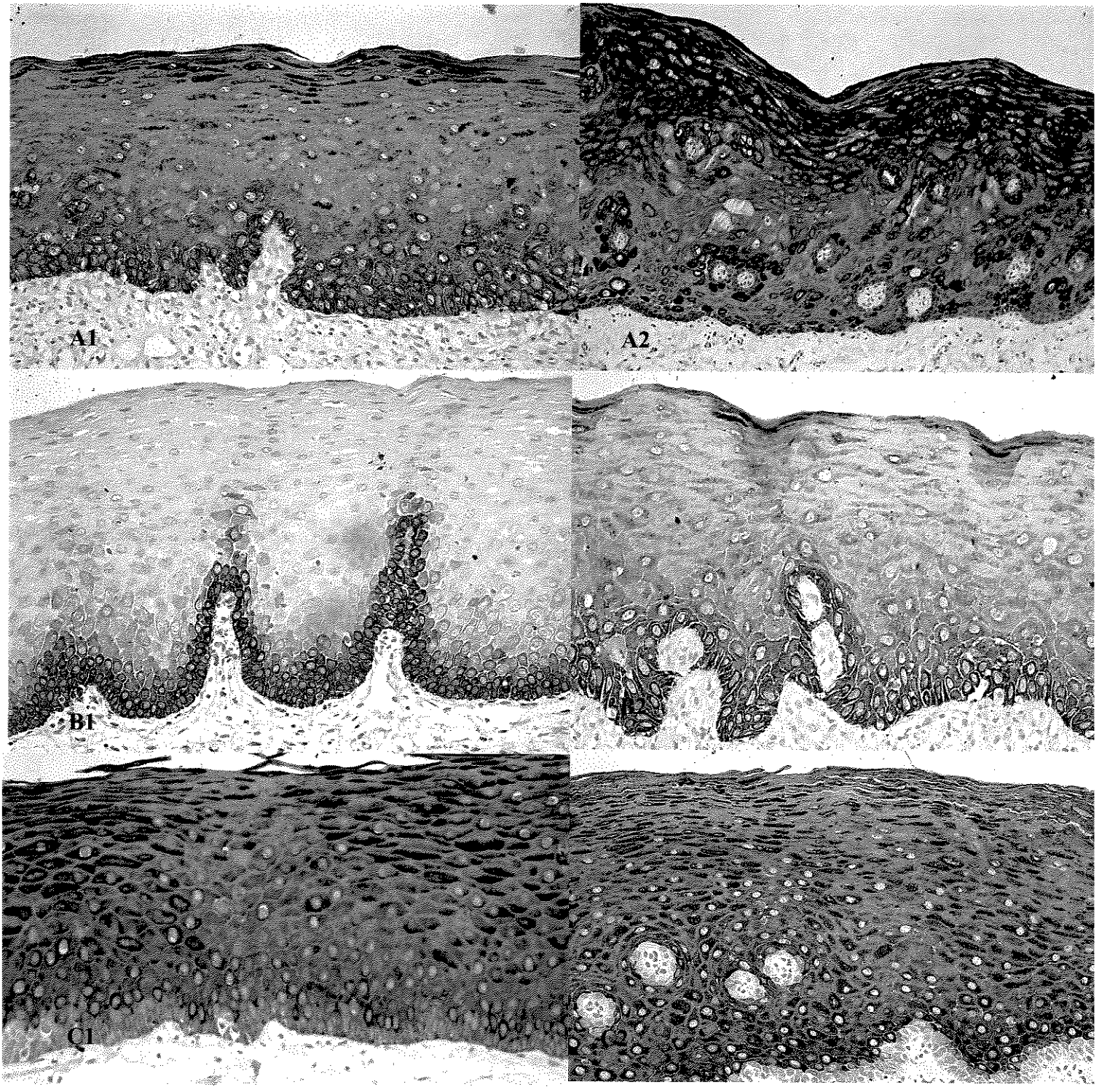

Figure 5. **A**. Ck 5, buccal mucosa. X200. Ck 5 expression was similarly positive in oral mucositis (A2) and normal mucosa (A1).

