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INFLUENCE OF LOW-LEVEL LASER THERAPY ON RANKL AND OPG LEVEL IN ALVEOLAR BONE OF OSTEOPOROTIC RATS

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ABSTRACT

Introduction: Osteoporosis is a bone disorder with significant alterations in bone biologic material and consequent bone structural distraction. Low-level laser therapy (LLLT) has been studied as a physical modality that influences cellular activity through photochemical, photophysical, and photobiological mechanisms, making it an effective intervention for osteoporosis. **Aim of the study:** The current study was conducted to evaluate the impacts of low-level laser therapy on glucocorticoid induced osteoporosis at different time intervals. **Methodology:** This investigation involved 40 adult male albino rats, which were evenly distributed into four groups, each containing ten rats; control, DEX, 7 days LLLT, and 25 days LLLT. At the end of the experiment, all the rats were humanely euthanized. Their lower jaws were then collected and subjected to staining with Hematoxylin & Eosin (H&E) and immunohistochemical (IHC) analysis for RANKL and OPG. **Results:** DEX group revealed massive alveolar bone destruction with strong RANKL and weak OPG immunoreactivities. However, 7 days LLLT and 25 days LLLT groups showed improvements in the alveolar bone architecture with decreased RANKL and increased OPG immunoreactivities. **Conclusion:** Through RANKL/OPG ratio downregulation, LLLT improved bone architecture, encouraged bone healing, and stimulated osteogenesis over time.

INTRODUCTION

Osteoporosis constitutes a significant global public health issue, with projections indicating an escalation in its prevalence within the coming decade [(1)]. Concerning our community, Egypt's demographic shift, characterized by a projected population exceeding 130 million by 2050 with over 30% above 50 years old, suggests a potential surge in the prevalence of osteoporosis within the nation [(2)].

According to the National Institutes of Health Consensus Development Panel on osteoporosis, it is defined as "a skeletal disorder characterized by compromised bone strength leading to an increased risk of fracture" [(3)].

Glucocorticoids (GC) are widely used to treat various conditions, with an estimated 1-2% of the population relying on long-term therapy. Despite the fact that GC therapy has demonstrable therapeutic benefits, prolonged use has been linked to a number of serious side effects, and as a result, glucocorticoid-induced osteoporosis is currently the most prevalent secondary cause of osteoporosis [(4,5)].

Direct effects of GC on bone resorption include an increase in the number and activity of osteoclasts as well as a decrease in the production of osteoprotegerin (OPG) by osteoblastic cells and osteocytes and an increase in the production of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [6].

The most concerning consequence of osteoporosis is pathologic bone fracture, which causes excruciating pain, disability, and a decline in quality of life and productivity. Fractures cause loss of independence and an increased risk of death, placing a heavy burden on healthcare systems [7].

Fortunately, the past decade has seen significant breakthroughs in how we diagnose and treat osteoporosis [1]. Among the various physical therapy modalities, LLLT is gaining attention recently because of its easy application, short treatment time and minimal side effects [8].

Low-level laser therapy is a novel, noninvasive and affordable approach in the field of physiotherapy. It is the process of exposing a biological system to light in order to encourage tissue repair, lessen inflammation, and ease pain [9,10].

It is believed that LLLT promote osteogenesis and contribute to fracture healing, its stimulating effect on bone is related to the proliferation of osteoblasts during mesenchymal differentiation. By releasing mediators, the increased vascularization caused by LLLT promotes the synthesis of bone matrix and enhances bone healing [11].

The RANK/ RANKL/ OPG system plays a crucial role in osteoclast differentiation and function. The cytokine RANKL, which is produced by osteoblasts and bone marrow stromal cells, is crucial for inducing osteoclastogenesis. Its specific receptor RANK, which is found on the cell surface of osteoclast progenitors, transduces the cytokine's signals. Through its role in the competitive binding of RANK with RANKL, OPG prevents osteoclastogenesis [12].

