Estimation of the Therapeutic Effect of Diode Laser on Bone Cells in Mandibular Distraction Osteogenesis: An Experimental Study in Adult Male Rabbits

Original Article

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ABSTRACT

Background: The use of the intraoral distractors has been associated with different drawbacks resulting in their untimely removal and treatment relapse.

Objective: This study aimed to estimate the therapeutic effect of diode laser on bone cells in the mandibular distraction osteogenesis (DO).

Materials and Methods: 28 adult rabbits were equally divided into 4 groups. The left and right mandibular corticotomy was performed with distractor fixation. The left side served as the non-laser subgroup whereas the right side served as the laser subgroup. After 3 days of latency and 7 days of the distractor activation (0.5mm/12h) to reach 7mm expansion limit, the distractor was removed after 2 weeks of consolidation. The laser sides were treated with 10 J/cm^2 per point every 48 hours during consolidation. The animals of the four groups were sacrificed 1, 2, 3 and 4weeks after the consolidation commencement respectively. The dissected hemimandibles were processed for histological and immunohistochemical (using anti-osteonectin (ONN) and anti-osteopontin (OPN) antibodies) examination.

Results: Histologically, the apparently increased new bone amount was higher in the laser subgroup at all time periods. Immunohistochemically, the ONN and OPN expressions were significantly increased in bone matrix and cells of groupIII>groupII>groupI but with insignificant difference between groups I and II for ONN. GroupIV revealed a decreased ONN and OPN reactivity which was significant compared to the laser side groupIII in particular, but still with increased ONN and OPN positivity compared to most subgroups of groups I and II with variation in the significance levels.

Conclusion: Diode low-level laser application exhibited superior cellular differentiation, ossification and amount of bone regeneration in mandibular DO over the unescorted conventional DO procedure thus reducing the consolidation time. Therefore, LLL can fasten tissue regeneration via the bio-stimulatory impact of laser on cells.

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Key Words: Bone cells, distraction osteogenesis; laser; osteonectin; osteopontin; .

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INTRODUCTION

Distraction osteogenesis (DO) is a bone-lengthening process using an external distractor for the gradual stretch of a surgically created osteotomy[1]. DO has been regularly used in oral and maxillofacial surgery since it was first reported by McCarthy in 1992[2]. It has been employed to treat many disarrays such as posttraumatic defects and congenital disturbances, based on the regenerative properties of bone[3]. Moreover, the physiological strain has a key function in skeletal growth, maintenance and bone remodeling. However, the use of intraoral distractors is unaesthetic and discomfortable as well as it can cause infection, fibrous malunion, transient labial numbness, impairment of oral function, articulation and mastication. All this can lead to premature removal of the device and treatment relapse[4].

Various types of laser are offered in the global market having different modes of action[5]. Low energy level laser (LLL) therapy was first applied to treat the non-healing and slow healing ulcers in 1971. This therapeutic approach has been used for improving postsurgical oedema, tissue healing and nerve regeneration. It is also utilized in orthopedics and dentistry in the cases of bone loss and fracture in addition to the treatment of variable distortions like rheumatoid arthritis and temporomandibular disorders[3,6]. LLL treatment can enhance tooth enamel conditioning as well as the functional attachment of titanium implants to the bone[7]. Diode laser (Gallium-Arsenide) is a type of LLL that is easily portable, inexpensive and readily available. It has better haemostatic outcomes, less thermal damage and less postoperative erythema than neodymium-doped yttrium aluminum garnet (ND: YAG) and CO2 lasers with

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a maximum effect of fast healing. It possesses a higher penetration depth through soft tissues to contact bone at the distraction osteogenesis site[8].

On the other hand, promising outcomes of LLL therapy in bone regeneration and healing were observed[3,9].

Non-collagenous matrix proteins like osteonectin (ONN) and osteopontin (OPN) are essential to regulate early osteogenesis, mineralization and remodeling[10]. ONN is a phosphorylated glycoprotein intimately related to matrix mineralization and stabilization of apatite crystals[11], while OPN is a multifunctional glycoprotein that regularly exists in bone matrix and enables bone cells adherence to the mineralized matrix[12]. Markers for osteoblast differentiation were described as early markers like collagen type I, midstage markers as ONN and finally late-stage markers as OPN[13]. Both ONN and OPN appear early in the process of DO and are upregulated in response to the gradual strain[4,14].

Thus, we estimated the therapeutic effect of the diode laser (LLL) on bone cells in mandibular distraction osteogenesis histologically and immunohistochemically using an adult male rabbit model.

MATERIALS AND METHODS

Ethical clearance

The guidelines of the Research Ethics Committee (FDASU-REC) which approved this work was followed, [Approval: FDASU-REC-IR121913] - Faculty of Dentistry, Ain Shams University, Egypt. The rabbits were housed in stainless steel cages 30 x 36 square inches individually to prevent wound infection and ensure proper healing. Housing was done under controlled temperature (24±2°C), relative humidity, proper ventilation, 12h dark-light cycle and adequate diet besides tap water throughout the experimental period. The animals' welfare was managed by the attending veterinarian to lessen any suffering of animals with daily confirmation of sickness nothingness.

Experimental design

Twenty-eight young adult male New Zealand rabbits, aged about 3 years and weighed 4 kg on average[15] after one week of acclimatization, they were endured distraction osteogenesis in both sides of the mandible.

Surgery

The animals were anaesthetized with 0.1 mg/kg 2% xylazine hydrochloride (XYLA-JECT®, ADWIA Co. S.A.E. 10th of Ramadan City, Egypt) and Ketamine hydrochloride (Ketamine®, Sigma-Tec Pharmaceutical Industries S.A.E. Egypt). Both right and left submandibular areas were shaved then cleaned by 4% chlorhexidine. Surgical sterile drapes were utilized for operation field isolation. Administration of 50 mg Enrofloxacin as a prophylactic antibiotic was done 1 hour prior to the procedure and for the subsequent three days. After lidocaine (0.9 ml) and epinephrine (2%) infiltration, a 2 cm incision was performed on the skin along the mandibular lower border in both left and right sides. The mandible was then exposed by careful elevation of the subperiosteal plane. The osteotomy was performed between the premolar and molars regions bilaterally with preservation of the inferior alveolar neurovascular bundle and thus the reflected periosteum. Osteotomes and burs were applied to produce the corticotomy with irrigation. The fixation of the distractor to the mandible by 4 screws (5 X 1.5 mm) was made perpendicular to the corticotomy. The wound was then irrigated using saline and closed in layers[16,17].

Discontinuous rhythmic distraction osteogenesis protocol

Latency period – (3 days; days 1 to 3): the distraction device was not activated; it was only inspected and cleaned with 1% iodophor alcohol. Activation period – (7 days; days 4 to 10): device activation started on the fourth postoperative day at a rate of 1 mm per day (0.5mm/12h) to obtain a total of 7mm at the end of the activation period. Bone consolidation period – (15days; days 11 to 26): after the activation period, the distractor was left to work as a rigid fixator, so that bone maturation was achieved[16,17].

Laser irradiation (during the consolidation period)

For each rabbit, the control side was the left side of the mandible that received no laser therapy. On the other hand, the experimental side was the right side of the mandible which received 10 J/cm^2 doses per point every 48 hours during the consolidation period[16,17].

