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ORIGINAL ARTICLE

Effects of imiquimod and low-intensity laser (λ660nm) in chemically induced oral carcinomas in hamster buccal pouch mucosa

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Abstract Squamous cell carcinoma (SCC) is the most common neoplasm of the oral cavity. It is aggressive, highly proliferative, and metastatic. This study aimed to evaluate the effect of LLLT and imiquimod on DMBA chemically induced lesions on the oral mucosa of hamsters. SCCs were induced on 25 hamsters. Animals of G1 (control 1) were killed and the presence of tumors confirmed; G2 (control 2) suffered no interventions for additional 4 weeks; animals of G3 (laser treatment) were irradiated (λ660nm, 50 mW, CW, Ø=3 mm, 0.07 cm^2, 714.2 mW/cm^2, 133 s, 95 J/cm^2, 6.65 J) at every other day for 4 weeks; animals of G4 (imiquimod treatment) received 5 % imiquimod three times a week for 4 weeks; and animals of G5 (imiquimod and laser treatment) received both treatments for the same period. Samples were taken and underwent histological analysis by light microscopy and were investigated using immunohistochemistry for $S\text{-}100^+$ dendritic cells. In G1, G2, and G3, the evaluations showed malignant tumors and the absence of $S\text{-}100^+$ dendritic cells in the tumor stroma. In G4, 60 % of the animals had no malignant tumors, and $S\text{-}100^+$ dendritic cells were present in the stroma of the tumors as well as dysplasia. In G5, 40 % of the animals presented SCC, with scarce or no $S\text{-}100^+$ dendritic cells. The imiquimod treatment played a direct effect on SCC, demonstrated by the increased number of $S\text{-}100^+$ dendritic cells, which could suggest an important role of immune surveillance against neoplastic proliferation. Furthermore, its association with laser needs to be further investigated.

Keywords Diode laser · Squamous cell carcinoma · Chemical carcinogenesis · Imiquimod

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Introduction

The golden Syrian hamster cheek pouch model of intraoral chemical carcinogenesis is one of the most used models for inducing experimental squamous cell carcinoma (SCC) as its mechanism occurs similarly to what is seen in the development of mucosal premalignant and malignant lesions in humans [1, 2]. Furthermore, the experimental development of SCC has been used to investigate new techniques of detection, early diagnosis, and treatment [3–5].

Modern immunotherapy involves the use of immune modulators, which are drugs that may either enhance or reduce immune response [6]. The imiquimod (1-(2-methylpropyl)-1-H-imidazole [4,5-c] quinolone-amine) is a new synthetic compound capable of activating the cells of the immune system, helping to control viruses, tumors, and intracellular parasites [7]. It possesses immune-modulatory action with anticancer activity [8, 9] and has two recognized properties: pro-apoptotic and immune modulatory [10].

There are three main hypotheses to explain the molecular mechanisms by which the imiquimod induces apoptosis of tumor cells. The first hypothesis is that the drug would directly activate cell death receptors present in the cell membrane, triggering the apoptotic cascade. The second suggests that the substance would penetrate the cell membrane acting later in the events that trigger apoptosis, independently from the receptors. In the third hypothesis, the compound would not depend on receptors either, acting in an intrinsic pathway that is dependent on the release of mitochondrial cytochrome C [11].

The pro-apoptotic action of imiquimod occurs through modulation of Bcl-2 family. It has been shown that tumor cells become more susceptible to apoptosis due to the reduction of the expression of Bcl-2 that occurs following the treatment with 5 % imiquimod cream [9, 12, 13].

With respect to the immune modulatory action, Bcl-2 may bind itself to specific receptors on dendritic cells resulting in both transcription and release of multiple pro-inflammatory local cytokines. This type of innate immune response is sufficient to induce also a tumor-specific cell immune response, as antitumor activity of imiquimod is based primarily on activation of the native immune system that seems to be controlled by the dendritic cells. These cells respond to lower concentrations of imiquimod better than many other cell types [9, 10], suggesting an important action of imiquimod on the efficiency of dendritic cells in presenting antigens [14].

