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Orthodontic Tooth Movement Activated By Laser and VEGF in Rabbets By Using Immuonhistochemical Application

KEYWORDS

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ABSTRACT *Immunohistochemical study for osteoblastogenesis and osteoclastogenesis include the followings*

Evaluation of the expression of tissue non specific alkaline phosphatase (ALPs) by osteoblast.

Evaluation of the expression of collagen type 1

Evaluation of the expression of macrophage colony-stimulating factor (M-CSF) toassessment for dental tissue response includes(periodontalligament, cementum,alveolar bone) to application of LLLT and VEGF in orthodontic treatment in experimental rabbits.

Introduction :

In an effort to meet the demands to increase the rate of orthodontic tooth movement, researchers have investigated the ways of orthodontic tooth movement following phototherapy using low level laser therapy as a recent method to accomplish treatment objectives in the timeliestmanner possible (Olson C. *et al*,.2012). Most of the investigators conclude that low level laser therapy (LLLT) can have an accelerator effect on tooth movement within a given range of parameters, but additional experimental data is needed (Yozgatian J. H. *et al.*, 2009).

Many questions have been raised; If LLLT does indeed increase the velocity of tooth movement, what are the ideal parameters? and/or are some subjects more susceptible to biostimulation than others? What are the risks associated with the procedure? (Yozgatian J. H. *et al.*, 2012).

Orthodontic tooth movement occurs in the presence of a mechanical stimuli sequenced by remodeling of the alveolar bone and periodontal ligament (PDL). Bone remodeling is a process of both bone resorption on the pressure site and bone formation on the tension site (Miaman) Orthodontic tooth movement can be controlled by the size of the applied force and the biological responses from the PDL (Meikle MC. 2006). The force applied on the teeth will cause changes in the microenvironment around the PDL due to alterations of blood flow, leading to the secretion of different inflammatory mediators such as cytokines, growth factors, neurotransmitters, colony-stimulating factors, and arachidonic acid metabolites. As a result of these secretions, remodeling of the bone occurs (Davidovitch Z. *et al.*, 1988; Krishnan V. and Davidovitch Z. 2006).

Vascular endothelial growth factor (VEGF)

This protein is a member of the PDGF/VEGF growth factor family and encodes a protein that is often found as a disulfide linked homodimer. This protein is a glycosylated mitogen that specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis(Teixeira C. Khoo K. Tran J.*et a.*,2010). This cytokines is involved in tissue neoformation that is strictly correlated with the presence of blood vessels. During orthodontic tooth movement, compressive forces induce angiogenesis of periodontal ligament together with the role of mediator of the VEGF. The localization of VEGF was analyzed in vivo in rat periodontal tissues during experimental tooth movement. In this analysis, 15 male Wister rats were used(Miyagawa*M.et al.*,2009;Teixeira C.*et al.*,2010).

Materials & Methods

Study Design: Seventy healthy male New Zealand -white rabbits, weighing (2.125 kg ± 0.375) aged 14-16 weeks were used for this experiment. The rabbits were kept in the animal department of (National Center for Drug Control and Research /Baghdad-IRAQ) in separate cages in a12:12 hour light/dark environment at a constant humidity and temperature of 23°C according to the National Research Council's guide for the care and use of laboratory animals and accessed to drinking water and libitum and standard laboratory rabbits pellets and green food.

Study population

Because of some rabbit have abnormality in M CI and the death some of them the actual number remaining are fifty four rabbits. The rabbits will be enumerated from one to fifty four as convenient samples. According to the local application of protocol the experimental rabbits will be grouping according to their serial number will be divided into followings

Experimental group (n=60) includes

- Orthodontically treated plus LLL therapy during orthodontic movement (n=15).

- Orthodontically treated plustapicalapplication of 0.1µm of VEGF (n=15).

- Orthodontically treated with a combination of LLL thera-

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pyand 0.1µm VEGF (n=15).

Control group orthodontically treated only (n=15).

- For each group the application days will be at (7^{th} $14^{th}$$21^{th}$)

The rabbits were sacrificed at end periods of application humanity with inhalation anesthesia

2.2. Study Setting : By permission from Al-Neelain University – College of postgraduate Studies,

Ethical clearness; we get the approval of laser institute for higher degrees /University of Baghdad for the application and uses of laser device. Thehistopathological study and immunohistochemical study in college of dentistry /University of Baghdad.

