

Evaluation of the effects of LLLT on biomechanical properties of tibial diaphysis in two rat models of experimental osteoporosis by a three point bending test

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Abstract Osteoporosis (OP) is a disease which causes bone loss and fractures, leading to severe pain and deformity. This study has aimed to assess the effects of pulsed wave low-level laser therapy (PW LLLT) on cortical bone in two experimental models of OP in rats. There were four ovariectomized (OVX-d) groups and four dexamethasone-treated groups. The healthy group were considered for baseline evaluations. At 14 weeks following ovariectomy, the OVX-d rats were further subdivided into the following: control rats with OP, OVX-d rats that received alendronate (1 mg/kg), OVX-d rats treated with LLLT, and OVX-d rats treated with alendronate and LLLT. The remaining rats received dexamethasone for 5 weeks and were divided into four groups: control, alendronate-treated rats (1 mg/kg), laser-treated rats, and

laser-treated rats with concomitant administration of alendronate. The rats received alendronate for 30 days. LLLT (890 nm, 80 Hz, 0.972 J/cm²) was performed on the tibias three times per week for 8 weeks. After 8 weeks, tibias were extracted and submitted to a three-point bending test. PW LLLT did not increase the biomechanical parameters of osteoporotic bones compared to controls and healthy rats. PW LLLT associated with alendronate treatment significantly increased stress high load in OVX-d rats compared to the healthy group. PW LLLT at the current study parameters failed to cause beneficial biomechanical effects in the examined osteoporotic cortical bones. PW LLLT associated with alendronate treatment produced a more remarkable effect on bone strength in the ovariectomized induced OP rat model.

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Introduction

Osteoporosis (OP) is a disease which causes bone loss and fractures and leads to severe pain, deformity, and in certain cases secondary complications that result in death [1]. In the USA, approximately 10 million adults older than 50 years are estimated to have OP, and another 34 million are at risk for this disease [2]. OP, in particular hip fractures, has a large economic impact; the direct costs of osteoporotic fractures in the USA in 2005 have been estimated at \$19 billion [3]. The disease is classified clinically as either primary or secondary OP. Primary OP refers to bone loss that occurs both in postmenopausal women (type I) or is attributed to the normal aging process (type II). Secondary OP refers to bone loss that ensues as a secondary effect of other diseases or drug treatment. Postmenopausal OP (type I) is the most common form of this disease and is believed to initiate with deficiencies in estrogen production that follow menopause [4]. During estrogen deficiency, osteoclasts remove excess bone without adequate formation by osteoblasts. Bone loss ensues when the basic trabecular bone units (trabeculae) become thin and resorb completely or fracture [5]; ultimately, bone fractures occur under minimal trauma in the bones of the hips, wrists, and spine [5]. Although glucocorticoids (GCs) effectively suppress inflammation, their prolonged use is accompanied by bone loss that leads to OP. GC-induced OP (GIOP) is the most common cause of secondary OP [6].

Various animal models have been established and used to investigate the pathogenesis of OP as well as to facilitate pre-clinical testing and new treatment options such as antiresorptive drugs [7]. Histomorphometric parameters and biochemical markers of bone metabolism in animal studies only indicate a decrease in bone formation and minimal changes in bone resorption. These parameters are less important with regards to OP-associated fractures and investigations in orthopedic surgery. Furthermore, histological studies do not give direct information about the mechanical strength of the bone. The ultimate reason for a bone fracture following minimal trauma is a reduction in mechanical strength [8]. On the other hand, bone densitometry is often used as a surrogate to evaluate bone fragility, but direct biomechanical testing of the bone undoubtedly provides more information about mechanical integrity [9].

There is an increasing need for strategies that prevent bone loss. Numerous treatments are planned and include estrogen therapy, bisphosphonate compounds, and physical activity programs [10]. Several researchers have determined that continuous wave (CW) low-level laser

therapy (LLLT) stimulates *in vitro* mineralization through increased IGF-I and BMP production, Runx2 expression, and ERK phosphorylation [11]. CW LLLT has been shown to stimulate bone nodule formation [12] in osteoblasts. Others reported that LLLT promoted the acceleration of bone strength and consolidation after a fracture, created new blood vessels, increased collagen fiber deposition, and promoted greater bone cell proliferation at the fracture site [13, 14]. Pinheiro et al. reported that LLLT resulted in increased mineralized bone tissue in the fractured femora [15]. Bossini et al. concluded that LLLT improved bone repair in the tibia of osteoporotic rats by stimulation of newly formed bone, fibrovascularization, and angiogenesis [16].