Using ASA IDEA Terza laser device, Gallium-aluminum arsenide (GaAlAs) laser was employed at 905 nm and 150 MW[18].

Rabbits were randomly divided into four groups (seven animals each) by Random Sequence Generator program (randomizer.org). Sacrification was done for group I after 1 week from laser therapy (received 3 laser sessions), group II after 2 weeks from laser therapy (received 6 laser sessions), group III after 3 weeks from laser therapy (received 9 laser sessions) and group IV after 4 weeks from laser therapy (received 12 laser sessions).

The sacrification was performed at morning by intracardiac injection of anaesthetic overdose of sodium pentobarbital (100mg/kg)[19] as recommended by the Universal Declaration on Animal Welfare. Once death was confirmed by the absence of vital signs, the distractor was removed (in case of groups I and II). Animal mandibles were dissected free and split into left control hemimandibles and right experimental hemimandibles using surgical scissors. Getting rid of the sacrificed rabbits' bodies was achieved according to the ethical committee rules in the incinerator of Ain Shams University Hospital. Immediate fixation using formaldehyde, rinsing and coding of specimens was accomplished.

Histopathological examination

The hemimandibles were immediately fixed for 72 hours in 10% formaldehyde solution. Then, the

decalcification was achieved at 4°C using 10% ethylene diamine tetra-acetic acid (EDTA) solution for about 5 weeks. Dehydration in rising alcohol concentrations was carried out, followed by alcohol clearance with xylene. Infiltration and specimens embedding in paraffin were done. 5µm thick sections were cut and mounted on regular glass slides. The routine histological examinations of the Hematoxyline and Eosin (H&E) stained sections were executed[9] by a research light microscope (Olympus® BX 60, Tokyo, Japan).

Immunohistochemical examination

Immunolabeling identification of osteonectin (ONN) and osteopontin (OPN) using (rabbit polyclonal antibodies) anti-osteonectin and anti-osteopontin respectively was implemented. Tissue blocks were sliced at 4-5 µm thick sections, which were deparaffinized with xylene. Antigen retrieval using citrate (pH 6.0) was achieved in the microwave oven followed by blockage of endogenous peroxidase using a solution of (1:1) hydrogen peroxide and 50% methyl alcohol. Afterwards, incubation in bovine serum albumin for 1 hour in a moist chamber was done to block the nonspecific antigens. Samples were then incubated with the primary antibodies (ONN: 1 hour, 1:400, room temperature and OPN: 1:100, 4°C overnight). Next to the incubation with the secondary antibody (Universal LSAB TM Kit/HRP, Rb/Mo/Goat – DAKO, Carpinteria, CA, USA) for 30 minutes, washing in PBS was done. The sections were then incubated with tertiary complex streptavidin peroxidase for 30 min. For reaction visualization, diaminobenzidine (DAB) was used followed by washing of the sections. Later, counterstaining was achieved with Mayer's hematoxylin. Subsequent to drying, the slides were coverslipped to be examined by a light microscope (Olympus® BX 60, Tokyo, Japan)[20]. The ONN and OPN results were expressed by brown coloration in the nucleus and/or cell cytoplasm.

Histomorphometric and statistical analysis

Immunohistochemical findings of the studied groups were explored by the digital camera and software (Leica Qwin 500) of Leica microscope. The change of the pixels to real micrometre units was created using an image analyzer program. The acquired data were analyzed by means of Statistical Package for Social Science software computer program version 26 (SPSS, Inc., Chicago, IL, USA). Then, data presentation in mean and standard deviation was accomplished. The Student's t-test (unpaired) was utilized to compare two different groups of parametric data. One-way Analysis of variance (ANOVA) and Tukey test were also used to compare more than two groups of parametric data. *P-value* < 0.05 was assessed as statistically significant[8,21].

RESULTS

Histopathological results

Group I (1 week)

Non-laser left side: The H&E sections after 1 week of consolidation demonstrated fibrovascular tissue in the distraction gap exhibiting five zones. The central fibrous interzone showed randomly oriented fibroblast-like cells associated with parallel collagen fibers. Bilaterally, the primary matrix front contained proliferating cells, most probably osteoblast progenitors and immature osteoblasts in a dense line (proliferation front). This zone was outlined by the microcolumn zones where intramembranous ossification would occur. Each microcolumn zone was found adjacent to the proximal/distal osteotomized bone edge (Figure 1a).

Laser right side: The distraction gap presented an expanded bone matrix around the osteoblasts/ osteocytes and into microcolumns. Few spicules of a highly cellular woven bone were surrounded by fibrovascular tissue in the microcolumn zones. These primitive bone trabeculae were lined by osteoblasts and showed large interposed osteocytes with relatively increased cytoplasm and large lacunae (Figure 1b).

Group II (2 weeks)

Non - laser left side: After 2 weeks of consolidation, the collagen fibers of the fibrovascular tissue in the distraction gap appeared in a parallel organization and contained blood capillaries. Irregular and highly cellular woven bone within the fibrovascular tissue was seen with no signs of inflammation (Figure 1c).

Laser right side: Areas of condensed fibrovascular tissue in the distraction gap were noticed. The apparently increased woven bone extended from the osteotomized bone margins towards the gap center. These interconnected primitive bone trabeculae revealed apparently increased lining osteoblasts plus interposed osteocytes and were surrounded by highly vascular wide marrow spaces. Few areas of osteoclastic activity were also shown (Figure 1d).

Group III (3 weeks)

Non-laser left side: Three weeks after the beginning of consolidation, dense fibrovascular tissue in the central interzone was displayed. Highly cellular woven bone with wide marrow spaces was perceived besides the evidence of intramembranous ossification represented by areas of lamellar trabecular bone with no cartilaginous tissue (Figure 1e).

Laser right side: Denser fibrovascular tissue in the diminished central interzone was presented. Besides the increasingly formed lamellar trabecular bone, the amount of the interconnected primitive bone trabeculae of woven bone was apparently greater in the distraction gap and extended close to the central interzone (Figure 1f).

Group IV (4 weeks)

Non-laser left side: Four weeks after the commencement of consolidation, clearly condensed fibrovascular tissue along the bone regenerate was still elucidated in the more diminished central fibrous interzone. The bone regenerate in this period was formed of new, irregular interconnected lamellar bone trabeculae with relatively wide marrow

spaces alongside the highly cellular woven bone areas close to the center of the distraction gap in particular. Apparent decrease of osteoblasts and entrapped osteocytes was perceived together with areas of osteoclastic activity (Figure 1g).

Laser right side: The distraction gap including the thinner and smaller central fibrous interzone was mostly obliterated by the bone regenerate. Few primitive bone trabeculae of woven bones in the central interzone were bounded by apparently denser, more regular, interconnected distal and proximal lamellar bone trabeculae in addition to the apparently narrower marrow spaces. The bone regenerate showed apparently decreased osteoblasts and osteocytes as well as areas of osteoclastic activity (Figure 1h).

Immunohistochemical and statistical results

Immunohistochemical and statistical results for osteonectin (ONN)

Group I (1 week)

Non-laser left side: Regardless of the intense ONN immunopositivity of the fibrovascular tissue in the distraction gap after one week of consolidation, mild diffuse reactivity was observed in scarce areas of new osteoid and newly mineralizing matrix bordered by the positive osteoblast-like cells (Figures 2a,3).

