

## Possible Role of Low Level Laser Therapy on Bone Turnover in Ovariectomized Rats

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**ABSTRACT:** The aim of this study was to assess the effect of low level laser therapy (LLLT) on bone turnover markers in ovariectomized rats. Thirty adult female albino rats were used in this study and divided into three groups: Group (1); 10 sham- operated rats served as controls group (2); 10 bilateral ovariectomized rats (OVX), and group (3); 10 OVX rats exposed to LLLT. LLLT was applied on the neck and shaft of femur, 5 times/week for 8 weeks. The dose applied on each point was 1000 Hertz, 5 Watts for 30 seconds with a total dose of 15 mJoule/cm<sup>2</sup>. At the end of the experiment, blood samples were collected and sera were separated for determination of serum calcium (Ca), inorganic phosphorus (Pi), osteocalcin and alkaline phosphatase (ALP). In addition, a 24 hour urine sample was also collected from each rat for the determination of urinary calcium, phosphorous, and deoxypyridinoline (DPD)/creatinine. Results showed significant increase in serum Ca, Pi, ALP, osteocalcin, and significant decrease in U-DPD/creatinine in LLLT exposed group as compared to the other two groups. Bone morphological results revealed increase in calcium deposition and alkaline phosphatase of femoral bones of LLLT exposed group in comparison to sham-operated and OVX rats. Using software image analysis showed increased osteoblast numbers, decreased osteoclast numbers and increased compact bone thickness in LLLT exposed group. Significant positive correlations were obtained between osteoblast numbers and serum Ca, Pi, ALP, and osteocalcin in LLLT exposed group, while a significant negative correlation was noticed with U-DPD. In conclusion, the use of LLLT was found to be effective in enhancing bone formation, decreasing bone resorption in the osteoporotic OVX rats. Further studies are necessary to investigate the effect of different parameters of LLLT as wave length, duration, and also numbers of sessions. The potential use of LLLT in postmenopausal women with osteoporosis is needed to be verified.

**Keywords:** Ovariectomy, Laser, Calcium, Phosphorus, Alkaline Phosphatase, Osteocalcin, Osteoporosis.

### INTRODUCTION

Osteoporosis is a generalized metabolic disease characterized by progressive loss of bone mass and micro-architectural deterioration of bone tissue, leading to bone fragility and increasing the risk of fracture.<sup>(1)</sup> Osteoporosis affects about 30% of postmenopausal women. It leads to an initial

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period of rapid bone loss, followed by lower rate of loss resulting in significant decrease in mineralized skeletal mass and mechanical resiliency thereby becoming prone to fracture.<sup>(2,3)</sup> The loss of estrogen at menopause is a major contributor to the pathogenesis of this disease as this hormone is a principal negative regulator of osteoclast activity and osteoclasts are the chief effector's cells responsible for bone remodeling in osteoporosis.<sup>(4)</sup>

The need to develop prevention strategies for bone loss has been increased. Many treatments have been proposed including estrogen replacement, bisphosphonate compounds, and physical activity programs.<sup>(5)</sup> Some studies highlighted the osteogenic effect of low level laser therapy (LLLT). Lasers in medicine and dentistry have dramatically improved the quality of life for patients. It is supposed

to reduce pain, accelerate wound healing and reduce inflammatory processes.<sup>(6,7)</sup>

The laser light is receiving increased attention not only regarding soft tissue, but also in bone metabolism. Stimulation with LLLT creates a number of environmental conditions that appear to accelerate the healing of bone defects in vivo and in vitro investigations.<sup>(8-10)</sup> While many studies have been published about the biostimulatory potential of LLLT during the process of bone repair, little attention has been given to the effect of LLLT on bone with osteoporosis or osteopenia.

A common experimental model of studying bone loss due to ovarian hormone deficiency is through the ovariectomized rat model (OVX). The characteristics of bone loss in rats after OVX are very similar to those found in postmenopausal women, and this model has proven to be informative to test

treatments against bone loss.<sup>(11)</sup>

Bone turnover is characterized by two tightly coupled activities, the degradation of old bone by osteoclasts followed by the formation of new bone by osteoblasts. The rate of resorption and formation of bone matrix can be assessed by measuring bone matrix components released into the circulation during remodeling, i.e., the biochemical markers of bone turnover.<sup>(12)</sup>

Biochemical markers that reflect remodeling or turnover of bone can be measured in urine or blood including formation and resorption markers.<sup>(13)</sup> Resorption markers include products of bone matrix degradation like hydroxyproline and telopeptides. Bone formation markers include alkaline phosphatase enzyme and products of bone matrix synthesis as osteocalcin.<sup>(14,15)</sup>

The purpose of our study was to investigate the possible role of LLLT on

bone turnover markers in ovariectomized rats with osteoporosis.

## **METHODS**

Thirty female albino rats aged 3-6 months and weighing 150-250 gm were used in this study. They were fed rat chow and water *adlibitum* and were maintained in a room with alternating light and dark. The acclimatized rats were randomly underwent laparotomy [(sham-operated, n = 10 (control group) or bilateral ovariectomy (OVX), n = 20)]. Six weeks after recovery. The OVX rats were divided into two groups; OVX, n = 10 (group II) and OVX exposed to LLLT, n = 10 (group III).

OVX was performed via abdominal incisions, under ketamine anesthesia. The uterine tubes were ligated and after removal of the ovaries, the incisions were closed.<sup>(16)</sup> Sham-operated rats were submitted to a skin incision and sutures. After the surgery, all animals were

conditioned for six weeks in cages with 5 animals/cage.

