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ORIGINAL ARTICLE

Effect of defocused infrared diode laser on salivary flow rate and some salivary parameters of rats

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Abstract This study aims to investigate whether infrared diode low-level laser therapy (LLLT) increased salivary flow rate and altered pH value, protein concentration, and peroxidase and amylase activities in saliva of rats. Wistar rats were used and divided into three groups. Experimental groups (A and B) had their parotid, submandibular and sublingual glands submitted to diode laser, 808-nm wavelength, on two consecutive days. The dose results were 4 and 8 J/cm^2, respectively. A red guide light was used to visualize the irradiated area. Group C was irradiated only with red pilot beam and served as control. The saliva samples were collected after each irradiation step (first and second collection days) and 1 week after the first irradiation (seventh day). Statistical analysis was performed, and differences were observed according to different days of salivary collection. The results showed that salivary flow rate for groups A and B was higher on the seventh day if it is compared to data obtained for the first day ($p<0.05$). LLLT applications on salivary glands are a therapy procedure that requires further studies.

Keywords Low-level laser therapy · Defocused laser · Saliva · Salivary glands and salivary flow rate

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Introduction

Saliva is an essential fluid for oral tissue health. Radiotherapy, medication, immunological and rheumatic diseases [13], as well as some genetic disorders like Down syndrome [25] cause a marked reduction in salivary flow rate. Patients with hyposalivation show a higher risk of infections and carious lesions, which leads to a decrease in life quality. Wetting agents or saliva substitutes have a palliative effect [3]. Therapies for hyposalivation also include systemic sialagogues, which stimulate salivary flow rate. However, they cause side effects [4, 19]. Therefore, it is necessary to find an efficient method to stimulate the salivary glands.

Saliva plays an important role due to its fluid characteristics and specific components. Cleansing of the oral cavity, solubilization of food substances, bolus formation, bacterial and food clearance are examples of functions at least in part related to fluid characteristics of saliva. Saliva components contribute to buffering action, provision of antimicrobial action (i.e., lysozyme and peroxidase enzymes), defense, as well as digestive action (salivary amylase) [18, 21].

Saliva is mainly produced by three pairs of major salivary glands: parotid, submandibular, and sublingual, which altogether accounts for about 90% of the fluid

production [18]. Serous cells are the predominant cells of the parotid gland and produce an aqueous and amylase-rich saliva [18]. Peroxidase enzyme is produced in acinar cells in both submandibular and parotid glands [28].

Low-level laser therapy (LLLT) is characterized by red or near infrared light, used in low-intensity spectrum, in such a way that the observed biologic effect is attributed to nonthermal events [5]. The LLLT produces basic effects such as anti-inflammatory, analgesic, and biomodulation [14, 24]. Previous reports showed its influence on protein synthesis and the number, growth, and differentiation of cells, when it is applied to different tissues [9].

Components of electron transport chain are the primary photo-acceptors of laser light. Changes in the redox properties, generation of singlet oxygen, localized transient heating of absorbing chromophores, and increased superoxide anion production are the primary action mechanisms of LLLT. The secondary effects occur after irradiation, and they are related to a cascade of biochemical reactions and changes in homeostasis parameters in the cell [10]. Nonthermal, photochemical reactions, the basis of LLLT, occur with power densities between 10^{-2} and 10^{0} W/cm^2 and doses between 10^{-2} and 10^{2} J/cm^2 [24].

Because laser therapy protocols for mucositis [6] and temporomandibular joint dysfunction [31] may have an effect on salivary glands, it is important to know the effects of this therapy on parotid and submandibular gland tissues. In addition, although the irradiation of salivary glands has demonstrated to be efficient in patients with hyposalivation and inflammatory process of the gland [30] and the radiation is known to affect the protein synthesis and secretion of guinea pig submandibular glands [20], the mechanism in which LLLT acts on salivary glands and its secretion is still unknown.

Therefore, this study aimed to investigate whether infrared LLLT is able to increase salivary flow rate and alter the pH value, protein concentration, and peroxidase and amylase activities in saliva of rats, after they had their parotid and submandibular glands irradiated.