Tumor regression occurs when T lymphocytes ($CD8^+$) recognize the peptide–MHC-I complex on the surface of tumor cells. This occurs when dendritic cells migrate to the tumor, capture tumor antigens and migrate to secondary lymphoid organs, generating cytotoxic effector T cells ($CD8^+$) directed against tumor-associated antigens. Dendritic cells may capture tumor antigens and present them on the surface or tumor cells, via MHC-II proteins to T cells ($CD4^+$), causing proliferation of specific T cells ($CD4^+$) and activating an immune response against tumors. However, in some cases, the process of presentation does not occur or is deficient causing then the development of tumors [15, 16].

It has been shown that low-intensity laser light induces immune cell activity in vitro. However, little is known about the effects of laser radiation on the immune cell activity in animal models [17]. In addition, the stimulation or inhibition of photoreceptor functions, which are part of the cellular respiratory chain, determines the magnitude of cell proliferation. The irradiation dose and the energy density are the most important parameters in photobiomodulation. If the dose is too high, a non-stimulatory or even inhibitory effect may occur [18, 19].

Laser light acts on cell immunity, as it has an immune modulatory action on T-lymphocytes and an immune stimulant action on B-lymphocytes [20]. Laser effect on the proliferative activity of cells is a controversial subject as the laser light may either stimulate or inhibits the proliferation of cell lines [21].

Although the use of low-intensity laser light has stimulatory or inhibitory effects that depend on the parameters of the device and the irradiated tissue. It is important to mention that most studies use cell cultures [21], which do not reflect the complex biological processes involved in cancer development. Therefore laser and imiquimod could be investigated on SCC induced in animal model as both activate immune responses [22]. Thus, this study assessed, the effect of the use of the imiquimod and/or low-intensity laser (λ660nm) in DMBA-induced SCC of the buccal cheek pouch of golden Syrian hamsters, as well as the participation of S-100 protein-positive dendritic cells in these cases, by immunohistochemistry.

Materials and methods

The Animal Experimentation Ethical Committee of the School of Dentistry of the Federal University of Bahia approved the present study. Twenty-five male 6 to 8 weeks old golden Syrian hamsters (*Mesocrietus auratus*) were obtained from the Animal House of the School of Veterinary Medicine of the Federal University of Bahia and were kept at the Laboratory of Animal Experimentation of the School of Dentistry of the Federal University of Bahia. The animals were fed with pelleted laboratory diet[1] and had water ad libitum. The animals were kept in individual plastic cages bedded with wood chips at controlled temperature (22 °C) in a 12/12 day/night cycle.

[1] Nuvital® NUVILAB, São Paulo, SP, Brazil

The animals were randomly selected, weighed, and divided into five groups of five animals as follows: Control 1—sacrifice after cancer induction of 8 weeks; control 2—8 weeks of cancer induction plus 4 weeks with no treatment, and death at the 12th week; laser—8 weeks of cancer induction plus 4 weeks of LLLT and death at the 12th week; imiquimod—8 weeks of cancer induction plus 4 weeks of imiquimod treatment and death at the 12th week; and laser and imiquimod—8 weeks of cancer induction plus 4 weeks of LLLT associated imiquimod treatment and death at the 12th week.

All animals were induced to develop tumors by using 0.5 % DMBA[2] in mineral oil, as described in a previous study [2].

At the end of 8 weeks of the tumor induction, animals of control group 1 were killed and the presence of tumors confirmed histologically. Animals of control group 2 suffered no further treatment during further 4 weeks. After this time, the animals of laser group were anesthetized[3] and irradiated using a diode laser[4] (Table 1) at every other day for 4 weeks. The cheek pouch was held manually. On animals of imiquimod[5] group, 50 mg of imiquimod cream was evenly applied on the lesioned site with a safety margin of 0.5 cm. The treatment was performed three times a week during the 4 weeks. Finally, animals of laser and imiquimod group received both treatments.

At the end of the experimental period animals were killed by an overdose of general anesthetics, samples taken, routinely processed to wax, cut, and stained with hematoxylin and eosin (HE). An experienced pathologist, in a blind manner, carried out the histological description using a light microscope[6] at the Laboratory of Surgical Pathology of the School of Dentistry of the Federal University of Bahia.

