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Effect of Low-Level Laser Irradiation on Salivary Gland Secretory Function and Histology

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Ola Abdullah A. Alshuhail, BDS

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THESIS COMMITTEE

Thesis Advisor

Driss Zoukhri, PhD

Professor

Department of Comprehensive Care

Tufts University School of Dental Medicine

Department of Ophthalmology

Tufts University School of Medicine

Committee Members

Noshir Mehta, BDS, MDS, DMD, MS

Associate Dean, Global Relations

Professor, Department of Public Health and Community Service

Senior Advisor, Craniofacial Pain, Headache and Sleep Center

Tufts University School of Dental Medicine

Athena Papas, DMD, PhD

Professor and Co-Head Division of Geriatric Dentistry

Director, Rubenstein Oral Medicine Clinic

Johansen Professor of Dental Research and Head Of the Division of Oral Medicine

Department of Diagnostic Sciences

Tufts University School of Medicine

Tofool Alghanem, BDS, MS, DMD

Assistant Director of Advanced and Graduate Education, Academic Affairs

Assistant Professor, Department of Public Health and Community Service

Tufts University School of Dental Medicine

Sarah E. Pagni, MPH, PhD

Instructor, Division of Biostatistics and Experimental Design

Tufts University School of Dental Medicine

ABSTRACT

Background: The purpose of this study was to investigate the effect of low-level laser irradiation on the secretory function and histology of the salivary glands of a murine animal model.

Material and methods: Sixteen 8 week-old, female BALB/c mice were divided randomly into two groups: control group (n=8) was irradiated with sham light, and laser group (n=8) was irradiated with low-level laser. The salivary flow rate (ml/min) and delay on salivation (min) parameters were measured at baseline and after 10 days of irradiation. Glandular weight (gm.) of parotid, submandibular and sublingual glands and cellular volume of salivary glands (sq. μm) were measured after 10 days of irradiation.

Result: There was no statistically significant effect of low-level laser irradiation on salivary flow rate, intra-group comparison for the experimental ($p = 0.44$) and for the control one ($p = 0.96$), and inter-group comparison of the mean difference between the two groups ($p = 0.747$). The delay in salivation was significantly higher in the experimental group compared to baseline ($p= 0.011$). The weight of the parotid glands of the experimental group was statistically significantly higher than the control group ($p = 0.003$). The results showed a non-statistically significant effect of the low-level laser irradiation on the cellular volume of the glandular duct area ($p = 0.094$). However, the study showed that both serous and mucous acini areas of the experimental group were significantly smaller than the area of the control group [(p-value = 0.006), ($p = 0.005$) respectively].

Conclusions: The used low- level laser irradiation protocol in the present study was able to neither significantly increase salivary flow rate nor decrease the delay on salivation.

However, it was able to increase the parotid glands weight and decrease the cellular volume of serous and mucous acini areas.

Future studies using different low-level laser irradiation protocols are needed to establish the ultimate protocol to effectively stimulate salivary secretion.

DEDICATION

"In the name of Allah, Most Gracious, Most merciful"

I dedicate this project to my supportive parents and siblings.

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Effect of Low-Level Laser Irradiation on Salivary Gland Secretory Function and Histology

Salivary Glands

Saliva is an important biological fluid that maintains integrity and function of the oropharyngeal tissues.[1] It is secreted mainly by three major paired salivary glands: parotid gland, submandibular gland and sublingual gland.[2] About 10% of saliva is secreted by minor salivary glands embedded in the submucosa of oral soft tissues.[3] Consistency and composition of saliva vary according to the type and the histology of salivary glands in which parotid secretion is serous, sublingual secretion is mucous and submandibular secretion is mixed. The parotid gland is the largest among the three-paired major salivary glands that is approximately double the size of submandibular gland and the sublingual gland being the smallest. Number of minor salivary glands is embedded in the oral submucosa of tongue, lips and palate.[4]

Salivary glands are composed of serous and/ or mucous acinar cells and ductal system which are responsible for the process of formation and secretion of saliva.[5] Saliva passes the lumen initially through the intercalated ducts then striated ducts and finally excreted through the excretory ducts. Salivary glands receive both parasympathetic and sympathetic nerves that control salivation. Neurotransmitters such as acetylcholine (ACh) released by parasympathetic nerves and noradrenaline (NA) released by sympathetic nerves stimulate secretion of both fluid and macromolecules from the surrounding blood capillaries. Saliva formation is a unidirectional process. The salivary acinar cells secretion is either a watery sero-mucous or a viscous mucin-rich modified interstitial fluid. This fluid is initially formed from the interstitial fluid of the surrounding blood capillaries, and then modified in the acini to be secreted into the lumen[4]. Further modifications occur while fluid passes through different salivary ducts, which are intercalated, striated or excretory.[6] Theses ducts are

responsible for the process of ion exchange of the fluid and finally the secretion of a hypotonic salivary fluid.[4] Salivary glands damage, interference with supply metabolites and failure in neural transmission could affect saliva formation and secretion altering saliva composition and/or reducing salivary flow rate.[4]

Saliva Composition and Function

Whole saliva is a hybrid of emulsions from the salivary glands (unstimulated and stimulated), the gingival sulcus, nasopharyngeal discharge, oral bacteria, hemolytic cells and the remnants of food and medications.[7] Saliva is not a well-mixed viscoelastic solution because it enters the oral cavity via various glands from different stimuli at variable times.[8, 9] It is composed of 99% water plus electrolytes such as sodium, potassium, chloride and bicarbonate.[5] Although salivary protein components such as immunoglobulin A, amylase, lipase, lysozyme, lactoferrin, cystatins, histatins, and mucins account for less than 2% of the total saliva, they play important roles in buffering, antimicrobial as well as antifungal action, digestion, lubrication, and protection of the oral mucosa.[10, 11]

Composition is crucial for a proper function of saliva. Several factors affect the composition and the volume of saliva such as the type of salivary gland in which parotid glands contribute approximately 50% of stimulated saliva while submandibular glands contribute approximately to 60% of unstimulated saliva.[4] Another essential factor to consider is that the increase in the salivary flow rate can elevate the pH and concentration of protein, sodium, bicarbonate and chloride and on the other hand demote the concentration of magnesium and phosphate.[4] Nature of stimulus, duration of stimulation and circadian rhythm

have important effect on the composition of saliva. Hormones, pregnancy as well as exercise also play roles in saliva formation and secretion.[4]