Studies of the influence of CW low-level lasers on ovariectomized-induced OP (OVX-d) in cortical bone have been few and with inconsistent results [17–21]. Ko et al. [17], Ko et al. [18, 19], and Renno et al. [20] reported that LLLT of osteoporotic bones produced beneficial effects on the prevention of bone loss. However, Medalha et al. [21], in a recent study, indicated that treatment of femurs and tibias of spinal cord injured rats with an 830-nm laser showed a trend toward significantly higher values of internal and external areas of tibial diaphysis. No increase was found in either mechanical or densitometric parameters. Consistently, Renno et al. reported that LLLT did not improve the stimulatory effects of the exercise on the osteopenic rats. [22].

The application of frequency is growing. A literature review produced three cellular studies on rat calvarial cells [23–25], one *in vivo* study on the tooth movement speed of rat molars [26], a study on bone turnover in ovariectomized rats [27], and a study on healing of a partial osteotomy of the tibia in streptozotocin-induced diabetic rats [28].

The aim of this study was to assess the effects of PW LLLT on cortical bone (tibia) strength of the following: (1) normal, healthy rats; (2) an OVX-d experimental model in rats; and (3) a GIOP experimental model in rats. Bone strength was evaluated by measuring the biomechanical properties of tibial diaphysis, which included bending stiffness (Young's modulus of elasticity), maximum force, and stress high load using a three-point bending test instrument.

Materials and methods

Experimental animals

A total of 66 adult male and female Wistar rats, aged 4.5 months were housed in standard rat cages on a 12-h light/dark schedule and provided water *ad libitum*. All procedures were approved by the Medical Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (protocol no 1392-1-115-1160). The rats body weights were

monitored weekly, and the volume of drugs administrated was calculated according to the most recent body weight.

Experimental protocols

In this study, normal, OVX-d, and GIOP rats received LLLT and alendronate, after which they were subjected to a three-point bending test.

Normal rats

There were 12 male rats divided equally into control and experimental groups. The right tibias of the experimental (laser-treated) rats were exposed to a pulsed infrared diode laser [MUSTANG 2000+ with LO7 pen (radiating head), Technica Co., Moscow, Russia] with the specifications shown in Table 1. During laser irradiation, irradiated animals were sedated by ketamine (25 mg/kg) and diazepam (2.5 mg/kg) anesthesia. Control rats received a placebo PW LLLT that was switched off. In the current study, the surface area of the bone was larger than the pen's spot size; therefore, we used sequential treatments (four shootings of the laser) to ensure that all areas of the bone received similar laser doses [29].

Ovariectomized-induced osteoporosis (OVX-d) and glucocorticoid-induced osteoporosis (GIOP) groups

The remaining 54 rats were randomly divided into 9 groups of 6 rats each. The groups consisted of the following: four OVX-d groups, four groups that received dexamethasone, and one healthy group considered for baseline studies (group 7). Ovariectomy was performed by two paravertebral skin incisions while rats were anesthetized with ketamine (50 mg/kg) and diazepam (5 mg/kg) anesthesia. The uterine tubes were ligated (catgut 4.0), and following removal of the ovaries, we closed the incisions (nylon 3.0). Ceftriaxone (Jaber ben Hayan, Tehran, Iran) at a dose of 50 mg/kg was

administrated immediately before and 24 and 48 h after surgery. All animals were kept for 14 weeks after surgery in cages in order to develop OP [30]. At the end of this period, the rats were submitted to different treatments as follows: group 3 (OC) comprised control rats with OP; group 4 (OA) were OVX-d rats treated subcutaneously with 1 mg/kg alendronate (Alborz Darou, Tehran, Iran); group 5 (OL) consisted of OVX-d rats treated with LLLT three times weekly (Table 1); and group 6 (OAL) were OVX-d rats treated with LLLT and concomitant administration of alendronate.