Materials and methods

Selection and preparation of the animals

The protocol of the study was approved by the Bioethics Committee of Animals. Male rats of the Wistar strain with approximately 200 g of body weight were used. For a period of 7 days, the animals were kept in individual plastic cages with free access to water and food to be acclimatized before being submitted to LLLT treatment.

The animals were divided into two experimental groups (A and B) and one control group (C). All of them were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg body weight) and sodium diethylbarbiturate (50 mg/kg body weight). All animals had their parotid, submandibular, and sublingual gland areas subjected to trichotomy and marked to be or not irradiated with infrared radiation.

Laser treatment

The laser applications were carried out on both submandibular glands at the same time and on each parotid separately. Therefore, three salivary gland areas of about 1.8 cm^2 were irradiated. A diode laser (ZAP Lasers®, Pleasant Hill, CA, USA), 808 nm, was used. To visualize the area irradiated by the infrared laser, a red pilot beam (650 nm) with 3-mW maximum power of the ZAP laser was used for the experimental groups. For the control group, the red pilot beam was the only device used (Fig. 1).

Laser irradiation was performed in continuous wave mode by means of an optical fiber of 400-μm diameter. It was perpendicularly fixed, by tripod, 4 cm away from the animal to cover an area of 1.8 cm^2. The exposure lasted 14 s for group A and 28 s for group B. The resulting dose was 4 and 8 J/cm^2, respectively. The power output (500 mW) and power density (277 mW/cm^2) were the same for both experimental groups. The power output was monitored by a power meter (Coherent Molectron®, Santa Clara, CA, USA). Groups A and B were submitted to infrared irradiation and red pilot beam at the same time.

Group C was irradiated with red pilot beam for 28 s under the same conditions in experimental groups and served as control because the dose of 0.05 J/cm^2 [24] is not bioactive. The irradiation of experimental and control groups was performed on two consecutive days.

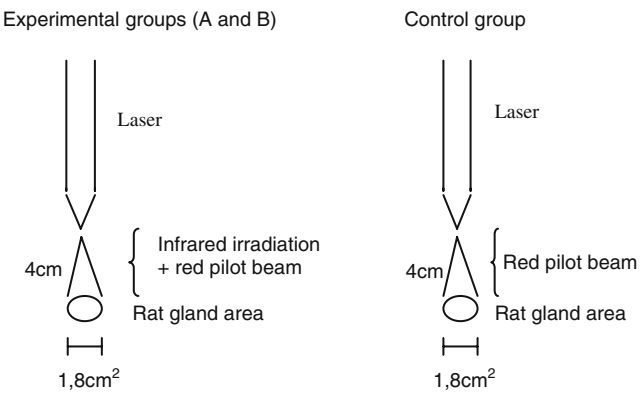

Fig. 1 Rat gland area irradiation for experimental groups (A and B) and control group (C). Laser beam was fixed at a distance of about 4 cm from the animal to cover an area of 1.8 cm^2. Experimental groups were irradiated by infrared light+red pilot beam. Group C was just irradiated by red pilot beam

Collection of saliva

Rats were ventrally positioned in a box, and their heads were kept out of it. Beneath their mouth, there was a graduated plastic tube maintained in ice. Stimulated whole saliva was collected after an intraperitoneal injection of pilocarpine (7.5 mg/kg body weight). Saliva was first collected 20 min immediately after each irradiation (first and second salivary collection days). It was collected again, 1 week after the first laser application (seventh salivary collection day), without the laser application procedure.

Analysis

The flow rate expressed by μl/min was calculated by using the relation between the volume of saliva collected and its collection time. Immediately after saliva collection, pH was measured by means of a pH meter E-520 (Metrohm Herisau®, Herisau, AR, Switzerland). Total protein concentration was determined by Folin-phenol reagent [12] using bovine serum albumin as standard. The intensity of the color was measured in a spectrophotometer (Beckman DU-68, Fullerton, CA, USA) at 660 nm.