Thus, RANKL and OPG regulate bone resorption by exerting a positive or negative control on the activation of RANK on osteoclasts. It has been demonstrated that in rats, the mechanical forces in conjunction with LLLT increase the expression of OPG, RANKL, and RANK [12].

The LLLT may potentially be effective in preventing and/or treating osteoporosis [13]. So, more studies are needed to fully understand how effective LLLT is for treating osteoporosis and to determine the most beneficial treatment settings [14].

MATERIALS AND METHODS

Approval of the Ethics Committee of Scientific Research, Faculty of Dentistry, Suez Canal University, had been obtained before starting the search with approval number (403/2021).

1. Materials and devices

Dexamethasone: dexamethasone phosphate 8mg/2ml, Medical Union Pharmaceuticals, Egypt.

RANKL IHC stain: rabbit primary antibody - catalog no. A13567, ABclonal®, USA.

OPG IHC stain: rabbit primary antibody - catalog no. A2100, ABclonal®, USA.

Diode laser: SL-202 portable diode laser device, Russia.

2. Study design and animals grouping

Forty adult male albino rats, weighing between 160-180 grams, were used in this experiment. The sample size was determined using G-power software (version 3.1.9.7) for sample size calculation [15].

The rats were acquired from Faculty of Veterinary Medicine, Suez Canal University. Each rat had a unique identification number, and they were housed in groups of five within a well-ventilated animal house at the Faculty of Dentistry, Suez Canal University. The rats were provided a balanced diet and water ad libitum.

Induction of osteoporosis: to induce osteoporosis, the rats were received a daily dose of dexamethasone (0.1mg/kg) for 60 days subcutaneously [16].

The rats were divided into four (4) groups as follows;

Group 1 (control group): containing 10 rats, didn't receive any drug or treatment.

Group 2 (DEX group): containing 10 rats with dexamethasone induced osteoporosis.

Group 3 (7 days LLLT): containing 10 rats with dexamethasone induced osteoporosis, then received LLLT (830nm, continuous wave, 100mW, 60J/cm^2 for 34 seconds) [17] every 48 hours for 7 days [18] directed on the lower molar area.

Group 4 (25 days LLLT): containing 10 rats with dexamethasone induced osteoporosis, then received LLLT for 25 days [18].

3. Methods of evaluation

Following euthanasia (achieved through an excessive amount of ether vapor), the rats' lower jaws were dissected, halved, and fixed in formalin for 72 hours, then decalcified in 10% EDTA with daily change of the solution for 2-3 weeks. After complete decalcification, the specimens were embedded in paraffin, sectioned (5 microns), and prepared for H&E and IHC staining for RANKL and OPG.

Immunohistochemical staining: following deparaffinization, rehydration, and HIER for optimal antigen retrieval, slides underwent IHC staining using the Mouse/Rabbit PolyVue PlusTM HRP/DAB detection system (Diagnostic BioSystems, USA). Primary antibodies against RANKL and OPG (diluted 1:100) were applied, followed by incubation with biotinylated secondary antibody and HRP-conjugated streptavidin**.** DAB chromogen was used for visualization, producing a brown precipitate at sites of antigen-antibody complex formation. Hematoxylin counterstaining provided nuclear contrast**.** Image analysis software (Leica QWin 500) quantified the area (%) of positive RANKL and OPG staining in each group.

Statistical analysis

Statistical Package for Scientific Studies (SPSS 26.0, USA) was used for the statistical evaluation. The numerical data were described as means ± standard error (S.E.) and the significance of differences between groups was assessed using a one-way ANOVA test. A P-value less than 0.05 was considered statistically significant.

RESULTS

Body weight assessment

The body weight of rats was determined during the experiment at different time points. **Initially**, there were no significant differences in body weight between groups. **Throughout the experiment**, the weight of the control group increased gradually, while the body weight of the other groups significantly decreased following dexamethasone injection compared to the control group. **At the end of the experiment**, the average body weight of the 7 days LLLT group showed a slight increase, while the 25 days LLLT group exhibited a marked increase compared to DEX group **(Table 1)**.