The remaining rats received intramuscular injections of dexamethasone (Alborz Darou, Tehran, Iran) at a daily dose of 1 mg/kg, 6 days per week for 5 weeks [31], with some modifications, i.e., 6 days per week instead of 7 days per week, and 5 weeks instead of 4 weeks. After 5 weeks, dexamethasone-treated rats were divided into four groups: group 8 (control) was OP rats treated with intramuscular injections of vehicle (distilled water; DC); group 9 (DA) or GIOP rats were treated with subcutaneous injections of 1 mg/kg alendronate (Alborz Darou, Tehran, Iran) [32]; group 10 (DL) was GIOP rats treated with PW LLLT (Table 1) three times per week; and group 11 (DAL) consisted of GIOP rats treated with LLLT and concomitant administration of 1 mg/kg/day alendronate (Table 2). In the laser-treated rats, both tibias (four points each) were irradiated with the laser pen held perpendicular to the bone from a distance below 1 cm. LLLT was administered for a period of 8 weeks, and alendronate injections were given for 30 days.

General examinations

At 8 weeks after the beginning of the treatments, we killed the rats with an overdose of anesthesia. Blood glucose concentrations were measured using distal tail vein blood samples (GM 300, Biomince, GMH, Heerbrugg, Switzerland). The right tibias were extracted and left tibias frozen for further examinations. The length of the tibia (mm) was measured using a sliding caliper, and the weight of the tibia was measured in grams.