Laser right side: A significant increase of ONN reactivity (*P-value* = 0.005) compared to the non laser side was observed in this group. Mild to moderate diffuse expression of ONN was displayed in osteoid and some newly mineralizing matrix areas surrounded by the apparently increased positive osteoblast-like cells in the distraction gap (Figures 2b,3).

Group II (2 weeks)

Non-laser left side: After two weeks of consolidation, moderate to intense ONN reactivity was demonstrated in string-like osteoid areas and dispersed over the mineralized osteoid foci in addition to the lining osteoblasts in the microcolumn zones (Figures 2c,3).

Laser right side: the amount of ONN matrix positivity was significantly increased compared to the non laser side (*P-value* = 0.005). ONN progressively stained the increased foci of osteoid and mineralized woven bone matrix as well as the lining osteoblasts in the osteotomy gap (Figures 2d,3).

Group III (3 weeks)

Three weeks after the start of consolidation, ONN reactivity reached the peak in this group for both non-laser and laser sides with the increase of the amount of woven bone in addition to the observed lamellar bone regeneration.

Non-laser left side: No or mild reactivity was elucidated in the more mature bone matrix areas while the less mineralized bone matrix illustrated a moderate diffuse ONN expression. More intense positivity was found in the new osteoid and newly mineralized foci, osteoblasts and large osteocytes. ONN was faintly or not expressed at the mineralization front (Figures 2e,3).

Laser right side: ONN staining was significantly increased compared to the non laser side (P-value = 0.001). Moderate to intense ONN positivity was presented in osteoblasts and large osteocytes. Mild to moderate diffuse expression was observed in the increasingly deposited woven bone in addition to the lack of reactivity in the increased areas of the more mature lamellar bone matrix (regenerate) (Figures 2f,3).

Group IV (4 weeks)

Four weeks after the initiation of consolidation, the relative decrease of ONN reactivity correlated with the apparent decrease of woven bone, enhanced the maturation of osteoblasts and osteocytes with the apparent increase of lamellar bone regeneration in this group compared to other groups.

Non-laser left side: Mild to moderate diffuse reactivity was noticed in the less mineralized bone matrix areas with the lack of ONN expression in the areas of the more mature lamellar bone matrix. Negatively stained osteocytes were mostly detected (Figures 2g,3).

Laser right side: ONN expression was significantly decreased compared to the non-laser side (*P-value* = 0.04). The lack of ONN reactivity was mostly illustrated in the more mature lamellar bone with negatively stained osteocytes. Nevertheless, mild diffuse reactivity was noticed in the areas of the less mineralized lamellar bone matrix (Figures 2h,3).

Among groups, ONN immunoexpression was increased in groupIII > groupII > groupI with significant differences (*P-value* < 0.001) in both sides except for the insignificant difference between groups I and II in the non-laser (*P-value* = 0.6) and laser (*P-value* = 0.62) sides. Regarding group IV, the ONN reaction decreased comparing to group III insignificantly (*P-value* = 0.086) in the non-laser side and significantly (*P-value* < 0.001) in the laser side. However, this staining still exhibited increased positivity compared to groups I and II with significant differences (*P-value* < 0.001) in the non-laser side while the laser side showed insignificant differences (*P-value* = 0.34) for group I and (*P-value* = 0.96) for group II (Figure 3).

Immunohistochemical and statistical results for osteopontin (OPN)

Group I (1 week)

Non-laser left side: After one week of consolidation, OPN expression was mild to moderate in some new osteoid and mineralized extracellular matrix areas besides the scattered fibroblast-like cells within the fibrovascular tissue of the distraction gap (Figures 4a,5).

Laser right side: A significant increase of OPN immunopositivity (*P-value* = 0.015) was demonstrated

compared to the non-laser side. Moderate to intense positivity was displayed in the apparently increased newly differentiated osteoblasts, surrounding areas of osteoid and the newly mineralized matrix of forming woven bone most probably in the microcolumn zone (Figures 4b,5).

Group II (2 weeks)

Non-laser left side: After two weeks of consolidation, moderate to intense reacted patches for OPN were elucidated in the unmineralized and increasingly in the mineralized matrix of the new woven bone islands. Variable existence of the OPN positive osteoblasts was detected in the three zones of the distraction gap (Figures 4c,5).

Laser right side: OPN expression was significantly increased (*P-value* = 0.003) compared to the non-laser side. In the callus region, OPN up-regulation was manifested in the matrix and the apparently increased bone cells of the apparently increased woven bone islands (Figures 4d,5).

Group III (3 weeks)

Akin to ONN reactivity, OPN expression reached the peak in both sides after 3 weeks of the consolidation beginning with the increase of the woven bone along with the lamellar bone formation (regeneration).

Non-laser left side: Wide areas of unmineralized/ mineralized woven bone matrix showed moderate to intense reaction and were deposited in the distraction gap and on the inner surfaces of the osteotomized bones. The apparently increased and intensely stained osteoblasts bordering the immature bone trabeculae as well as the enclosed positive osteocytes were seen. In addition, OPN reactive fibroblast-like cells were also observed in the wide bone marrow spaces. Few areas of regenerated lamellar bone with decreased OPN reactivity were also evident (Figures 4e,5).

Laser right side: Comparing to the non-laser side, OPN positivity was significantly increased (*P-value* = 0.002) in this side. OPN expression was enhanced in the apparently increased woven bone, osteoblasts and osteocytes. Also, the regeneration areas of mature lamellar bone which showed less OPN staining were increased (Figures 4f,5).

Group IV (4 weeks)

Four weeks after the start of consolidation, the decrease of OPN expression in this group was associated with the apparent increase of lamellar bone regeneration, an apparent decrease of woven bone, osteoblasts and osteocytes compared to other groups.

Non-laser left side: The moderate to intense stained woven bone was apparently decreased and was perceived close to the central interzone. Likewise, mild OPN expression was elucidated in the mature lamellar bone matrix areas. Intense reactions of the apparently decreased osteoblasts and osteoclasts were also noticed along with the large and small osteocytes of woven and lamellar bone respectively. Reacted discrete cement lines were found (Figures 4g,5).

Laser right side: The OPN positivity was significantly decreased (*P-value* = 0.009) compared to the non-laser side. The areas of the apparently diminished and immunoreacted woven bone in this side were found in the central interzone. However, the weakly reacted mature lamellar bone was apparently increased in the distraction gap. The further decrease of the intensely stained bone cells including osteoclasts was evident. Likewise, reacted cement lines were also presented (Figures 4h,5).

Among groups, OPN reactivity was increased in groupIII > groupII > groupI with significant differences (*P-value* < 0.001) among the three groups in both non-laser and laser sides. Regarding group IV, OPN staining in the non-laser side was significantly decreased (*P-value* <0.001) compared to groupIII but still showed increased positivity with significant differences for groupI (*P-value* < 0.001) and for groupII (*P-value* < 0.047). Concerning laser side group IV, the decreased OPN expression was significant (*P-value* < 0.001) compared to groupIII and insignificant (*P-value* = 0.69) in relation to group II but still revealed a significant increase (*P-value* < 0.001) when compared to groupI (Figure 5).