### **Laser exposure**

Laser exposure started six weeks after surgery, and was performed 5 times a week for 8 weeks. After shaving, the irradiation points (neck and shaft) were localized. Each femur was exposed to infra-red pulsated low level laser therapy in the two points. Each point is exposed to LLLT of 1000 Hertz, 5 watts for 30 seconds with a total dose 15 m joule/cm<sup>2</sup>.<sup>(17)</sup> At the end of the experiment, each rat was isolated in a separate metabolic cage for collecting the 24 h urine samples. Then, animals were sacrificed, and blood samples were also collected, immediately centrifuged, and sera were aliquotted and stored at -70°C. Femora were dissected, cleaned from adherent materials, and were prepared for bone morphological examination.

### **Biochemical analysis**

- Serum and urine calcium (Ca) and

inorganic phosphorus (Pi) levels were estimated using commercially available kits<sup>(18,19)</sup>. Urinary Ca and Pi were expressed as mg/mg creatinine (Cr).

- Serum alkaline phosphatase was measured using a kinetic procedure (Chemroy, biochemical trade, Inc USA).<sup>(20)</sup>

- Serum osteocalcin was determined using electrochemiluminescence immune assay (ECLIA).<sup>(21)</sup>

- Urinary deoxypyridinoline (DPD) levels were determined using a solid phase chemiluminescent enzyme labeled immunoassay.<sup>(22)</sup> Values are expressed as n moles DPD/mg creatinine.

### **Bone morphometry**

The femoral bones were separated from muscles, decalcified in 0.5 nitric acid in 10% formaline, dehydrated in descending series of alcohol, cleared in

xylene and then embedded in wax for preparation of paraffin sections using microtome 5  $\mu$  thick. The slides were stained by H & E and were examined by optical light microscopy. The thickness of compact bone was measured at the two edges, number of osteoblasts, osteoclasts, and osteocytes were determined by morphometry of histological sections at X10 magnification using the Optus image analyzer (Histochemistry and Cell Biology Department, Medical Research Institute, Alexandria University).

#### **Histochemical determination of calcium<sup>(23)</sup>**

Slides were deparaffinized, rehydrated and incubated in media containing 2% Alizarin red S in buffer phosphate pH 5. Then slides were differentiated in acetone, cleared in xylene, and mounted by DPX. The golden yellow deposit of dye indicates

calcium deposits.

#### **Histochemical determination of alkaline phosphatase:<sup>(24)</sup>**

The deparaffinized and rehydrated slides were incubated in media containing 2% glycerophosphate, barbitone pH 8.4 and 2% calcium chloride. Then the slides were treated with ammonium sulfide and cobalt nitrate. After washing by distilled water, slides were counter stained by nuclear fast red. The alkaline phosphatase appeared as black granules in the cytoplasm and the nucleus was given a red color.

#### **Statistical analysis**

Results were presented as means  $\pm$  standard deviations (SD). One way analysis of variance (ANOVA) F test was done to compare changes among groups using SPSS software and  $P < 0.05$  was considered statistically significant.

## **RESULTS**

Table (1) shows the effect of LLLT on

some bone turnover markers in the three studied groups. Using ANOVA test, significant difference was found among the three groups in serum calcium, phosphorus, osteocalcin, Alkaline phosphatase, urinary calcium, and U-DPD/Cr. F &  $p$  values were 6.193,  $p=0.006$ , & 13.275,  $p<0.001$ , & 11.321,  $p<0.001$ , & 6.130,  $p<0.001$  & 23.088,  $p<0.001$ , and 44.215,  $p<0.001$ , respectively. Serum calcium and phosphorous levels were significantly decreased in OVX and increased in LLLT exposed group as compared to sham-operated group. Serum osteocalcin was also significantly decreased in OVX group and increased in LLLT exposed group as compared to sham-operated controls. Serum ALP was significantly increased in LLLT as compared to the other two groups. Urinary calcium/Cr & U-DPD/Cr were significantly increased in OVX as compared to sham-operated

controls and significantly decreased in LLLT as compared to OVX group.

Table (2) shows the effects of LLLT on osteoblast numbers, osteoclast numbers, and compact bone thickness. ANOVA test showed significant differences among the three groups ( $p<0.001$ ). F values were 11.469, 29.728 & 11.062, respectively. In group (3), LLLT exposed rats showed significant increase in osteoblast numbers and bone thickness as compared to OVX and sham-operated controls. The osteoclast numbers were significantly increased in OVX as compared to control and LLLT exposed rats.

Table (3) shows correlations between both osteoblast and osteoclast numbers and some bone turnover markers in LLLT exposed group. Osteoblast numbers were significantly positively correlated with serum calcium, serum phosphorus, serum osteocalcin, and serum alkaline phosphatase

( $r=0.694$ ,  $P = 0.018$ , &  $r=0.655$ ,  $p=0.029$ , &  $r = 0.868$ ,  $p=0.001$  &  $r =0.624$ ,  $p = 0.04$ , respectively and significantly negatively correlated with U-DPD ( $r=-0.672$ ,  $p=0.023$ ). However, osteoclast numbers were negatively correlated with serum calcium, serum osteocalcin, and serum alkaline phosphatase ( $r= - 0.608$ ,  $p=0.047$  &  $r = -0.62$ ,  $p=0.042$  &  $r= -0.834$ ,  $p=0.001$ , respectively) and significantly positively correlated with U-DPD ( $r=0.708$ ,  $p=0.015$ ).