Table (1) *Illustrates one-way Anova test*

Group	Initial weight	60th day	67th day	85th day
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Group 1	174.19 ± 3.22	290.20 ± 2.10	304.99 ± 1.39	346.90 ± 4.75
Group 2	170.98 ± 3.27	212.27 ± 2.38	220.73 ± 1.41	228.01 ± 1.19
Group 3	170.78 ± 2.76	210.49 ± 2.67	235.66 ± 3.64	
Group 4	171.91 ± 2.40	212.21 ± 2.75		322.03 ± 1.68
F level	0.283	248.632	351.770	438.333
Significance	0.837	0.000	0.000	0.000

1. Histological results (Figure 1)

Group 1 (control group): exhibited histologic evidence of normal alveolar bone with smooth and regular alveolar margin as well as dense and well-connected bony trabeculae with narrow marrow cavities lined by a uniform osteoblastic rim. Higher magnification revealed normal Haversian systems and osteocytes within their lacunae.

Group 2 (DEX group): revealed severe alveolar bone resorption with markedly thinned, fragmented bony trabeculae enclosing enlarged marrow spaces. Higher magnification demonstrated an eroded and irregular alveolar margin with numerous multinucleated osteoclasts within their Howship's lacunae. Additionally, cellular degeneration was evident, with widened and occasionally empty osteocyte lacunae.

Group 3 (7 days LLLT): showed partial alveolar bone recovery although the alveolar margin remained somewhat eroded and irregular with osteoclastic activity. The bony trabeculae demonstrated increased thickness and enclosed narrower marrow spaces, approaching their normal size and configuration. Higher magnification revealed enhanced osteoblastic activity along the alveolar margins and a relative increase in the number of osteocytes was observed.

Group 4 (25 days LLLT): exhibited significant improvement and dramatic recovery of alveolar bone architecture. The alveolar margin showed a relatively smooth, minimally undulated, and regular configuration, indicating a decrease in osteoclastic activity. The bone trabeculae displayed increased density and thickness, arranged in a well-organized lamellar pattern, and enclosing markedly narrowed marrow cavities. Higher magnification revealed plump osteoblasts with normal morphology and osteocytes exhibiting normal size and distribution.

2. Immunohistochemical results for RANKL and OPG (Figure 2)

Group 1 (control group): the bone cells exhibited weak immunoreactivity for RANKL, indicated by light color intensity. Conversely, the bone cells, primarily osteoblasts, displayed strong immunoreactivity for OPG, evident by intense color staining. This signified a normal rate of bone turnover, likely due to the balanced activity of RANKL and OPG.