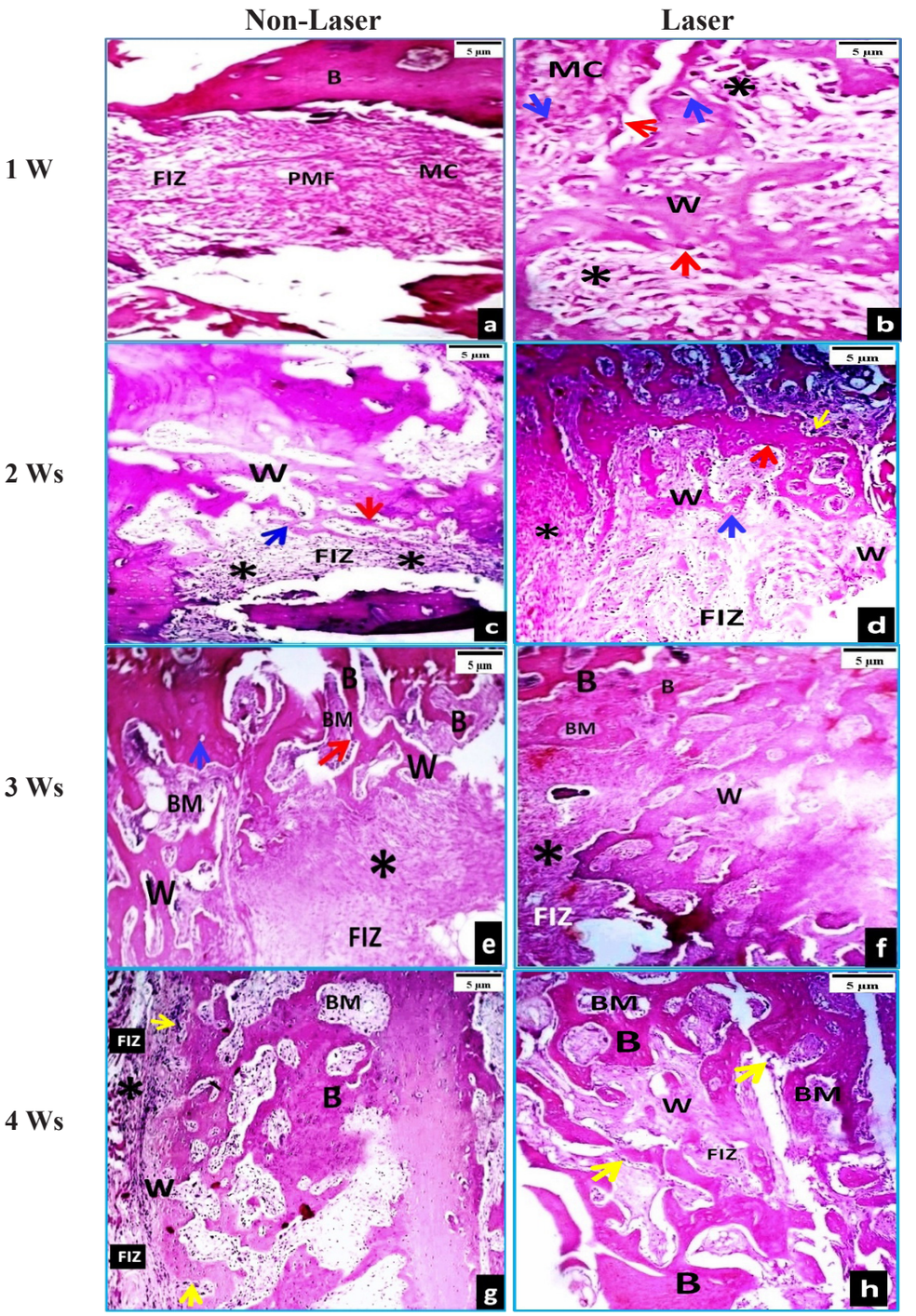

Fig. 1: Photomicrographs of mandibular distraction gap in rabbit, (a, c, e, g) laser side and (b, d, f, h) non-laser side; (a) groupI non-laser side showing the gap zones including central fibrous interzone (FIZ), primary matrix front (PMF) and microcolumn zone (MC) adjacent to osteotomized bone margins (B) (H&E, X 100), (b) groupI laser side showing expanded bone matrix around osteoblasts/osteocytes in microcolumn zone (MC) and few woven bone spicules with osteoblasts and large osteocytes (H&E, X 400), (c) groupII non-laser side showing fibrovascular tissue in FIZ and other gap zones with irregular woven bone trabeculae (H&E, X 100), (d) groupII laser side showing condensed fibrovascular tissue in FIZ, apparent increase of woven bone, wide marrow spaces, apparently increased osteoblasts, large osteocytes and few osteoclasts in other zones (H&E, X 100), (e) groupIII non-laser side showing dense fibrovascular tissue in central FIZ, woven bone with wide marrow spaces and areas of lamellar bone (H&E, X100), (f) groupIII laser side showing diminished FIZ, greater amount of woven bone, areas of lamellar bone (H&E, X100), (g) groupIV non-laser side showing much diminished FIZ, woven bone near/at the gap center, apparent increase of lamellar bone with relatively wide marrow spaces, apparent decrease of bone cells and areas of osteoclastic activity, (H&E, X100), (h) groupIV laser side showing few woven bone in the thin central FIZ, regular lamellar bone trabeculae with narrower marrow spaces, apparently decreased bone cells and osteoclastic activity areas in other zones (H&E, X100). Fibrovascular tissue (black asterisk), woven bone (W), lamellar bone trabeculae (B), osteoblasts (red arrows), osteocytes (blue arrows), osteoclasts (yellow arrows), bone marrow (BM).

