Journal of Photochemistry and Photobiology B: Biology 126 (2013) 33–41

Contents lists available at SciVerse ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Effect of topical 5-ALA mediated photodynamic therapy on proliferation index of keratinocytes in 4-NQO-induced potentially malignant oral lesions

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ARTICLE INFO

Article history:
Received 8 February 2013
Received in revised form 19 June 2013
Accepted 21 June 2013
Available online 2 July 2013

Keywords:
Potentially malignant oral lesion
5-ALA-mediated photodynamic therapy
Caspase-3 expression
Proliferating cell nuclear antigen expression
Immunohistochemistry

ABSTRACT

Fractionation can improve photodynamic therapy (PDT) efficacy for potentially malignant oral lesion treatment. The aim of this study was to demonstrate the apoptosis/proliferation index of oral keratinocytes after two sessions of topical 5-ALA-mediated PDT in 4-Nitroquinoline-1-oxide-induced potentially malignant oral lesion, and to suggest the ideal interval between PDT sessions. Immuno-histochemical tests for proliferating cell nuclear antigen and caspase-3, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay were performed at 6 h, 24 h, 48 h, and 72 h time intervals after PDT. The number of positive cells showing caspase-3 expression was significantly higher, mainly at 6 h after PDT. In the first cycle of PDT, the highest frequency of positive cells for TUNEL was found at 24 h. At 72 h after PDT, proliferating cell nuclear antigen positive cells increased significantly, indicating that there was an epithelial response in direction towards DNA repair and cell proliferation at this time. Because cell proliferation increases and cell death index decreases at 72 h after PDT, it is recommended that the interval between the PDT sessions must not be longer than 2 days up to total lesion remission.

1. Introduction

Oral leukoplakia exhibiting dysplasia is considered a potentially oral malignant lesion (PMOL) with significant risk for malignant transformation. The recommended management of this lesion is surgical excision and careful follow-up of the patient [1]. Surgical procedures involve both traditional and laser-based excision, but there is no consensus about total excision of the lesion, irrespective of the technique, being effective in avoiding the malignant transformation or recurrence of the lesion [2].

Recently some reports have described the efficacy of topical and systemic photodynamic therapy (PDT) on the partial or complete reduction of oral leukoplakias [3–8]. The advantages of this therapy are reduced scar formation associated with low frequency of recurrences [6]. With particular regard to routine oral management in the dental office, topical PDT is more interesting than systemic therapy, because topical application of the photosensitizer does not require the patient to be isolated from light, and minimizes other systemic side effects, such as hypersensitivity reaction to the photosensitizer compounds [9]. However protocols using topical PDT have not yet been well-established, especially concerning the type and concentration of the photosensitizer, and number of sessions in relation to the lesion characteristics [5].

Fractionation of PDT has been considered essential for therapeutic optimization in neoplastic disorders. PDT is based on the induction of oxygen reactive species (ROS) in the cell by luminous sensitization of certain chemical molecules with affinity to light, such as protoporphyrin IX (PpIX). During PDT, oxygen molecules are the substrate for ROS production. Thus, for PDT, it is fundamental to maintain oxygen tension in the tissue. Fractionation of this technique (multiple sessions and/or multi-stop irradiation) has been adopted in order to promote the recovery of oxygen tension and PpIX in the tissue and to guarantee ROS production after laser irradiation [5]. However the ideal interval between the sessions needs to be established, particularly with regard to oral leukoplakias.

To understand the cell proliferation cycle after topical PDT, focusing on the cell proliferation/death index may contribute to establishing the protocol for fractionation. Cell kinetics after PDT in oral mucosa or in oral malignant and premalignant disorders is poorly understood. One study demonstrated that there was

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http://dx.doi.org/10.1016/j.jphotobiol.2013.06.011

maximum damage and repopulation of the tumor cells in oral squamous cell carcinoma transplanted in the rat dorsum at 24 and 48 h, respectively, after the PDT performed with hematoporphyrin oligomers injected intraperitoneally and irradiation with N:YAG dye laser (630 nm) [10]. The authors used the proliferating cell nuclear antigen (PCNA) labeling index (Li) to detect the proliferation index of the tumor cells, and concluded that the second laser irradiation should be performed within a period of 24 h, when the photosensitizer used was also active within the cell and when the peak level of tumor necrosis and the lowest PCNA-Li were achieved.

In the present study, the kinetics of keratinocytes in 4-NQO-induced PMOL submitted to topical 5-ALA-mediated PDT was analyzed. The purpose was to demonstrate the apoptosis/proliferation index of oral keratinocytes immediately after PDT treatment, and to suggest the ideal interval between the PDT sessions for PMOL treatment.

2. Materials and methods

The following experimental protocols were approved by Ethics Committee for Animal Research of our institution.

2.1. Experimental groups

Fifty-four female Wistar rats (*Rattus norvegicus*), 150 g body mass, were maintained under controlled conditions (24 ± 2 °C temperature and light–dark periods of 12 h) and were fed with water *ad libitum* and commercial diet (Labina®, Purina, Brazil). The animals were randomly divided into the following groups:

- *Normal mucosa* – six animals without any treatment.
- *Only PDT* – six animals submitted to 5-ALA mediated PDT in the ventral mucosa of the tongue.
- *Only 4-NQO* – six animals submitted to PMOL induction by means of daily topical application of 4-NQO solution.
- *PDT first cycle* – 24 animals submitted to PMOL induction in the tongue mucosa using 4-NQO, and subsequently treated with one session of 5-ALA mediated-PDT.
- *PDT second cycle* – 12 animals submitted to PMOL induction in the tongue mucosa using 4-NQO, and subsequently treated with two sessions of 5-ALA mediated-PDT.