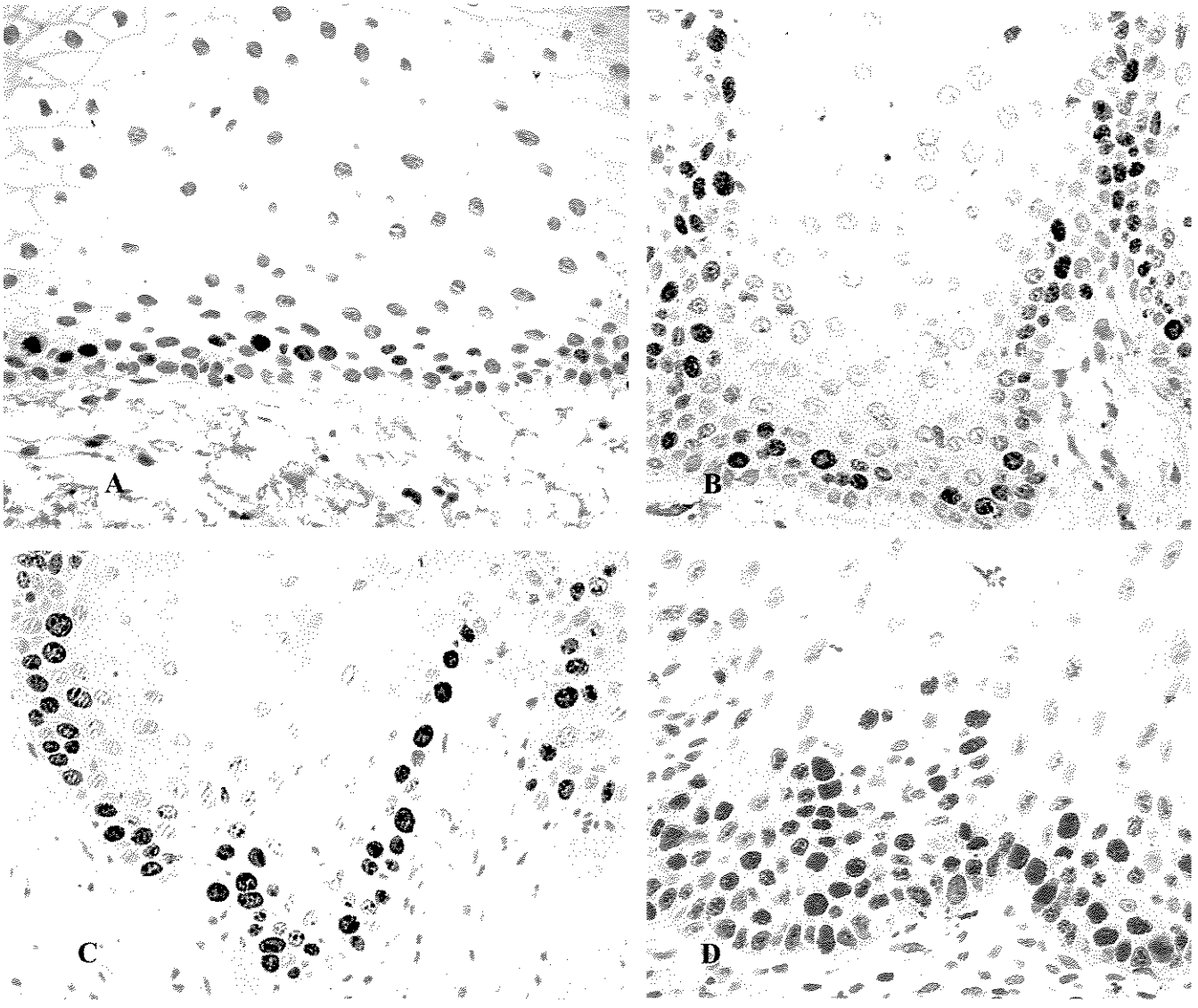

Figure 4. Expression of Ki-67 positive cells, Immunoperoxidase (200X). **A**. Normal mucosa (NM). NM showed few positive cells in parabasal layer.