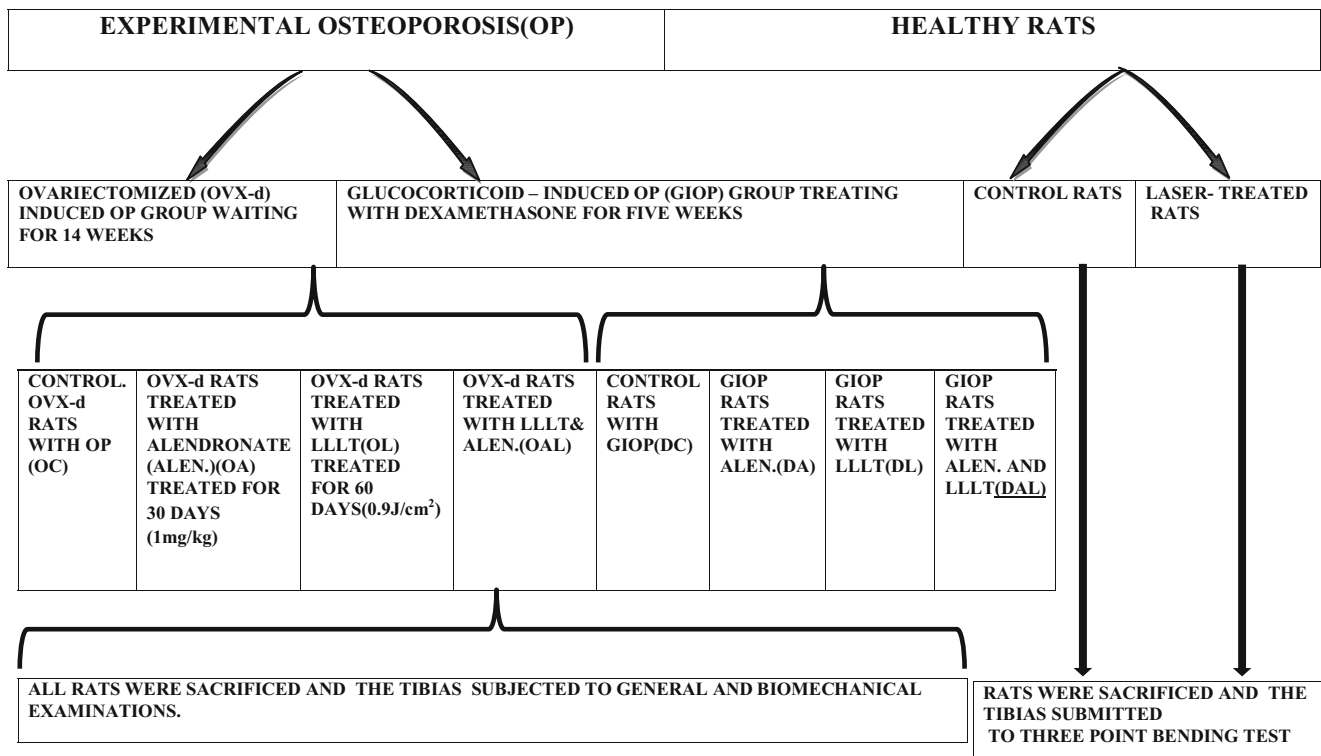
Biomechanical examinations

The bones were subjected to three-point bending on a material testing device (Zwick/Roell, Germany) until fracture, defined as separation of the bone into two pieces occurred. All the bones were oriented similarly in the testing machine, and we calculated each bone's surface area. Two loading points, 19 mm apart, were used to mount each bone, and a press head was subsequently activated to compress the midline of the bone shaft until a fracture occurred. The compressive loading speed was 0.08 mm/s for all tests. Specimens were

Table 1 Specifications of the laser used

Parameters	Dose and unit
Peak power output	75 W
Average power	1.08 mW
Power density	1.08 m W/cm ²
Wavelength	890 nm
Pulse frequency	80 Hz
Spot size	1 cm ²
Pulsed duration	180 μs
Duration of exposure for each point	900 s
Energy density	0.972 J/cm ²

Table 2 Distribution of rats in the studied groups



loaded uniaxially so that the fracture and complete load-deformation curve could be recorded by transducers coupled to bridges and sampled in a personal computer by an analog-to-digital convertor (PC-software 27005). Load characteristics were directly plotted on an x-y chart recorder. From the load-deformation curve, the following biomechanical properties were automatically calculated: bending stiffness (N/mm), maximum force (N), and stress high load (N/mm²). We defined these biomechanical parameters as follows. Bending stiffness is the slope of the linear portion of load-deformation curve (i.e., the ratio of loading to deformation in the elastic region of the curve). Maximum force is the force needed to break the bone microscopically. The stress high load was calculated by dividing the maximum force value by surface area (mm²) of the bone at the site of the fracture [33].

Statistical analysis

All data were expressed as mean±standard errors of mean (SEM).

Normal distribution of data was analyzed using the one-sample Kolmogorov-Smirnov test. Parametric and nonparametric statistical methods were used. Significance of differences between the normal control and laser-treated normal groups was assessed using the Student’s *t* test. The analysis of variance (ANOVA) test was used to compare changes among groups with normal distribution of data and LSD’s test to identify differences. A *p* value of ≤0.05 was considered statically significant. Nonparametric methods were used for statistical analysis of other groups. These data were analyzed using the Kruskal-Wallis and Mann-Whitney *U* tests. Differences were regarded as significant if *p*<0.005 for analyses between groups 3–11. The differences were also regarded as

Table 3 Mean ± SEM of blood glucose (ml/dc) of studied groups compared by ANOVA test

Groups	OC	OA	OL	OAL	DC	DA	DL	DAL
Blood glucose	114±3.06	115±2.48	114.5±3.03	111.2±8	115.7±2.56	116.3±2.14	113.4±4	112.5±2.7

Ovariectomized (OVX-d) control rats (OC), OVX-d rats treated with alendronate (OA), OVX-d rats treated with LLLT (OL), OVX-d rats treated with LLLT and alendronate (OAL), healthy rats (H), dexamethasone-treated control rats (DC), dexamethasone-treated rats received alendronate (DA), dexamethasone-treated rats received LLLT (DL), dexamethasone-treated received alendronate and LLLT (DAL). There was no any significance difference between the studied groups

Table 4 Mean \pm SEM of initial body weight and last body weight of all groups

Groups→ Weights↓	OC	OA	OL	OAL	DC	DA	DL	DAL
Initial	232.2 \pm 4.3	237.4 \pm 4.5	241 \pm 4.1	238.6 \pm 4.7	245.7 \pm 6.9	233 \pm 4.2	247 \pm 4.2	235.7 \pm 5.9
Last	233 \pm 3.5	248.4 \pm 3.3**	234 \pm 4.6	233.3 \pm 3.6	221.8 \pm 5.8***	229.7 \pm 6.7	239.8 \pm 2.8*	231 \pm 3.9

Student's *t* test showed significant differences between initial and last body weight of OC, DC, and DL groups

* <0.05 , ** <0.01 , *** <0.001

significant if $p \leq 0.01$ for analyses between groups 3–7 and analyses between groups 7–11, respectively.