Study area;dioid laser application at laser institute for higher degrees /University of Baghdad the histopathological study and immunohistochemical study in college of dentistry /University of Baghdad.

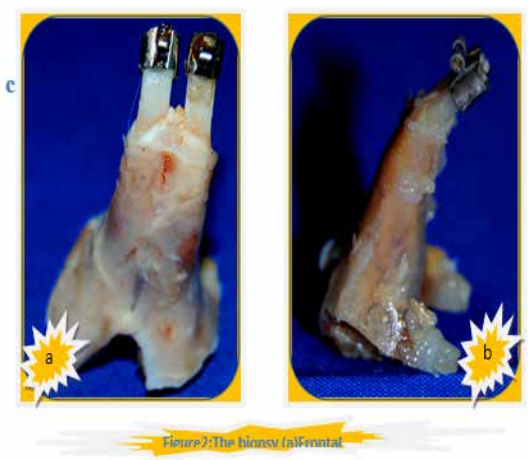

Figure2:The biopsy (a)Frontal

Orthodontic Appliance Design and LLL therapy
The orthodontic appliance consists of orthodontic bands, arch wires, and NiTi open-coil spring. The bands were customized for each rabbit. Briefly, the animals were anesthetized with general anesthesia, induced by an intramuscular injection of a ketamine (50 mg/ml) at a dose of 50mg/kg body weight and muscle relaxant Orbarcaine 2% at a dose of 5mg/kg body weight. The two drugs were mixed at the ratio of 2:1 (Ketamine: Orbarcaine), impression for mandibular central incisors (MCIs) of each rabbit was taken first with silicone material ,study stone models of the MCIs and the surrounding region were made ,which used for preparing of individual resin trays for each rabbit; that then used to take precise final impressions with alginate material and the master stone models .Orthodontic bands were prepared to fit the teeth sizes ,using band strips(Dentaurum-Germany) and then welded under pressure by using of Welder device**.** Then a round buccal tube with wings welded to the hand made bands in a horizontal direction and used as labial tube**.** The bands were cemented to its co-related MCIs after the removing of the orthodontic elastic separator, so that the superior border of the cemented bands was 3mm away from the incisal edge to allow for wear of the teeth and the lower border about 2mm away from the cervical area to avoid a trauma of the surrounding tissue.Orthodontic-tooth movement was generated by the insertion of a stainless steel arch wire with diameter of 0.016" and 15 mm in length through the labial tubes and the NiTi open-coil spring(ORTHO. TECHNOLOGY USA) with 3-4 mm in length (about 4-6 coils) was inserted along the arch wire from the non-bend end and subjected to constrict pressure with tucker in order to be inserted between the labial tubes, so that it will apply a pushing force on both MCIs (in distal direction) with a total orthodontic force of (100 gm)divided into two teeth so that each incisor receive a light continuous force of (50gm) according to **Proffitet *al.*** [6]**.**This force was measured by pressure-gauge (CORBLX , Dentarum –Germany). Two coils at both ends of the arch wire were made in one plan **,**and it serves as stopper for the arch wire and as non-traumatic end. Experimental tooth movement was conducted for 21 days. The experimental group (A) was received the LLL therapyat 810 nm, with an output of 250 mW, and exposure of 20 s for each 7 days. While the experimental group(B) was received the0.1µm of VEGF in pressure side and about 0.2 mm subgingivally,for each 7 day.

Experimental group (C) treated with a combination of LLL therapyand 0.1µm VEGF and for each 7 days.

RESULT;Immunohistochemical findings for expression of Alkaline phosphatase (ALP).
Localization of positive expression of **ALP** and the cells that associated with their immune reaction ,were studied in control, laser, VEGF and combination groups.

Immunoreactions views for **laser group** show positive expression of ALP by fibroblast cells and osteoclast ,figure(1,2).

For VEGF group immunoreactions photograph show positive expression of ALP by interstitial tissue in compressed periodontal ligament, figure(3,4).

Immunoreactions photograph for **combination group**(compressed site) shows positive expression of ALP by cementoblast cells),osteoclast and progenitor cell, figure(5)

Statistic analysis for Expression of Markers in working sideamong studied groups .