Salivary Flow Rate

Maintaining adequate salivary flow rate is fundamental for food tasting and swallowing and for the health of oropharyngeal tissues. The average daily production of whole saliva ranges between 0.5 to 1.5 liters.[12] Unstimulated salivary flow rate is influenced by different variables including degree of hydration, body posture, medications and the biological rhythms.[4] Unstimulated salivary flow rate ranges between 0.2 to 0.4 ml/min that reaches the peak during late afternoon and drops to almost zero during sleep.[8] Stimulated salivary flow rate is widely studied and measured using different stimuli and ranges between 3.15 to 4.94 ml/min during food chewing and can reach up to 7.07 ml/min after an infusion of 5% citrus acid in the mouth.[8] Gustatory stimuli, especially citric acid, promote a higher stimulated salivary flow rate compared to mechanical stimuli such as the gag reflex.[7] Other factors such as tobacco smoking and olfactory stimuli have minimal effect on salivary flow rate.[7] Salivary flow rate is considered abnormal when the unstimulated salivary flow value is less than 0.1ml/min and the stimulated salivary flow value is less than 0.5 ml/min.[4]

Sialometry and sialochemistry are sensitive but not specific investigational methods. They have been widely used to diagnose some systemic diseases and to assess medication's metabolism and elimination.[7] Salivary flow rate as well as saliva composition have been studied widely and salivary biomarkers could be used as prognostic/ diagnostic tool for Sjögren's syndrome.[8, 13] Alteration in salivary flow rate or salivary components due to age, radiotherapy, medications, rheumatic and immunological disease as well as genetic disorders

such as Down syndrome can affect a person's quality of life.[14] Hyposalivation and xerostomia have obvious clinical complications on hard as well as soft oral tissues.[11]

Xerostomia

Xerostomia or mouth dryness is one of the most common complaints in geriatric dental clinics.[15] Xerostomia is defined as "a subjective sensation of dry mouth due to a reduction of saliva production or due to an alteration of its chemical composition that does not necessarily explain a true hyposalivation[16]". Hyposalivation can be clinically assessed when the salivary flow rate is reduced.[17] The diagnosis of hyposalivation is confirmed when the unstimulated salivary flow rate is less than 0.1 ml/min and the stimulated salivary flow rate is less than 0.5 ml/min.[18] The prevalence of xerostomia generally ranges between 5.5% - 39%, which is higher among females and elderly.[19] Individuals with xerostomia are at high risk of oral hard and soft tissue diseases such as dental caries and periodontitis.[20] Several studies suggested different etiological factors of xerostomia such as age, disease or syndromes such as Sjögren's and some medications including antidepressants, anticholinergic, antispasmodic, antihistamines, antihypertensive, sedatives, and bronchodilators.[19, 21-24]

Xerostomia is the most common side effect of prescription drugs with a published 80% incidence of the 200 most commonly prescribed medications in the United States.[25,26] Although xerostomia is a temporary side effect, its prevalence increases with the number of drugs taken per a day for different common chronic conditions and diseases among elderly including depression, high blood pressure, and chronic fibromyalgia.[25]

A literature review reported that 64% of patients with head and neck cancers complained of moderate to severe xerostomia due to radiotherapy.[27] Salivary flow rate decreased rapidly by almost 50-60% after the first week of radiotherapy that continues to decreases to almost 80% by the 8th week of therapy. Temporary and/or permanent hyposalivation affects cancer patient's quality of life that correlates with chronic mucositis, burning mouth syndrome, difficulty of speech and swallowing.[28]

Sjögren's Syndrome

Sjögren's syndrome can be defined as a chronic systemic inflammatory autoimmune disorder affecting the salivary and lacrimal glands.[21, 29, 30] The function of the exocrine glands is altered and decreased because of lymphocyte-mediated destruction of the acinar cells leading to dry mouth and dry eyes.[31, 32] The syndrome is either primary in which the function of the exocrine glands is impaired or secondary and accompanies other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematous.[33] The prevalence of Sjögren's syndrome is estimated to be 1-3% and is more common in women than men in a ratio of 9:1.[21] Although Sjögren's syndrome is considered to be a benign disease, morbidity and mortality rates are affected by the development of glomerulonephritis, peripheral neuropathy and B-cell lymphoma.[30, 34] Many studies have been conducted; however, the definitive etiology and pathogenesis are still unknown and seem to be multifactorial.[21, 35, 36]

Diagnosis of Sjögren's syndrome is challenging because of the progressive nature of the syndrome and the number of tissues that can be affected.[31] Moreover, clinical manifestations and extensive laboratory tests are required for the diagnosis that often delay

the detection and early treatment.[31] Mouth dryness can lead to dysphagia, burning mouth sensation, induced stomatitis and fissured tongue as well as atrophy of tongue papillae.[25]

Patients with Sjögren's syndrome complain of mouth dryness that is either due to reduced salivary flow rate or increased saliva viscosity or both and are associated with increased risk level of caries as well as bacterial and fungal infections affecting the periodontal tissues.[39, 40] Bacterial and fungal species such as *Lactobacillus acidophilus, Streptococcus mutans,* and *Candida albicans* are prominent among Sjögren's syndrome patients with reduced salivary flow rate.[41, 42] The association between periodontal diseases and Sjögren's syndrome is still controversial although others found that periodontal condition and gingival index are worse among Sjögren's syndrome patients.[43]

The early detection of Sjögren's syndrome is key to maintaining the integrity of the affected organs and preventing further destruction.[37] Dental clinicians might be the first ones to detect and manage the syndrome if they preform sufficient investigations.[44]

Different biological agents have been tested to manage primary Sjögren's syndrome, however; limited data is available to support one over the other.[46] Rituximab, which depletes the hyperactivity of B-lymphocytes, has been extensively studied and approved to treat Sjögren's syndrome, however long term side effect and drug safety are not completely known yet.[47]

Sjögren's syndrome symptoms such as eye and mouth dryness can be managed using local or/and systemic therapies. The local treatment of eye dryness is variable and it is approached based on the severity of eye symptoms, and include artificial tears, local

corticosteroids, sealed glasses, and blocking the lacrimal punctum.[25] Mouth dryness can be locally managed by stimulating salivation mechanically via chewing gum and gustatory via consumption of sugar-free sweet.[25]

The most commonly prescribed systemic agents are saliva substitute and salivary gland stimulants (sialagogues) such as pilocarpine and cevimeline.[14, 48] Pilocarpine (Salagen) is widely studied and was found to significantly improve salivary flow rate.[49] Pilocarpine side effects on the cardiovascular and respiratory systems have always been an issue for long-term use.[44] Other documented adverse effects of pilocarpine are flushing and increased urinary frequency. Cevimeline (Evoxac) treatment leads to improvement of salivary flow rate, and eye and mouth dryness.[46] There is no evidenced recommendation regarding both medications efficacy and safety but cevimeline demonstrated severe gastrointestinal side effects beside the increased sweeting and urination.[46]

Although systemic cotricosroids successfully managed some autoimmune diseases including systemic lupus erythematous and Sjögren's syndrome, they increase individuals' risk to cardiovascular disorders and diabetes mellitus.[46] Antifungal medication has been used to treat oral candidiasis among patients with Sjögren's syndrome, however; its management evidence is vey low.[50]

