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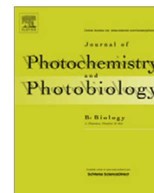

Temperature measurement and Hsp47 immunoexpression in oral ulcers irradiated with defocused high-energy diode laser

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ABSTRACT

Heat shock proteins (HSPs) are conservative protective proteins responsible for protein integrity during transcription in the cell under stress. Hsp47 is one of the most important chaperonins for collagen synthesis and release, and is up-regulated during wound repair. The aim of this study was to verify whether defocused high-energy diode laser (DDL) causes sufficient increase in local temperature to cause Hsp47 up-regulation during repair of oral ulcers. Chemically-induced ulcers in the rat tongue, and non-ulcerated tongue mucosa were irradiated using a high energy diode laser (non-contact – 4 mm from surface, 500 mW, 10 Hz for 40 s, energy density 80 J/cm^2, fixed ulcer area of 0.25 cm^2). Afterwards the specimens were submitted to immunohistochemical test for Hsp47. Temperature oscillation during DDL irradiation was also measured using a thermographic camera. Irradiated specimens exhibited transient mild increase in local temperature and significant up-regulation of Hsp47 in the mucosa from the superficial region ($p = 0.035$) to 1.7 mm deep ($p = 0.049$). In the deepest region of the mucosa Hsp47 was up-regulated only in ulcerated specimens mainly at 24 h ($p = 0.049$) and 72 h ($p = 0.029$) after ulcer induction. Conclusion: DDL increases local temperature and Hsp47 expression, which may contribute to wound repair by improvement collagen synthesis and release.

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1. Introduction

Heat shock proteins (HSPs) constitute a family of conservative protective proteins responsible for protein integrity during transcription, preventing aggregate formation in the cell under stress conditions. Induction of these molecules is started by a broad spectrum of stressors, such as hyper/hypothermia, ischemia/reperfusion, hypoxia/hyperoxia, energy depletion, acidosis, viral infection, and reactive oxygen species/reactive nitrogen species [1]. The protective effect of HSP seems to lead to improvement in wound healing, especially in the normal reparative process [2]. In the repair of oral and gastrointestinal ulcers, some HSPs, such as Hsp27, Hsp47, and Hsp70 are up-regulated [3,4]. This up-regulation is associated with cell proliferating stimuli and protein synthesis, mainly collagen, and is restricted to specific cells, such as keratinocytes, endothelial cells, and fibroblasts [5]. Hsp47 is the HSP directly linked to collagen synthesis and is released by fibroblasts, and is one of the most important constitutive and inducible Hsp for tissue repair [6].

Thermal lasers used to stimulate tissue repair also seem to induce the expression of HSP during wound repair. Mild heat production (about 45–50 °C) associated with CO_2 laser irradiation of the skin using controlled hyperthermia is correlated with increase in Hsp70 expression, which in turn could play a role in the coordinated expression of growth factors, such as tumor growth factor β [7]. HSP, up-regulated during laser irradiation, has been considered one of the main protein factors that contribute to wound repair.

High power diode laser can be used as low power laser when it is defocused. The advantages of this adaptation are to enable both high and low power functions with same laser device. Defocused diode laser (DDL) is based on physical parameters that produce only biomodulatory effects on the tissue without thermal damage or ablation. DDL has been applied to induce analgesia and acceleration of wound repair, mainly of minor aphthous stomatitis [8], oral mucositis [9], and labial herpes lesions [10]. It is not known whether DDL irradiation increases the inducible HSP and if this induction is present in a favorable scenario in tissue repair. It is also not clear if this induction could be associated with increase in local temperature.

In this study we tested the hypothesis that DDL irradiation increases local temperature and this thermal oscillation may be

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related to change in Hsp47 expression in ulcerated and non-ulcerated rat tongues. Therefore, the aim of this study was to measure the *in vitro* DDL temperature oscillation in irradiated tongue ulcers and to analyze *in vivo* the wound repair process and Hsp47 expression in different experimental periods.

2. Material and methods

The methodological steps described below were approved by Ethical Committee in Animal Experimental Research of our institution and are in accordance with International Ethical Committee of Animal Research.

2.1. Experimental groups for in vivo analysis

Fifty female rats (*Rattus norvergicus*, Wistar), with body weight of 200–250 g, 4 months of age, were randomly separated into four groups, as follows: (a) Laser treated, ulcerated group (LU) – 20 animals with ulcers induced in the ventral tongue, and afterwards treated with 810 nm diode laser; (b) Laser untreated, ulcerated group (UU) – 20 animals with ulcers induced in the ventral tongue but without treatment afterwards; (c) Laser treated, non-ulcerated group (LG) – 5 animals with 810 nm laser application on the normal surface (non-ulcerated) of ventral tongue mucosa; (d) Laser untreated, non-ulcerated group (UG) – 5 control animals, without ulceration and laser treatment.