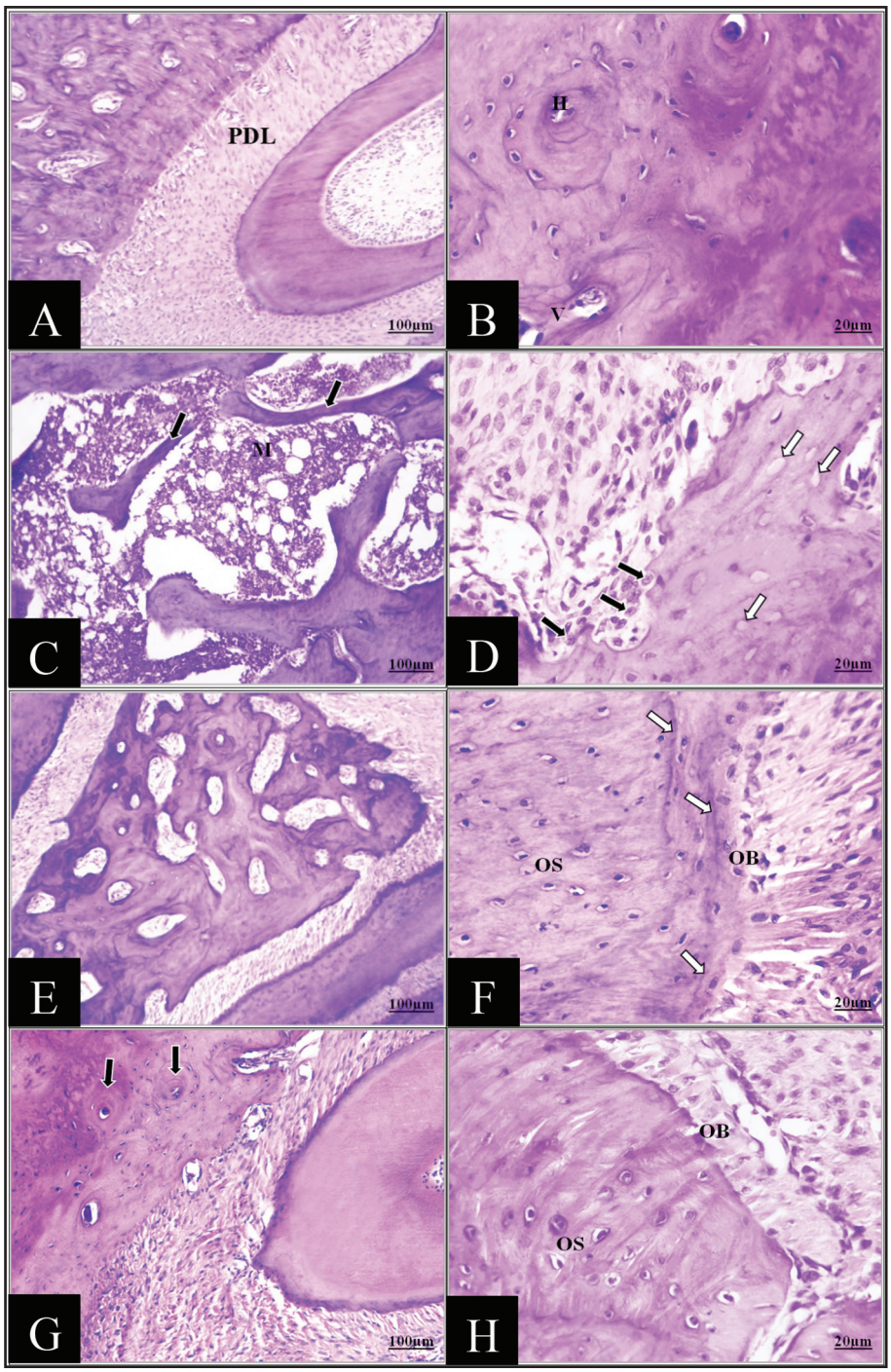

Fig. (1) Photomicrographs of the alveolar bone. **A:** Control group showing alveolar bone with smooth, regular alveolar margin and well-organized PDL fibers (PDL). **B:** Control group showing Haversian system with its Haversian canal (H), concentric bone lamellae and Volkmann's canal (V). **C:** DEX group showing widening in the marrow cavities (M) associated with fatty tissue infiltration and the presence of thin bony trabeculae was also observed (arrows). **D:** DEX group showing cellular degeneration with some osteocytes' lacunae appeared widened and others were empty (white arrows) in addition to osteoclasts with multiple resorption pits were highly detected (black arrows). **E:** 7 days LLLT group showing partial improvement of the alveolar bone architecture with degeneration in some areas of the alveolar bone was still detected. **F:** 7 days LLLT group showing plump osteoblasts lining the alveolar margin (OB) with a relative increase in the number of normal osteocytes (OS) and the presence of resting lines (arrows). **G:** 25 days LLLT group showing newly formed bone with smooth margin and could be detected from old bone being formed in concentric lamellae of Haversian systems (arrows). **H:** 25 days LLLT group showing normal osteoblastic activity (OB) and normal distribution of osteocytes (OS). (H&E, orig. mag. 100, 400).