### **Histological results**

Light microscopic examination of femoral bone of sham-operated controls (group 1) revealed the mineralized bone matrix (compact bone) which appeared as a pink stained layer having a thin basophilic cement lines that reflect the amount of bone remodeling, the inner surface of bone is covered by a layer of osteoblast (OB) called endosteum. The osteoblastic layer is formed of cuboidal

or flat cells that are loosely arranged in sheet. Their cytoplasm is intensely basophilic. Osteocytes are present deep within the mineralized bone. They are housed in small cavities called lacunae. Osteoclasts are large multinucleated cells present on the surface of bone matrix and are tightly associated with the calcified matrix, (Fig. 1A). The histological examination of rat femoral bone of OVX rats (group 2) illustrated a decreased compact bone thickness which appeared as faint color with absence of the cement line, as well as reduction in size of osteocytes. The osteoblasts lost its arrangement and were reduced in number, the presence of marrow cavities in the bone matrix were seen, (Fig. 1B). Animals exposed to LLLT after OVX (group 3) showed mild change of both compact bone and osteocytes. Osteoblasts appeared to be separated from bone by dark-pink

stained band which represents osteoid (non-mineralized matrix), (Fig. 1C)

### **Histochemical results**

The calcium demonstration on rat femoral bone illustrated a moderate positive staining in control group, (Fig. 2A). Whereas a reduction of calcium content was observed in OVX rats (group 2) compared to controls, (Fig. 2B). The strongest positive staining of calcium content was observed in group 3 which is exposed to LLLT compared to other groups, (Fig. 2C).

The alkaline phosphatase staining of rat femur showed strong reactions in the most bone marrow cells, osteocytes, and compact bone. A moderate reactions was observed in osteoclasts and osteoblasts, (Fig. 3A). The reaction was decreased in the OVX rats group (2), the reaction was very weak in the compact bone, (Fig. 3B). Animals exposed to LLLT (group 3) showed a moderate

positive reaction mostly in the osteocytes and compact bone. A strong activity was noticed in osteoblasts and osteoclasts, (Fig. 3C).

(Table 4) illustrates the intensity of the different positive results of histochemical alkaline phosphatase activity in the three studied groups. In sham-operated controls strong activity was found in osteoblasts and compact bone and moderate in osteoclasts and osteocytes. In OVX group, ALP activity was absent in osteocytes and weak in compact bone. In the LLLT exposed group, strong activity was found in osteoblasts and osteoclasts and moderate activity in osteocytes and compact bone

### **DISCUSSION**

Since osteoporosis has become one of the most important public health problems affecting postmenopausal women, it is important to seek effective

treatments with osteogenic potential able to stimulate bone formation and to prevent bone loss.<sup>(25)</sup> The development of laser technology represents one of the most promising treatment modalities. LLLT, as a bone attachment stimulating factor was reported to have a role in the biostimulation of bone repair.<sup>(26)</sup> However, little attention has been given to its effect on bones with osteoporosis.

Previous research found that OVX in rats leads to osteopenia and osteoporosis, with a decrease in bone mass and an increase of bone fragility.<sup>(27,28)</sup> In this study the lack of estrogen in ovariectomized rats increased bone turnover, with an imbalance between resorption and formation which could result in the decrease of bone mass. Moreover, the lack of estrogen can also affect the activity of bone cells with osteoblast

insufficiency and excessive osteoclasts activation.

In the present study, the use of LLLT for 8 weeks in ovariectomized rats demonstrated positive osteogenic effects. Significant higher levels of serum calcium, phosphorus, osteocalcin, and alkaline phosphatase activity and significant lower U-DPD values were obtained in the laser exposed group as compared to OVX and sham-operated controls. In addition bone histological findings revealed increased osteoblast , decreased osteoclast numbers, and increased bone thickness in LLLT exposed group as compared to the other two groups.

The osteoblasts are primarily known to be responsible for bone formation by producing type I collagen and non-collagenous proteins as well as a wide variety of biomolecules.<sup>(29)</sup> The power of

stimulating bone cell proliferation and accelerating bone fracture consolidation has been attributed to LLLT<sup>(30)</sup>. Two principal mechanisms are proposed for the beneficial role of LLLT in early bone healing; namely stimulation of osteoblast precursor proliferation and later stimulation of cell differentiation thus increasing the number of osteoblasts.<sup>(31)</sup> In cultures of osteoblasts, LLLT promoted stimulation of DNA synthesis, increased liberation of proteins associated with cell maturation, resulting in increased concentrations of calcium, phosphorus, and ALP activity with proliferation of osteoblast cells<sup>(32)</sup>

In the present work, osteoblast numbers in the laser exposed group was found to be significantly positively correlated with serum calcium, phosphorus ALP, and osteocalcin and negatively correlated with U-DPD, while the correlation between osteoclast numbers and the same parameters showed opposite results. These finding suggest that the role

of LLLT in enhancing bone formation or inhibiting bone resorption occurs through its action on bone cells. Moreover, findings of bone histochemistry demonstrated increased ALP and Ca deposition in femoral bones of laser irradiated group as compared to OVX and sham-operated groups which confirm also the promotion of bone formation by LLLT. Similar to our study, a histochemical analysis by Ninomiya *et al.*, 2007<sup>(33)</sup> revealed increased bone volume by laser that was primarily achieved by increased osteoblasts and decreased osteoclasts. They suggested that active bone formation is induced by laser as osteoblasts and preosteoblasts around trabecular bone appear on day 1 and day 3 after laser irradiation, in the same time, osteoclasts are inactivated and decreased. They speculated that the mechanism of increased bone volume as a result of laser radiation was explained by reduced resorption of the calcified cartilage,

inactivation of osteoclasts, then osteoblasts actively form bone matrix on the remaining calcified cartilage. As a result, many cartilage matrices in the trabecular area arise and trabecular thickness and bone volume then increase.