Immunohistochemistry was performed on paraffin wax-embedded sections (3-μm thick). The tissue sections were routinely deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using hydrogen peroxide. Polyclonal antibody against S-100[7] was used with the EnVision™ System.[8] The sections were incubated with the antibody[9] for 30 min at room temperature. The immunohistochemical reactions were developed with diaminobenzidine as the chromogenic peroxidase substrate and the slides were counterstained with Meyer's hematoxylin. Oral pyogenic granuloma tissue sections were used as a positive control.

[2] Sigma-Aldrich Lab, St. Louis, MO, USA
[3] 0.5 mg/kg intramuscular Zoletil® 50, Zolazepam, Lab Virbac do Brasil, São Paulo, SP, Brazil
[4] BioWave®, Kondortech, São Paulo, SP, Brazil
[5] Aldara® 5 % cream—3 M Health Care Limited, Loughborough, Leicestershire, UK
[6] Axiolab®, Zeiss, Germany
[7] clone Z0311; Dako Cytomation, Califórnia, USA; dilution 1:700
[8] Dako Cytomation, Califórnia, USA
[9] 1:700

Table 1 Device and parameters used on LLLT

Parameter	Settings
Wavelength (nm)	660
Average power (mW)	50
Irradiance (mW/ cm^2)	714.2
Mode	Continuous
Fluence (J/cm^2)	95
Energy (J)	6.65
Exposure time (s)	133
Area (cm^2)	0.07
Spot diameter (mm)	3
Power meter	Thorlabs PM30
Beam area measurement (cm^2)	0.07
Beam profile measurement	Round

The criteria used on the present study to grade the lesions were based upon the histological grading system of the World Health Organization (WHO) [23]. Thus, the oral epithelial dysplasia was graded into: mild, moderate and severe. Squamous cell carcinoma of oral cavity was graded into: well, moderately, and poorly differentiated SCC. This classification was adapted and included micro invasive SCC (tumors in a precocious stage of invasion) [22]. In addition the inflammatory infiltrate, when present, was graded in discrete (<15 of inflammatory cells/field), moderate (15 to 50 inflammatory cells/field), and intense (>50 inflammatory cells/field).

For the description of the immunostained sections, the presence or absence of $S\text{-}100^+$ dendritic cells in the normal mucosa of the hamster cheek pouch in both the stroma and in the mucosa adjacent to the lesions of the experimental groups was determined. For semi quantitative study of $S\text{-}100^+$ dendritic cells, four most confluent (hot spot) fields (×40) were examined on each slide, using high definition light microscope[10] and a specific software,[11] located in the stroma of tumors in each study group and also in the normal mucosa. The total number of immunostained cells per square millimeter was counted and the average number of $S\text{-}100^+$ cells in each animal, as well as the standard deviation, was calculated. The identification of the $S\text{-}100^+$ dendritic cells was based on their fusiform or dendritic shapes, excluding those cells sorrounding the blood vessels and those close to nerves. Differences between groups were tested using either Fisher or Mann–Whitney tests. All statistical calculations were performed using Bioestat®.[12] Significance level was that of 5 %.

[10] Axiostar Plus, Zeiss, Germany, ×400
[11] Axiovision Rel 4.8, Zeiss, Germany 2008
[12] version 5.0, Manaus, AM, Brazil

Results

Before applying the carcinogen, it was observed that the hamster cheek pouch buccal mucosa was normal. After the initial experimental period of the tumor induction (first 8 weeks), the animals showed multiple exophytic papillomatous lesions of various sizes across the buccal mucosa.

Four weeks after induction, animals treated only with the laser showed no clinical significant changes compared to the aspect observed in the final period of tumor induction. On the other hand, animals treated only with imiquimod still showed the presence of exophytic papillomatous lesions that were somewhat smaller than the observed before treatment. Animals treated with the association of imiquimod and laser showed variable clinical aspect and a more hyperemic mucosa was observed in some animals. The number of animals per study group and the percentage of the different histological grades of squamous cell carcinoma and dysplasia may be seen in Table 2.