Results of statistics evaluation of Positive Expression of 3 markers /Mean of positive cell No/mm2 at the working Side of studied groups at 3 intervals with comparisons significant were listed in the tables(1; 2;3. 45; 6; 7; 8; 9)and as followings:

1. Collagen I
At the end of 1^{st} week , 2^{nd} week and 3^{rd} week the results show that combination group records a high expression of collagen I (36.8± 1.14 ; 33.3 ± 1.17; 14.7 ± 0.42 ,respectively)

followed by laser ,M ± SD (35.8± 0.70; 26.8±1.28; 10.7±0.80 ,respectively) and with a high significant value in comparison to control.

2. M-CSF
At the end of 1^{st} week , 2^{nd} week and 3^{rd} week the results show that combination group records a high expression of M-CSF (9.8±0.60; 9.2±0.48; 8.0± 0.26 ,respectively)

followed by laser ,M ± SD (8.2± 0.31; 7.7±0.42; 5.5±0.34 ,respectively) and with a high significant value in comparison to control.

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3. ALP

At the end of 1st week and 3rd week the results show that combination group records a high expression of ALP (9.0±0.37 ; 7.5± 0.43 ,respectively) ,followed by laser ,M ± SD (7.2± 0.40; 6.2±0.31 ,respectively) and with a high significant value in comparison to control.

While the end of 2nd week shows a high expression of ALP by laser group , M ± SD (19.0± 1.13)

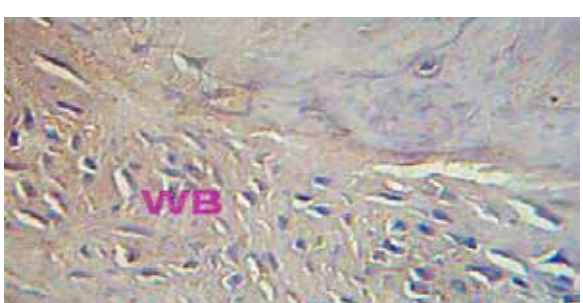
followed by combination group (18.3± 0.61)

Figure(4) shows Stem-Leaf Plots for Expression of Markers in working sideamong studied groups at different periods.

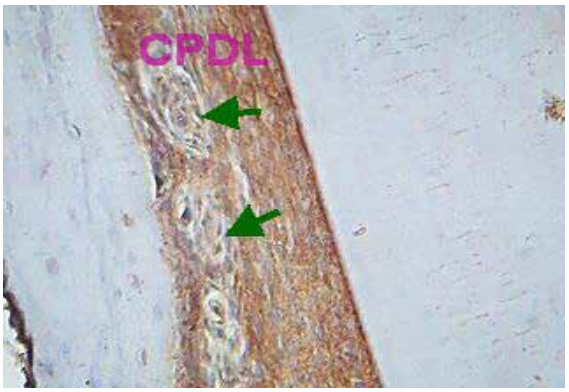
Table(1) and figures(6,7,8) show the Area under the curve (ROC) for Expression of Markers in working sideamong studied groups at different periods (1st week,2nd week and 3rd week).A high record for the area (0.472) was reported toM-CSF marker at the 2nd week.

Results also show high sensitivity(100) and specificity (100;94.4) for collagen marker in all three periods.

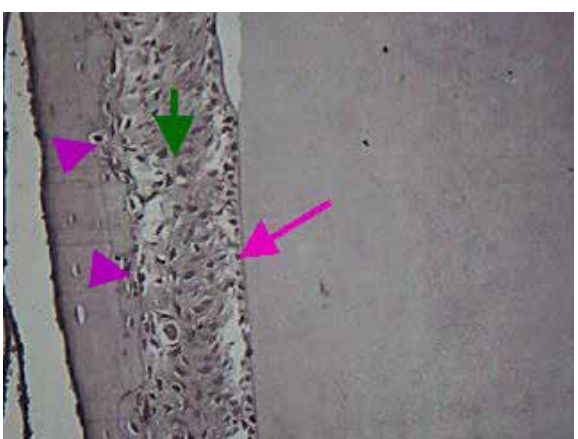
Figure(1) Immunoreactions photograph for laser group shows positive expression of ALP by fibroblast cells (arrow heads),osteoclast(arrow).DAB stain ×40.