The animals of the PDT first cycle group were euthanized at time intervals of 6 h, 24 h, 48 h and 72 h after laser irradiation (six animals for each experimental period). The animals of the PDT second cycle group were killed 6 h and 72 h after the irradiation (six animals per period). The animals of the Only PDT group were killed 6 h after the irradiation. The animals of the Normal mucosa group, and Only 4-NQO group were euthanized at the end of the experiment.

2.2. Induction of potentially malignant oral lesion

An ointment composed of 4-NQO (Sigma, Aldrich, USA) added to propylene glycol (5 mg/ml) was applied on the dorsal and ventral mucosa of the animal's tongue, using a microbrush, in a regimen of three times per week for 16 weeks. Each topical application contained about 0.15 mg of the ointment.

After 16 weeks of 4-NQO application, in the majority of the individuals the lateral border of the tongue exhibited a white plaque compatible with oral leukoplakia (Fig. 1). For the purpose of standardization, the intention was to use the animals to compose the PDT groups or Only 4-NQO group only when the lesion measured $\geqslant$5 mm. All the animals treated with 4-NQO exhibited large lesions after 16 weeks. One operator randomly selected the animals to create the two PDT groups and the Only 4-NQO group. All 4-NQO-treated animals were included.

2.3. PDT procedure

A cream composed of 5% 5-ALA with saline EDTA homogenized with lanolin and petroleum jelly was applied to the ventral and dorsal tongue mucosa using a cotton brush (an average of approximately 0.189 g per application). To apply the cream and later perform the irradiation, the animals were previously anesthetized with an intraperitoneal injection of ketamine and xylazine (0.1 ml/g and 0.01 ml/g, respectively) and their tongues were immobilized. Two hours after 5-ALA application, laser irradiation was performed in two points (one in the middle of the dorsum, other in the middle of the ventrum) using a commercial diode laser (MMOptics, São Paulo, Brazil) at a wavelength (660 nm) similar to that described by Jerjes et al. [7] for PMOL. The parameters adopted were 40 mW power, 90 J/cm^2 fluency, 0.04 spot area, for 1.5 min in each point (total time of 3 min) [11]. All the PDT sessions were performed by the same operator. The second PDT cycle was performed 3 days after the first PDT application using the same protocol.

2.4. Euthanasia and tissue processing

Euthanasia was performed with a lethal dose of anesthesia at the previously cited experimental time intervals. The tongues were then excised, fixed in 10% formalin solution and submitted to routine tissue processing for paraffin-embedding. For each lesion, 8–10 3 μm sections were obtained: three slices were stained with hematoxylin and eosin; and the other sections were stretched onto 3-aminopropyltriethoxysilane-treated glass slides, and submitted to immunohistochemical tests.

2.5. Histological analysis

Three histological sections for each specimen were stained with hematoxylin-eosin for analysis of the morphological alterations in

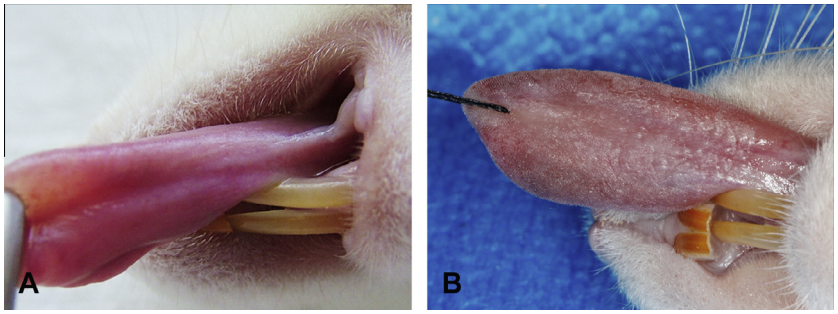

Fig. 1. Clinical aspect of white lesion induced by 4-NQO in the ventral tongue region after 16 weeks. (A) Ventral surface of tongue without 4-NQO and PDT treatment (Normal mucosa group). (B) Ventral surface of tongue after 16 weeks of 4-NQO treatment (Only 4-NQO group): presence of extensive non detachable white plaque (>5 mm).

the keratinocytes, in order to detect the influence of PDT on epithelial morphology. The following characteristics were observed: hyperkeratosis; hyperplasia of the spinous layer; hyperplasia of the basal layer; loss of basal cell polarity; drop-shaped rete ridges; nuclear vacuolization; nuclear hyperchromatism; and epithelial necrosis. The sections were examined by two calibrated pathologists who observed the presence or absence of these morphological alterations in a blinded manner and classified the intensity of the alterations in the cells and architecture according the proportion occupied by the morphological alteration in the field (X400 magnification): 0 = absent (0% of morphological alteration); 1 = mild (1–25% of morphological alteration in the field); 2 = moderate (25–50%); 3 = intense (>50%). In addition, cellular dysplasia was graded in the Only 4-NQO group, using the WHO criteria [12] and a binary classification [13].