Zero point of the experiment was 24 h after acid-induced ulcers. The animals with ulceration were euthanized at zero point and 24, 48, 72, and 120 h (5 animals per experimental period). All the control animals (LG and UG) were euthanized only at 120 h.

2.2. Ulcer induction

Ulcers were induced in the ventral surface of the tongue following a protocol adapted from Fujisawa et al. [11]. The animals were anesthetized with an intraperitoneal injection of ketamine (Dopalen®, Vetbrands, Paulinia, SP, Brazil) and xylazine (Anasedan®, Vetbrands, Paulinia, SP, Brazil) (0.1 ml/kg and 0.01 ml/kg, respectively). The tongue was pulled out of the mouth and a filter paper of 5 mm $\times$ 5 mm containing 20 μl of acetic acid 50% was applied to the ventral surface for 60 s. The animals were kept in individual cages with commercial feed and water *ad libitum*. This acid application induces formation of blisters on the ventral epithelium that later rupture and ulcerate. Confirmation of the ulcer was performed after 24 h of the acid treatment, time considered as zero point of the experiment.

2.3. Diode laser irradiation for in vivo experiment

DDL irradiation (ZAP Lasers®, Pleasant Hill, CA, USA, 810 nm) was performed at zero point of the experiment, at a distance of 4 mm from the ulcer surface (no contact) and perpendicular to it. The parameters used were adapted from Bello-Silva et al. [10] for oral herpetic lesions. Irradiation was restricted to the ulcer area, in continuous-wave mode, with 500 mW and 10 Hz pulse frequency for 40 s using horizontal scanning movements (energy density 80 J/cm^2, considering a fixed ulcer area of 0.25 cm^2). For each irradiation, the output power was measured with a power meter (Coherent Molectron®, Santa Clara, CA, USA). Irradiations were performed by a single operator. The animals were briefly maintained under sedation during irradiation, to facilitate access to the lesion. The same sedation was applied to non-laser groups.

2.4. Euthanasia and tongue processing

The animals were euthanized with a lethal dose of ketamine (Dopalen®, Vetbrands, Paulinia, SP, Brazil). Immediately after this, the tongue was extirpated and fixed in buffered formalin for 24 h. After this a longitudinal cut was made through the largest diameter of the ulcer, and the two fragments were submitted to routine tissue processing and embedded in paraffin. Five 3 μm histological slices were obtained from each tongue fragment, totaling 50 slices for each experimental period. Two slices were randomly chosen and stained with hematoxylin-eosin; the other three slices were stretched on glass slides, treated with 3-aminopropyltrietoxysilane (3-APTS) and submitted to immunohistochemical tests.

2.5. Immunohistochemical tests

The streptavidin–biotin–peroxidase technique was used for the tests with polyclonal antibodies against Hsp47 (Abcam, Cambridge, MA, USA). The slides were dewaxed and rehydrated in a series of descending grades of alcohol. Slides were then subjected to endogenous tissue peroxidase blocking. Incubation was performed in primary antibody at a dilution of 1:1000. Afterwards the samples were incubated with a biotinylated swine-anti-rabbit/goat antibody, as well as a streptavidin–biotin peroxidase conjugate (LSAB System, Dako®, Carpenteria, CA, USA) for 30 min each. The reaction was then revealed by diaminobenzidine (Dako®, Carpenteria, CA, USA); 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.

2.6. Histological analysis and semi quantitative analysis

The repair process was analyzed by observation of the hematoxylin-eosin stained ulcer slides. The intensity of the following tissue elements was considered: thermal damage, edema, hyperemia, inflammation, necrosis, young fibroblasts, angiogenesis, re-epithelialization and collagenization. The intensity was classified according the percentage of tissue element in the field: 0 = absent (0%); 1 = mild (0.1–25%); 2 = mild to moderate (26–50%); 3 = moderate to intense (51–75%); and 4 = intense (76–100%). Immunohistochemical expression of Hsp47 was evaluated by means of the above intensity classification observed in the following tissue regions: layers of preexistent (2 mm distant from the ulcer) and newly formed (migrating) epithelium; fibroblasts; vessel wall cells; inflammatory cells; extracellular matrix; skeletal muscle fibers. These two analyses were blinded and performed by two pathologists. The histological pattern corresponding to each graduation was established beforehand by the two pathologists using a representative sample of histological slices.