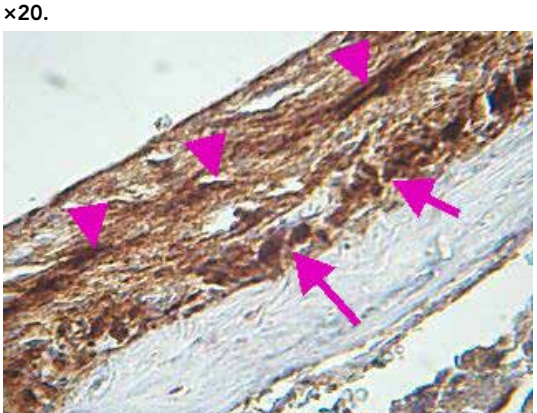
Figure(2) Positive expression of ALP by woven bone cell(WB). DAB stain ×20.



Figure(3.) Immunoreactions photograph for VEGF group shows positive expression of ALP by interstitial tissue (arrows) in compressed periodontal ligament(CPDL). DAB stain ×20.



Figure(4) Immunoreactions photograph for combination group(compressed site) shows positive expression of ALP by cementoblast cells (pink arrows),osteoclast(pink arrow heads),progenitor cell(green arrow).DAB stain ×20.



Figure(5) Positive expression of ALP by osteoblast cells(arrows),fibroblast(arrow heads). DAB stain

Expression of Markers in working side

Table (1): Descriptive statistics of Positive Expression of Collagen /Mean of positive cell No/mm2at the working Side of studied groups at 1 week interval with comparisons significant

Descriptive Statistics / Marker/Mean	Studied groups			
	Control	Lazer	VEGF	Combination

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Collagen/Mean of positive cell No/ mm2	3.5	35.8 a*** b***	10.8a***,	36.8a***, bNS, c***
± SD	0.34	0.70	0.31	1.41

aANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

bANOVA test: VEGF and Comb.vsLazer***p<0.001 , NS: Not

cANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

Table (2): Descriptive statistics of Positive Expression of M - CSF /Mean of positive cell No/mm2at the working side of studied groups at 1 week interval with comparisons significant

Descriptive Statistics	Studied groups			
Marker/ Mean	Control	Lazer	VEGF	Combination
M - CSF / Mean of positive cell No/mm2	4.2	8.2a*** b***	6.0a***,	9.8a***, bSc***
± SD	0.17	0.31	0.52	0.60

aANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

bANOVA test: VEGF and Comb.vsLazer***p<0.001 , S: **p<0.05

cANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

Table (3): Descriptive statistics of Positive Expression of ALP/Mean of positive cell No/mm2at the working Side of studied groups at 1 week interval with comparisons significant

Descriptive Statistics	Studied groups			
Marker/ Mean	Control	Lazer	VEGF	Combination
ALP/Mean of positive cell No/ mm2	3.5	7.2a*** bNS	6.0a***,	9.0a***, bS c***
± SD	0.34	0.4 0	0.26	0.37

aANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

bANOVA test: VEGF and Comb.vsLazerS: **p<0.05, NS: Not

cANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

Table (4): Descriptive statistics of Positive Expression of Collagen /Mean of positive cell No/mm2at the working Side of studied groups at 2 week interval with comparisons significant

Descriptive Statistics	Studied groups			
Marker/ Mean	Control	Lazer	VEGF	Combination
Collagen/ Mean of positive cell No/mm2	9.2	26.8a*** b***	14.8a***,	33.3a***, bS c***
± SD	0.54	1.28	0.75	1.17

aANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

bANOVA test: VEGF and Comb.vsLazer***p<0.001 , S:**p<0.05

cANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

Table (5): Descriptive statistics of Positive Expression of M - CSF /Mean of positive cell No/mm2at the working side of studied groups at 2 week interval with comparisons significant