Amylase and peroxidase activities were determined as described elsewhere [25]. Briefly, amylase activity was measured after incubation of samples with 1% starch solution in 20-mM phosphate buffer, pH 7.0, for 5 min at 30°C. The reaction was interrupted by adding an alkaline solution of dinitrosalicylic acid, and the mixture was maintained in boiling water for 5 min. After the dilution of the mixture with distilled water, the intensity of the developed color was measured at 530 nm in a Beckman DU-68 spectrophotometer. One unit of enzyme activity corresponds to the amount of enzyme producing 1 μmol of the product in 1 min. Peroxidase was measured on a medium containing 8-mM phosphate buffer, pH 6.0, 1-mM *o*-dianisidine, and saliva samples. The absorbancy was measured in a Beckman DU-68 spectrophotometer at 460 nm. A solution of lactoperoxidase was used as standard.

For statistical analysis, the data are presented as mean±standard deviation (SD). Based on the normal distribution of all parameters studied under different conditions, analysis of variance and Student's *t* test were performed taking 5% as the level of significance to compare the data among groups A, B, and C and also within the same group (to analyze the data in different collection days).

Results

Table 1 shows the mean and SD of amylase and peroxidase activities in saliva of different groups (A, B, and C) in different experimental periods (first, second, and seventh salivary collection days). No statistical difference was observed when the experimental groups (A and B) were compared with control (C) on each collection day. However, differences were noted in amylase and peroxidase activities within the same group, when the three collection groups were compared. Amylase activity was higher on the second collection day in all groups. However, when the laser was applied (group A and B), the increase was smaller, and it was not statistically significant for group B. Peroxidase activity was statistically lower on second collection day for groups A and C if compared with the first day of collection. Although, on second day, the value of peroxidase for the B group was not statistically significant, it was considerably lower than the first collection day. It was numerically comparable with the other groups. However, on the third collection day, that is, 7 days after the first laser irradiation, the values of all three groups increased, reaching the same value measured on the first collection day.

Table 2 shows the data on amylase and peroxidase output. No statistical difference significantly important was observed between the experimental groups (A and B) and the control group (C), except that group A amylase output on the second collection day was higher than the output of control and B groups. However, within the same group, we

Table 1 Mean and SD results obtained from amylase and peroxidase activities in saliva of rats that had their salivary glands submitted to LLLT

Collection day	Amylase (U/mg protein)			Peroxidase (μg/mg protein)		
	C	A	B	C	A	B
First	13.15±4.01a (11)	16.62±8.94a (10)	16.62±8.29ab (10)	6.58±1.63a (11)	7.28±1.99a (10)	7.12±3.03ab (11)
Second	31.14±10.42b (11)	28.26±12.16b (10)	21.99±10.32a (11)	3.46±3.33b (10)	3.31±1.70b (9)	4.03±3.72a (10)
Seventh	9.25±2.44c (11)	8.27±2.93c (9)	10.56±2.82b (10)	6.51±1.97a (11)	5.26±2.13c (9)	7.40±4.25b (10)

Stimulated saliva with pilocarpine was collected after the first and second laser applications and 7 days after the first one. The numbers of irradiated samples are expressed in parenthesis. Different letters mean statistically different considering the same column ($p<0.05$).
C Control group, *A* 4 J/cm^2, *B* 8 J/cm^2

Table 2 Mean and SD of results obtained from amylase and peroxidase activities output in saliva of rats that had their salivary glands submitted to LLLT

Collection day	Amylase (U/min)			Peroxidase (μg/min)		
	C	A	B	C	A	B
First	2.66±0.53a (11)	2.79±0.76a (10)	2.63±0.53a (10)	1.38±0.49a (11)	1.33±0.39a (10)	1.13±0.33a (11)
Second	3.66±0.64b (11)	4.90±0.94b (10)[a]	3.92±0.38b (11)	0.49±0.54b (10)	0.58±0.47b (9)	0.75±0.64a (10)
Seventh	3.43±0.82b (11)	3.59±1.10a (9)	3.67±0.71b (10)	2.46±1.16c (11)	2.52±1.48c (9)	2.69±1.51b (10)

Stimulated saliva with pilocarpine was collected after the first and second laser applications and 7 days after the first one. The number of samples is in parenthesis. Different letters mean statistically different considering the same column ($p<0.05$).
C Control group, *A* 4 J/cm^2, *B* 8 J/cm^2
[a]Results were statistically different when compared to corresponding groups.

noticed some differences regarding the peroxidase output. There was a reduction in peroxidase output on the second day of saliva collection. However, the output of this enzyme was greater on the third saliva collection day for all groups.