2.6. Immunohistochemical analysis and establishing labeling index

Immunohistochemical tests were performed for analysis of PCNA and caspase-3 expression. Histological sections were stretched onto 3-aminopropyltriethoxysilane-treated glass slides, and maintained at 60 °C for 24 h. Dewaxing and rehydrating were performed in a series of descending grades of alcohol. Antigen retrieval was performed with citrate (4 mM), pH 6.0 in a water bath at 95 °C for 30 min. Endogenous peroxidase was inhibited by treatment with H_2O_2 20% in methanol (1:1) for 30 min. PCNA expression was then analyzed by incubating sections with anti-PCNA monoclonal mouse antibody (Clone PC10, 1:100 diluted; DAKO M0897) at room temperature for 60 min. To analyze caspase-3 expression, the slices were incubated with anti caspase-3 rabbit monoclonal antibody (E87 1:50 diluted, Abcam ab32351) also at room temperature for 60 min. Afterwards the samples were incubated with a biotinylated swine-anti-rabbit/goat antibody, and a streptavidin–biotin peroxidase conjugate (LSAB System, Dako®, Carpenteria, CA, USA) for 30 min each. The reaction was then revealed by diaminobenzidine (DAB) (Dako®, Carpenteria, CA, USA). After this the sections were stained with Mayer hematoxylin, dehydrated in a series of increasing grades of alcohol, immersed in xylol, and mounted in resin for conventional light microscopy. For the negative control, sections were incubated in a buffer without primary antibody.

The labeling index (Li) was obtained for PCNA and caspase-3. Positive and negative keratinocytes in the ventral epithelium were counted up to the total count of 1000 cells. Li was expressed by the percentage of positive cells in relation to negative cells.

2.7. TUNEL assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) assay of DNA strand break (DeadEnd™ Colorimetric TUNEL System, Promega Corporation, Madison, WI, USA) was performed in accordance with the guidelines for paraffin embedded tissue described by the manufacturer. Histological sections of each group and experimental time interval were deparaffinized in xylol and rehydrated in ethanol solutions from 100% to 50% concentration. After this, the sections were washed in 85% NaCl solution and in PBS solution. They were then treated with proteinase K working solution (1:500 diluted in PBS) for 10 min at 37 °C and then washed in PBS. After this, the sections were treated with TUNEL reaction mixture composed of rTdT enzyme, equilibration buffer, and nucleotide mix containing biotin. The sections were incubated with these components in a humidified chamber at 37 °C for 60 min and then washed in PBS. After this a streptavidin solution was applied on the slices, which were then revealed with DAB solution. At the end, the slices were counterstained with Mayer hematoxylin, dehydrated in a series of increasing grades of alcohol, immersed in xylol, and mounted in resin for conventional light microscopy.

Positive and negative cells were counted up to the total count of 1000 cells. Li was expressed as the percentage of positive cells in relation to negative cells.

2.8. Epithelial area measurement

The same slices used for histological analysis were also used for quantification of the epithelial area, to verify whether there was sufficient effect of PDT on the cell kinetics to cause significant epithelial atrophy. Three fields of the epithelium of the ventral surface (X5 magnification) were chosen and the epithelial area was measured by means of morphometric software (ImageLab®, Softium, Brazil). The areas in three slices of each tongue were quantified. The final area of each ventral epithelium was represented by the average of the values found in the three slices. The same procedure was separately performed for the keratin layer, in order to verify the influence of hyperkeratosis on the change in epithelial area.

2.9. Statistical analysis

Descriptive analysis was based on the mean and standard-deviation of the numerical data obtained for Li. The grades of morphological alterations were described as median and minimum/maximum values. The Kruskal–Wallis test followed by Mann–Whitney test was performed for numerical data. The level of significance was 5%.

3. Results

3.1. Histological analysis

Table 1 shows the morphological alterations observed in all the groups. The Normal mucosa, and Only PDT groups exhibited a similar histological pattern (Fig. 2A and B). However, the two above-mentioned groups and the Only 4-NQO group (Fig. 2C) showed significant differences in comparison with the PDT groups.

Table 1
Median values (range) of morphological alteration grading observed in the epithelium of the ventral surface of the tongue.

Morphological alteration	Normal	Only	Only 4-NQO	First PDT cycle				Second PDT cycle		*p* Value
	Mucosa	PDT		6 h	24 h	48 h	72 h	6 h	72 h	
Hyperkeratosis	0 (0–1)	0 (0–1)	1 (0–1)	2 (1–3)	2 (1–3)	1 (1–2)	2 (2–2)	3 (2–3)	2 (2–3)	0.002
Hyperplasia of basal layer	1 (1–0)	1 (1–1)	2 (1–2)	3 (2–3)	3 (2–3)	1 (1–3)	2 (1–2)	3 (2–3)	2 (2–3)	0.004
Atrophy	0 (0–0)	0 (0–0)	2 (1–3)	1 (2–0)	1 (0–3)	2 (1–3)	2 (1–3)	2 (1–3)	1 (1–3)	0.039
Loss of polarity of basal cells	0 (0–0)	0 (0–0)	2 (1–3)	3 (2–3)	2 (2–3)	1 (1–1)	1 (1–1)	1 (1–1)	1 (1–1)	0.000
Drop-shaped rete ridges	0 (0–0)	0 (0–0)	2 (2–2)	3 (3–3)	3 (2–3)	1 (1–2)	2 (2–2)	2 (1–2)	1 (1–2)	0.000
Cell vacuolization	1 (0–1)	1 (0–1)	1 (1–2)	3 (2–3)	2 (2–3)	1 (0–2)	1 (1–2)	2 (2–3)	2 (2–3)	0.019
Nuclear hyperchromatism	1 (1–0)	1 (1–1)	3 (3–3)	3 (3–3)	3 (3–3)	2 (1–2)	2 (2–3)	3 (3–3)	3 (3–3)	0.000
Loss of cell cohesion	0 (0–0)	0 (0–0)	0 (0–0)	1 (1–2)	1 (0–3)	2 (1–3)	1 (0–1)	1 (0–3)	1 (0–3)	0.038
Inflammation	0 (0–0)	0 (0–0)	2 (1–2)	2 (0–2)	1 (0–3)	2 (1–3)	2 (1–3)	2 (1–3)	2 (1–3)	0.002

Grading: 0 = absent; 1 = discrete; 2 = moderate; 3 = intense. *P* value obtained from Kruskal–Wallis test.