The action of LLLT has been studied in many tissues and its stimulatory effects have been proven. However, The exact regulatory mechanism of LLLT on tissues is not fully understood.<sup>(34)</sup> The effects of LLLT are based on the absorption of light by tissues, which generates a series of modifications in cell metabolism. When LLLT is applied to a tissue, the light is absorbed by chromophores and will induce acceleration of chemical reactions; altering the production of molecular oxygen and ATP which can promote the increase of DNA and RNA synthesis.<sup>(35)</sup> In bone defects, LLLT may increase local blood flow, enhancing the supply of circulating cells with nutrition, oxygen, and inorganic salts

resulting in growth and differentiation of bone forming cells<sup>(36)</sup>

Although, there are positive reports from both *in vitro* and *in vivo* studies, the results of some studies of LLLT effects on bone healing are contradictory. Some studies showed no relevant effect, others demonstrated stimulation of bone matrix synthesis by LLLT.<sup>(37,38)</sup> Such discrepancies might be attributable to variations in the irradiation protocols. Queiroga *et al.*, 2008<sup>(39)</sup> have evaluated bone repair process of standardizing bone defects in rat femurs submitted to red laser (660nm) and infrared (780nm). They reported that bone defects submitted to infra red laser had a more advanced repair as compared to controls and red laser exposed rats. They added that a significant amount of newly formed bone occurs within 15 days that shows the biomodulated effect of laser therapy in the early stages of the repair process in which there is a large

quantity of cells, mainly osteoblasts and undifferentiated cells suggesting the biostimulatory effects of laser on these cells.

It is well known that wavelength defines the depth of penetration in the target tissue and that lower wave lengths are less resistant to the dispersion and don't penetrate deeply into tissue while, higher wave lengths penetrate more deeply and are more effective at stimulating bone tissue<sup>(40)</sup>. Moreover, the red light penetrates 0.5-1 mm before losing 37% of its intensity, while the infrared wave lengths penetrate 2 mm before losing the same percentage of energy. In addition, the radiation emitted in the infrared region (830nm) showed a low coefficient of absorption resulting in a maximum penetration and the interrupted laser light has been reported also to be more effective for bone formation than

continuous laser.<sup>(41)</sup> This could explain the choice of using pulsed infrared LLLT with high wave length in this study.

Therefore, it is important to select the optimal laser therapy for experimental and clinical applications. The use of infrared laser radiation could be the best indication when investigating the biomodulatory effects on bone tissue taking into account other parameters as the fluency, power, density, frequency as well as the wave length.

In conclusion, LLLT is effective in enhancing bone formation and preventing high bone turnover in ovariectomized rats. Further studies are necessary to investigate the effects of different parameters as wave length, duration, and number of sessions on bones of OVX rats. The potential use of LLLT in postmenopausal women with osteoporosis is needed to be verified.

**Table (1): Effect of LLLT on some bone turnover markers in the studied groups (mean±SD)**

	Group I (Sham) (n=10)	Group II (OVX) (n=10)	Group III OVX + LLLT (n=10)	F value & P value
Serum calcium (mg/dl)	10.67±0.19	10.12±0.48 <sup>a*</sup>	10.97±0.76 <sup>b**</sup>	6.193 P=0.006
Serum phosphorus (mg/dl)	6.84±1.18	5.91±0.8 <sup>a*</sup>	8.00±0.68 <sup>a&amp;b**</sup>	13.275 <0.001
Serum osteocalcin ( ng/ml)	65.06±9.05	45.21±3.98 <sup>a**</sup>	64.61±14.32 <sup>b**</sup>	11.231 P<0.001
Alkaline phosphatase (U/L)	98.8±24.96	85.44±14.22	121.18±26.98 <sup>a,b**</sup>	6.130 P=0.006
Urinary calcium / creatinine (mg/mg Cr)	0.03±0.01	0.05±0.00 <sup>a**</sup>	0.03±0.01 <sup>b**</sup>	23.088 P<0.001
Urinary phosphorus/ creatinine (mg/mg Cr)	0.5±0.09	0.51±0.06	0.54±0.1	0.698 p=0.506
U-DPD/Cr (nmol/mg Cr)	7.09±0.61	11.24±0.82 <sup>a**</sup>	7.33±1.5 <sup>b**</sup>	44.215 p<0.001

F: F test (ANOVA)

a: comparison to Group I.

b: comparison to Group II.

\*: significant p&lt;0.05

\*\*: p&lt;0.001.

**Table (2): Effect of LLLT on osteoblast numbers, osteoclast numbers and bone thickness (mean±SD) in the studied groups.**

	Group I (Sham) (n=10)	Group II (OVX) (n=10)	Group III OVX + LLLT (n=10)	F value & P value
Osteoblast numbers / 10 field	82.3±10.76	65.67±12.29 <sup>a</sup>	94.9±16.54 <sup>a,b**</sup>	11.469 p<0.001
Osteoclast numbers / 10 field	5.7±1.42	14±2.69 <sup>a**</sup>	7±3.07 <sup>b**</sup>	29.728 p<0.001
Bone thickness (µm)	276.95±62.23	173.46±24.85	357.9±128.74 <sup>a,b**</sup>	11.062 p<0.001

F: F test (ANOVA)

a: comparison to Group I.

b: comparison to Group II.