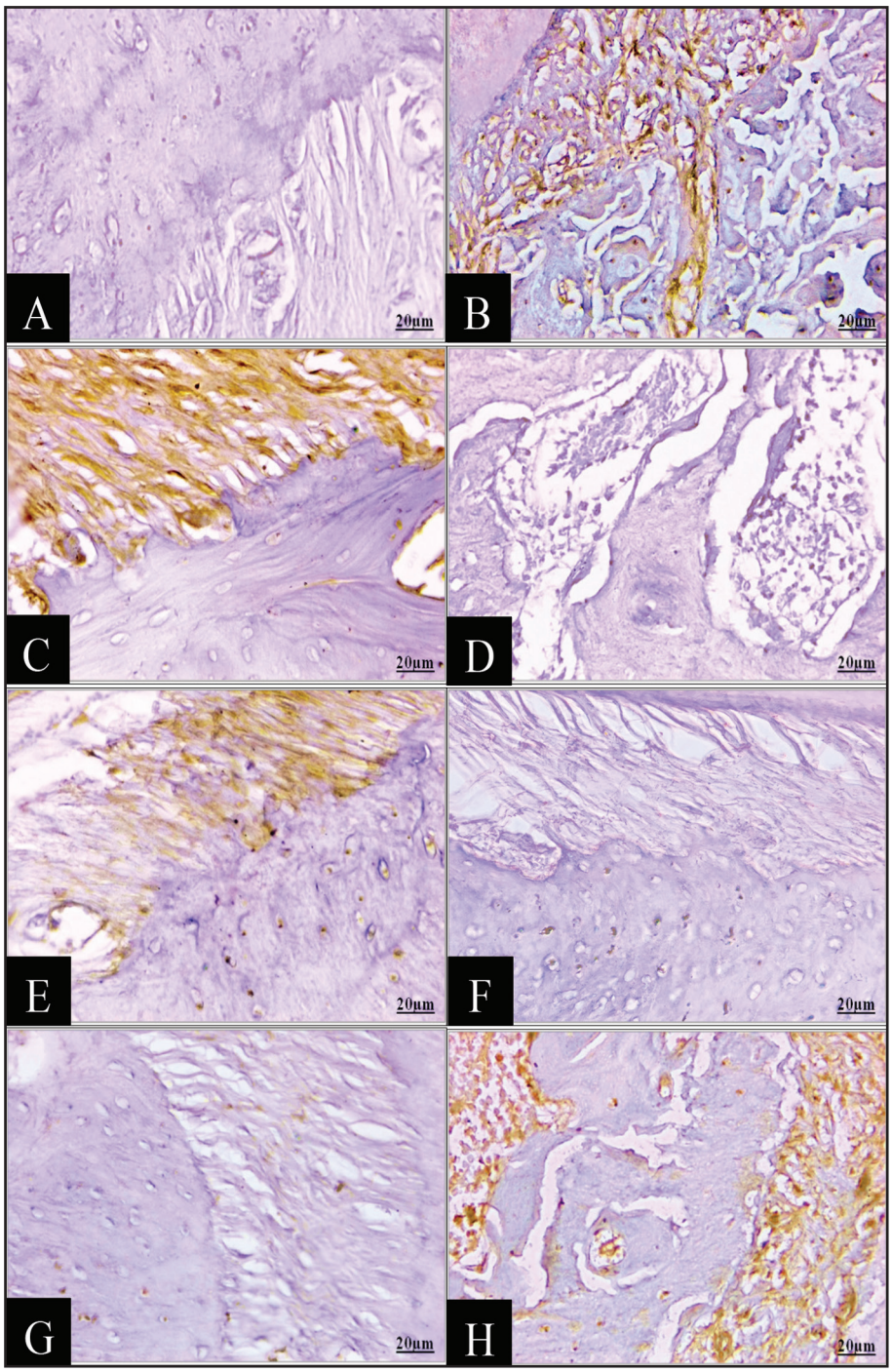

Fig. (2) Photomicrographs of the IHC evaluation expressed by alveolar bone cells. **A:** Control group showing weak positive staining immunoreactivity of RANKL. **B:** Control group showing markedly strong positive staining immunoreactivity of OPG. **C:** DEX group showing markedly strong positive staining immunoreactivity of RANKL. **D:** DEX group showing weak positive staining immunoreactivity of OPG. **E:** 7 days LLLT group showing mild decrease in the positive expression of RANKL with moderate staining immunoreactivity compared to DEX group. **F:** 7 days LLLT group showing mild increase in the positive expression of OPG with weak to moderate staining immunoreactivity compared to DEX group. **G:** 25 days LLLT group showing marked decrease in the positive expression of RANKL with weak staining immunoreactivity of the bone cells. **H:** 25 days LLLT group showing marked increase in the positive expression of OPG with strong staining immunoreactivity of the bone cells compared to DEX group. (H&E, orig. mag. 400).

Group 2 (DEX group): displayed a significant imbalance in the RANKL/OPG system compared to the control group. The bone cells exhibited very strong immunoreactivity for RANKL, evident by intense color staining, indicating an abnormally high rate of bone resorption. Conversely, OPG immunoreactivity was markedly weak, with light color intensity, suggesting a significantly reduced rate of bone deposition.

Group 3 (7 days LLLT group): compared to DEX group, this group exhibited a shift towards a more balanced bone remodeling environment. The bone cells displayed moderate immunoreactivity for RANKL, with a decrease in color intensity, suggesting a reduction in the rate of bone resorption. Additionally, OPG expression increased, with a moderate color intensity, indicating a slight improvement in the rate of bone deposition.

Group 4 (25 days LLLT group): demonstrated a more pronounced effect on the RANKL/OPG system compared to DEX group and 7 days LLLT group. The bone cells exhibited markedly decreased immunoreactivity for RANKL, with light color intensity, suggesting a significant reduction in bone resorption. Conversely, OPG expression was markedly increased, with strong color intensity, indicating a substantial improvement in the rate of bone deposition. These findings suggested that a longer course of LLLT might be even more effective in promoting a balanced bone remodeling environment.