Descriptive Statistics	Studied groups			
Marker/ Mean	Control	Lazer	VEGF	Combination
M - CSF / Mean of positive cell No/mm2	2.8	7.7a*** b***	5.7a***,	9.2 a***, bSc***
± SD	0.48	0.42	0.49	0.48

aANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

bANOVA test: VEGF and Comb.vsLazer***p<0.001 , S: **p<0.05

cANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

Table (6): Descriptive statistics of Positive Expression of ALP/Mean of positive cell No/mm2at the working Side of studied groups at 2 week interval with comparisons significant

Descriptive Statistics	Studied groups			
Marker/ Mean	Control	Lazer	VEGF	Combination
ALP/Mean of positive cell No/ mm2	5.2	19.0a*** b***	12.2a***,	18.3a***, bNS c***
± SD	0.31	1.13	0.65	0.61

aANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

bANOVA test: VEGF and Comb.vsLazer***p<0.001, NS: Not

cANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

Table (7): Descriptive statistics of Positive Expression of Collagen /Mean of positive cell No/mm2at the working Side of studied groups at 3 week interval with comparisons significant

Descriptive Statistics	Studied groups			
Marker/ Mean	Control	Lazer	VEGF	Combination

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Collagen/ Mean of positive cell No/mm2	3.5	10.7[a***] [b***]	6.8[a***],	14.7[a***],[b***] [c***]
± SD	0.34	0.80	0.48	0.42

[a]ANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

[b]ANOVA test: VEGF and Comb.vsLazer***p<0.001

[c]ANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

Table (8): Descriptive statistics of Positive Expression of M - CSF /Mean of positive cell No/mm2at the working side of studied groups at 3 week interval with comparisons significant

Descriptive Statistics	Studied groups			
Marker/ Mean	Control	Lazer	VEGF	Combination
M - CSF / Mean of positive cell No/mm2	1.8	5.5[a***] [b***]	3.7[a***],	8.0[a***], [b***c***]
± SD	0.31	0.34	0.21	0.26

[a]ANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

[b]ANOVA test: VEGF and Comb.vsLazer***p<0.001

[c]ANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

Table (9): Descriptive statistics of Positive Expression of ALP/Mean of positive cell No/mm2at the working Side of studied groups at 3 week interval with comparisons significant

Descriptive Statistics	Studied groups			
Marker/ Mean	Control	Lazer	VEGF	Combination
ALP/Mean of positive cell No/ mm2	2.8	6.2[a***] bS	5.2[a***],	7.5[a***],[b***] [c***]
± SD	0.17	0.31	0.31	0.43

[a]ANOVA test: Lazer,VEGF and Comb.vs Control group: ***p<0.001 Significant.

[b]ANOVA test: VEGF and Comb.vsLazer***p<0.001, S:p<0.05

[c]ANOVA test: Combination vsVEGF: ***p<0.001, SD: standard of deviation.

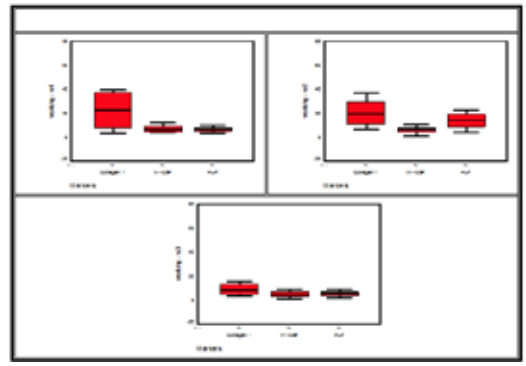

Figure6: Stem-Leaf Plots for Expression of Markers in workingsideamong studied groups atdifferent sources of variation

Table 10: Area under the curve (ROC) for Expression of Markers in workingsideamong studied groups atdifferent sources of variation

Area Under the Curve								
Test Result: Compressed Side –at diff. intervals								
weeks	Contrasts	Area	Std. Error	Asymptotic Sig.	95% C.I. L.B.	95% C.I. U.B.	Sen.	Spec.
W1	Collagen 1	0.000	0.000	0.000	0.000	0.000	100	100
	M - CSF	0.056	0.047	0.001	-0.037	0.148	100	88.9
	ALP	0.028	0.033	0.001	-0.038	0.093	100	94.4
W2	Collagen 1	0.000	0.000	0.000	0.000	0.000	100	100
	M - CSF	0.472	0.135	0.841	0.207	0.737	100	5.6
	ALP	0.000	0.000	0.000	0.000	0.000	100	100
W3	Collagen 1	0.028	0.033	0.001	-0.038	0.093	100	94.4
	M - CSF	0.278	0.104	0.110	0.073	0.482	100	44.4
	ALP	0.139	0.074	0.009	-0.007	0.284	100	72.2