Results

Normal rats

The independent sample *t* test showed a significantly lower maximum force in the control group (25 ± 1.3) compared to the experimental group (37.3 ± 3.6 ; $p=0.01$).

Ovariectomized-induced osteoporosis (OVX-d) and glucocorticoid-induced osteoporosis (GIOP) rats

General observations

Blood glucose All blood glucose values were less than 120 mg/dc. According to the ANOVA test, there were no significant differences in blood glucose values between the studied groups (Table 3).

Body weight As seen in Table 4, the use of dexamethasone led to a significant decrease in body weight ($p < 0.001$). Although rats from all groups that received dexamethasone lost body weight, the weight loss was more severe in rats from groups DC and DL. In all groups, five rats died during dexamethasone administration, and three rats died during alendronate treatment and LLLT. In the OVX-d groups, four rats died during the study. The dead rats were replaced. The OA group showed a significant increase in body weight ($p < 0.01$).

Tibia length According to the LSD test, the tibial lengths of the OA ($p=0.011$), OL ($p=0.009$), OAL ($p=0.012$), DC ($p=0.023$), DA ($p=0.002$), DL ($p=0.001$), and DAL ($p=0.015$) groups significantly increased compared to the healthy group. DC group showed a significant increase in tibial length compared to the DA (LSD test, $p=0.041$) and OL ($p=0.004$) groups. There were significantly greater tibial length values in the DL group compared to the DA ($p=0.23$), OC ($p=0.039$), OAL ($p=0.000$), and OL ($p=0.003$) groups (LSD test; Fig. 1). Laser treatment administered to dexamethasone-treated rats had a positive effect on tibial length.

Fig. 1 Mean \pm SEM of tibial length (mm) of studied groups compared by LSD test; * <0.05 , ** <0.01 , *** <0.001 . The DC group showed a significant increase in tibial length compared to the DA and OL groups. Tibial length values of the DL group were significantly higher than the DA, OC, OAL, and OL groups

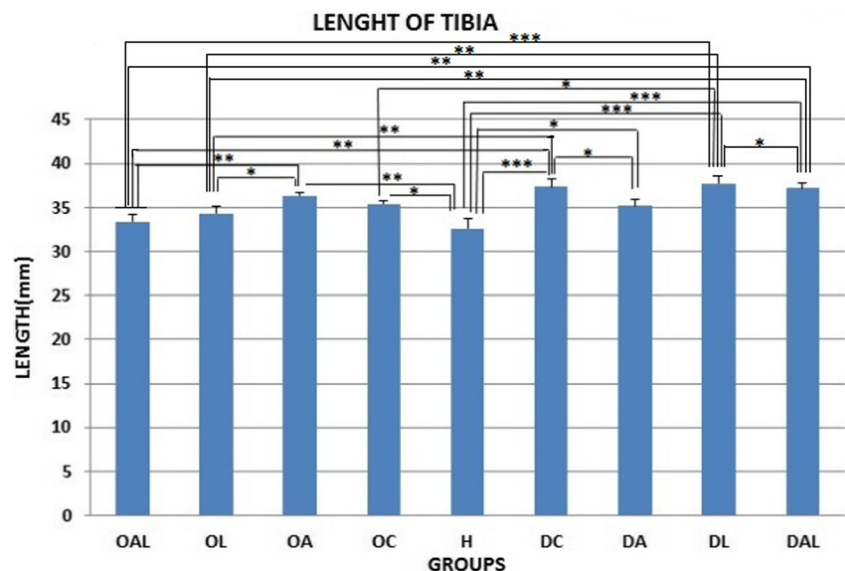
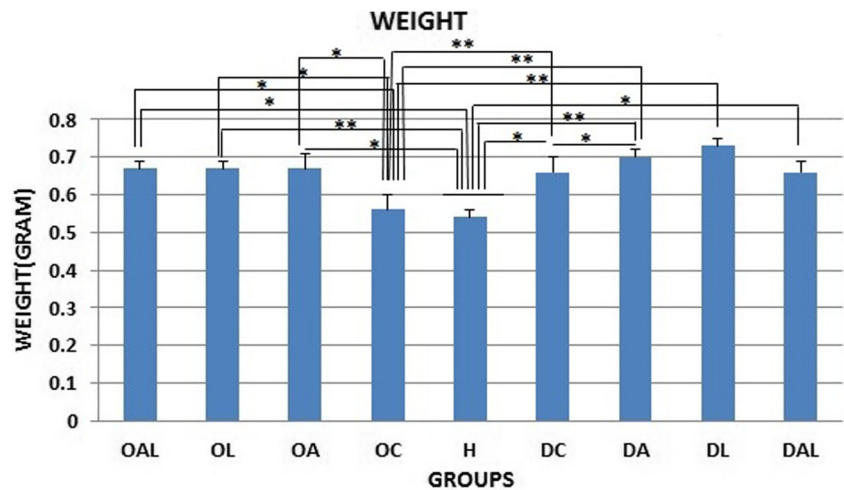