Table 3 shows the mean and SD of salivary flow rate and whole protein concentration for experimental and control groups on different salivary collection days (first, second, and seventh). Similarly, the results for amylase and peroxidase activities showed no statistical differences when the groups were compared. However, within the same group, on different saliva collection days, some differences were noticed. The flow rate was statistically higher on the seventh day for groups A and B when it was compared to the first and second days. However, group C did not present the same results. The whole protein concentration was lower on the second collection day for groups C and B, when it was compared to concentration obtained on the first and seventh days. On the seventh day, there was an increase in protein in all groups.

By analyzing the output of protein (Table 4), it is observed that the output values on the third collection day were higher than those measured on the other two collection days. One important difference regarding this group was that it received no laser application when saliva was collected. The pH values (data not shown) were similar when all groups and all three collection times were compared.

Discussion

The aim of this study was to examine the effect of infrared defocused diode laser irradiation on salivary flow rate and on salivary composition of healthy rats. Few histological and clinical reports that analyzed LLLT effect on salivary glands are available. However, no biochemical studies concerning laser irradiation effect on salivary flow rate and saliva composition of rats are available.

Preliminary results of a study carried out with Sjögren's syndrome patients showed a fast response of the glands to laser irradiation [30]. Moreover, in humans aged 20–45, He–Ne laser irradiation of the oral cavity with final 10-mW output for 3 min (1.8 J) applied three times caused a significant rise in the salivary lysozyme activity [7]. When rats and Ga–As semiconductor laser were used, it reported an increase in ductal epithelial cells mitoses [26]. In this study, the submandibular glands were surgically exposed to laser irradiation. Furthermore, a study using guinea pig [20] whose submandibular glands were surgically exposed to He–Ne laser at 7-mW power under continuous irradiation

Table 3 Mean and SD results obtained from salivary flow rare and total protein concentration of rats that had their salivary glands submitted to LLLT

Collection day	Flow rate (μg/min)			Protein (μg/min)		
	C	A	B	C	A	B
First	41.64±19.63a (11)	42.77±18.01a (10)	39.78±10.86a (9)	4.99±0.97a (11)	4.53±1.70a (10)	4.65±2.23a (11)
Second	44.09±18.00a (11)	60.50±21.66ab (10)	54.44±16.16ab (11)	2.89±0.93b (11)	3.28±1.39a (10)	2.78±0.99b (11)
Seventh	55.11±17.45a (11)	62.09±18.33b (10)	60.77±21.59b (9)	6.96±1.45c (11)	7.41±2.13b (9)	6.02±1.61a (10)

Saliva production was stimulated by pilocarpine. The samples were collected immediately after the first and second laser treatments and 7 days after the first one. The numbers of collected samples are in parenthesis. Different letters mean statistically different considering the same column ($p<0.05$).
C Control group, *A* 4 J/cm^2, *B* 8 J/cm^2

Table 4 Mean and SD results obtained from the whole protein output of rats that had their salivary glands submitted to LLLT

Collection day	Protein (mg/min)		
	C	A	B
First	0.209±0.041a (11)	0.195±0.073a (10)	0.186±0.089a (11)
Second	0.127±0.041b (11)	0.199±0.084a (10)	0.143±0.051a (11)
Seventh	0.383±0.080c (11)	0.452±0.130b (9)	0.364±0.097b (10)

Saliva production was stimulated by pilocarpine. The collected samples were collected immediately after the first and second laser treatments and 7 days after the first one. Different letters mean statistically different considering the same column ($p<0.05$).
C Control group, *A* 4 J/cm^2, *B* 8 J/cm^2

for 2 min (11.2 J/cm^2) showed that laser effect was progressive as well as had a trophic stimulant effect after 2-h irradiation, leading to vasodilatation, vascular congestion, perivascular infiltrate, and a necrotic picture of glandular parenchyma at longer times.