Statistical analysis comparing the control groups (1 and 2) with the other groups evidenced a significantly greater number of well-differentiated SCC cases (Fig. 1) in the control groups (p=0.04). There was no statistical difference between the cases of mild dysplasia, which was observed only in the groups treated with imiquimod and imiquimod associated with laser. In the groups treated with laser and imiquimod associated with laser, poorly differentiated SCCs were observed (Fig. 2). However, further studies are needed to clarify this matter, as statistical difference was not observed between groups.

The histological grade, the inflammatory infiltrate, and the average number of S-100$^+$ dendritic cells in the stroma of both carcinomas and dysplasia of both experimental groups and normal mucosa are shown in Table 3.

As no S-100$^+$ dendritic cells were detected in the control 1, control 2, laser (λ660nm; Fig. 3), and imiquimod and laser groups, they could not be used in statistical analysis. Statistical analysis was only performed in the imiquimod group (Fig. 4), with comparison of the means obtained with the values of the means found for the tissue of normal hamster cheek mucosa. Despite animals of the imiquimod group showing S-100$^+$ cells on both stroma of the tumors and in the connective tissue subjacent to dysplastic areas, when comparing this group with the normal mucosa (Fig. 5), a significant reduction on the number of the cells was observed (p=0.007). The inflammatory infiltrate was considered chronic and discrete in 76 % of the specimens, and no significant differences were observed between groups.

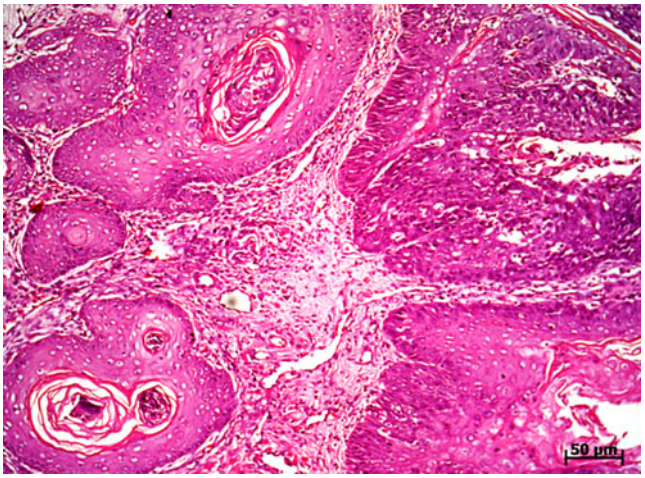

Fig. 1 Photomicrograph showing well-differentiated SCC histological aspect

Discussion

In the present study, the golden Syrian hamster cheek pouch model was used to induce experimental carcinogenesis with DMBA, according to the methodology used in previous studies [2, 5, 24]. This method was selected because of its great similarity to the events involved in human oral cancer, especially the SCC [1].

The animals in the control 1 group, sacrificed 48 h after the last application of DMBA showed the presence of 100 % of malignant tumors, corroborating the findings of a previous report [25] that obtained 100 % tumor incidence in the hamster cheek pouch after administration of DMBA using the same protocol used on the present investigation.

Table 2 Number and percentage of animals per experimental group according to the histological grade of carcinoma and dysplasia; *n*, number of animals; SCC, squamous cell carcinoma

Group	Mild dysplasia		Well-differentiated SCC		Moderately differentiated SCC		Poorly differentiated SCC	
	n	%	*n*	%	*n*	%	*n*	%
Control 1	0	0	4	80	1	20	0	0
Control 2	0	0	5	100	0	0	0	0
Laser	0	0	2	40	1	20	2	40
Imiquimod	3	60	2	40	0	0	0	0
Laser + imiquimod	2	40	2	40	0	0	1	20