2.7. Positive cell counts for Hsp47 immunolabeling

Positive cells for Hsp47 immunolabeling were counted in order to achieve an objective index of Hsp expression. Hsp47 expression was correlated with the change in temperature data obtained in an *ex vivo* experiment (see below). Three regions of the tongue were analyzed: A – at 0.5 mm from ulcer surface (in the lamina propria of ventral tongue); B – at 1.5–1.7 mm from ulcer surface (in the submucosa of ventral tongue); C – at 2.7–3.0 mm from ulcer surface (in the lamina propria of dorsal tongue, i.e., immediately below the specialized epithelium of the dorsal tongue) (Fig. 1). Only the Hsp47 positive cells with fibroblast morphology were counted by one (blinded) pathologist. Vessel wall cells and inflammatory cells were not included in the count. Eight fields at $\times$400

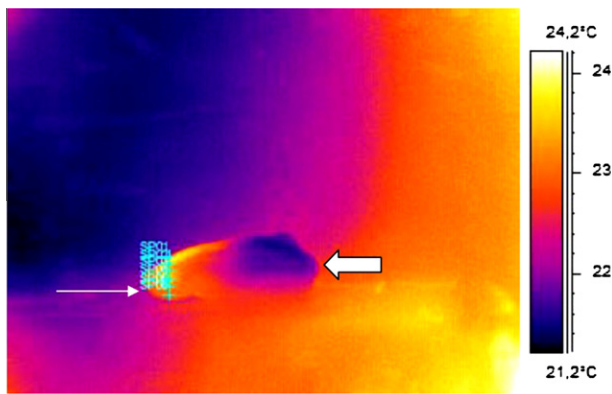

Fig. 1. Lateral view of a half tongue specimen under thermographic camera analysis. Temperature was measured in the points localized in the apical region (thin arrow) of the tongue irradiated with defocused diode laser. This image was obtained immediately after the laser irradiation.

original magnification of each region (area of 24,816 μm^2) in three histological slices of each animal were digitized and analyzed by means of morphometric software using a manual counting tool.

2.8. Ex vivo temperature measurement during laser irradiation

Additional ventral ulcers were induced in 6 animals for *ex vivo* analysis of temperature oscillation during DDL irradiation. After these animals were euthanized, the ulcerated tongues were extirpated at zero point and then frozen at −20 °C. At the time of temperature measurement, the tongues were thawed in a water bath until the temperature rose to 22 °C, and then they were cut through the longitudinal axis in the middle of the ulcer. This cut was made to obtain the temperature oscillation in the deep region. To confirm that the tongue was thawed, its initial temperature was measured with a thermographic camera (ThermaCam FLIR SC3000 Systems, Boston, MA, USA) with accuracy: 0.001 °C and response time: 0.02 s. The camera was set to 60 Hz capture frequency and temperature range of −5 °C to +98 °C. Recording was done at 21.5 °C room temperature and 50% relative humidity. Emittance of the tongue was considered as 0.98, the same as for muscle tissue [12] since most of the tongue volume was composed of striated muscle. After the first temperature measurement, the specimens were fixed in a plate and the ulcers were irradiated with the same DDL parameters as those used in the *in vivo* experiment, and also performed by the same operator. The laser power emission was previously checked with a Power Meter (Coherent Molectron®, Santa Clara, CA, USA). During irradiation, the temperature oscillation was recorded by the thermographic camera. The specimens were positioned at 10 cm from camera lens, in order to obtain focus on the lateral surface of the tongue. Perpendicular points in relation to irradiated area were analyzed using the thermographic camera software (ThermaCam®, ThermaCam Research 2001, Boston, MA, USA). First point was on the ulcer surface, and another six points were measured at a depth of 0.5 mm from this surface (Fig. 2). These points were coincident with those analyzed in the cell counts. One software operator calculated the temperature values at the points in accordance with the irradiation time (30 s before, 40 s during, and 30 s after the laser irradiation).

The thermographic analysis described above was also performed *in vivo* at the superficial point in order to verify if the temperature variation was similar to the *in vitro* tests.

2.9. Statistical tests

Descriptive statistical analysis was performed for all the variables using median, medium, standard-deviation, and minimum–maximum values. The Mann–Whitney test was used for numerical data in semi quantitative analysis of the repair process analysis and of Hsp47 immunohistochemical labeling. For the cell counts, the Kruskal–Wallis variance analysis was used to detect differences considering all the groups together. The Mann–Whitney test was applied for the pairs of groups, and Friedman's test was adopted to identify differences between the experimental periods and histological regions analyzed (A, B, and C) for each group individually. Calculations were made using SPSS® software (IBM, NY, USA). The level of Significance was 5%.