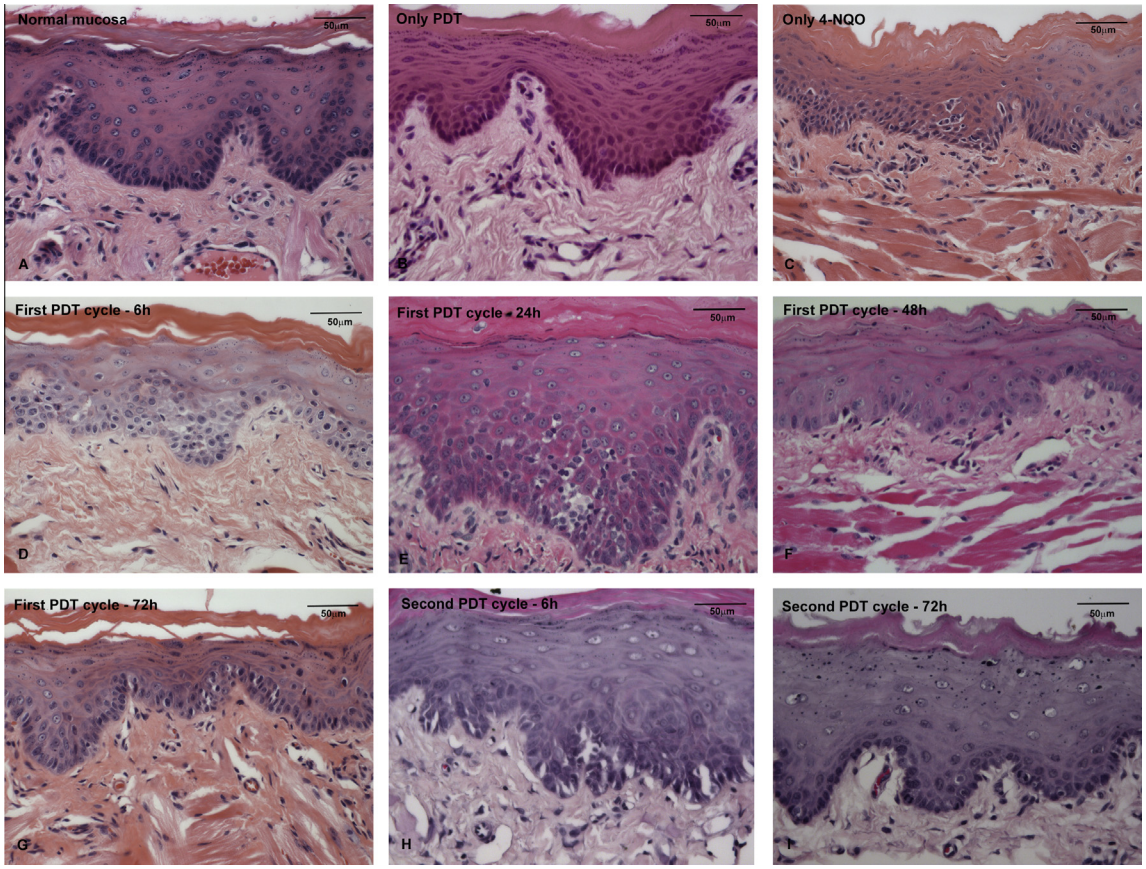

Fig. 2. Morphological alterations in the keratinocytes of ventral epithelium observed in HE slices (HE, original magnification X40). Similar histological pattern is observed between Normal mucosa and only 4 PDT group (A and B). Only 4-NQO group shows mild dysplasia mainly characterized by hyperplasia of the basal cell layer, nuclear hyperchromatism, and cell vacuolization (C). Intense hyperchromatism and loss of polarity of basal cells are evident in first PDT cycle at 6 h (D). At 24 h of the first PDT cycle the keratinocytes show loss of cohesion (E). 48 h and 72 h exhibit intense nuclear hyperchromatism and epithelial atrophy (F and G).

In general, at the time intervals of 6 h and 24 h (Fig. 2D and E), the PDT first cycle showed more intense alterations, and also in comparison with PDT at 48–72 h (Fig. 2F and G). In the PDT second cycle, the main difference was more intense hyperkeratosis in comparison with the experimental time intervals of the first PDT cycle (Fig. 2H and I). It is important to mention that clinically, there was no alteration in the tongue mucosa in the PDT first cycle group throughout all the experimental periods. In the PDT second cycle the lesions exhibited notable reduction, but the there was no total remission (data not shown).

Table 2 shows the cellular dysplasia observed in the Only 4-NQO group. In general, induced lesions exhibited discrete or moderate dysplasia (in accordance with WHO criteria) or had low risk of malignant transformation (in accordance with the binary system).