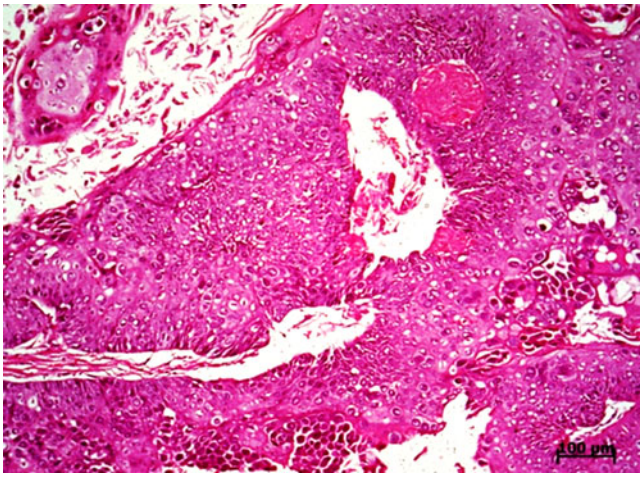

Fig. 2 Photomicrograph showing poorly differentiated SCC histological aspect after laser treatment

All the animals of the control 2 group, even after a 4-week period without any kind of induction or treatment, showed the presence of malignant tumors, demonstrating the irreversible effects of chemical carcinogenesis, even in the absence of any stimulus [26].

Many studies have reported the use of imiquimod in the treatment of cutaneous SCC [27, 28], including invasive types [29]. The present study involved the application of 5 % imiquimod cream in the oral mucosa of hamsters, based on published studies describing its use in other lesions of the oral [30] and nasal [31] mucosae.

In this study, DMBA-induced hamster buccal pouch precancerous lesions treated only with imiquimod showed lower numbers of malignant tumors compared to the other study groups and an increased number of mild dysplasia (60 %). Although other studies using different methodologies are needed for the description of a more clarifying point of

Table 3 Histological grading, inflammatory infiltrate, mean counts, and standard deviation of the S-100^{+} cells counts

Experimental groups	Animals	Histological grading	Inflammatory Infiltrate	S-100-positive dendritic cells mean counts ± SD
Control 1	1	SCC/WDm	Chronic/discrete	0
	2	SCC/WD	Chronic/moderate	0
	3	SCC/WDm	Chronic/intense	0
	4	SCC/WDm	Chronic/intense	0
	5	SCC/MD	Mixed/moderate	0
Control 2	6	SCC/WD	Chronic/discrete	0
	7	SCC/WD	Chronic/moderate	0
	8	SCC/WD	Mixed/moderate	0
	9	SCC/WD	Chronic/discrete	0
	10	SCC/WD	Chronic/discrete	0
Laser	11	SCC/WDm	Chronic/discrete	0
	12	SCC/PD	Chronic/discrete	0
	13	SCC/WDm	Chronic/discrete	0
	14	SCC/PD	Chronic/discrete	0
	15	SCC/MD	Chronic/discrete	0
Imiquimod	16	SCC/WDm	Chronic/discrete	5±4.08
	17	Md	Chronic/discrete	2.5±1.29
	18	SCC WDm	Chronic/discrete	10.5±4.5
	19	Md	Chronic/discrete	4±2,7
	20	Md	Chronic/discrete	7.5±2.08
Imiquimod and laser	21	SCC/PD	Chronic/discrete	0
	22	Md	Chronic/discrete	1±1.15
	23	Md SCC/WD	Chronic/discrete	1±0.81
	24	SCC/WD	Chronic/discrete	0
	25		Chronic/discrete	0
Oral mucosa	26	–	–	23.5±9.32
	27	–	–	14.25±6.18
	28	–	–	13.75±5.73
	29	–	–	15±5.31
	30			23.75±5.67

SCC squamous cell carcinoma, *WD* well-differentiated, *WDm* well-differentiated micro-invasive, *MD* moderately differentiated, *PD* poorly differentiated, *Md* discrete dysplasia, *SD* standard deviation

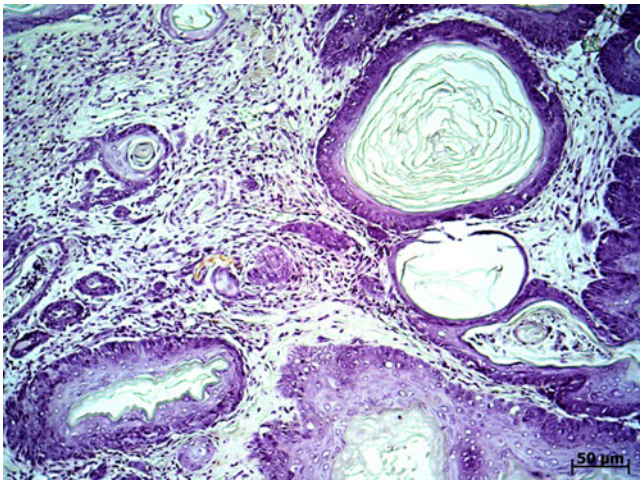

Fig. 3 Photomicrograph showing well-differentiated SCC with absence of dendritic cells after laser treatment