3. Results

3.1. Temperature variation during laser irradiation

There was variation in the tongue temperature during DDL irradiation in all the points. Fig. 2 shows an example of a standard graph of the temperature curve observed in all the specimens. The initial temperature (the first 30 s) was about 22 °C. The graph shows there was a temperature peak at 30 s (when irradiation started) from the ulcer surface to 1 mm deep. In the deepest regions, this oscillation was minimal at this time. From 40 s to 70 s, progressive temperature increase was observed in all the points, except the surface, which maintained the mean values presented at 40 s. The temperature declined progressively from 70 s (when

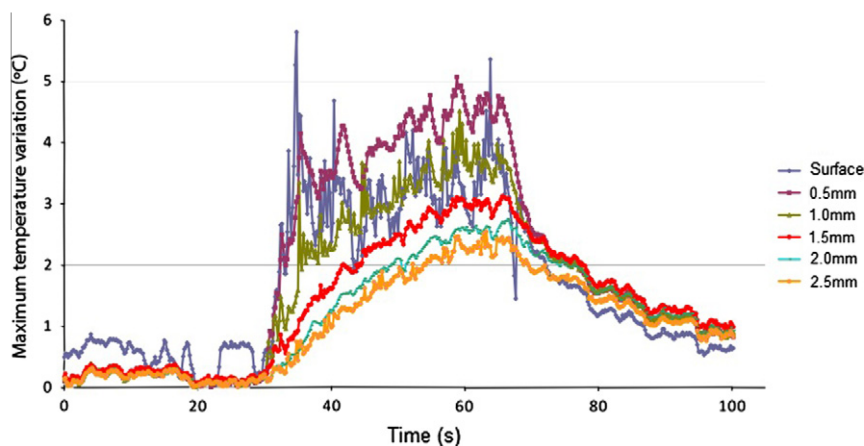

Fig. 2. Temperatures measured in *ex vivo* experiments, previous (0–30 s), during (30–70 s), and after (70–100 s) laser irradiation.

Table 1
Average (±standard error) of temperature variation according to the different depth of measured points.

Depth of measured points	Temperature variation (°C)
Surface	6.70 ± 0.50
0.5 mm	5.20 ± 0.60
1.0 mm	4.30 ± 0.50
1.5 mm	3.20 ± 0.30
2.0 mm	2.85 ± 0.10
2.5 mm	2.77 ± 0.23

laser irradiation stopped) to the end of the experiment (100 s), but only the surface point achieved the initial mean temperature values. The other points showed about +1 °C temperature at 100 s. The temperature impairment was more pronounced in the surface point. Table 1 shows the temperature variation means during laser irradiation in the analyzed points for the six tongues. The lowest variation values were observed at deeper points (about 2.5 °C). The highest variation (about 7.20 °C considering the standard error) was present in the surface point. This variation was similar to that observed *in vivo* test (8.12 °C), without statistically significant differences ($p = 0.500$). Considering the maximum temperature value, there was significant difference between the surface point and deepest point ($p = 0.0455$).

3.2. Analysis of the repair process

Table 2 contains the mean values of semi quantitative analysis for each tissue element during ulcer repair with or without DDL irradiation. At 0 h there were no significant differences between the groups. The tongue mucosa showed an ulcerated epithelium covered by necrotic tissue with intense inflammatory exudate composed mainly of neutrophils. Moderate to intense hyperemia and edema were observed in the lamina propria. Discrete to moderate angiogenesis was noted mainly in the ulcer basis. At 24 h granulation tissue, angiogenesis, and collagen deposition in ulcers treated with laser were significantly more intense than in non-treated ulcers. However these differences were not maintained in the other experimental periods, in which the tissue elements were similar (Fig. 3A and B). At 120 h, 97% of the ulcers in both groups had undergone complete re-epithelialization, with a large number of fibroblasts and mature granulation tissue in the lamina propria (Fig. 3C and D). No difference in speed of repair was observed between the groups.

3.3. Analysis of Hsp47 immunolabeling

Table 3 contains the data of semiquantitative analysis for Hsp47 immunolabeling in laser treated and non-treated ulcers. Inflammatory cells, muscle fibers, and epithelium >2 mm distant from the ulcer were negative. In general the intensity of Hsp47 expression increased with time, mainly in migrating epithelium, fibroblasts, vessel wall, and extracellular matrix. The effects of laser irradiation on Hsp47 expression was early observed at 0 h, when the immunolabeling in the ulcers treated with laser was significantly stronger than that in the non-treated ulcers. At 24 h, the ulcers treated with laser exhibited moderate to intense Hsp47 expression for fibroblasts and vessel wall, which was significantly more intense in comparison with the non-treated ulcers (Fig. 3E and F). The strong positivity in fibroblasts was maintained in both groups until 120 h without significant differences (Fig. 3G and H).