3.2. Labeling index (Li)

The Li for PCNA, caspase-3, and TUNEL are shown in Fig. 3. In the Only 4-NQO group the PCNA, caspase-3, and TUNEL Li values were significantly higher than those observed for the Normal mucosa and Only PDT groups. The latter two groups exhibited similar PCNA and TUNEL LIs, but with regard to caspase-3, the Only PDT group showed a significantly higher Li.

Table 2
Grading of dysplasia observed in Only 4-NQO group.

Animal	WHO graduation	Binary graduation
#1	Discrete	Low risk
#2	Moderate	Low risk
#3	Moderate	Low risk
#4	Moderate	Low risk
#5	Intense	Low risk
#6	Discrete	Low risk

In the first PDT cycle, the 6 h time interval showed the highest PCNA and caspase-3 LIs, which differed significantly from the other analyzed periods in this cycle. The TUNEL Li at 6 h, however, was significantly lower than that presented at 24 h. At the end of this first PDT cycle (72 h time interval) the PCNA Li values increased significantly in comparison with those at 48 h. This trend, in addition to the fact that there was stabilization of caspase-3 Li and decrease in TUNEL Li in this period, may indicate that the action of PDT had ceased.

In the second PDT cycle, the PCNA Li value was significantly higher than it was in the first PDT cycle, but the caspase-3 Li did not accompany this trend. The caspase-3 Li value in the second PDT cycle was not higher than that present at 6 h of the first cycle, although it was higher than the value observed in the other experimental periods. The TUNEL Li value, however, confirmed an intense PDT action in this cycle. At 6 h of the second PDT cycle, the TUNEL Li value was the highest, when up to 59% (average of 37.5%) of the epithelial cells exhibiting DNA fragmentation was noted. Similarly to that observed at the end of the first PDT cycle, at 72 h of the second cycle the TUNEL Li value sharply decreased, differing statistically from the other periods of the first and second cycles.

Figs. 4 and 5 show the microscopic characteristics of PCNA and caspase-3 expression.

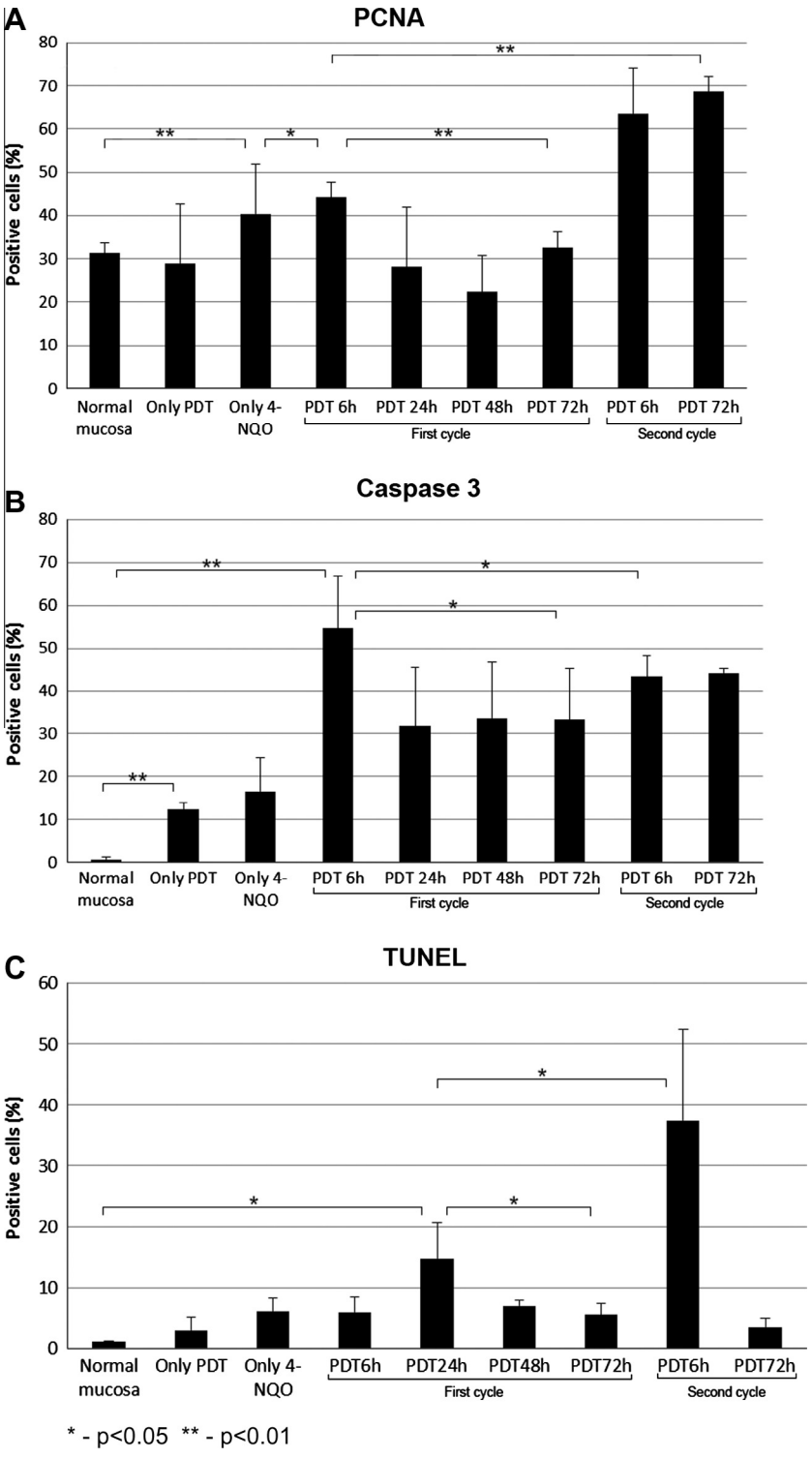

Fig. 3. Labeling index (%) mean and standard deviation of PCNA, caspase-3, and TUNEL for all groups and experimental time intervals.