Fig. 2 Mean \pm SEM of tibial weight (gram) of studied groups compared by LSD test; * <0.05 , ** <0.01 . According to the LSD test, the OC group had a significant decrease in tibial weight compared to the OA, OL, DL, DA, DAL, and DC groups. The DA group showed a significant increase in tibial weight compared to the DC and OC groups



Tibial weight Tibial weights of all groups, with the exception of the OC group, significantly increased compared to the healthy group. The OC group had a significant decrease in tibial weight compared to the OL (LSD test, $p=0.022$), OA ($p=0.025$), OL ($p=0.028$), DL ($p=0.001$), DA ($p=0.004$), DAL ($p=0.033$), and DC ($p=0.05$) groups. The DA group showed a significant increase in tibial weight compared to the DC ($p=0.049$) and OC ($p=0.004$) groups. All results are shown in Fig. 2.

Biomechanical results

The results of biomechanical analyses are shown in Figs. 3, 4, and 5.

Bending stiffness

There was a significant increase in bending stiffness in all study groups compared to the healthy group (Mann-Whitney test, $p=0.004$ for all groups). The DA group showed significant increase in bending stiffness compared to the DA and DAL groups (both $p=0.004$). LLLT had no positive effect

on bending stiffness of osteoporotic bones. All results are shown in Fig. 3.

Maximum force

According to the LSD test, the maximum force observed in all groups significantly increased (OC, OL, DC, and DL: $p=0.000$ for all; OA and OAL: $p=0.002$ for both; DL: $p=0.006$) compared to the healthy group. The DA group showed a significant increase in maximum force compared to the DL group ($p=0.035$). LLLT did not show any positive effect on the maximum force of osteoporotic bones. All results are shown in Fig. 4.

Stress high load

There was a significant increase in the OAL group for stress high load compared to the healthy, DL, and DAL groups (Mann-Whitney test, $p=0.004$ for all). Stress high load values for the OA and DA groups were significantly higher than the DL group (Mann-Whitney test, $p=0.004$). LLLT had no positive effect on the stress high load. As seen in Fig. 5, LLLT did

Fig. 3 Mean \pm SEM of tibial bending stiffness (N/mm) of studied groups compared by Mann-Whitney test; ** <0.01 . The DA group showed a significant increase in bending stiffness compared to the DA and DAL groups