Low-level laser therapy

Lasers are classified based on the power output that is measured in Watts and on the risk for causing biological damage.[51] The laser light amplification process (stimulated emission) occurs when power is applied to a lasing medium in an optical chamber to produce

photons that are emitted as concentrated light beam via the laser applicator.[51] Each laser device emits different light color in the electromagnetic spectrum depending on the lasing medium used. Each Laser light beam has a single wavelength range that determines tissue penetration capability.[51]

Low-level laser or cold laser emits a light beam with a single wavelength range in the optical window (600-1070 nm) of the electromagnetic spectrum that is perceived as red, infrared or invisible color. Low-level laser uses a lower level of power densities compared to other types of laser, which need higher power to thermally coagulate or cut the tissues. Low-level laser has been used widely for wound healing, pain reduction and treatment of inflammation. Light wavelength, power density, pulse structure and timing are the therapy parameters to be considered for each individual laser-irradiated tissue. Despite the fact that the World Association of Laser Therapy (WALT) created dosage guidelines, the complexity of dosage calculation is still an issue that is not solved yet due to different parameters and tissues to be irradiated.[51]

The low-level laser therapy has been used to treat different clinical conditions in dentistry, dermatology, rheumatology and physiotherapy.[51] Numerous effects of the low-level laser therapy can be summarized as wound healing and tissue repair; inflammation alleviation of an injury or chronic condition; and curing or analogizing neurological problems.[51] The effect of low-level laser therapy on human osteosarcoma cells was studied and demonstrated increased proliferation of osteoblasts and enhanced growth factors too.[52] Bone healing was studied on rats receiving low-level laser therapy and showed better and accelerated bone healing compared to control rats.[52] Analgesic effect and pain reduction were

reported after the application of low-level laser to manage different pain conditions such as rheumatoid arthritis, temporomandibular joints pain, low-back pain, shoulder pain and neck pain.[52] Trigger point release and acupuncture using low-level laser probe were tested and showed an activation of the taut band of the muscle fibers.[51]

The low-level laser technology has been used in the dental medicine and many clinical studies were conducted to manage different conditions such as temporomandibular disorders, burning mouth syndrome and dentin hypersensitivity.[51,52] Recently, low-level laser therapy was recommended to prevent oral mucositis in cancer patients receiving hematopoietic stem cells with chemotherapy or/and radiotherapy.[52] Moreover, low-level laser therapy is being studied to manage bisphosphonate-induced osteonecrosis of the jaws with promising results.[53] Chronic advanced periodontitis was managed successfully using low-level laser therapy.[52] Low-level laser therapy also successfully managed erosive oral lichen planus lesions by reducing pain and inflammation with no reported side effects.[54]

A recent study documented the benefit of low-level laser therapy in managing oral pain as well as oral mucositis in cancer patients.[55] In another study, it was suggested that low-level laser could be used as an alternative therapy for acute temporomandibular disorders.[56] Another group has studied and compared the effect of low-level laser versus CO_2 laser on oral lichen planus lesions, and low-level laser showed better results in reducing pain and size of the lesions without any side effects.[57]

The beneficial effect of low-level laser therapy on managing various medical and dental conditions with no or mild temporary side effects made it an attractive target for many researchers and clinicians to treat symptoms such as pain and xerostomia.[14,49,55-57,58]

The effect of the low-level laser therapy on the salivary glands is still under investigation on animals and humans. Improvement of xerostomia, parotid gland swelling and pain were reported in Sjögren's syndrome patients treated with low-level laser.[49] Another study reported stimulating as well as regenerative effect of low-level laser therapy on patients with xerostomia.[14] Low-level laser therapy was also used as an auxiliary palliative intervention to reduce radiotherapy-induced xerostomia.[59] Moreover, low-level laser irradiation was able to increase salivary flow rate in healthy rats and decrease catalase activity in parotid gland of diabetic rats.[58] On the other hand, a non-significant effect of low-level laser therapy on salivary flow rate was found in patients suffering form xerostomia due to head and neck radiotherapy.[60] Another study found a non-significant effect of laser irradiation on salivary flow rate of the submandibular glands of hypothyroid rats.[61]

In summary, low-level laser therapy was tested to treat various dental conditions resulting in either no complication or interim side effects such as skin redness or discomfort. However, data regarding the effect of low-level laser on salivary flow rate and histological changes are still limited and further laboratory studies are needed. Therefore, the purpose of this study was to assess the effect of low-level laser irradiation on salivary flow rate, glandular weight and cellular volume of the salivary glands of a murine animal model.

SPECIFIC AIMS AND HYPOTHESIS

The aim of this study was to investigate the effect of low-level laser irradiation on the secretory function and histology of the salivary glands of a murine animal model. This was accomplished by measuring the following parameters:

- Stimulated salivary flow rate
- Time before first sign of salivation after pilocarpine stimulation
- Glandular weight
- Cellular volume (ducts, serous and mucous acini)

We hypothesized that low-level laser irradiated mice would show increased salivary flow rate, decreased time before first sign of salivation, increased glandular weight and increased cellular volume compared to the animals irradiated with red non-active sham-light.

RESEARCH DESIGN AND METHODS

The study was designed as a randomized controlled animal study, and was conducted at Tufts University School of Dental Medicine. Tufts-Medical Center Institutional Animal Care and use Committee (IACUC) approved the study.

1. Study Animals

Sixteen 8-week old, female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in cages under controlled temperature of 72.5 F and constant humidity of 27%, under 12-hour intervals of light/dark, and were fed *ad libitum*. For the purpose of the study the mice were randomized into two groups: 8 BALB/c mice for the control group and 8 BALB/c mice for the experimental one.

Salivary flow rate and delay in response (until the first sign of salivation) were measured on the first day (the baseline pre-treatment measurements) and on the tenth day (the outcome post-treatment measurements).

Salivary secretion was stimulated by an intra-peritoneal injection of pilocarpine hydrochloride solution (100 μg/ml) on the first day for baseline measurement before the first irradiation and on the tenth day for outcome measurement after the final irradiation. Delay in response until the first sign of salivation was recorded in minutes and saliva samples were collected from the oral cavity, over a period of 5 minutes, using a micropipette. Mice were anesthetized by isoflurane gas inhalation for a total period of 6 minutes. Irradiation was applied on three separate areas including the right parotid gland, the left parotid gland and the submandibular and sublingual glands together. Each area received either low-level laser or

sham light for 2 minutes per day for ten consecutive days. By the 10th day, the outcomes (salivary flow rate and delay in response) were measured. At the end of the experiment animals were euthanized and all major salivary glands (parotid, submandibular and sublingual glands) were carefully removed for glandular weight measurement. Then, the salivary glands were processed for histological examination and digital analysis of cellular volume.