3.3. Epithelial area

Table 3 shows the mean and standard deviation of the epithelial and keratin layer area in the positive control and PDT groups. There were significant differences between the groups. For keratin layer and epithelium as a whole, the main difference occurred in the First PDT cycle at 48 h, which showed the smallest area. This time interval differs significantly from the majority of other groups and time intervals. The smaller keratin layer area was present in the Normal mucosa group, which significantly differs from First PDT cycle (24 h and 72 h). In the second PDT cycle, the epithelial area was smaller than that observed in the Normal mucosa, but was significantly larger than that detected in the Only 4-NQO group.

4. Discussion

In this study PCNA, caspase-3, and TUNEL expressions in the oral keratinocytes of induced premalignant lesions were analyzed

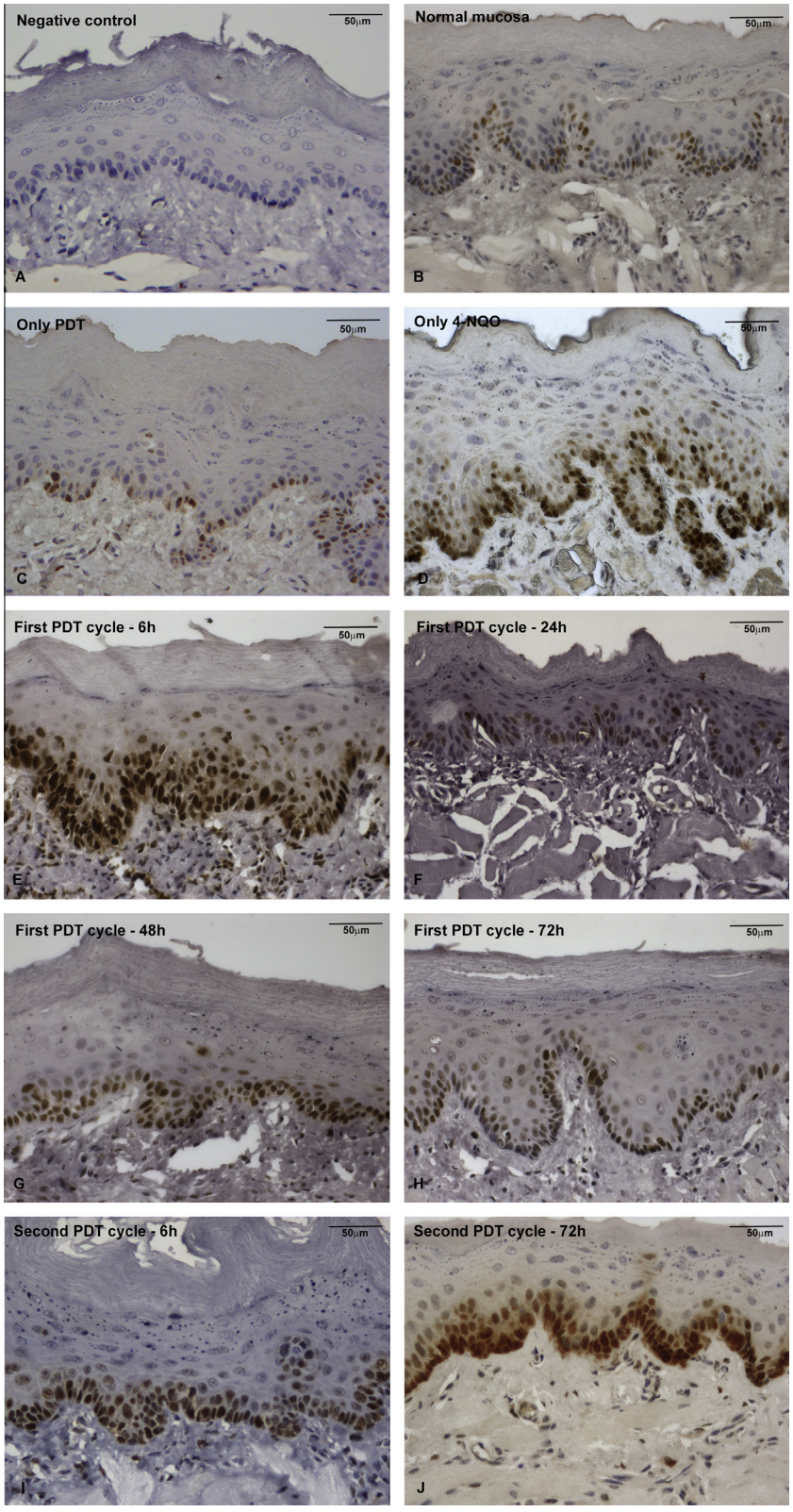

Fig. 4. Immunohistochemical expression of PCNA (streptavidin–biotin, original magnification X40). Negative control (A). PCNA expressions in the Normal mucosa (B) and only PDT groups (C) are similar, but Only 4-NQO group shows intense expression (D). First PDT cycle – 6 h (E) shows intense PCNA expression, as well as second PDT cycle (6 h and 72 h) (I and J), mainly in suprabasal and basal layers. At 24h, 48h, and 72h (F, G, and H) of First PDT cycle the PCNA expression is decreased in relation to 6h.