Cutoff Point (5) at w1; Cutoff Point (10) at w2; Cutoff Point (5) at w

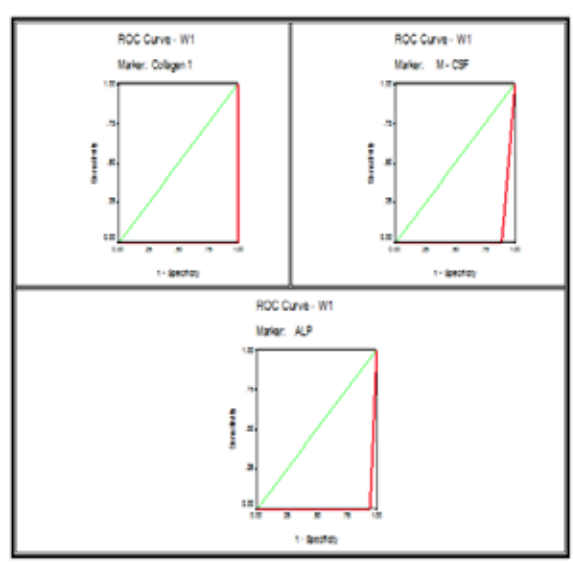

Figure 7: Receiver Operating Characteristic (ROC) curve of different Markers in workingside in contrast of control group at first week

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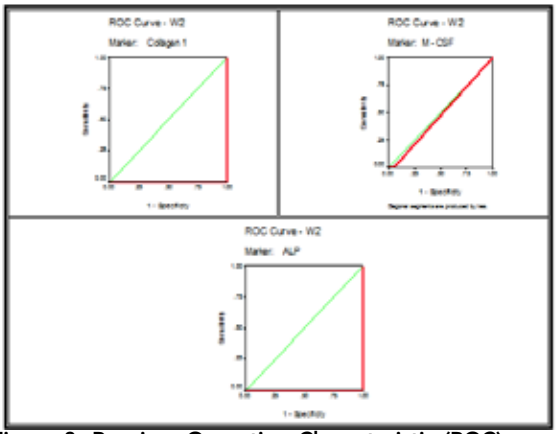

Figure 8: Receiver Operating Characteristic (ROC) curve of differentMarkers forworkingside in contrast of control group at second week

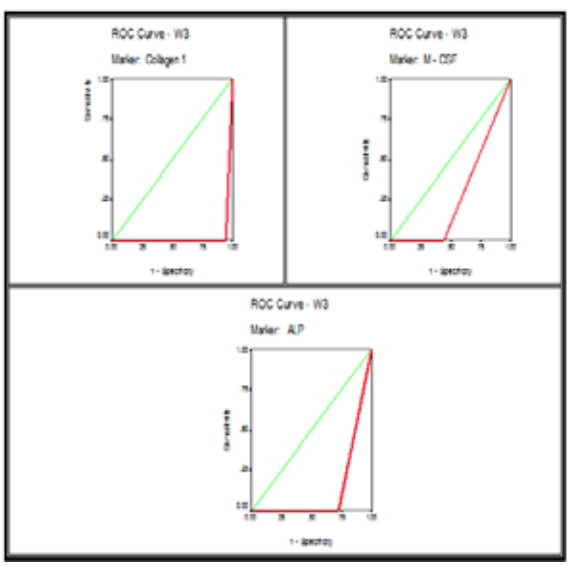

Figure9: Receiver Operating Characteristic (ROC) curve for different Markers forworkingside in contrast of control group at third week

Discussion;

Semiconductor diode lasers are compact and have a high conversion efficiency from electrical energy to laser energy.In the present study the laser probe was held perpendicular for 20 seconds in direct contact with the alveolar mucosa on each five points ,in order to allow LLL penetrate soft and hard tissues from 3 mm to up to 15 mm. As the energy penetrates tissues, there is multiple scattering by both erythrocytes and microvessels. Because of this, both blood rheology and the distribution of microvessels in the tissue influence the final distribution pattern of laser energy**(Medrad*et al.*,2003).**