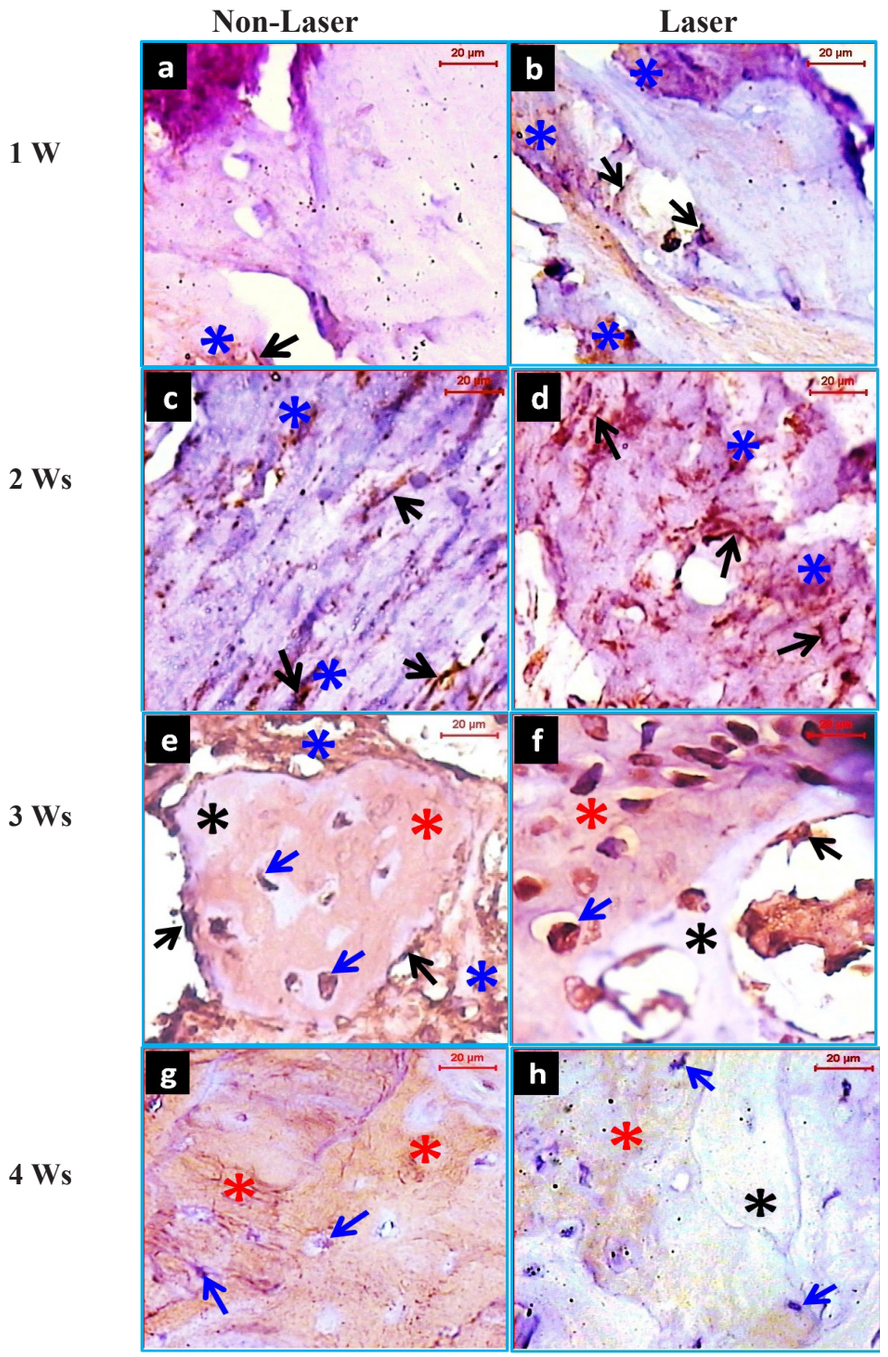

Fig. 2: Photomicrographs of mandibular distraction gap in rabbit showing the immunohistochemical reactivity to anti-osteonection, (a, c, e, g) laser side and (b, d, f, h) non-laser side; (a) groupI non-laser side showing intense fibrovascular tissue expression, mild reactivity of scarce osteoid and newly mineralizing areas, positive osteoblast-like cells (b) groupI laser side showing increased mild/moderate expression, (c) groupII non-laser side showing moderate/ intense positivity in string like osteoid areas, newly mineralized foci and lining osteoblasts, (d) groupII laser side showing apparent increase of the positive foci and osteoblasts, (e) groupIII non-laser side showing moderate expression in less mineralized woven bone and more intense in osteoid, newly mineralized foci, osteoblasts plus large osteocytes, none/mild expression in the more mature bone areas, none at the mineralization front, (f) groupIII laser side showing mild/moderate positivity in the apparently increased mineralized matrix areas, positive bone cells with no reactivity of the more mature bone matrix, (g) groupIV non-laser side showing decreased ONN positivity, mild/moderate in the less mineralized lamellar bone matrix, none in more mature matrix areas, negative osteocytes, (h) groupIV laser side showing down regulated ONN expression, none/mild in mature lamellar bone trabeculae with negative osteocytes, (DAB, X400). Osteoblasts (black arrows), osteocytes (blue arrows), newly mineralized matrix (blue asterisk), less mineralized matrix (red asterisk), more mature matrix (black asterisk).

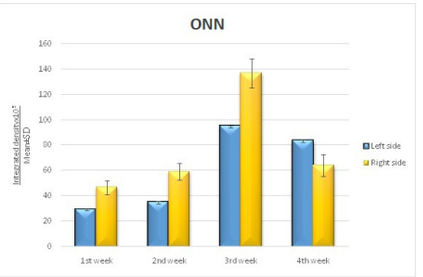

Fig. 3: Bar chart showing mean ±SD of ONN immunoreaction (Integrated density x10^5) among all studied groups.