after two topical applications of ALA-mediated PDT. Significant increase in caspase-3 and PCNA expression was found at 6 h after PDT, but DNA fragmentation was only detected 24 h after the first irradiation and 6 h after the second irradiation. This result may indicate that the PDT performed in the present study caused activation of the caspase cascade and DNA fragmentation derived from

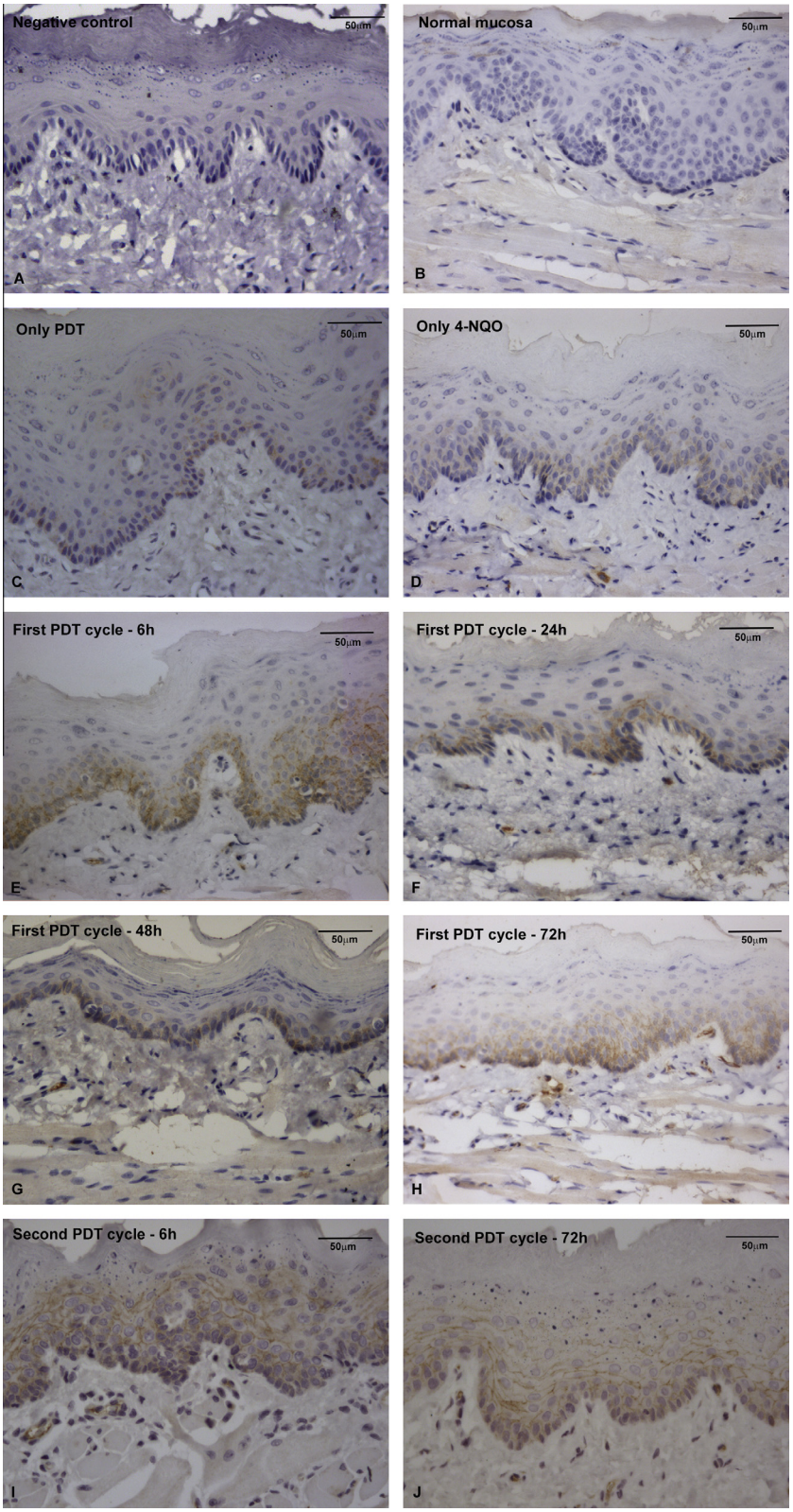

Fig. 5. Immunohistochemical expression of caspase-3 (streptavidin–biotin, original magnification X40). Negative control (A). In Normal mucosa (B), the expression is negative, but in only PDT (C) and Only 4-NQO (D) groups there is mild expression. First PDT cycle – 6 h (E) shows intense caspase-3 expressions in the basal and suprabasal epithelial layers, as well as second PDT cycle (6 h and 72 h) (I and J). In first PDT cycle – 72 h (H) these expressions are less intense, as well as at 24h (F) and 48h (G).

apoptosis. However, the cell death was accompanied by intense cell proliferation and DNA repair, shown by the high levels of PCNA positive cells. The intense morphological alterations observed in the keratinocytes at 6 h, mainly high levels of basal layer hyperplasia, nuclear hyperchromatism, and cell vacuolization, contributed to visualizing this high cell proliferation accompanied by cell

Table 3
Mean (±standard deviation) of epithelial and keratin layer area (mm^2).