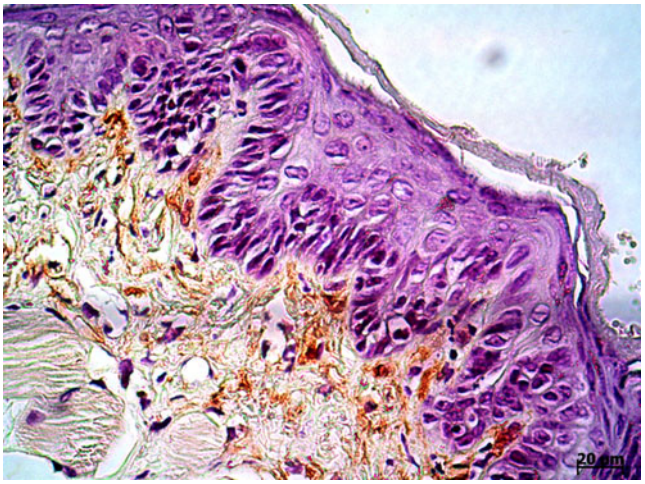

Fig. 5 Oral mucosa exhibiting dendritic cells S-100^{+}

view, the analysis of other results of the treatment could help us to infer the role of imiquimod [8–14].

The effects of low level laser therapy in the cell proliferation are influenced by the exposure time, energy density and wavelength, as well as by the type of tissue and its absorption capacity [32]. The stimulation or inhibition of photoreceptor functions, which are part of the cellular respiratory chain, determines the magnitude of cell proliferation or inhibition. The irradiation dose and the energy density are the most important parameters in photobiomodulation. If the dose is too high, a non-stimulatory or even inhibitory effect may occur [18, 19].

Castro et al. [33] and Werneck et al. [34] conducted studies on regards the effect of LLLT (λ685nm, 4 J/cm^2) on KB and H.Ep.2 cells and found that LLLT had a stimulatory effect on cell proliferation. In the present study, all the animals in the group treated only with laser (λ660nm) showed malignant tumors, being 40 % of them poorly differentiated SCC. However, this difference was not significant.

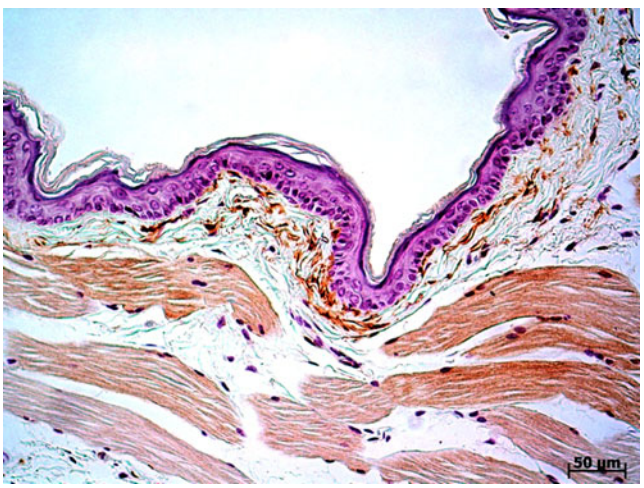

Fig. 4 Dendritic cells S-100^{+} in mild dysplasia case

It has been shown that low-intensity laser light induces immune cell activity in vitro. However, little is known about the effects of laser radiation on the immune cell activity in animal models [17]. Previous studies indicated that laser light is able to increase ATP production in lymphocytes, increasing its proliferation [35], and inducing the stimulation of cell secretory activity: stimulation of IL-2 and nitric oxide, and also increased NK cell activity and enhanced production of IFN-γ, TNF-α, and IL-6, improving the immune response [17, 35]. In the present study, chronic inflammation was found in the chemically induced lesions.