\*\*: significant at p&lt;0.001

**Table (3): Correlations between both osteoblast and osteoclast numbers and some bone turnover markers in LLLT exposed group.**

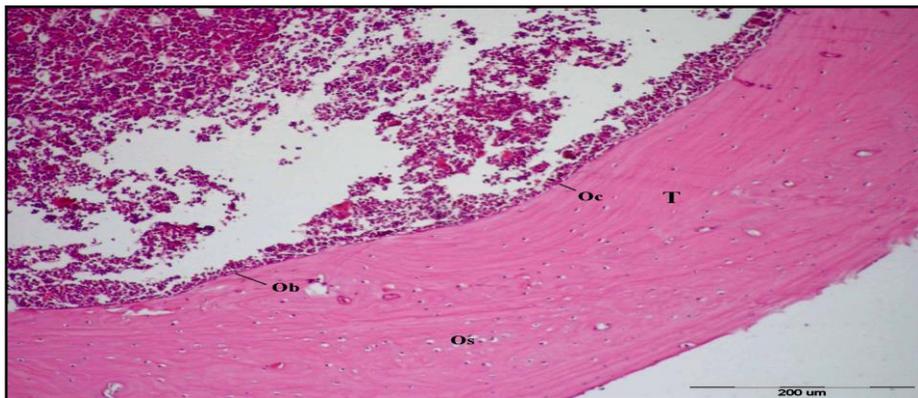
	Osteoblast numbers	Osteoclast numbers
Serum calcium	r=0.694* p=0.018	r= - 0.608* p = 0.047
Serum phosphorus	r = 0.655* p= 0.029	r =- 0.381 <sup>N.S</sup> p=0.247
serum osteocalcin	r=0.868** p=0.001	r=-0.62* p=0.042
Serum alkaline phosphatase	r=0.624* p=0.04	r=- 0.834** p=0.001
U-DPD	r= -0.672* p=0.023	r=0.708* p=0.015

\* Significant at p <0.05.

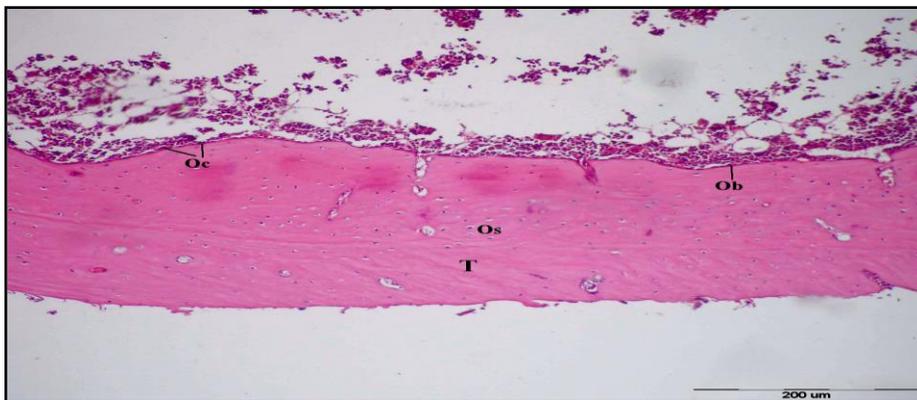
\*\* significant at p<0.001.

**Table (4): Semiquantitative measurements of histochemical ALP in femoral bones of the studied groups**

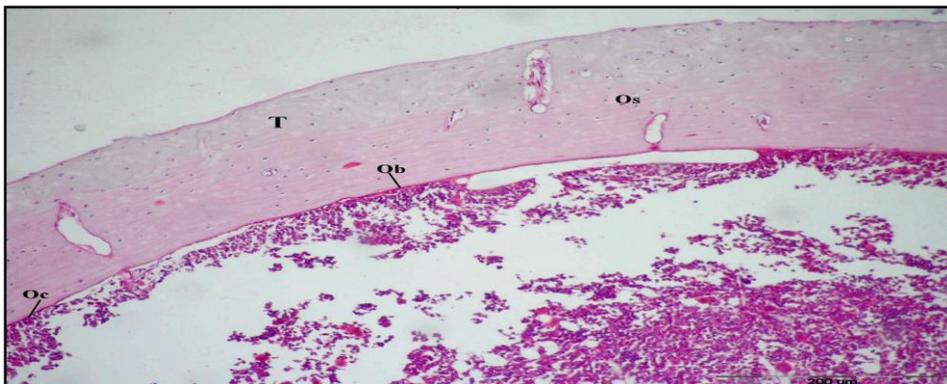
	Group I Sham-operated controls	Group II (OVX)	Group III OVX + LLLT
Compact bone	+++	+	++
Osteocytes	++	0	++
Osteoblasts	+++	++	+++
Osteoclasts	++	++	+++