B. Normal mucosa of cancer patients (NMCP). NMCP showed parabasal positive cells more commonly than NM.

C. Oral mucositis. Oral mucositis presented basal and parabasal positive cells.

D. Oral dysplasia. Oral dysplasia presented basal, parabasal and suprabasal positive cells.

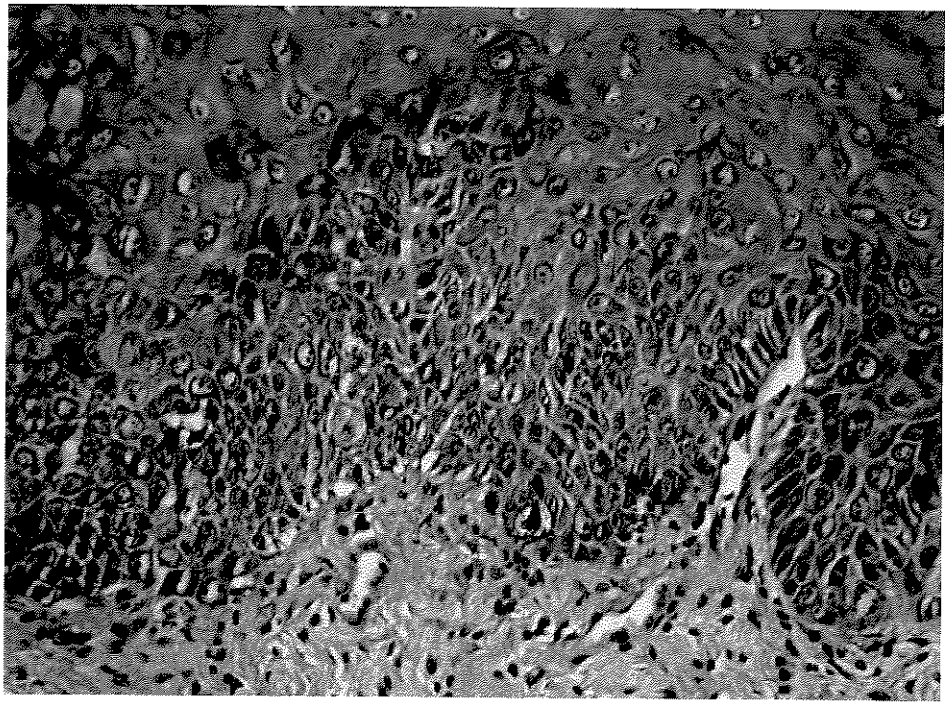

Figure 1. Dysplasia-like findings induced by radiation showing pleomorphic nuclei, nuclear hyperchromasia, picnosis, loss of nuclear polarization, eosinophilia and vacuolization. HE (X100).