In the present study, salivary glands were not surgically exposed. Amylase, a metalloenzyme that catalyzes the hydrolysis of the glucosidic linkages from starch, comprises about 50% of the protein produced by salivary glands [12]. Salivary amylase may play an important role in colonization and metabolism of streptococci, leading to the formation of dental plaque and caries [1, 23]. In this study, significant statistical differences (higher specific amylase activity) were noticed when comparing the data of the second saliva collection with the first or third one, even for control group. It was reported that pilocarpine stimulation of the parotid gland of rats caused discharge of amylase from the gland [15]. On the other hand, the rat parotid gland does not secrete saliva spontaneously, normally requiring stimulation via autonomic innervation to elicit secretion [2]. Changes on salivary enzyme amylase have been proposed to indicate stress-reactive bodily changes [16]. Considering that the first and second saliva samples were collected less than 24 h apart and that, to make collection easier, the animals were submitted to both anesthesia and sialagogue injections, it is our opinion that both procedures may have worked as stressors, explaining the increase in amylase activity and the output on the second saliva collection. As the variation observed for the control group on the second saliva collection day was about 135% higher if compared with the first saliva collection, and considering the experimental groups in which the increase in amylase activity was 70 and 33% for group A (4 J/cm^2) and B (8 J/cm^2), respectively, we may assume that LLLT caused some changes in this parameter. In short, pilocarpine effect on amylase activity was lower when laser was applied (group A and B). The values related to the third collection day are similar for all three groups, as no laser was applied to any group.

Peroxidase is an enzyme with antimicrobial properties. It catalyzes the oxidation of thiocyanate (SCN^-), in presence of hydrogen peroxide (H_2O_2), producing hypothiocyanate (OSCN−), which inhibits bacterial growth [27]. By consuming H_2O_2, it prevents the accumulation of this toxic substance. The analysis of protein concentration of saliva can be used to observe if there is a change in protein synthesis of irradiated salivary glands. In the present study, the total protein concentration of saliva showed altered values when different collection days were compared. The decrease in protein concentration observed on the second day of saliva collection may be caused by the fact that the animals were under stress of irradiation procedure, injections of anesthetics and sialagogue. One week after the first irradiation, groups A and C showed an increase in protein concentration. Group B, that is, the group that received 8 J/cm^2 of LLLT irradiation, showed a value 30% higher than the one measured in the first saliva samples, although the difference was not statistically significant.

Protein metabolism stimulation is related to LLLT irradiation [17, 22]. However, secreted protein in saliva was not increased by LLLT irradiation in this study. On the second saliva collection, the protein concentration and output were reduced, and on the third saliva collection, the protein output was higher than the values measured on the other two saliva collections. This may explain in part the reduction in peroxidase activity on the second saliva collection and its increase on the third saliva collection. We have also to consider that the enzyme amylase is mainly produced by the parotid glands, while the peroxidase is mainly produced by the submandibular glands. Both glands show histological and biochemical differences.

About 30% of the population reports some degree of dry mouth [8]. Hyposalivation may cause inadequate preparation of food for digestion and taste, and increase susceptibility of oral structures to disease [13]. Consequently, it is important to find an efficient stimulation method for salivary glands. In this study, flow rate was statistically higher on the seventh day than it was on the first day (about 45 and 51%) for group A and B, respectively, but the same was not reported for group C. This flow rate stimulation by laser irradiation on salivary glands is in agreement with clinical studies previously reported [11, 29]. However, more studies using healthy and xerostomic animals should be conducted.

Based on these results, further studies on LLLT should be conducted, regarding its effect on salivary flow rate stimulation, as it can be used as an auxiliary therapy for hypofunction or inflammatory process of salivary glands.

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