2. Experimental Design

Stimulated salivary flow rate and delay in response were measured at baseline for all groups. Animals were then randomly divided into two groups: control and experimental. The control group received sham light irradiation. The experimental group received low-level laser irradiation. Each mouse was irradiated with either sham light or low-level laser for 2 minutes on each parotid gland separately and on the submandibular and sublingual glands together for a total of 6 minutes per a day. The irradiation with either sham light or low-level laser was carried out for a total period of ten consecutive days (Figure 1).

Three BALBc mice suffocated and could not recover after the intra-peritoneal xylene injections. Therefore, the sedation protocol was modified using isoflurane gas instead of intra-peritoneal xylene injections. Another BALBc mouse was lost on the 7th day of laser irradiation due to handling difficulties.

Animal Grouping

A total of sixteen BALBc mice were randomly divided into 2 groups:

Group 1: Eight BALB/c mice were irradiated with sham light (control group 1)

Group 2: Eight BALB/c mice were irradiated with low-level laser (experimental group 1)**.**

Laser Application

As there was no definitive low-level laser irradiation protocol for saliva stimulation in BALB/c mice (experimental models), the laser equipment (Lumix® 2 DENTAL) was used and the irradiation parameters were adjusted and followed the parameters of previous studies that reported positive effect on tissue stimulation[59,61,14]. Low-level laser beam was applied in a non-contact mode to the parotid glands separately and to the submandibular and sublingual glands together. For the experimental groups, a single application of an active dose of low-level laser beam was applied daily for a total period of ten days; each application lasted for 2 minutes. For the control group, a single application of a non- bioactive dose of red sham light beam was applied daily for a total period of ten days; each application lasted for 2 minutes. The distance between the probe and the irradiated area was kept constant at ~ 0.5 cm throughout the treatment period to cover the gland area which is approximately ~1.2 cm^2 (Table 1).

3. Study Parameters

a. Stimulated salivary flow rate and delay in response

Stimulated salivary flow rate was expressed as ml/min and delay in response expressed in minutes. Both parameters were measured at baseline (the first day before the irradiation) and on the tenth day (the last day after the last irradiation). A single blinded investigator collected the saliva samples and recorder the delay in response. Before measuring the outcomes, animals were anesthetized with isoflurane for a total of 6 minutes daily during the irradiation. Salivary secretion was stimulated by an intra-peritoneal injection pilocarpine hydrochloride

of 100 µg/ml. Delay in response was recorded in minutes and saliva samples were collected from the oral cavity, over a period of 5 minutes, using a micropipette and placed in labeled pre-weighted microfuge tubes. Volume of saliva samples was determined, after a brief centrifugation, using a precision scale. On the last day of treatment, animals were euthanized and the salivary glands were carefully removed.

b. Glandular weight

After removal of all salivary glands (parotid, submandibular and sublingual) at the end of the experiment, glandular weight, expressed in milligrams per body weight, was determined using a high precision scale and tissues were processed for measurement of cellular volume.

c. Histological Preparation

The submandibular and sublingual glands from each animal were surgically removed. Samples were prepared for the digital analysis to determine the cellular volume of the glands. The right lobes of the glands were carefully separated from the left ones and immediately fixed in 4% paraformaldehyde made in phosphate-buffered saline (PBS), pH 7.4 and stored at 4^{o}C for 24 hours. Next, samples were washed with PBS two times for 20 minutes and dehydrated in graded ethanol solutions. Samples were embedded in 1:1 xylene: paraffin followed by embedding in fresh paraffin wax. Paraffin blocks were sectioned using a Reichert Jung Autocut 1150 microtome (Nussloch, Germany) to obtain a series of 5 µm thick sections. A range of twenty to twenty-five slides was prepared for each sample. Four slides of each sample were selected (#5, 10, 15, and 20) for histological evaluation. Sections were deparaffinized and stained with hematoxylin and eosin for analyses using a light microscope.

Two sections of each slide were examined at a 40x magnification with a light microscope in order to identify secretory components of the salivary glands (mucous acini, serous acini and ducts). Images were taken of each section for each component of the salivary glands for cellular volume measurement.

Histological evaluations were performed using The SPOT Basic™ image capture software version 5.2. With precise tools of The SPOT Basic™ software, the salivary gland components (mucous acini, serous acini and ducts) were marked and measurement of the components' (area of the cell) was calculated. All measurements were transferred to an excel sheet for further data analyses.

4. Sample Size Calculation

The primary aim of this study was to compare the salivary flow rate across the two different groups of animals (control and low-level laser irradiation groups). Based on data from other studies[62], we anticipated that the mean (SD) difference of salivary flow rate between baseline and the end of the study will be 0.03 (0.009) mL/min for the control group. For the laser to be beneficial, we will require a 50% increase in the change of salivary flow rate. Thus, a sample of 8 mice per group was required to have 85% power to detect a difference between the control sham-treated group and the laser-treated group, setting α=0.05 (nQuery Advisor, 7.0).

5. Data Presentation and Statistical Analysis

Normality was assessed using the Kolmogorov-Smirnov test and the assumptions of normality were not violated. Thus, means and standard deviations were reported. Intergroup

comparison of the change in salivary flow rate from baseline to the end of study was conducted using two-sample *t*-test. Intragroup comparisons (baseline to the end of study) were performed using paired *t*-test. All p-values less than 0.05 were considered statistically significant. Analyses were performed using SPSS, Version 22 (IBM Corp, Armonk, NY).

RESULTS

- **Effect of low-level laser irradiation on salivary flow rate**

Salivary flow rate was measured at baseline and after 10 days of irradiations to determine the effect of low-level laser irradiation. The results showed that low-level laser irradiation did not result in a statistically significant increase in salivary flow rate in the experimental group compared to baseline [mean (SD): 0.16 (0.03)] baseline versus values after 10 days irradiation 0.13 (0.06), (p =0.44)]. The control group results also showed no statistically significant difference (p = 0.96) in salivary flow rate between baseline and after 10 days of irradiation [mean (SD): 0.13 (0.04) and 0.11 (0.06), respectively] (Table 2 and Figure 2).

Inter-group comparisons of the mean difference, also revealed that low-level laser irradiation has no statistically significant effect on salivary flow rate (p = 0.747) when comparing the experimental group [mean (SD): - 0.029 (0.077)], with the control one [mean (SD): - 0.015 (0.074)] (Table 3).

- **Effect of low level laser irradiation on the delay in salivation**

The delay in salivation in response to pilocarpine stimulation was measured in minutes at baseline and after 10 days of irradiation. The delay in salivation was significantly higher in the experimental group (p= 0.011) compared to baseline [mean (SD): 4.98 (0.34) and 7.73 (1.43), respectively]. On the other hand, in the control group, the delay in salivation was not significantly affected when compared to baseline values [mean (SD): 5.15 (0.19) and 7.11 (1.10), respectively, p = 0.119] (Table 4 and Figure 3).