3.4. Hsp47 positive cell counts

Fig. 4 shows the average number of Hsp47 positive cells in non-ulcerated groups (LG and UG). The number of positive cells decreased from region A (at 0.5 mm from epithelial surface) to region C (at 2.7–3.0 mm from epithelial surface) with significant differences between these regions only in the laser-irradiated group ($p = 0.039$). In the comparison of groups, the number of positive cells was significantly higher in irradiated than in non-irradiated specimens considering the regions A ($p = 0.035$) and B ($p = 0.049$).

Figs. 5–7 show the distribution of Hsp47 positive cells in relation to experimental periods of ulcerated groups (LU and UU) for each histological region (A, B, and C). For region A (at 0.5 mm from epithelial surface) (Fig. 5), at 0 h, the number of positive cells was significantly higher in the laser-irradiated than in the non-irradiated group ($p = 0.049$). At 120 h this condition was inverted, with significantly higher frequency of positive cells in non-irradiated group ($p = 0.039$). The average number of positive cells increased from 0 h to 72 h with peak at 120 h in non-irradiated groups, whereas in irradiated group the peak was at 72 h. For region B (at 1.5–1.7 mm from epithelial surface) (Fig. 6) there was also a

Table 2
Median (minimum–maximum) values in semiquantitative analysis for ulcer repair in the laser treated- (LU) and laser untreated- (UU) ulcerated groups.

Experimental period	0 h		24 h		48 h		72 h		120 h	
Groups	UU	LU	UU	LU	UU	LU	UU	LU	UU	LU
Thermal damage	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Edema	4 (4–4)	3 (2–4)	3 (2–3)	3 (2–3)	3 (2–3)	2 (2–3)	2 (2–2)	2 (2–2)	1 (0–1)	0 (0–1)
Hyperemia	3 (3–4)	3 (3–3)	3 (2–3)	3 (2–3)	2 (1–3)	3 (3–3)	2 (1–2)	2 (2–2)	2 (2–2)	2 (2–2)
Inflammatory infiltrate	4 (4–4)	4 (4–4)	3 (2–3)	3 (2–3)	3 (3–3)	2 (2–3)	2 (1–2)	2 (2–2)	0 (0–1)	0 (0–0)
Necrosis	3 (3–3)	3 (3–3)	3 (3–3)	3 (2–3)	2 (2–2)	2 (2–3)	1 (1–2)	1 (0–1)	0 (0–1)	0 (0–0)
Granulation tissue	0 (0–0)	0 (0–0)	**1** (**1–1**)	**2** (**2–2**)	2 (2–2)	3 (2–3)	4 (4–4)	4 (4–4)	4 (4–4)	4 (4–4)
Angiogenesis	1 (1–2)	2 (2–2)	**2** (**2–2**)	**3** (**3–3**)	3 (3–3)	3 (3–3)	3 (3–3)	3 (3–3)	3 (3–3)	3 (3–3)
Reepithelization	1 (1–1)	1 (1–1)	2 (2–2)	2 (2–2)	2 (2–2)	2 (2–2)	3 (3–3)	3 (3–3)	4 (3–4)	4 (3–4)
Collagen deposition	0 (0–0)	0 (0–0)	**1** (**1–1**)	**2** (**2–2**)	2 (2–2)	3 (2–3)	3 (3–3)	3 (3–3)	4 (3–4)	4 (4–4)

0 = absent (0%); 1 = mild (0.1–25%); 2 = mild to moderate (26–50%); 3 = moderate to intense (51–75%); and 4 = intense (76–100%). Pair of values in the bold had significant statistical differences (Mann–Whitney test, $p < 0.05$).