Groups	Epithelial area	Keratin layer area
Normal mucosa	0.63 ± 0.29[a,b]	0.13 ± 0.04[d]
Only PDT	0.57 ± 0.13[a,b]	0.25 ± 0.10[a]
Only 4-NQO	0.42 ± 0.09	0.15 ± 0.03[d]
First PDT cycle – 6 h	0.48 ± 0.13	0.20 ± 0.11
First PDT cycle – 24 h	0.57 ± 0.13[a,b]	0.24 ± 0.05[a,b,c]
First PDT cycle – 48 h	0.41 ± 0.10	0.17 ± 0.08[d]
First PDT cycle – 72 h	0.58 ± 0.22[a,b]	0.25 ± 0.09[a,b,c,e]
Second PDT cycle – 6 h	0.52 ± 0.06[a,b]	0.18 ± 0.04[c]
Second PDT cycle – 72 h	0.55 ± 0.15[a,b]	0.17 ± 0.10

[a] Statistically different from First PDT cycle – 48 h.
[b] Statistically different from only 4NQO group.
[c] Statistically different from Normal mucosa group.
[d] Statistically different from only PDT group.
[e] Statistically different from second PDT cycle – 72 h.

degeneration at the 6 h time interval. However epithelial atrophy was present only at 48 h of the first PDT cycle, a condition that is expected due to the intense DNA fragmentation at 24 h.

The effect of 5-ALA-mediated PDT on the induction of apoptosis has been extensively described. The 5-ALA induces PpIX synthesis in the mitochondria [14], which can lead to the activation of apoptosis mainly due to the increase in membrane permeability in this organelle, with consequent release of cytochrome c and Ca^{2+} [15,16]. Moreover the interaction of PpIX with light promotes the formation of singlet oxygen and reactive oxygen species, and the activation of *Bax* and *Bak* proteins, which in turn promotes the stimulation of a cascade of sequential activation of initiator and effector caspases [16]. Caspase-3 is considered an effector caspase, responsible for a large portion of the biochemical and ultrastructural alterations observed during apoptosis [17].

The results of the present study for PCNA Li differed from those described by other authors, who mentioned significant reduction in PCNA expression in squamous cell carcinoma at 24 h, and significant increase in this expression at 48 h, returning to nearly original levels [10]. In the present study, the lowest PCNA Li expression was found at 48 h, and the highest Li at 72 h. These differences were probably derived from the distinct cell proliferation pattern observed in a carcinoma and in a potentially malignant lesion, in addition to variables inherent to the PDT technique (such as type and amount of photosensitizer, irradiation parameters, etc.). Another difference between the study of the cited authors and the present study was that in the latter, the PCNA Li at 72 h did not recover the baseline levels observed in the positive control. As there was no difference in caspase-3 Li and PCNA Li expression at this experimental time interval, the compensatory cell death/cell proliferation system may probably be responsible for maintaining the cell proliferation index at a level lower than the baseline levels. However the rising curve of PCNA Li after 48 h does not exclude the possibility of it returning to baseline levels or even to higher values in time intervals after 72 h. In fact, after the second PDT cycle, there was a substantial increase in the PCNA positive cells, which may indicate that the epithelium did not lose its repair capacity. Absence of epithelial atrophy at this PDT cycle corroborates this hypothesis. The intense DNA fragmentation detected at 6 h after the second PDT cycle may be responsible for this epithelial response. Therefore, as it was demonstrated that there was intense epithelial repair after two cycles of PDT, and considering that there was significant reduction in apoptosis mainly at 48 h and 72 h, it is strongly recommended that the interval between the PDT sessions must not be longer than 2 days.

The 5-ALA-mediated PDT causes a change in PCNA expression, mainly due to the massive DNA damage/repair. PCNA coordinates replication factors during DNA replication, in addition to participating in the process of recognition and repair of DNA damage. During DNA replication and repair, PCNA is loaded onto DNA and acts a sliding clamp that interacts with and enhances the processivity of the DNA polymerases and endonucleases [18].

Although the cell proliferation and apoptosis indexes may be important for the establishment of PDT protocols for potentially malignant oral lesions, the lesion characteristics must also be carefully considered. Clinical studies with oral verrucous hyperplasia have demonstrated that at least 3–4 PDT sessions are necessary for total remission of a lesion up to 1.5 cm in size; well-orthokeratinized lesions of a larger size could demand a higher number of sessions, but for lesions with dysplasia and with a keratin layer$\leqslant$40 μm the number of sessions can be reduced [19]. The lesions analyzed in the present study were no larger than 7 mm and the maximum keratin layer thickness was about 12 μm (data not shown). Two sessions of 5-ALA-mediated PDT using an energy density of 90 J/cm^2 was not sufficient for total clinical remission of the lesion. Therefore the fractionation rule of 3–4 PDT sessions could probably also be applied to the conditions of the present experiment. More PDT sessions must be performed with the lesions shown in the present study, in order to confirm that total remission of the lesions can be achieved with a protocol using PDT fractionation.

In conclusion, the cell kinetics of potentially malignant lesions after two sessions of 5-ALA-mediated PDT indicated that there are oscillations in apoptosis and DNA repair by epithelial cells. As there is a trend toward epithelial repair after 72 h of irradiation, a maximum interval of 2 days between PDT sessions is recommended up to the time of total remission of the lesion.

Acknowledgments

The authors thank FAPESP (São Paulo Research Foundation) (#2011/07437-0), for the financial support.

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