Similarly, when comparing the differences of the experimental group [mean (SD): 2.76 (1.39)] with the control group [mean (SD): 1.96 (2.85)], the effect of the low-level laser

irradiation on the delay in salivation between the two groups was not statistically significant (p = 0.574) (Table 5).

- **Effect of low level laser irradiation on glandular weight**

The weight of the salivary glands was measured at the end of the 10-day sham or laser treatment. The results showed that there was no statistically significant difference (p = 0.757) in submandibular and sublingual salivary glands weight when comparing the experimental group [mean (SD): 0.12 (0.01)] to the control group [mean (SD): 0.12 (0.01)]. On the other hand, the weight of the parotid glands of the experimental group [mean (SD): 0.06 (0.01)] was statistically significantly (p = 0.003) higher than the control group [mean (SD): 0.05 (0.01)] (Table 6 and Figure 4).

- **Effect of low-level laser irradiation on the cellular volume of submandibular and sublingual salivary glands cells**

A. Glandular duct area

The cellular volume of the glandular duct was determined by measuring the area (sq. μm) of the duct. By comparing the glandular duct area of experimental group [mean (SD): 26350 (3563)], to the control group [mean (SD): 29839 (2966)], the results showed a non-statistically significant effect of the low-level laser irradiation on the cellular volume of the glandular duct area (p = 0.094) (Table 7, and Figure 5 and 8).

B. Serous acini area

The results showed that the serous acini area (sq. μm) of the experimental group was significantly smaller (p-value = 0.006) than the area of the control group [mean (SD): 23485 (1338) and 26119 (1257), respectively] (Table 7, and Figure 6 and 8).

C. Mucous acini area

By comparing the mucous acini area of the experimental group [mean (SD): 51043 (7085)] to that of the control group [mean (SD): 63239 (4961)] we found that the mucous acini area of the experimental group was significantly smaller (p = 0.005) than the control ones (Table 7, and Figure 7 and 8).

DISCUSSION

Low-level laser therapy has been widely used in medicine to manage various medical and dental conditions and symptoms such as wound healing and pain management. Massive move towards the use of low-level laser therapy was because of its known effectiveness and minimal side effects.[51] The mechanism of action of the low-level laser irradiation on various type of tissues is still not fully understood. Thus, numerous investigational studies using the low-level laser therapy on different conditions have been conducted and still in progress in order to determine specified guidelines and protocols for use of low-level laser therapy.[51,52,54,55]

This study was conducted to investigate the effect of the low-level laser irradiation on the secretory function of the salivary glands of BALB/c mice and hypothesized that low-level laser irradiation will increase salivary flow rate and decrease the delay in salivation. The data of our study showed that there was no statistically significant difference in the salivary flow rate between the low-level laser group and the sham light group. Moreover, there was no significant difference in the salivary flow rate between before and after low-level laser irradiation of the experimental group. Our data suggest that low-level laser irradiation has no effect on the salivary flow rate of BALBc mice. This was consistent with the result of the study of De Jesus et al. who reported a non-significant effect of the laser therapy on the salivary flow rate of the submandibular glands of the hypothyroid rats.[61] Moreover, Saleh et al. reported a supportive finding where they found a non-significant effect of laser irradiation on the salivary flow rate of patients that received head and neck radiotherapy and complaining of xerostomia.[60] On the other hand, Simon et al. found a significant effect of the low-level laser in increasing the salivary flow rate of healthy rats.[11] Loncar et al. also

reported that low-level laser therapy significantly stimulated saliva secretion in patients with xerostomia, which is opposite to our study's result.[14]

Another outcome we measured was the delay in salivation in response to pilocarpine and low-level laser irradiation. Our data showed a non-statistically significant difference between the experimental and the control groups. On the other hand, the delay was statistically increased after 10 days of low-level laser irradiation in the experimental group; where mean of delay before the laser (4.976) was lower compared to the mean of delay after laser (7.732).

Laser irradiation has a known effect on cellular metabolism and energy production, as it stimulates epithelial and fibroblast proliferation, and increases the amount of mitochondria and energy produced.[63] Simoes et al. found a significant increase in the salivary protein concentration of laser-irradiated rats with no morphological alterations of the salivary glands tissues.[64] Our result showed a supportive finding of a non- statistically significant difference in the weight of the submandibular and sublingual glands of the experimental group compared to the control one. On the other hand, we found a significant increase in the glandular weight of the parotid glands of BALB/c mice that received low-level laser irradiation compared to the control group. This result could be explained by the limited known benefits and side effects of the low-level laser therapy, which requires further investigations using different protocols and laser parameters.[64] The mechanism of action of low-level laser therapy on salivary glands is a very crucial area to study, which will help researchers in establishing proper use-protocol and eventually frame all previous experimental variations.

Histomorphometric analyses of the salivary glands were performed and the cellular volume of the salivary glands components' (glandular ducts, serous and mucous acini) was analyzed. The result showed that there were no significant differences comparing the glandular duct area of the control group with the experimental one. This finding is in agreement with that of Simon et al, who reported no difference in the acinar size of the parotid and submandibular glands of control and irradiated rats.[64] On the other hand, we found that the area of both serous and mucous acini of submandibular gland's been statistically smaller in the low-level laser group compared with the control ones.

There are certain limitations of this study that should be considered. First, the study was conducted on animals and generalizing the result to the human conditions needs further investigation. Although animal models were widely used to study the effectiveness of drugs and the pathophysiology of diseases with predictive validity, animal studies fall short in predicting human responces.[65] Second, the mechanism of action of low-level laser irradiation on salivary glands is still not fully understood and need further investigation. Third, therapeutic protocol of low-level laser irradiation on the salivary glands is lacking and yet not published, which made the methodological design uncertain.

CONCLUSIONS

Based on the result of this study and considering its limitations, we can conclude that the low-level laser irradiation protocol used in this study was neither able to increase salivary flow rate nor decrease the delay time in salivation. However, we found that the parotid glands weight was increased using the same laser protocol. Moreover, the cellular volume of mucous and serous acini after laser irradiation was lower compared to the control group. These two findings might indicate alterations and changes in the salivary glands at the cellular level. Theses results may enable future histomorphometric analyses and investigations of other parameters that were not assessed in our study.

Future studies using different low-level laser irradiation protocols are needed to establish the ultimate protocol to effectively stimulate salivary secretion on healthy as well as NOD mice, and animal model of Sjögren's syndrome.

APPENDIX A

Table 1. Summery of the Laser Parameter Used in this Study.