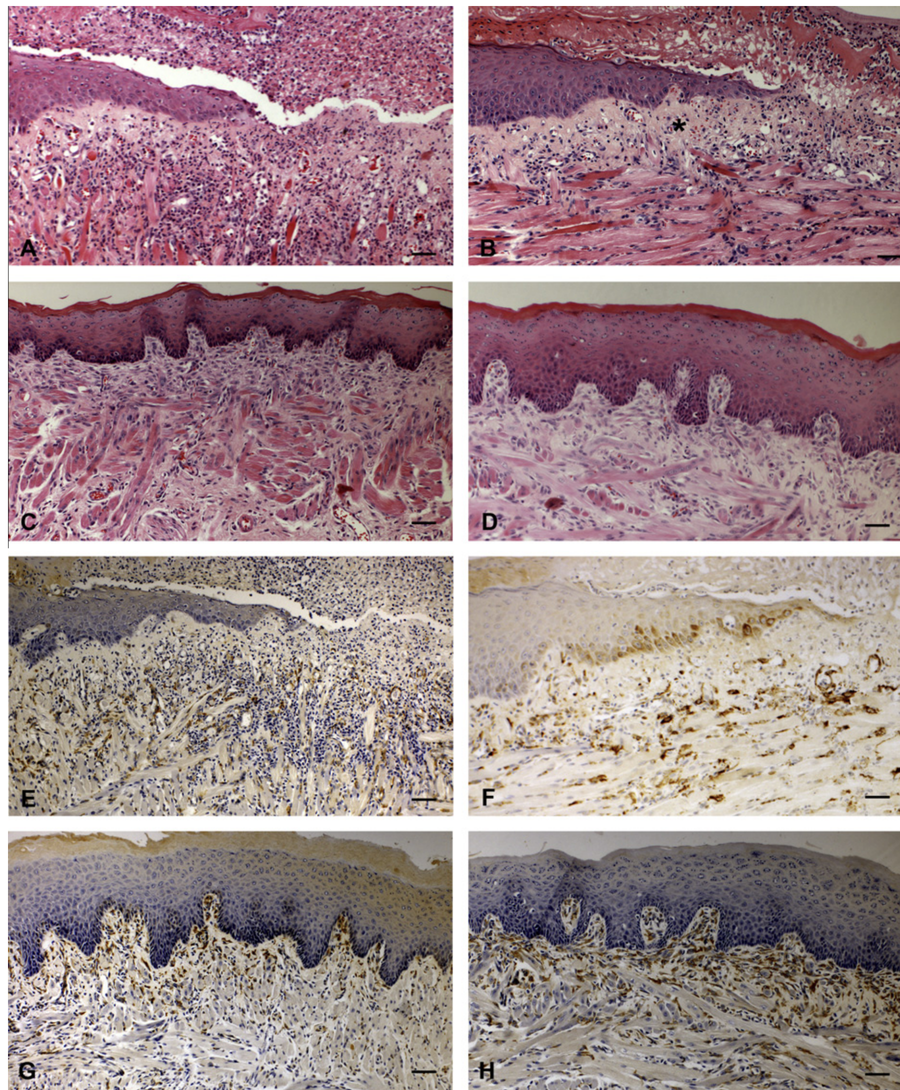

Fig. 3. Histological sections of oral ulcers from laser-treated and laser-untreated groups. (A–D): Hematoxylin-eosin stain (×100). Intense collagen deposition and angiogenesis in the laser-treated group (B) in comparison with laser-untreated group (A) at 24 h. Similar reepithelization degree and connective tissue remodeling at 120 h for both laser-untreated (C) and laser-treated (D) groups. (E–H): Streptoavidin–biotin stain (×100). Intense Hsp47 expression at ulcer basis 24 h after laser irradiation (E) in the comparison with laser-untreated ulcer (F). Similar Hsp47 expression in the both laser-untreated (G) and laser-treated (H) at 120 h.

Table 3
Median (minimum–maximum) values in semiquantitative analysis for Hsp47 immunolabeling in the laser treated- (LU) and laser untreated- (UU) ulcerated groups.

Experimental period	0 h		24 h		48 h		72 h		120 h	
Groups	UU	LU	UU	LU	UU	LU	UU	LU	UU	LU
Preexistent epithelium	0 (0–0)	3 (0–3)	2 (2–3)	2 (1–3)	2 (1–2)	1 (1–1)	1 (1–2)	2 (2–2)	0 (0–1)	1 (0–2)
Migrating epithelium	**1** (**1–1**)	**2** (**2–2**)	2 (2–2)	3 (2–3)	3 (2–3)	3 (3–4)	3 (3–3)	3 (3–4)	2 (1–2)	2 (2–3)
Fibroblasts	**2** (**1–2**)	**3** (**3–3**)	**3** (**3–3**)	**4** (**4–4**)	4 (4–4)	4 (4–4)	3 (3–4)	4 (3–4)	3 (3–4)	4 (3–4)
Vessel wall	1 (0–1)	2 (1–2)	**2** (**2–2**)	**3** (**3–3**)	3 (3–3)	3 (3–3)	2 (2–3)	3 (3–4)	2 (2–3)	2 (2–3)
Extracellular matrix	**0** (**0–0**)	**1** (**1–1**)	2 (0–2)	2 (1–2)	3 (3–3)	3 (1–3)	2 (2–3)	3 (2–3)	1 (1–1)	1 (1–3)

0 = absent (0%); 1 = mild (0.1–25%); 2 = mild to moderate (26–50%); 3 = moderate to intense (51–75%); and 4 = intense (76–100%). Pair of values in the bold had significant statistical differences (Mann–Whitney test, $p < 0.05$).

higher average number of Hsp47 positive cells in the laser-irradiated than in the non-irradiated group at 0–72 h. The differences were statistically significant between the groups at 24–72 h ($p = 0.049$). At 120 h, the number of the positive cells was similar in the groups. In region C (at 2.7–3.0 mm from epithelial surface) (Fig. 7), the average number of positive cells in laser-irradiated