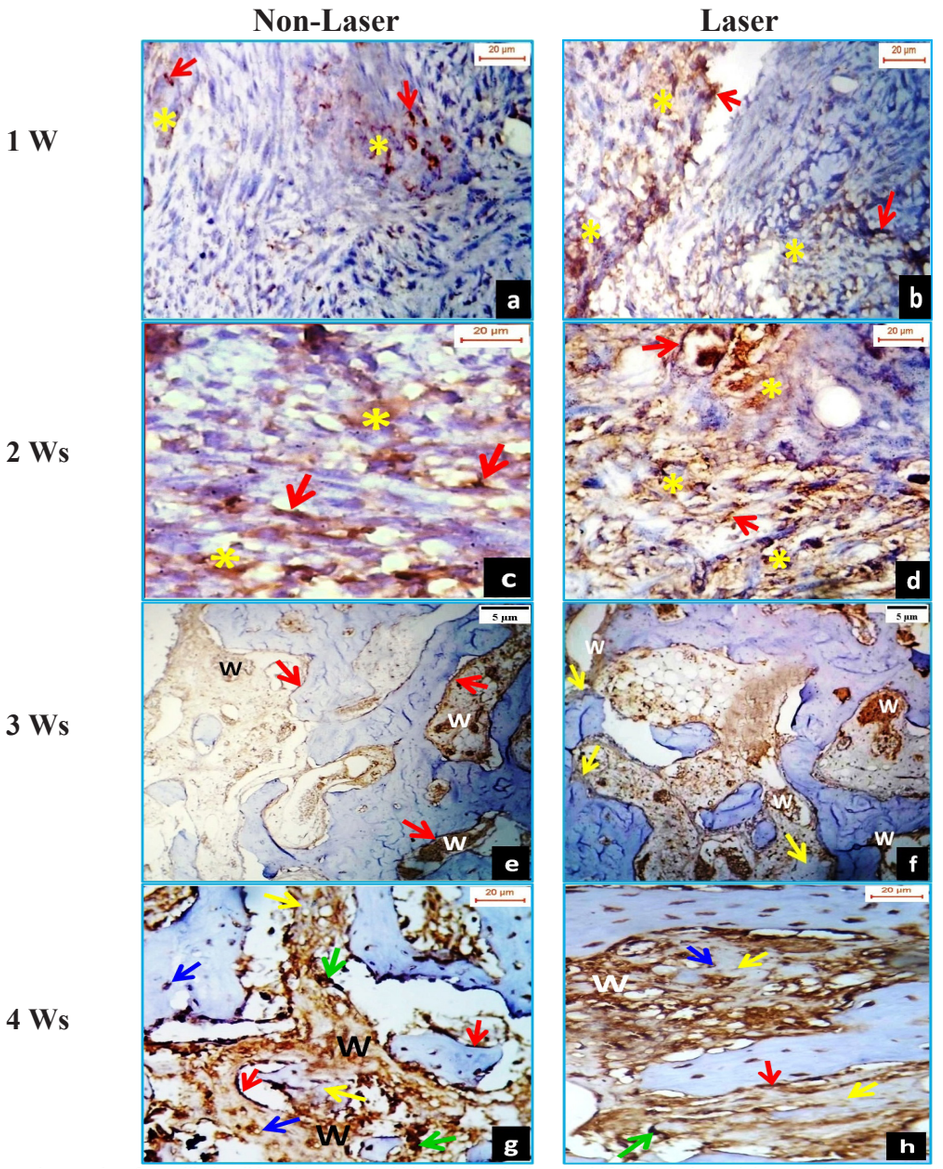

Fig. 4: Photomicrographs of mandibular distraction gap in rabbit showing the immunohistochemical reactivity to anti-osteopontin, (a, c, e, g) laser side and (b, d, f, h) non-laser side; (a) groupI non-laser side showing mild/ moderate positivity in new osteoid and mineralized marix areas besides fibroblast-like cells, (b) groupI laser side showing moderate/intense reaction in the increased newly differentiated osteoblasts and unmineralized/mineralized matrix areas, (c) groupII non-laser side showing islands of moderate/ intense reaction of unmineralized and mineralized woven bone matrix with positive osteoblasts, (d) groupII laser side showing OPN up-regulation in the apparently increased bone matrix islands and cells, (e) groupIII non-laser side showing moderate/intense reaction in wide areas of woven bone and in the apparently increased osteoblasts plus entrapped osteocytes, less reactive mature lamellar bone areas, positive marrow fibroblast- like cells, (f) groupIII laser side showing enhanced OPN expression in the apparently increased woven bone and cells, apparent increase of the less reactive mature bone areas, (g) groupIV non-laser side showing moderate/intense reaction of apparently decreased woven bone, osteoblasts and osteocytes, some osteoclasts, mild positivity of mature lamellar bone areas and discrete reacted cement lines, (h) groupIV laser side showing apparent diminish of positive woven bone, apparently increased weakly stained mature bone, reacted cement lines and further decrease of positive bone cells. (a, b, c, d, g, h; DAB,X400) (e, f ; DAB,X100). Osteoblasts (red arrows), osteocytes (blue arrows), osteoclasts (green arrows), unmineralized/mineralized woven bone matrix (yellow asterisk), woven bone (W), mature lamellar bone areas (yellow arrows)..

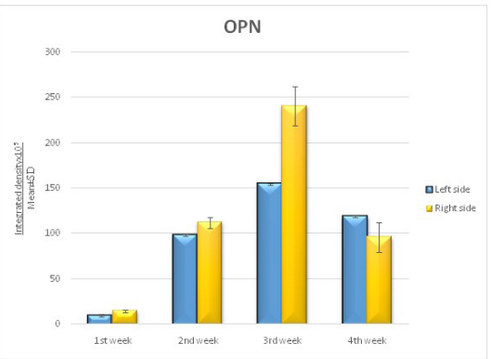

Fig. 5: Bar chart showing mean ±SD of OPN immunoreaction (Integrated density x10^5) among all studied groups.

DISCUSSION

Low-level diode laser (LLL) is a non-ionizing and non-invasive radiation. It possesses photochemical, thermal and non-linear promoting effects on soft and hard tissues[22]. An important concern regarding the distraction osteogenesis (DO) of the mandible is to maximally shorten the consolidation and total treatment time for best patient compliance. Rabbit model was used in this work due to the greatest compromise between its mandible size and cost in addition to having bone accrual patterns, mass profiles and skeletal maturity similar to those of humans[23]. LLL applied rabbits presented a greater degree of bone formation compared to other animals[9]. We attempted to estimate the therapeutic effect of the low-level laser during distraction osteogenesis on bone tissue regeneration histologically and on the changes of two osteogenesis related factors, osteonectin and osteopontin in bone tissue and cells immunohistochemically.

In consensus with the herein DO procedure, the disrupted integrity and vascularity occurred following osteotomy were restored during the latency period. This was achieved by the action of the hypoxia-inducible factor generated in the hypoxic environment which was created by the released lysosomal enzymes from the necrotic bony edges and soft tissues. The applied dynamic microenvironment and traction forces during callus distraction could trigger endothelial proliferation, angiogenesis, tissue oxygenation[4,10], fibroblastic proliferation and activity besides the osteogenic differentiation of mesenchymal cells[4,24]. DO is based on the Law of Tension-Stress which results in tissue regeneration[25], but its success depends on optimizing the distraction parameters such as stability, latency time, the rhythm and daily rate of distraction besides the consolidation time[4,26]. In harmony with the histological findings of group I, Bouletreau *et al*.[4] distinguished the gradual fibrous replacement of the formed hematoma, at the osteotomy time, throughout the first 7–10 days of DO with the initiation of osteogenesis in the distraction gap at the proximal and distal osteotomized bone edges. In rat studies, the distraction gap beyond seven days showed five active histomorphologic zones similar to our results[1].

In group I laser sections, the enhanced tissue repair together with the few woven bone spicules in the microcolumn zone could be explained by the accelerated resorption of hematoma and the significant suppression of the inflammatory responses by inhibiting the pro-inflammatory mediators including TNF-α, IL-6 and IL-1β[9,22]. Moreover, LLL could accelerate the initial phases of osteogenesis as it modulates the diverse metabolic activities existed during DO[16,17] via potentiating the proliferation of irradiated cells and increasing the protein synthesis[7,22]. The non-thermal and thermal laser influences enhance the growth factors generation[3], for example, the basic fibroblast growth factor that stimulates fibroblastic plus mesenchymal cells proliferation and differentiation along with collagen deposition[8,27]. Additionally, the vascular endothelial growth factor (VEGF) expression was also promoted by LLL providing endothelial proliferation and migration besides the formation of a capillary network[6,22]. At the subcellular level, these influences were achieved by the absorbing ability of the intracellular chromophores like cytochromes and porphyrins for light energy which was transformed into metabolic energy. Furthermore, the acceleration of mitochondrial metabolism, nuclear DNA and RNA synthesis , change of redox status by reactive oxygen species generation in small physiological amount, alteration of the membranous enzymatic activity like ATPase, enhancement of intracellular ATP levels and cell differentiation were all achieved in response to LLL[5,6,22].

In agreement with the H&E results of group II, the cyclic mechanical strain in the discontinuous DO was reported to induce structural alteration, proliferation, differentiation, migration and activity of the strained osteoblasts in a linear manner[1,14,25]. The subsequently formed bone microcolumns, along the tension vector, would grow towards the osteotomy gap center[4]. The levels of angiogenic factors and vascularization increased during early consolidation and decreased during its late phase suggesting that vasculogenesis preceded osteogenesis[25]. The histological outcomes of the laser side group II were akin to those of other studies[3,8]. LLL biostimulation for two weeks of consolidation potentiated more angiogenic and osteogenic activities involving matrix regeneration and mineralization in addition to intramembranous rather

than endochondral ossification in bone defects[3,9,17]. This would shorten the consolidation period and permit the earlier distractor removal after the interposed osteoid mineralization[3,16,17]. Numerous doses of laser irradiation were more effective for osteogenesis rather than the applied intensity[5]. Moreover, LLL enhances rhodopsin-kinase enzyme (photosensitive at definite wavelengths) and NF-kB transcription factor which induce other genes transcription as well as repair associated proteins[3]. After the LLL application, cyclo-oxygenase-2 as a repair-associated chemical mediator could escalate angiogenesis, regulate osteoblastic differentiation genes like osterix plus Cbfa1, enhance osteoblastic proliferation and also prevents cell apoptosis during the early osteogenesis in bone repair[5].