Parameter	
Laser classification	3b
Wavelength (nm)	665
Pulse Energy (mW)	300
Power density (mW/cm^2)	250
Illuminated area (cm^2)	1.2
Time (per session)(s)	360
Total treatment time(s)	3600

Table 2. Effect of Low-Level Laser Irradiation on Salivary Flow Rate (Intra-group Comparison).

	N	Baseline	After 10 days	*P- value*
Laser group	5	0.16 (0.03)	0.13 (0.06)	0.444
Control group	7	0.13 (0.04)	0.11 (0.06)	0.957

Data represent mean (SD) of salivary flow rate (ml/min). Data was analyzed using paired *t*-test.

Table 3. Effect of Low-Level Laser Irradiation on Salivary Flow Rate (Inter-group Comparison).

	N	Difference	*P- value*
Laser Group	5	- 0.03 (0.08)	0.747
Control Group	7	- 0.02 (0.07)	

Data represent mean (SD) of the difference in salivary flow rate (ml/min). Data was analyzed using independent sample *t*-test.

Table 4. Delay in Salivation Following Low-Level Laser Irradiation (Intra-group comparison).

	N	Baseline	After 10 days	*P- value*
Laser group	5	4.98 (0.34)	7.73 (1.43)	0.011*
Control group	7	5.15 (0.19)	7.11 (1.10)	0.119

Data represent mean (SD) Delay on salivation (min). Data was analyzed using paired *t*-test.
*Statistically significant (*P- value <0.05)*

Table 5. Delay in Salivation Following Low-Level Laser Irradiation (Inter-group comparison).

	N	Difference	*P- value*
Laser Group	5	2.76 (1.40)	0.578
Control Group	7	1.96 (2.85)	

Data represent mean (SD) of the difference of delay on salivation (min). Data was analyzed using independent sample *t*-test.

Table 6. Effect of Low-Level Laser on Submandibular/Sublingual and Parotid Glands Weights.

Weight Difference	Laser Group N = 5	Control Group N =7	*P- value*
Submandibular/ Sublingual Glands	0.12 (0.01)	0.12 (0.01)	0.757
Parotid Glands	0.06 (0.00)	0.05 (0.01)	0.003*

Data represent mean (SD) of the difference in weight of the submandibular/sublingual and parotid glands in grams. Data was analyzed using independent sample *t*-test.
*Statistically significant (*P- value <0.05).*

Table 7. Effect of Low-Level Laser on the Cellular Volume of Glandular Duct, Serous and Mucous Acini.

	Laser Group	Control Group	*P-value*
Duct Area	26350 (3563)	29839 (2966)	0.094
Serous Area	23485. (1338)	26119 (1257)	0.006*
Mucous Area	51043 (7085)	63239.63 (4961)	0.005*

Data represent mean (SD) of the cellular volume of glandular ducts, serous and mucous acini.
Data was analyzed using Independent sample *t*-test.
* Statistically significant (*P- value* <0.05).

Appendix B

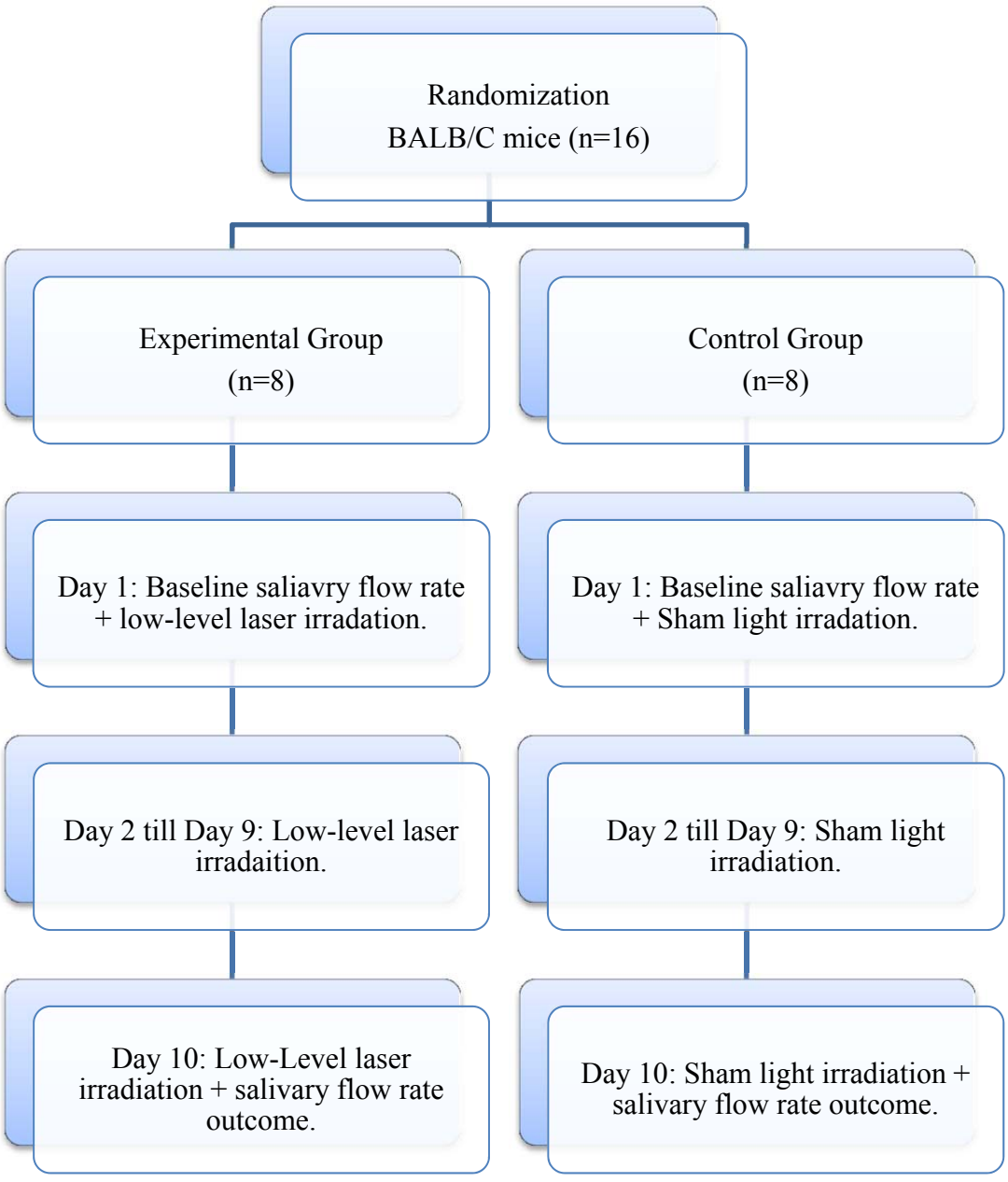


Figure 1. Flow Chart of The Study Design.