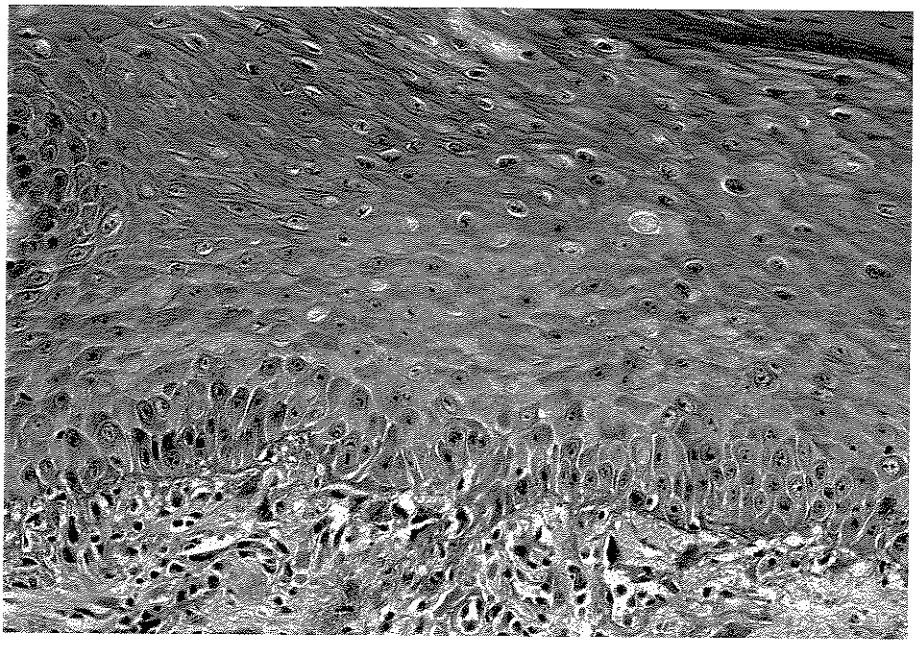

Figure 2. Mild to moderate epithelial dysplasia with pleomorphic nuclei, loss of nuclear polarization and slight hyperchromasia. HE (X100).

Conclusões

1) A mucosite oral é um processo patológico complexo, com aspectos histopatológicos e biológicos ainda sendo esclarecidos e com manejo paliativo e sintomático.
2) Os pacientes portadores de carcinomas espinocelulares em cabeça e pescoço apresentaram, em sua maioria, péssimas condições dentárias.
3) O tratamento odontológico mais realizado em pacientes com carcinomas espinocelulares em cabeça e pescoço foi exodontias seriadas.
4) Embora exodontias seriadas prévias a radioterapia tenham sido realizadas na maioria dos casos, não impediram o surgimento de 5 casos de osteorradionecrose, os quais apresentaram etiologia multifatorial.
5) Atrofia, redução do perímetro e área do epitélio; alterações com características de displasia e aumento de vasos no conjuntivo e no infiltrado inflamatório, formado principalmente por macrófagos, foram observados na mucosite oral inicial.
6) Na mucosite oral inicial houve aumento na expressão das Ck 1, 6, 10, 14, 16 em relação ao epitélio normal.
7) A marcação imunohistoquímica para Ki-67 evidenciou alto índice da proliferação camada basal na mucosite oral e não foi estatisticamente diferente da displasia oral.

Anexos

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

O presente estudo intitulado "Avaliação dos mecanismos envolvidos na mucosite oral induzida por radioterapia" está sendo realizado tendo como objetivo compreender melhor os fenômenos relacionados com a queimação de boca devido a radioterapia e propor novos tratamentos para esse problema que resulta em dor, em dificuldade de se alimentar, falar e ingerir líquidos. Isso só será possível se analisarmos como a mucosa bucal se comporta durante as diferentes fases do tratamento. Visando a compreensão desse processo diretamente nos pacientes e não em animais (isso torna os resultados mais significativos) será realizada 1 biópsia em bochecha, antes da radioterapia, e/ou em alguns casos uma segunda biópsia aproximadamente na metade do tratamento. Os pacientes serão previamente avaliados através do seu estado físico e psicológico anteriormente a realização dos procedimentos. Todos os procedimentos serão realizados sob anestesia local, em área previamente anestesiada com anestésico tópico, onde será removido um pequeno fragmento de tecido de bochecha ou da parte de fundo de sulco, sendo leve o desconforto durante a cirurgia e após sua realização. O risco de desenvolvimento de infecções secundárias é mínimo. Analgésicos para uso do paciente serão fornecidos. Os pacientes terão acompanhamento do pesquisador Paulo Rogério Ferreti Bonan no Orocentro da Faculdade de Odontologia de Piracicaba. O telefone do pesquisador é (19) 9153-0213 e serão acompanhados durante e após o tratamento oncológico. O paciente terá todo o direito de questionar sobre a metodologia da pesquisa e não aderir ou abandonar a realização da pesquisa sem nenhuma espécie de retaliação. O paciente irá receber os laudos histopatológicos das biópsias realizadas. Terá o direito à preservação de sua privacidade visto que os espécimes histológicos são identificados por números e não por nome. Conforme supracitado os pacientes receberão medicação analgésica gratuita, acompanhamento e tratamento odontológico antes, durante e após o tratamento oncológico, gratuitamente.

Ciente,

_____ _____

Nome e RG Assinatura

_____, de _____ de _____