The histological findings of the non-laser side groupIII three weeks after the consolidation commencement (after the distractor removal) were confirmed by Djasim *et al*.[25] As they proved the increased biosynthetic activity of osteoblasts in this time period by the insignificant increase of osteoblastic numbers alongside the significant rise of mineral apposition rate. In this work, the bone regeneration was accomplished only by intramembranous ossification. This most probably occurred when the daily distraction rate not exceeded 2 mm and also indicated the stable fixation of the distractor[10]. Distraction strain promotes the production of growth factors plus cytokines by the strained osteoblasts and mechanosensor osteocytes such as nitric oxide signaling molecules that contribute to the strain distribution via cellular communications[4,14]. Concerning the mechanism of the observations in laser side group III, laser could enhance bone formation by decreasing osteoclastic numbers and activity and increasing the osteoblastic activity[6]. The increase of collagen plus non-collagenous proteins synthesis and alkaline phosphatase activity in the relatively mature osteoblasts motivated the bone regenerative process in response to LLL[4,6]. A significant positive correlation was found between bone volume and blood vessels quantity particularly in the central part of the regenerated bone, which is the youngest and last part to mineralize[25].

The H&E results of group IV non-laser and laser specimens 4 weeks after consolidation initiation (after the distractor removal) were akin to those of Taha *et al*.[8]. In concurrence, the released osteogenic cytokines in DO as BMPs and Runx2 could accelerate bone regeneration and inhibit its resorption[10,24] with the increase of their role during latency, distraction and early stages of consolidation. This role was returned to normal at 4 weeks after consolidation[28]. The signal transduction pathways of these cytokines have been activated by laser irradiation of bone cells[6]. In parallel to the apparently higher bone content of the osteotomy gap in the laser side group IV, Taha *et al*.[8]elucidated the prominent effect of laser therapy on the quantity and quality of the regenerated bone in DO via the increased intramembranous ossification with more mature bone trabeculae and more bone mineral density than that of the non-laser bone defects. Bone maturation and crystallinity were inversely related to the phosphate ions amount, which was lower in the LLL-treated specimens giving mature new bone with better quality and superior as well as homogenous crystallinity[16]. In contrary to laser results, the distraction strain could potentiate osteogenesis from early to maturity phases but with prolonged immature osteogenic phase, noticeable fluctuation of bone matrix composition and alterations in mineralization features[8]. Miloro *et al*.[3] confirmed that optimal bone stability in the distraction gap was detected during the 4th week of consolidation in the LLL-treated bone defects but was not reached until the 6th week in the non treated defects reflecting the more enhanced bone repair with LLL therapy thus evading the prolonged fixation.

We used both ONN and OPN markers to detect the changes of bone tissue and cells in the distraction gap secondary to the discontinuous DO and LLL treatment. The expression of ONN in the fibrovascular/granulation tissue in the distraction gap of the non-laser side groupI could be explained by Young *et al*.[29] who affirmed that ONN was produced by fibroblasts plus macrophages at the repair sites. Revealing a few areas of the mildly reacted new osteoid and mineralizing matrix along with the positive osteoblasts-like cells in this group may prove the relation of ONN tissue distribution to the strain-associated osteogenesis[11]. The secreted ONN by earlier osteogenic cells could regulate collagen fibrillogenesis plus configuration, matrix assembly and osteoblastogenesis-related transcription factors and osteogenic signals as TGFβ, Runx2 and BMP-2 through the p38 signaling pathway which in turn regulates the alkaline phosphatase activity. It also has a high affinity to collagen type I, calcium and hydroxyapatite confirming its role in the nucleation of the apatite crystals[30,31], mineralization initiation and regulation of the apatite crystals size and growth[4,11]. Additionally, the significant enhancement of ONN staining in the laser side coincided with its moderate to strong positivity in other studies in the newly differentiated cells and deposited osteoid preventing excessive mineralization[32,33]. It also binds to cytokines and growth factors in a Ca2 + dependent manner controlling cell-matrix plus cell-cell interactions, differentiation, proliferation, survival and migration of some cells, for instance, the VEGF for endothelial cells[29].

The ONN results exhibited a significant increase in the laser side compared to the non-laser side in group II with more significant increase in group III. In synchronization, ONN stained extracellular matrix in the distraction gap could mirror that the ONN functionality and reactivity could exceed the physiologic limit during the early consolidation phase of DO in the direction of the tension vector, confined to the centers of the mineralized bone matrix and hardly found at the mineralization front[11,28]. ONN reactivity was distinguished only during active osteogenesis, in active osteoblasts and young osteocytes[32,34]. Therefore, the upregulation of ONN mRNA and protein was enhanced with

intramembranous ossification[11] which was accelerated by laser irradiation[8,9]. The LLL-associated ONN upregulation was strongly related to the enhanced osteogenic signals[6], improved osteoblastic differentiation[28], recruitment and activity as well as the rapid collagen maturation increasing the surface areas for bone matrix deposition and mineralization according to Meyer *et al*.[11].

A significant decrease of ONN reactivity was illustrated in group IV, particularly in the laser side. In harmonization, Ishigaki *et al*.[31] affirmed that the mineralized bone matrix would lack ONN reactivity with progressive matrix mineralization. In contrast, some rat studies revealed that ONN forms a minor component of the mineralized matrix or may not be incorporated into the newly formed mineralized bone[31]. The lack of ONN reactivity in quiescent and aged osteocytes in their small spindle-shaped lacunae together with the abovementioned reactiveness of young osteocytes in their large round lacunae and other osteogenic cells confirmed that the ONN expression reflected the gradual functional differentiation of osteogenic cells[32,34].

In parallel to the OPN results in group I where the laser side showed a significantly increased expression compared to the non-laser side, the fibroblast-like cells within the distraction fibrous area in other reports exhibited intermittent low OPN levels after 1 week of consolidation that was accelerated in the laser-irradiated cells[1,6]. The OPN expression in the extracellular matrix of each callus zone proved its distinct function in each zone. It was deposited in the bone matrix earlier to mineralization. The localized discrete foci of OPN were intracellularly cytoplasmic in the mature osteoblasts and perimembranous in the less differentiated migratory cells (from central fibrous interzone to the primary matrix font and/or to other locations). Its binding to the cell surface integrins, in the proliferative front, for example, could activate numerous signaling cascades increasing cell proliferation, migration, adhesion and survival in DO. The initially decreased OPN expression was directly related to the increased cell proliferation; hence it can act as a negative regulator of osteogenic cells proliferation and differentiation at both early and late stages of osteogenesis. It can also prevent the premature mineralization of the matrix that would entrap osteoprogenitors by inhibiting apatite crystals formation[1,33,35]. At the proliferation front/ microcolumns leading edge, the decreased OPN expression by post-proliferative osteoblasts has been crucial for mineralization commencement of new bone columns[1,33]. A significant increase of OPN reactivity in the laser subgroup in correlation to the non-laser one was presented in group II with further significant increase in group III. The intensely OPN reacted patches in the newly formed woven bone islands (not as mineralized as lamellar bone) in group II was enhanced to be bulky amount of OPN in the apparently increased new bone tissue and active cells in group III akin to Morinobu *et al*. study[12]. In parallel, the OPN concentration in these time periods from consolidation start was least in the central fibrous interzone and greatest in the intramembranous primary matrix front and forming microcolumns particularly with the commencement of maturation of the new bone and proximity to the osteotomized bone edges[1,12]. Referring to Irie *et al*.[36], these OPN patches corresponded to the large integrated amount of OPN among the loosely arranged collagen fibers and matrices in the new osteoid and mineralized foci of the woven bone in early mineralization[36]. The generated OPN mRNA and protein by the newly differentiated and immediately embedded osteoblasts were found to be increased by mechanical strain[1] and further amplified by the LLL irradiation of bone cells[6,7]. OPN could bind to other matrix components[1,31], mediate the mechanical strain signaling to osteoblasts[12] and support the rapid intramembranous osteogenesis during DO[1]. However, the OPN distribution would change with the less packing density of collagen, speed of osteogenesis and mineralization degree, all accelerated by laser radiation as least OPN reactions were found on the rich mineralized collagen fibers[6,7,36]. Thus, its biphasic expression during intramembranous ossification was exhibited at a peak related to cell proliferation and differentiation while the other peak occurred with matrix mineralization. The latter peak can be accomplished by posttranslational modifications or enzymatic cleavages of OPN that limit its initial inhibitory role in premature crystallization and prevent the interference with the later stages of bone maturation[1].