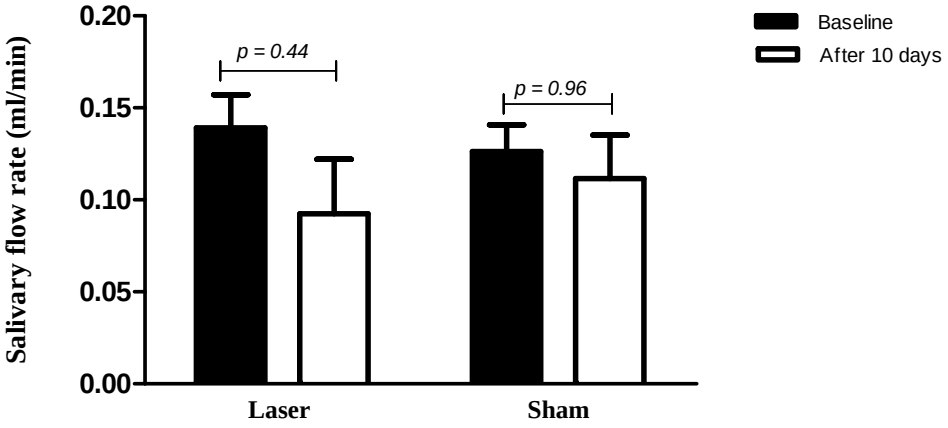


Figure 2. Effect of low-level laser irradiation on salivary flow rate (ml/min)(Intra-group comparison). Data represents means ± SD and was analyzed using paired *t*- test. The difference in salivary flow rate after 10 days of low-level laser irradiation was not statistically significant in both laser and sham groups. Laser group (N= 5) and sham group (N= 7).

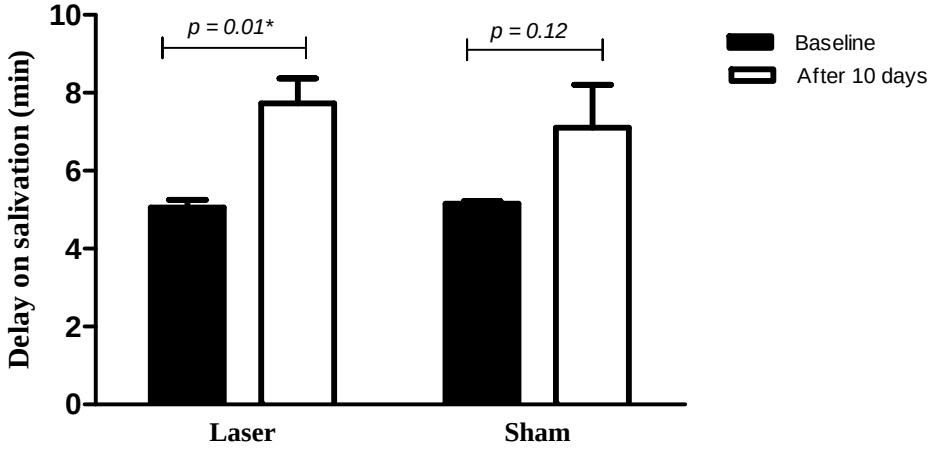
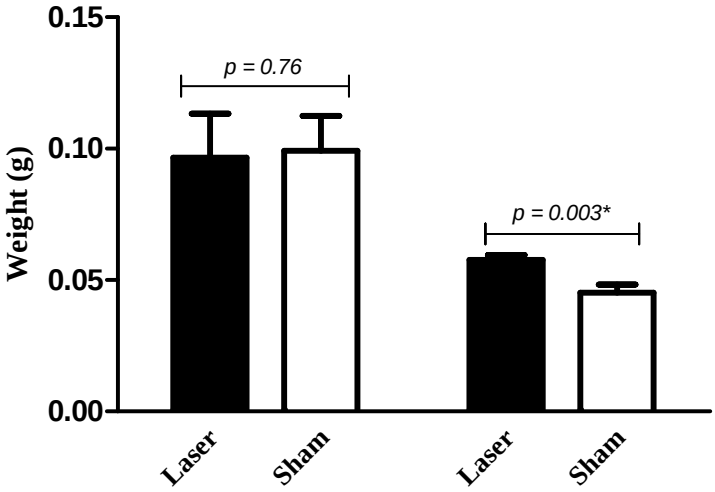


Figure 3. Effect of low-level laser irradiation on delay in salivation (min) (Intra-group comparison). Data represents means± SD and was analyzed using paried *t*- test. *Denotes a statistically significant increase in the delay on salivation of the laser group after 10 days of low-level laser irradiation. Laser group (N= 5) and sham group (N=7).



A. Submandibular glands B. Parotid glands

Figure 4. Effect of the low-level laser on glandular weight of A. Submandibular glands and B. Parotid glands in grams (Inter-group comparison). Data represents means ± SD and was analyzed using independent sample *t* -test. *Denotes a statistically significant enlargment of parotid glands of the laser group compared to the sham one. Laser group (N= 5) and sham group (N=7).

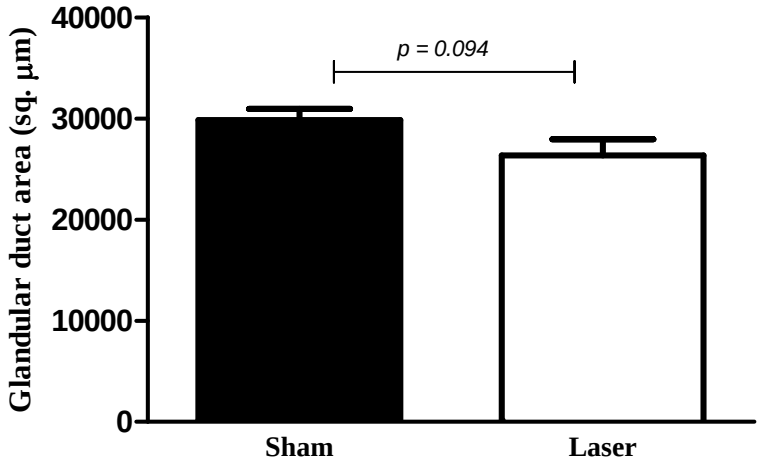


Figure 5. Effect of low-level laser irradiation on cellular voulme of glandular duct area (sq. µm) (Inter-group comparison). Data represents means ± SD and was analyzed using independent sample *t* -test. There was no statistically significant diffrence in the glandular duct area of the laser group compared to the sham one. Laser group (N= 5) and sham group (N=7).