1A

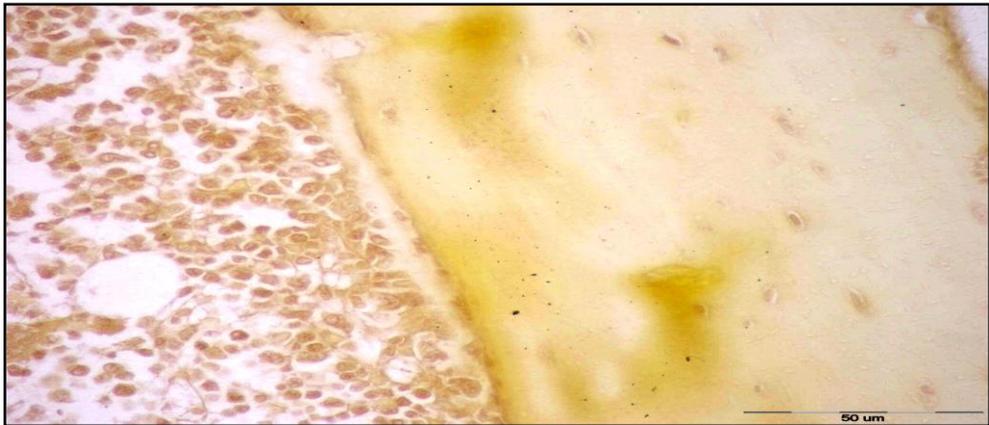


1B

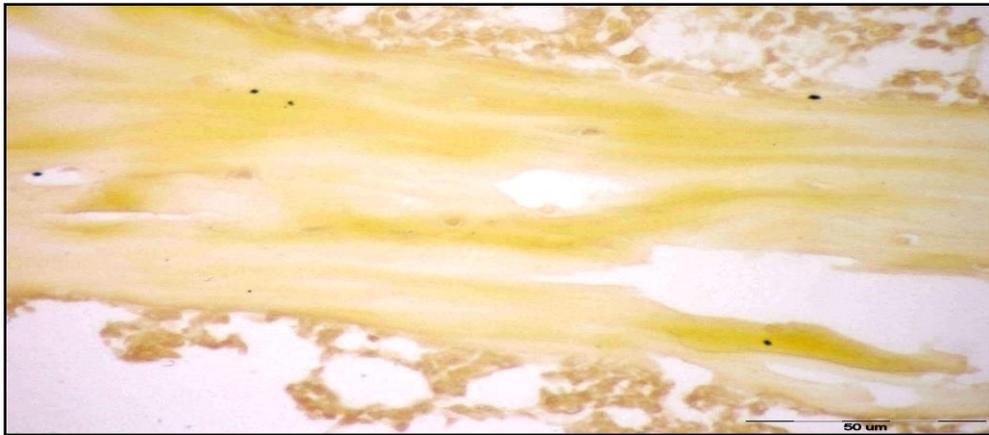


1C

**Figure (1): (1A):** Paraffin section micrograph of femoral bone of sham-operated control group, showing compact bone, covered with a row of osteoblasts, cement lines are present as dark stained lines, osteocytes (Os) are reduced in size, increase number of osteoblasts (Ob) arranged in a sheet over the bone matrix. **(1B):** Femoral bone of OVX rats showing reduction of compact bone thickness, absent cement lines, Osteocytes are reduced in size, separation between osteoblasts with lost architecture with presence of primary marrow cavities in bone matrix. **(1C):** Femoral bone of LLLT exposed rats showing mild reduction of compact bone thickness, increased osteoblast numbers. (H & E, Bar = 200µm).