The decreased OPN positivity in the distraction gap of group IV specimens that was significant in the laser subgroup compared to the non-laser one could be ascribed to the apparently increased mature lamellar bone which displayed less integrated OPN in the closely packed collagen fibers as stated in other studies. In synchronization, the OPN mRNA and protein expression was reported to gradually decrease with the slower osteogenesis rate in this time period and fade with bone remodeling. Additionally, OPN is also involved in the osteoclastic migration and cell-matrix adhesion that aid in bone resorption and remodeling[36,37].

What is more, the different matrix composition around osteoblasts and osteocytes designated their dissimilar relationship with the surrounding matrix. Osteoblasts interact with the surrounding proteins and minerals in the first mineralization phase while crystals maturation and matrix remodeling are achieved via the osteocytic network in the secondary phase thus regulating the ONN and OPN production and function[13,33].

Regarding the statistical results of the herein groups, OPN results reinforced and almost simulated ONN results. A significant increase of ONN and OPN expressions was detected in groupIII > groupII > groupI but with an insignificant difference between groups I and II for ONN. The decrease in both ONN and OPN positivity in group IV was significant compared to the laser side group III in particular but with the still detected increased reactivity in relation to most subgroups of both groups I and II with

variation in the significance levels. These findings were consistent with other OPN[36,37] and ONN[28] studies. Yet regarding ONN, Kim *et al*.[28] illustrated that the gradual decrease of the ONN gene expression from its peak began after 2 weeks of the consolidation onset unlike the herein decrease of the ONN protein expression which began after 3 weeks in both laser and non-laser sides.

CONCLUSION AND RECOMMENDATIONS

We conclude that the intramembranous ossification was the regenerative process of the herein distraction gap in the rabbit mandible. This process was more active beyond the 7th day of consolidation almost around the 21st day despite the earlier removal of the distractor but with the continued LLL application. LLL afforded higher cellular differentiation and activity, ossification, maturation and amount of the regenerated bone than the unescorted conventional DO procedure across all time periods of this study thus reducing the consolidation time. Hence, LLL can be a convenient adjuvant therapy that fastens tissue regeneration including hard tissues via the bio-stimulatory impact of laser on different cell types such as bone cells.

DATA AVAILABILITY

"All data underlying the results are available as part of the article and no additional source data are required."

All authors shared equally in this work (A-F)

A – Research plan and design.

B – Collection and/or assembly of data.

C – Practical work.

D – Data analysis and interpretation.

E – Writing the article.

F – Critical revision and final approval.

ABBREVIATION

DO: Distraction osteogenesis; **LLL:** Low level laser; **ONN:** Osteonectin; **OPN:** Osteopontin.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تقييم التأثير العلاجي لليزر ديود على خلايا العظم في استطالة العظم الهابط للفك السفلي: دراسة تجريبية فى ذكر الأرنب البالغ

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نبذة مختصرة: ارتبط استخدام جهاز الشداد العظمي داخل الفم بعيوب مختلفة مما أدى إلى إزالته وانتكاسة العلاج في الوقت الغير المناسب .

الهدف من التجربة: هدفت هذه الدراسة إلى تقدير التأثير العلاجي لليزر ديود (منخفض الطاقة) على خلايا العظام في استطالة العظم الهابط للفك السفلي في نموذج الأرانب.

التجربة: تم تقسيم ٢٨ من الأرانب البالغة مقسمة بالتساوي إلى ٤ مجموعات. تعرضت الحيوانات للاستئصال القشري بالجانب الايسر والايمن للفك السفلي مع تثبيت جهاز الشداد العظمي.أُعتبر الجانب الأيسر كجانب غير الليزر (مجموعة فرعية) بينما كان الجانب الأيمن كجانب الليزر (مجموعة فرعية). بعد ٣ أيام كفترة كُمُون ، ٧ أيام من فترة التنشيط للجهاز (٠,٥ مم / ١٢ ساعة) للوصول إلى حد التوسع ٧ مم ، تمت إزالة جهاز الشداد العظمي بعد أسبوعين من فترة الدمج (الاندمال). تم معالجة جانب الليزر بـ ١٠جول/سم٢ لكل نقطة كل ٤٨ ساعة منذ فترة الدمج (الاندمال). تم التضحية بحيوانات المجموعات الأربع بعد ١ و ٢ و ٣ و ٤ أسابيع بعد بدء الدمج (الاندمال) على التوالي. تمت معالجة انصاف الفك السفلي التي تم تشريحها للفحص الهستولوجي الروتيني وللفحص المناعي باستخدام الأجسام المضادة لـكلاً من الاستيونيكتين و الأُستيوبونتين .

النتائج: هستوباثولوجياً، كانت الزيادة الظاهرة لكمية العظام الجديدة أعلى في مجموعة الليزر الفرعية في جميع الفترات الزمنية. بينما من الناحية الكيميائية المناعية ، زاد تعبير كلا من الاستيونيكتين والاستيوبونتين بشكل ملحوظ في نسيج وخلايا عظم المجموعة الثالثة> المجموعة الثانية> المجموعة الأولى ولكن مع اختلاف بسيط بين المجموعة الأولى والثانية في حالة الاستيونيكتين. اظهرت المجموعة الرابعة انخفاضًا في التفاعل لكلا من الاستيونيكتين والاستيوبونتين والذي كان ملحوظاً مقارنةً بجانب الليزر في المجموعة الثالثة على وجه الخصوص ولكن مع الزيادة الواضحة في الإيجابية لكلا من الاستيونيكتين والاستيوبونتين مقارنة بمعظم المجموعات الفرعية من المجموعتين الأولى والثانية مع التباين في الدلالة الاحصائية.

الاستنتاج: أظهر تطبيق الليزر منخفض المستوى ديود (LLL) اثناء الشد العظمي تمايز خلوي وتحجر وتجديد عظام فائق في استطالة العظم الهابط للفك السفلي عن إجراء الشد العظمي التقليدي غير المصحوب بالليزر وبالتالي تقليل فترة الدمج (الاندمال). لذلك ، فإن الليزر يدعم تجديد الأنسجة من خلال تأثير التحفيز الحيوي لليزر على الخلايا