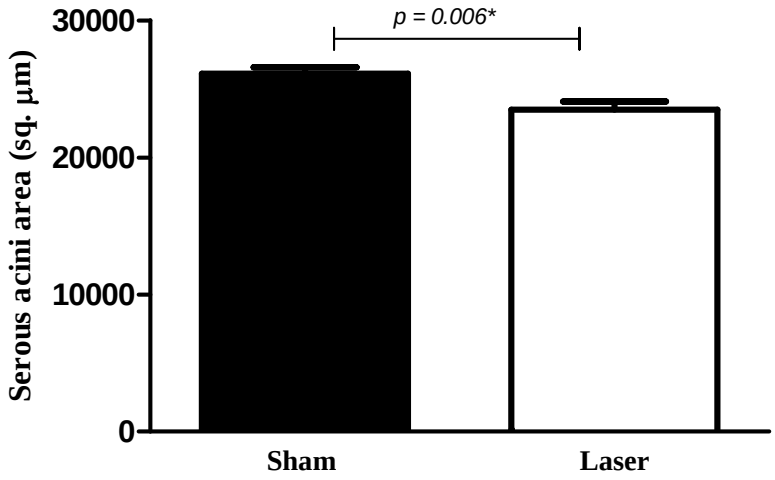


Figure 6. Effect of low-level laser irradiation on cellular voulme of serous acini area (sq. µm) (Inter-group comparison). Data represents means ± SD and was analyzed using independent sample *t* -test. * Denotes a statistically significant decrease in the serous acini area of the laser group compared to the sham one. Laser group (N= 5) and sham group (N=7).

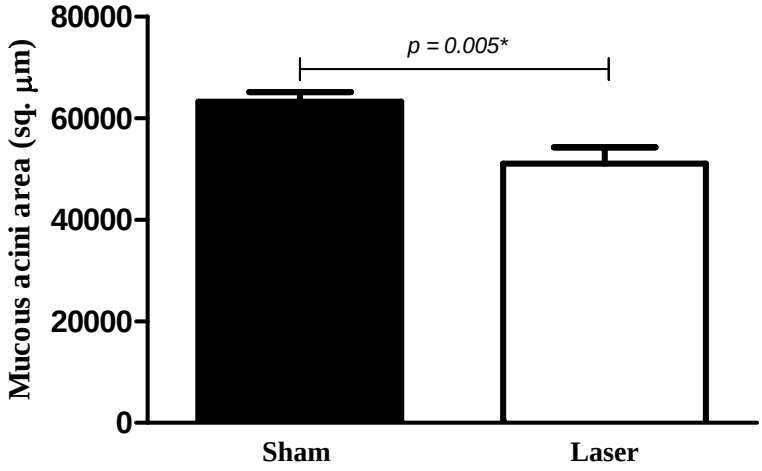


Figure 7. Effect of low-level laser irradiation on cellular voulme of mucous acini area (sq. µm) (Inter-group comparison). Data represents means ± SD and was analyzed using independent sample *t* -test. * Denotes a statistically significant decrease in the mucous acini area of the laser group compared to the sham one. Laser group (N= 5) and sham group (N=7).

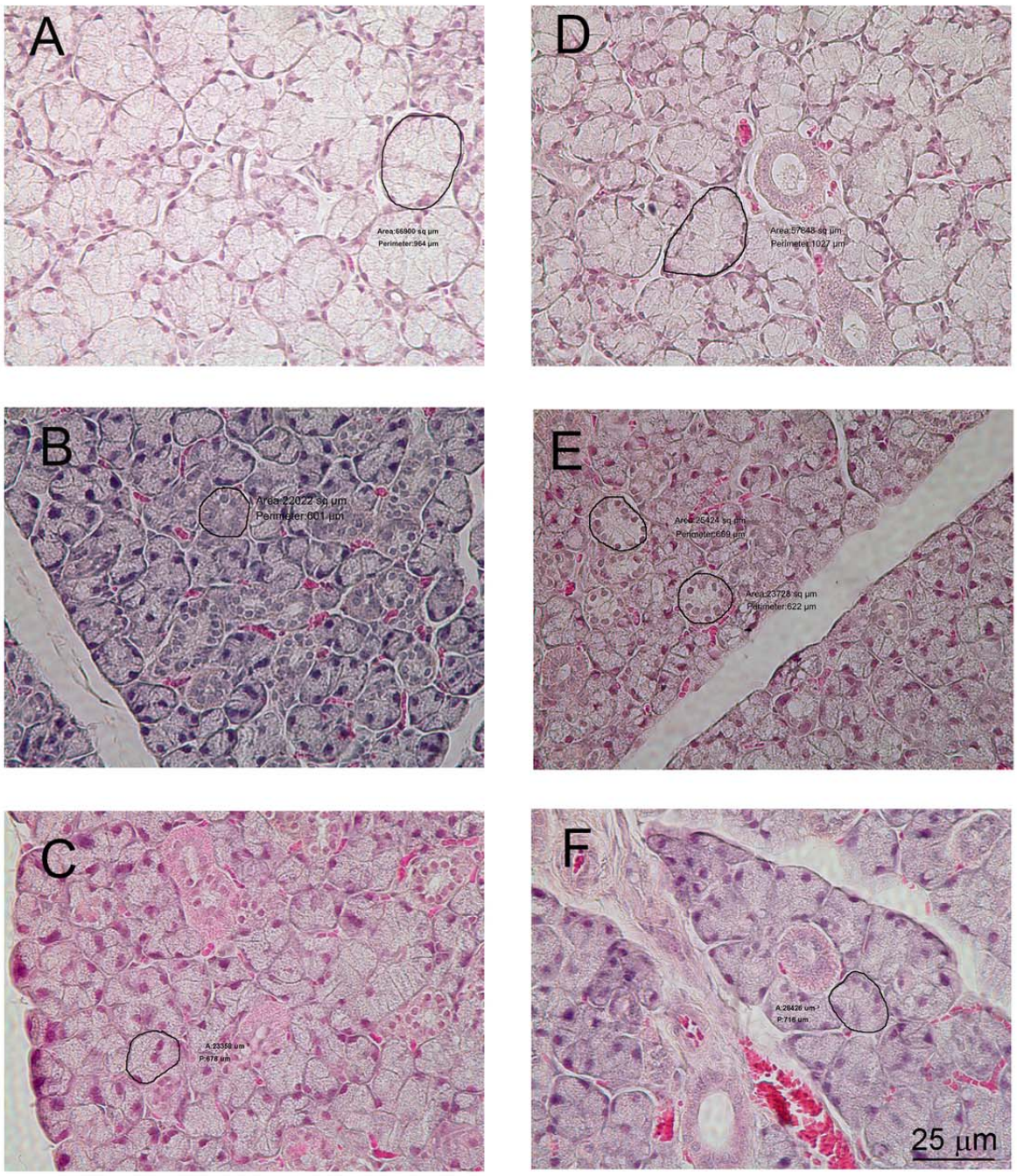


Figure 8. Light photomicrograph of H& E stained submandibular glands.
Laser group areas A. Mucous acini, B. Ducts, C. Serous acini.
Control group areas, D. Mucous acini, E. Ducts, F. Serous acini.

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