Table (2) *illustrates one-way Anova test for RANKL and OPG*

Group	RANKL	OPG
	Mean ± SE	Mean ± SE
Group 1	3.59 ± 0.43	35.90 ± 1.02
Group 2	29.86 ± 0.64	5.94 ± 0.84
Group 3	15.95 ± 0.78	20.07 ± 1.07
Group 4	8.97 ± 0.83	30.17 ± 0.74
F level	270.505	198.774
Significance	0.000	0.000

Statistical analysis for RANKL and OPG immunohistochemical results:

DISCUSSION

Osteoporosis is regarded as a worldwide bone disease that diminishes the quantity and quality of mandibular and maxillary bones in addition to affecting weight-bearing bones. Furthermore, both clinical dentistry and basic research are focused on osteoporosis due to its effects on jaw bones and oral health in general [19].

This study attempted to evaluate the impacts of LLLT on the treatment of glucocorticoid induced osteoporosis at different time intervals through histological and RANKL/OPG immunohistochemical assessments.

Despite the fact that histology in bone is known to be highly difficult, we selected these approaches because they enable the visual assessment of the regenerated structure. They also make it possible to quantify the levels of expression of the immunohistochemical antibodies under investigation and to ascertain their distribution pattern via image analysis. The significance of these methods was highlighted by ***Matos et al.*** [20]

and ***Duraiyan et al.*** [21] who critically reviewed the applications and importance of histology and immunohistochemistry.

The laboratory albino rat was chosen as an osteoporotic model for this study because it is readily available, reasonably priced, and easy to handle, additionally the pathophysiologic responses of the human and rat skeletons are similar which was supported by ***Soussaa et al.*** [19]. It is the most commonly used animal model for glucocorticoid-induced osteoporosis, with mice coming in second[22].

In this study, male albino rats were used to induce osteoporosis in order to prevent hormonal changes in female rats, as suggested by ***Ibrahim and Abdow***[23], and to exclude the estrogen effect, which is crucial for preserving normal bone turnover [24].

One of the most catastrophic side effects of GC, and the main cause of secondary osteoporosis, is glucocorticoid-induced osteoporosis. It is characterized, like the other kinds of osteoporosis, by a decrease in bone mass and a breakdown in the microarchitecture of the bone tissue, leading to increased bone fragility [25].