2A

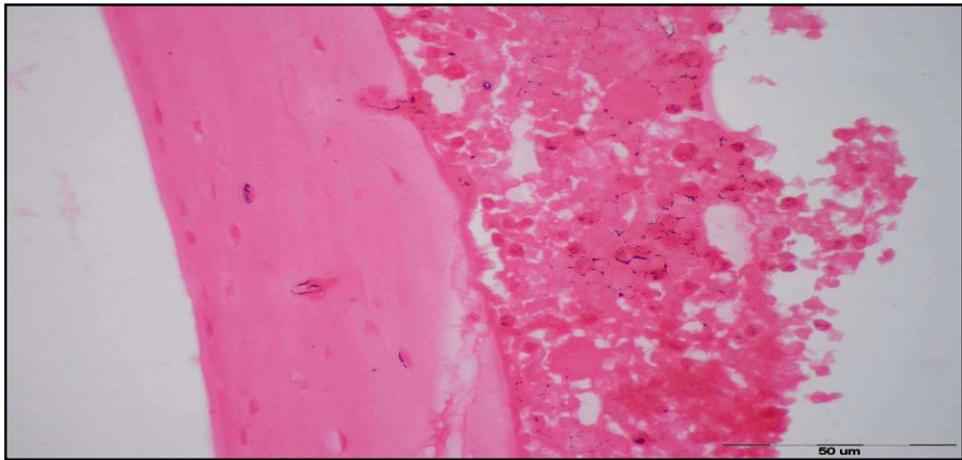


2B

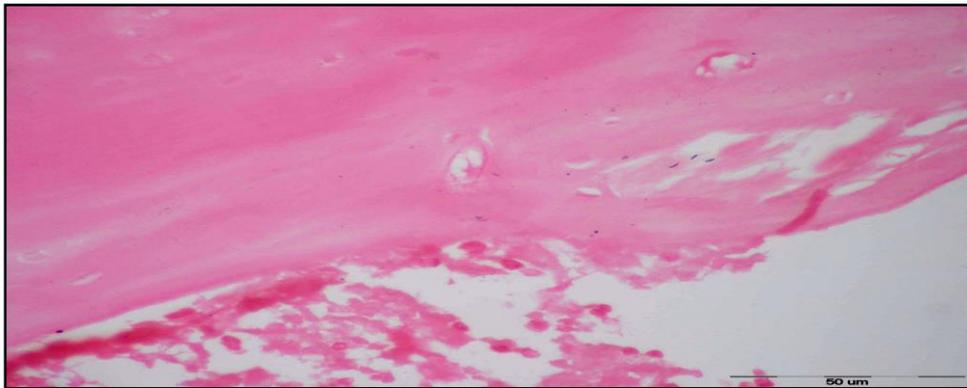


2C

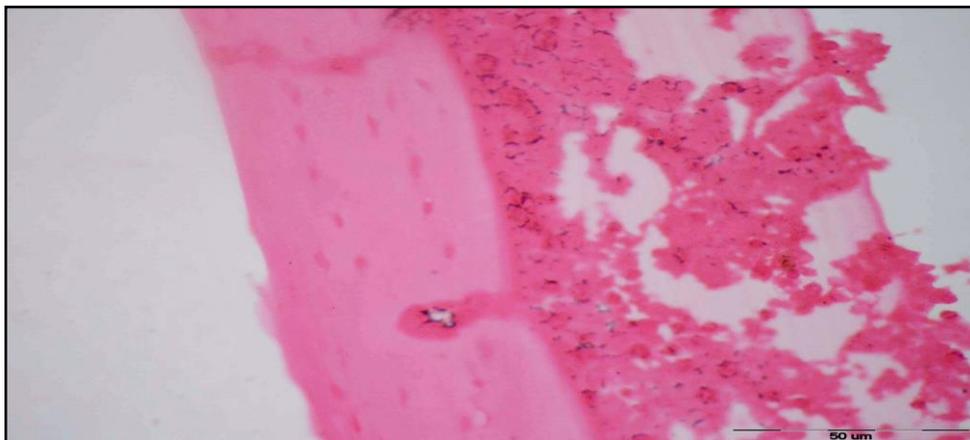
**Figure (2): (2A):** Paraffin section micrograph of femoral bone of sham-operated control group, showing moderate content of calcium appeared as diffuse golden color. **(2B):** In OVX rats, reduction of calcium content in bone matrix. **(2C):** In LLLT group increased calcium content in compact and trabecular bone matrix. (Alzarin red, Bar = 50μm).



3A



3B



3C

**Figure (3): (3A):** Paraffin section micrograph of femoral bone of sham-operated control group, showing strong ALP activity in osteocytes, compact bone and bone marrow cells. **(3B):** In OVX rats showing decreased ALP activity **(3C):** In LLLT group, strong increased ALP activity in osteoblast, osteoclasts. (□-glycerophosphate, Bar = 50µm).

## REFERENCES

1. Diniz J, Nicolau R, Ocarino N, Magalhares F, Pereira R, Serakides R. Effect of low-power gallium-aluminum-arsenium laser therapy (830nm) in combination with bisphosphonate treatment on osteopenic bone structure: an experimental study. *Lasers Med Sci*. 2009;24: 347-52.
2. Johnell O, Kanis J. Epidemiology of osteoporotic fractures. *Osteoporos Int*. 2005; 16: S3-S7.
3. Fan Y, Liu J, Wang S, Wang H, Shi F, Xiong L, *et al*. Functional proteome of bones in rats with osteoporosis following ovariectomy. *Life science*. 2005; 76: 2893-901.
4. Roodman G. Advances in bone biology: the osteoclast. *Endocr Rev*. 1996; 17: 308-32.
5. Renno A, Moura F, Dos Santos N, Tirico R, Bossini P, Parizotto N. Effects of 830 laser light on preventing bone loss after ovariectomy. *Photomed Laser Surg*. 2006; 24: 642-5.
6. Abo Elsaad N, Soory M, Gadalla L, Ragab L, Dunne S, Zalata K, *et al*. Effect of soft laser and bioactive glass on bone regeneration in the treatment of bone defects. *Lasers Med Sci*. 2009;24:387-95.
7. Walsh L. The current status of laser applications in dentistry. *Aust Dent J*. 2003; 48: 146-55.
8. Silva J, Pinheiro A, Oliveira M, Weismann R, Ramalho P, Nicolau R. Computerized morphometric assessment of the effect of low level laser therapy on bone repair: an experimental study. *J Clin Laser Surg*. 2002; 20: 83-7.
9. Stein A, Benayahu D, Maltz L, Oron L. Low level laser irradiation promotes proliferation and differentiation of human osteoblasts in vitro. *Photomed laser surg* 2005; 23: 161-6.
10. Merli L, Santos M, Genovese W, Faloppa F. Effect of low intensity laser irradiation on the process of bone repair. *Photomed laser surg*. 2005; 23: 212-5.
11. Renno A, de Moura F, Dos Santos N, Tirico R, Bossini P, Parizotto N. The effects of infrared 830nm laser on exercised osteopenic rats. *Laser Med Sci* 2006; 21: 202-7.
12. Brown J, Albert C, Nassar B, Adashi J, Cole D, Davison K, *et al*. Bone turnover markers in the management of postmenopausal osteoporosis. *Clin Biochem*. 2009; 10: 929-42.
13. Watts NB. Clinical utility of biochemical markers of bone remodeling. *Clin Chem*. 1999; 45: 1350-8.
14. Civitelli R, Armamento-Villareal R, Napoli N. Bone turnover markers: understanding their value in clinical trials and clinical practice. *Osteoporos Int*. 2009; 20: 843-51.
15. Pagani F, Francucci C, Moro L. Markers of bone turnover: Biochemical and clinical perspectives. *J Endocrinal invest*. 2005; 28: 8-13.
16. Thompson D, Simmons H, Pierrie C, Ke K. Guidelines and animal model for osteoporosis. *Bone*. 1995; 17: 125S-33S.
17. Fulga C, Fulga IG, Predescu M. Effect of laser therapy on experimental inflammation in rats. *Rom J Intern Med*. 1995; 33: 257-61.