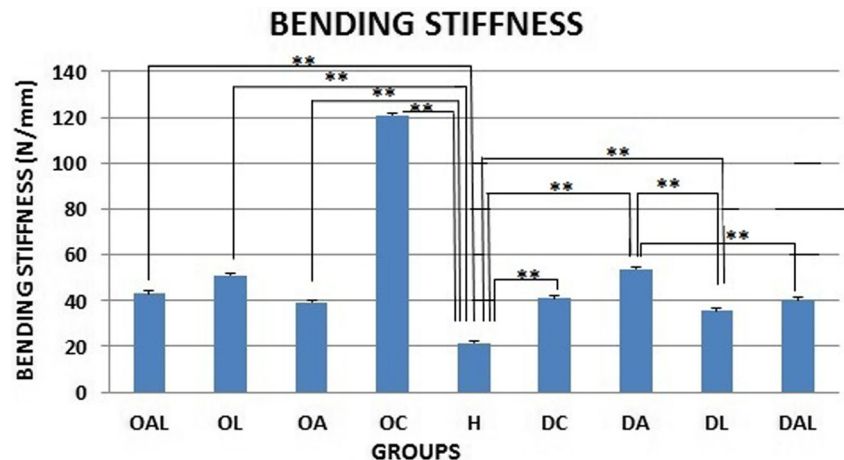
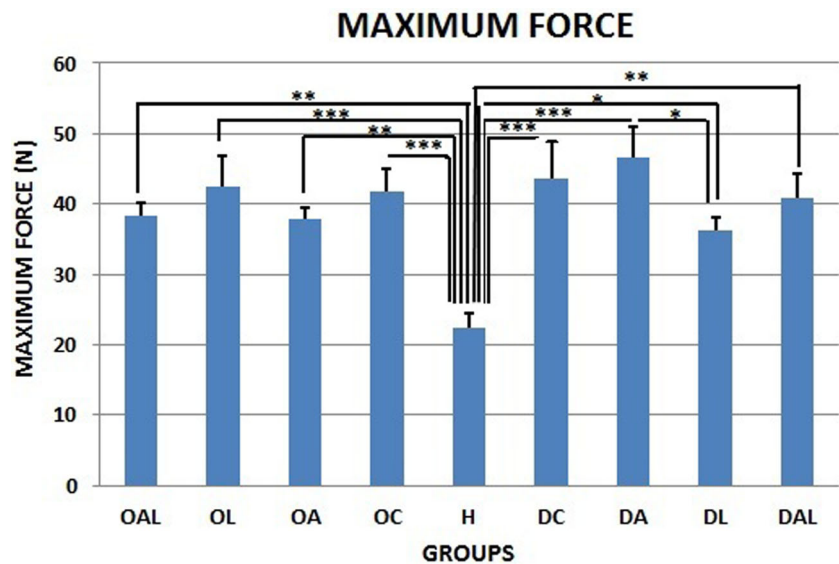


Fig. 4 Mean \pm SEM of tibial maximum force (N) of studied groups compared by LSD test; * <0.05 , ** <0.01 , *** <0.001 . The DA group showed a significant increase in maximum force compared to the DL group



not show any positive effect on the stress high load of osteoporotic bones.

Discussion

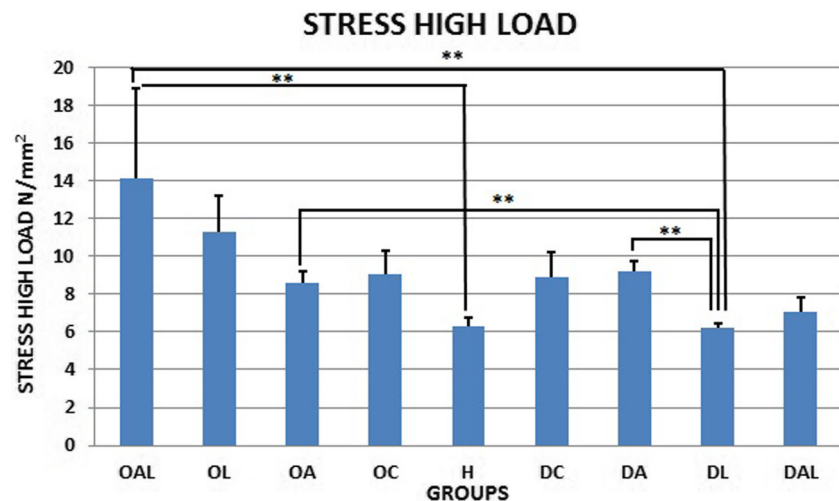
In the current study, we observed significant body weight loss in GC administered groups which resulted in a considerable mortality rate of 10 % among the rats. We observed significantly higher weights and biomechanical properties of the tibia from control rats treated with dexamethasone compared to healthy rats. With respect to the increased bone weights and biomechanical properties of dexamethasone-treated rats, we concluded that these findings might be attributed to the anabolic effects of GC administration at the level of the cortical bone [34, 35].

These data contradicted findings in patients with supraphysiologic GC administration. Those patients had OP, bone loss, increased bone fractures, and increased body weight [1, 36].

OP is a cause of bone loss and fractures which can lead to severe pain, deformity, and in certain cases, secondary complications that result in death [1]. Therefore, it is important to investigate treatments that have osteogenic potential, the capability to stimulate bone formation, and prevent bone loss [22]. The osteogenic effects of PW LLLT on osteoblastic cell proliferation and bone metabolism are well known [23–27].