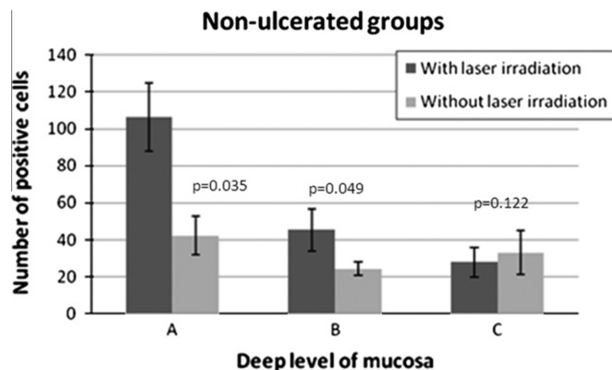

Fig. 4. Average number of Hsp47 positive cells in accordance with the deep level in the mucosa for non-ulcerated groups treated or not with laser.

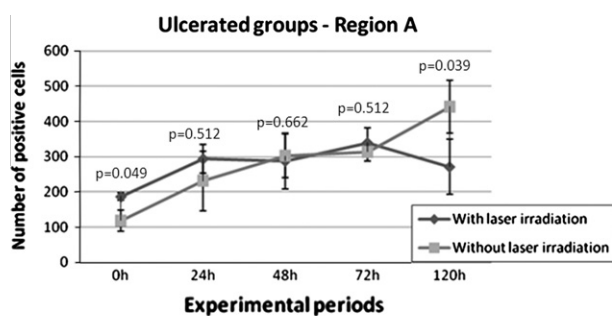

Fig. 5. Average number of Hsp47 positive cells for region A in ulcerated group submitted or not to defocused diode laser irradiation.

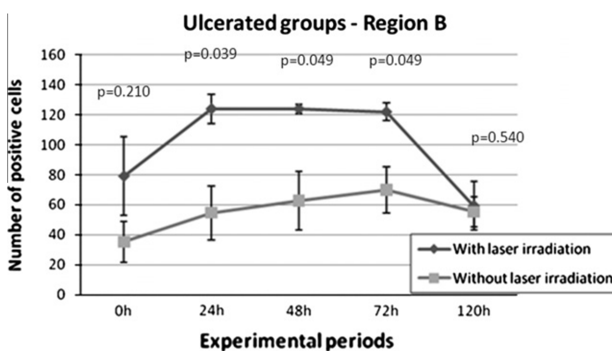

Fig. 6. Average number of Hsp47 positive cells for region B in ulcerated group submitted or not to defocused diode laser irradiation.

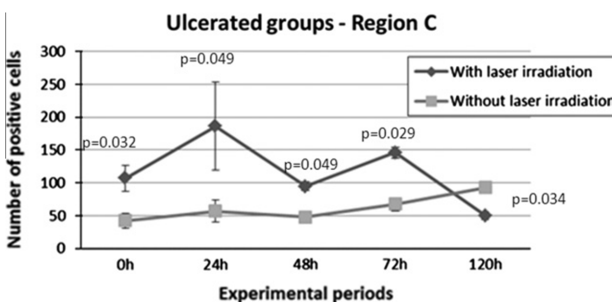

Fig. 7. Average number of Hsp47 positive cells for region C in ulcerated group submitted or not to defocused diode laser irradiation.

group continued to be higher than it was in the non-irradiated group, except at 120 h. All the experimental periods showed statistically significant differences between the groups.

4. Discussions

In this paper we tested the hypothesis that DDL may produce local temperature increase and that this laser irradiation would modify the Hsp47 expression both in inflammatory and non-inflammatory situations. In addition, we intend discussing the possibility that the beneficial effects of DDL on tissue repair may be associated with minimal temperature increase and Hsp47 expression.