18. Balmert RN, Skodon SB, Goldberg MH. Performance of kits used for clinical chemical analysis of calcium in serum. *Am J Clin Pathology*. 1973; 59: 836-43.
19. Goodwin JF. Quantification of serum inorganic phosphorus, phosphate and urinary phosphate without preliminary treatment. *Clin Chem*. 1970; 16: 776-80.
20. Tietz NW, Burtis CA, Duncan P, Erin K, Petittlenc CJ, Rinker AD, *et al.* A reference method for measurement of alkaline phosphatase activity in serum. *Clin Chem*. 1983; 29: 751.
21. Rosenquist C, Qvist P, Bjarnason N, Christiansen C. Measurement of a more stable region of osteocalcin in serum with two monoclonal antibodies. *Clin Chem*. 1995; 41: 1439-45.
22. Robins SP, Woitge H, Hesley R, Tu J, Seyedin S, Seibel M. Direct enzyme linked immunoassay for urinary deoxypyridinoline as a specific marker for measuring bone resorption. *J Bone Miner Res*. 1994; 9: 1943-9.
23. Kiernan JA. Histological and histochemical methods "theory and practice" 3<sup>rd</sup> ed. Arnold. London: Hodder Headline Group; 2001. 268-9.
24. Lillie RD, Furth HM. Method for alkaline phosphatase in: *Histopathologic technical and practical histochemistry*. New York: McGraw-Hill; 1976. 265-7.
25. Dempster D. Treatments of osteoporosis: how do they reduce the risk of fracture? *Osteop Int*. 2003; 14: S1.
26. Obradovic R, Kesic L, Pesevska S. Influence of low level laser therapy on biomaterial osseous integration: a minireview. *Laser Med Sci*. 2009;24:447-51
27. Kalu D. The ovariectomized rat model of postmenopausal bone loss. *Bone Miner*. 1991; 23: 301-6.
28. Peng Z, Tuukkanen H, Zhang H. The mechanical strength of bone in different rat models of experimental osteoporosis. *Bone*. 1994; 15: 523-32.
29. Kim K, Kim J, Kim S, Lee J, Ko S, Shimizu M. Effects of low level laser irradiation with 904nm pulse diode laser on osteoblasts: A controlled trial with the rat osteoblast model. *Laser Therapy*. 1996; 8: 223-32.
30. Yamamoto M, Tamura K, Hiratsuka K. Stimulation of MCM3 gene expression in osteoblasts by low level laser therapy irradiation. *Laser Med Sci*. 2001; 16: 213-7.
31. Khadra M, Lyngstadaas S, Haanaes H, Mustafa K. Effect of laser therapy on attachment, proliferation and differentiation of human osteoblast cells cultured on titanium implant material. *Biomaterials*. 2005; 26: 3503-9.
32. Khadra M, Kasem M, Haanaes H, Ellingsen J, Lyngstadaas S. Enhancement of bone formation in rat calvarial bone defects using low level laser therapy. *Oral Surg Oral Med Oral Radiol Endod*. 2004; 97: 693-700.
33. Ninomiya T, Hosoya A, Nakamura H, Sano K, Nishisaka T, Ozawa H. Increase bone volume by a nanosecond pulsed laser irradiation is caused by a decreased osteoclast number and an activated osteoblasts. *Bone*. 2007; 40: 140-8.
34. Karu T, Lubart R. Effects of low power light on biological systems. *Spie Proc*. 2000; 4159: 1-17.
35. Stein A, Benayahu D, Maltz I. Low-level laser irradiation promotes

- proliferation and differentiation of human osteoblasts invitro. *Phomed Laser Surg.* 2005; 23: 161-6.
36. Pretel H, Rosane F, Lizeti T. Effect of low-level laser therapy on bone repair: histological study in rats. *Lasers in surgery and Med* 2007; 39: 788-96.
  37. Do rtbudak O, Haas R, Pokorny M. Biostimulation of bone marrow cells with a diode soft laser. *Clin Oral Imp Res.* 2000; 11: 540-5.
  38. Coombe A, Ho C, Darendeliler M, Philips J, Chapple C, Yum L. The effects of low level laser irradiation on osteoblastic cells. *Clin Orthop Res.* 2001; 1:3-14.
  39. Queiroga A, Sousa F, Araujo J, Santos S, Sousa D, Quintans T, Almeida T, *et al.* Evaluation of bone repair in the femur of rats submitted to laser therapy in different wavelengths: An image segmentation. *Method of analysis: Laser Methods in Chemistry, biology and Medicine.* 2008; 18: 1087-91.
  40. Karu T, Pyatibrat L, Kalendo G. Irradiation with He-Ne laser increases ATP level in cells cultivated in vitro. *Photochem Photobiol.* 1995; 27: 219.
  41. Kucerova H, Dastalova T, Himmlova L. Low level laser therapy after molar extraction. *J Clin Laser Med Surg.* 2000; 18: 309.