Previous studies have shown that in vitro application of PW LLLT on bone cells stimulates bone formation [23, 25], promotes osteoblast proliferation and differentiation, and indirectly inhibits osteoclast differentiation by downregulation of the RANKL/OPG mRNA ratio in osteoblasts [24]. Thus, PW LLLT irradiation may play an important role in bone

Fig. 5 Mean \pm SEM of tibial stress high load (N/mm²) of studied groups compared by Mann-Whitney test; ** <0.01 . Stress high load values of the OA and DA groups were significantly higher than the DL group (Mann-Whitney test). LLLT did not have any positive effect on maximum force or stress high load of osteoporotic bones



remodeling and should be a valuable option for the treatment of bone diseases such as OP. In vivo studies have shown that PW LLLT treatments led to faster orthodontic tooth movement [26], effectively enhanced bone formation, and decreased bone resorption in osteoporotic OVX-d rats [27].

In contrast, PW LLLT at an 80-Hz pulse frequency, 900-s duration, 0.972-J/cm² energy density, and other delivery parameters used in the current study failed to cause any beneficial effects in the biomechanical parameters of osteoporotic bones. These results were supported by other studies [21, 22].

Medalha et al. found no increase in either mechanical or densitometric parameters of laser (830 nm, 250 J/cm²)-treated spinal cord-injured rats' bones [21]. Renno et al. showed that CW LLLT at 120 J/cm² did not enhance the effect of exercise on the femora of OVX-d rats. They concluded that since LLLT has osteogenic properties, higher delivery parameters should be investigated to verify if increased energy could create an extra-osteogenic stimulus in addition to exercise [22].

Possibly, the stimulatory effects of the PW LLLT protocol used in the current study was insufficient in decreasing the detrimental effects of the experimental OP induced by dexametason and the ovariectomy. Furthermore, the combination of alendronate (1 mg/day) and PW LLLT in OVX-d rats partially preserved bone function and formation. This combination significantly increased the stress high load of the osteoporotic tibia compared to the healthy group. The results of this study confirmed data from a previous study [37]. Diniz et al. investigated the effect of CW LLLT in combination with bisphosphonate on osteoporotic trabecular bone structure [37]. Laser-treated rats received a gallium-aluminum-arsenium laser at 830 nm, 50 m Watt, and 4 J/cm² on the femoral neck and vertebral segments. Remarkably, in the association between laser and alendronate, the trabecular bone volume was significantly greater in the vertebrae. They concluded that LLLT associated with alendronate administration was the best method for reversing vertebral OP caused by the ovariectomy [37].

We recently studied the capability of PW LLLT to increase bending stiffness in repairing an osteochondral defect [38]. The experimental group received PW LLLT at a frequency of 1500 Hz and energy density of 4.8 J/cm². The defects were examined biomechanically. There was significantly higher equilibrium indentation stiffness observed in the experimental group compared to the control group. Kamali et al. have concluded that PW LLLT significantly enhanced the stiffness of repairing tissue at the 8-week postinjury in rabbits with osteochondral defects [38]. Bending stiffness reflects the mineral fraction of the bone [39]. It seems that the effect of LLLT on tissue is dependent both on energy density and other parameters [40]. The PW LLLT devices provide more laser (light) parameters than CW LLLT devices. It is assumed that by investigating different values of these parameters, research models can be more effectively studied and compared with

CW LLLT devices in an attempt to achieve better outcomes [41–43].

Although we observed better biomechanical results in the DA group compared to other dexamethasone-treated rats, these differences were not significant. Possibly, if the amount of alendronate administration to the rats was adequate, there would be significant changes between those groups.

Conclusion

PW LLLT at the parameters used in this study failed to cause any beneficial biomechanical effects in the examined osteoporotic cortical bones. PW LLLT associated with alendronate treatment produced a more remarkable effect on bone strength in the ovariectomized induced OP model in rats.

Further studies that investigate different values of PW LLLT delivery parameters are needed to demonstrate the probable positive effects of PW LLLT on preventing bone loss in osteoporotic bones.

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Conflict of interest No competing financial interests exist.

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