By means of a thermographic camera and *ex vivo* tongues, we showed that the DDL parameters induced an increase in local temperature during irradiation. The maximum oscillation was about 6.7 ± 0.5 °C, which represents 42–43 °C as maximum temperature *in vivo* (normal basal temperature about 36 °C). This temperature is sufficient to induce Hsp release since temperatures between 37 °C and 47 °C can cause cellular stress with or without reversible injuries [13]. As the experiment with the use of the thermographic camera was performed *in vitro* (only the superficial point was measured *in vivo*, demonstrating that the temperature oscillation was similar to that observed in the superficial point of the *in vitro* test), we did not consider the thermoregulation of the body, which can maintain the local temperature under normal levels. However, even in this case the inducible Hsp may be present since the heat proteins participate in the thermoregulation and thermotolerance processes [1]. As laser-irradiated tongues showed significantly higher numbers of Hsp47 positive cells than non-irradiated tongues in the surface regions (A and B), we can assume that temperature increase caused by laser irradiation may be related to this modification. In this case the effects of DDL may be related to absorption of photon energy accompanied by increase in heat. Increase in reactive oxygen species caused by low energy lasers is one of the factors that lead to HSP induction. The larger portion of the HSPs is inactive in the cells because they are compounded with heat shock factors (HSFs) forming inactive complexes. High levels of ROS break these complexes, and HSF migrates to the nucleus activating HSP gene encoding [14]. As HSF is a highly redox-sensitive transcriptional factor and both luminous and thermal energy are ROS-inductive, this mechanism may be one of the most important to explain the effect of low energy lasers on HSP induction. Further biochemical studies are necessary to demonstrate this association and the exact role of photochemical and photothermal effects on HSP activation.

It is important to consider that the temperature increase produced by DDL irradiation does not cause cytotoxicity. The low levels of temperature elevation and the absence of thermal injury analyzed in the histological sections in the *in vivo* experiment confirmed the absence of protein coagulation.

A mild increase in temperature was also observed in deep regions of the tongue (up to 3.0 mm from the irradiation point, region C) but with less oscillation (2.77 ± 0.23 °C) than in the surface regions. The increase and decrease curve demonstrated that the superficial region absorbs and dissipates heat more rapidly as a function of the laser irradiation period. On the other hand, the deep regions raise the maximum temperature at the end of laser irradiation and it takes longer for the temperature to decrease. This trend seems to be similar to the case of sun burn, in which there is solar energy accumulation in the deep region of the dermis [15]. Differently from regions A and B, this transient temperature increase in the region C probably does not change the cellular status since the Hsp47 expression did not differ from that of the non-irradiated non-ulcerated tongue. The lower temperature oscillation may explain the absence of Hsp47 up-regulation.

We also used an *in vivo* model with ulcerated tongues to analyze whether the laser irradiation was superior to ulcer inflammation in the induction of Hsp47. We observed that DDL produces stimulation of vessels, granulation tissue, and collagen deposition in the first periods of ulcer repair. The intense expression of Hsp47 in the fibroblasts, vessel wall, and extracellular matrix in the same experimental periods contributes to explaining this stimulatory effect of DDL. Inducible Hsp47 has been correlated with triple-helical formation of collagen with transient binding to procollagen. Hsp47 is resident in the endoplasmatic reticulum and is the most important collagen-specific chaperone. It is constitutive in the oral mucosa only in the connective tissue, but during oral mucosa repair some migrating keratinocytes can express this protein [5]. DDL induces the Hsp47 both in the connective tissue and in the migrating epithelium, and these expressions may compound the wide range of factors that explain the stimulatory effects of low energy lasers.

Hsp47 expression was also analyzed in terms of tissue depth in the *in vivo* analyses. Similarly to the *ex vivo* experiment, regions A and B showed significantly more Hsp47 positive cells in the DDL group. However, there were differences regarding the chronological distribution of the positive cells. In region A, Hsp47 up-regulation was also brief and restricted to some minutes after laser irradiation, behavior that is coincident with the rapid heat dissipation in this region. But in regions B and C, the number of Hsp47 positive cells was significantly higher in the DDL group for most of the time (from 24 h to 48 h). This perpetuation of Hsp47 up-regulation in the comparison with non-irradiated ulcerated specimens may be related both to the temperature oscillation curve discussed previously, and to the cascade of stress events promoted by laser irradiation. This cascade may promote an intense activation of Hsp induction, which is superior to ulcer inflammation alone. Therefore, we can say that the laser irradiation increases the natural inflammation-evoked Hsp47 up-regulation during tissue repair. The results of Hsp47 expression in region C of the ulcerated specimens (about 3.0 mm from the surface) can confirm that Hsp47 up-regulation during tissue repair may be caused by laser irradiation in addition to the tissue inflammation. Considering the high penetration of the diode laser at 810 nm, the effects of irradiation are potentially expected. Only in the ulcerated groups was the number of Hsp47 positive cells higher in irradiated than non-irradiated specimens, which indicates that an additional input of for intense Hsp47 activation in the deeper regions of laser-irradiated biological tissue.

In conclusion, DDL within the described parameters can cause a slight increase in local temperature without the induction of thermal damage to the tissue. DDL irradiation can also increase the Hsp47 expression both in non-ulcerated and ulcerated mucosa, which may contribute directly to ulcer repair by means of collagen synthesis and release. The mild increase in local temperature caused by DDL could be associated with Hsp47 up-regulation.

5. Abbreviations

HSP	heat shock protein
DDL	defocused diode laser

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