There are several pathophysiologic mechanisms underlying glucocorticoid-induced osteoporosis, which lead to a decrease in bone formation and an increase in bone resorption[26]. The principal mechanism of GC-induced bone loss is thought to be the damage GC causes to the viability and function of osteoblasts[27]. Accumulating evidence suggests that the pathogenesis of glucocorticoid-induced osteoporosis could be the upregulation of M-CSF and RANKL, which leads to increased osteoclastogenesis, and the inhibition of osteogenesis-related signaling pathways, which results in decreased osteoblastogenesis and downregulation of OPG [28].

In the present study, the rats received dexamethasone subcutaneously at 0.1mg/kg for 60 days to induce osteoporosis coincided with that found by ***Hozayen et al.*** [16] to simulate long-term human GC administration. Dexamethasone was used to cause osteoporosis as it was noted to be the most effective osteoporosis inducer in animal models, among other corticosteroids. Moreover, it is characterized by its long-term action [29,30].

In the present research, the osteoporotic rats were exposed to LLLT sessions (830nm, continuous wave, 100mW, 60J/cm^2 for 34 seconds) as a therapeutic dose which was reported by ***Bossini et al.*** [17] on the lower molar area every 48 hours for 7 days for the 7 days LLLT group and for 25 days for the 25 days LLLT group which was also reported by ***Fávaro–Pípi et al.*** [18].

Bossini et al. [17] observed that laser dosage that was utilized demonstrated effectiveness in promoting bone healing, indicating that bone fractures could be treated with this dosage, especially in cases of osteoporosis by promoting formation of new bone. Moreover, ***Fávaro–Pípi et al.*** [18] demonstrated favorable effects of LLLT on bone repair at early (7 days), intermediate (13 days), and late (25 days) instances following bone injury, as shown by histopathological and gene expression analysis.

The rats body weight assessment during the experiment at different time points showed that, throughout the experiment, the weight of the control group rats increased gradually, while the rats body weight of the other groups significantly decreased following dexamethasone injection. In agreement with ***Zhang et al.*** [31] who reported that after five weeks of the experiment, animals in the DEX group noted significantly less weight gain than those in the control group. These findings demonstrated that dexamethasone decreased bone mineral density and prevented weight gain.

At the end of the experiment, the average body weight of the 7 days LLLT group showed a slightly increase, while the 25 days LLLT group exhibited a marked increase compared to the DEX group. In accordance with ***Bayat et al.*** [32] who showed that the rats' body weight significantly decreased after receiving dexamethasone. Furthermore, the majority of the weight loss caused by dexamethasone in rats ceased when laser treatment was administered. Thus, LLLT acted as an anabolic agent on bones.

In the present research, H&E stain was used as it has been always considered the gold standard in the histological evaluation of different tissues[24]. The histological results of the control group showed normal architecture of the alveolar bone. In agreement with ***Nabil et al.*** [33] who reported that, H&E examination of alveolar bone in the control group announced alveolar bone proper with a regular shape and a normal distribution of osteoblasts and osteocytes.

According to the current study results, bone resorption was the most observed effect in the DEX group with markedly thinned bony trabeculae enclosing enlarged marrow spaces. In addition to numerous multinucleated osteoclasts within their Howship's lacunae. ***Sherif et al.***[22] reported that the GC group alveolar bone in rats exhibited conspicuous bone resorption and a decrease in the thickness of bony trabeculae, featuring a relatively irregular margin outline filled with multiple osteoclasts in Howship's lacunae.

Yao et al. [34] discovered that the administration of GC caused the osteocytes' lacunae to enlarge, while also demineralizing and decreasing the elasticity of the matrix encircling the lacunae. Additionally, it was discovered that these transformed osteocytes could generate proteins that inhibit osteoblast differentiation and matrix mineralization.