The effects of imiquimod and laser on malignant tumors are reported separately in the literature. Whereas imiquimod inhibits tumor growth [28], laser therapy has controversial results sometimes inhibiting [36] or sometimes stimulating cell proliferation [33, 34, 37]. There are no studies using the association of both therapies and the present study suggested roles of both therapies, especially as shown by imiquimod influence on the number or average cell count lesions induced in dendritic cells. The association to treat DMBA-induced hamster buccal pouch cancer has shown distinct histological results, showing dysplasia in some animals and carcinoma in others, stressing the existence of complex biological processes, that demands further studies to clarify these events and the involvement of these treatments.

Dendritic cells are major targets of stimulation in the immune response to cancer cells, as well as in cancer immunotherapy [38]. Our results demonstrated a reduction in the number of dendritic cells in both stroma and connective tissue of the treated mucosae. This is consistent with the findings of a previous study [39] that found a significant reduction in the density of these cells following DMBA-induced carcinogenesis (0.5 % in mineral oil, three times per

week) for 8 weeks. It is important to state that the hamster cheek pouch is considered a site with immune tolerance as a consequence of lymphatic drainage practically absent, and few dendritic cells. However, this study demonstrated the cancer induction that triggers an antigenic response against tumors, including dendritic cells as previously described [39].

In the control group 1, control group 2, and laser group, the counting of S-100$^+$ dendritic cells was absent in tumor stroma, which is in agreement with previous findings [40] in which it was suggested that the referred cells were scarce or absent in most skin or mucosal SCC of the head or neck. It is possible that the C-reactive protein may have influenced the dendritic cells in this study as this protein plays an important role in the innate immune system and is a known indicator of the malignant potential. High C-reactive protein levels are related to a poor prognosis of the tumor, because they interfere with the differentiation, maturation, and biological functions of dendritic cells and decrease their migration into the tumor [41]. Other mechanisms that could explain the lack of dendritic cells in the tumor stroma are: insufficient chemotaxis of dendritic cells in the tumor microenvironment and dendritic cell apoptosis induced by the tumor [42]. The different factors produced by tumor stromal cells such as VEGF, IL-10, TGF-β, gangliosides, and others may induce dendritic cell apoptosis and stimulate spontaneous apoptosis contributing to the observed decrease in the number of dendritic cells in the tumor [43].

In the group treated only with imiquimod a significant number of S100$^+$ dendritic cells were quantified in the tumor stroma and dysplasia compared to the other study groups. This result supports the imiquimod therapy because the referred compound plays immune modulatory action on dendritic cell specific receptors [9, 10]. Another important beneficial effect of imiquimod is the enhanced antigen presentation by the dendritic cells, inducing a more effective immune response against tumor [14]. However, there was a statistically significant decrease in the number of S100$^+$ dendritic cells when the imiquimod group was compared to the normal mucosa (p=0.0079). It has been suggested that the lack of Langerhans cells in malignant salivary gland tumors impairs the presentation of tumor antigens and consequently facilitates neoplastic development [44].

In our study, the inflammatory infiltrate was characterized as chronic and discrete in 76 % of the cases, but this seemed does not play influence on the mean number of dendritic cells. According another study [45], tumor-associated inflammation is a phenomenon frequently observed and is regarded as one of the most important characteristics of the progression of neoplastic disease. In the early stages of cancer, inflammation supports the malignant change and the survival of tumor cells. However, it also allows their recognition by dendritic cells. In more advanced stages of cancer, inflammation contributes to tumor cell migration and invasion and angiogenesis.

The imiquimod treatment played a direct effect on SCC induced by DMBA on hamster cheek pouch. This finding was demonstrated by an increased number of S-100$^+$ dendritic cells, suggesting a key role of immune surveillance against tumors. Under the parameters of the present study, LLLT has shown distinct results regarding the presence of SCC and should be further investigated, since the level of differentiation of tumors is an important aspect for its prognosis. The influence of LLLT on the differentiation tumor still deserves speculation. Therefore, the association of imiquimod with laser needs to be further investigated.

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