The histological examination of the 7 days LLLT group showed partial alveolar bone recovery although the alveolar margin remained somewhat eroded and irregular with osteoclastic activity. ***Matsumoto et al.*** [35] reported that at the 7th day of LLLT, the rats' tibia's bony walls showed signs of new bone formation. It was also noted by ***Pires-Oliveira et al.*** [36] that the immature and haphazardly growing new bone trabeculae in the osteoporotic group treated with LLLT for 7 days indicated that laser had an inductive effect on bone repair in the tibias of rats.

Pires-Oliveira et al. [36] documented that the osteoblast cell line was stimulated by laser irradiation to proliferate and differentiate, which increased the number of osteoblasts and the production of bone. Because of the early onset of the inflammatory response and increased vascularization, LLLT may have accelerated bone repair by promoting the synthesis of bone matrix.

In the present study, the 25 days LLLT group exhibited significant improvement of the alveolar bone architecture with relatively smooth, and regular configuration of the margin indicating a decrease in the osteoclastic activity. Consistent with the current study findings, ***Hamza et al.*** [2] demonstrated that, following 4 weeks of treatment, the mandibles of the laser-irradiated group showed narrower marrow spaces and thicker bone trabeculae than the osteoporotic group. These results were also supported by ***Fávaro–Pípi et al.*** [18] who noted that tibias exposed to laser radiation on day 25 of the therapy showed a significant amount of bone deposition.

According to this study, the long-term application of LLLT may have more favorable effects against the osteoporotic alveolar bone of rats than the short-term application. This was supported by ***Barbosa et al.*** [37] who revealed that LLLT had a time- and

wavelength-dependent beneficial biomodulatory effect on the healing process of bone.

The histological observations from the H&E-stained sections could be explained in the light of the IHC results. The control group showed weak immunoreactivity for RANKL and strong immunoreactivity for OPG. This was confirmed by ***Wu et al.*** [38] who observed that in the control group, osteoblasts expressed weak RANKL and evident OPG immunoreactivities.

As evidenced by a decline in the RANKL/OPG ratio, ***Hendrijantini et al.*** [39] reported that bone formation may be linked to either decreased RANKL expression and/or increased OPG expression. On the other hand, elevated RANKL expression and/or decreased OPG expression may cause pathological bone resorption, which is indicated by an increase in the RANKL/OPG ratio, as in osteoporosis.

The IHC results of the DEX group displayed a significant imbalance in the RANKL/OPG system, by which the bone cells exhibited very strong immunoreactivity for RANKL and markedly weak immunoreactivity for OPG. This was corroborated by ***Sousa et al.*** [40], who observed that in comparison to the control group, the GC group had strong RANKL immunostaining and decreased OPG immunostaining. Due to the persistence of inflammatory cytokines, it was proposed that using GC concurrently with inflammatory disorders could stimulate the resorptive process by upregulating RANKL.

The IHC results of the 7 days LLLT group exhibited a moderate immunoreactivity for RANKL while OPG expression was increased compared with the DEX group. Moreover, the 25 days LLLT group demonstrated a more pronounced effect on the RANKL/OPG system as the bone cells exhibited markedly decreased immunoreactivity for RANKL and OPG expression was markedly increased.

Bayer-Alinca et al. [41] documented that when comparing the LLLT group to the positive untreated group, there were more OPG-stained cells but fewer RANKL-stained cells. These findings implied that LLLT promotes bone formation and promotes bone healing by decreasing bone resorption.

Overall, the histological and immunohistochemical findings of this investigation demonstrated that the application of LLLT significantly improves the healing of the alveolar bone of osteoporotic rats, with the longer therapy duration having more advantageous effects. However, research on the mechanism of action of laser irradiation in organisms is still ongoing, and the specific functions of laser irradiation in bone remodeling remain unclear.

CONCLUSION

Low-level laser therapy can possibly be used as a good alternative local treatment strategy with minimal side effects and superior outcomes in the case of osteoporosis, as it can improve bone strength by faster bone deposition. Through RANKL/OPG ratio downregulation, LLLT improved bone architecture, encouraged bone healing, and stimulated osteogenesis over time.

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