Vascular endothelial growth factor therapy

The first stages before the orthodontic tooth movement are characterized by an acute inflammatory that appeared in the periodontium and involves the vasodilatation of capillaries which allows the migration of leucocytes in the periodontal tissue, where they are induced by biochemical signals to synthesize and to secrete several proinflammatory cytokines and chemokines, growth factors and enzymes**(Römer *et al.*, 2014)**.Therefore, and up to above knowledge the present research was designed to use experimental group with orthodontic appliance that received 0.1µm of VEGF in working side as VEGF is a biological active substance that is expressed by cells within the periodontium in response to mechanical stimuli from orthodontic appliances with several possible of other biomarkers representing biological modifications during specific phenomena of inflammatory process**(d'Apuzzo*et al.*,2013)**.

Immunohistochemical Finding For Collagen I

The present results show that combination group records a high expression of collagen I and for all studied periods ,followed by laser group and then VEGF group, and with high significant differences in comparison with control group .These findings could be explained as follows:

1.According to explanation that was done by**HU *etal.*,(2010),**

when an osteogenic enhancer material or light is used, it activates aseries of cellular events, including chemotaxis of pluripotentialmesenchymal cells into the defect site, differentiationof these cells into chondroblasts and osteoblasts, and the population of thenew bone with bone marrow elements. Osteoblasts playa major role in bone formation and produce type I collagen,which is the major organic component of the mineralizedbone matrix. Osteoblasts also express many kindsof noncollagenous proteins, and these include osteocalcin(OC) osteonectin (ON), bone sialoprotein (BSP), and osteopontin(OPN). All of these are acidic proteins that havea strong affinity for hydroxyapatite and most of theseproteins bind calcium ion.

2.The present findings suggest that cell contact with precise exogenous extracellular matrix molecules include VEGF protein influence and change cell behavior by regulating the quantity and quality of matrix deposition mainly collagen I**according to Hu *et al.*(2005) results.**

3.Exogenous application of VEGF stimulate vascular endothelial cell proliferation. These cells secrete a variety of growth factors(VEGF, βFGF, PDGF), which promote angiogenesis. With a vascularized ECM, apposition of collagen I is achieved. Collagen cleavage products in proliferative phase also stimulate cell migration and proliferationof fibroblast.Fibroblasts secrete a variety of GFs (IGF-1, bFGF, TGF-b, PDGF, and KGF), which guide the formation of the ECM**.(Fraser *et al.*, 2008)**.

4.Application of low level laser therapy (LLLT) improves tissue perfusion and fibroblast proliferation, that increases collagen synthesis, these results are coincide with findings of **da Silva *et al.*(2010) and Figuróváet *al.*(2016).**

5.It was found that LLLT may accelerate metabolism and/or mineralization during early bone healing.**Khadra,2005** showed that in vitro experiments, cellular responses to LLLT were studied in two cell types: primary cultures of human gingival fibroblasts and human osteoblast-like cells, with special reference to attachment, proliferation, differentiation and production of transforming growth factor beta1 (TGF-beta1).

2 Immunohistochemical Finding forMacrophage colony-stimulating factor (M-CSF)

Orthodontic tooth movement is dependent on efficient remodeling of periodontal ligament and alveolar bone, correlated with several biological and mechanical responses of the tissues surrounding the teeth. A periodontal liga-

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ment placed under pressure will result in bone resorption whereas a periodontal ligament under tension results in bone formation. In the primary stage of the application of orthodontic forces, an acute inflammation occurs in periodontium. Several proinflammatory cytokines are produced by immune-competent cells migrating by means of dilated capillaries includes interleukin-1β ,vascular endothelial growth factor, and the macrophage colony stimulating factor**(Di Domenico *et al.*,2012;Kitaura *et al.*,2014).**

The present resultsshow that combination group records a high expression of M-CSF and for all studied periods ,followed by laser group and then VEGF group, and with high significant differences in comparison with control group .These findings could be explained as follows:

1.Results suggest that local administration of VEGF enhances the number of osteoclasts, and may increase the rate of orthodontic tooth movement**(Kaku*et al.*,2001).**Our results in agreement with**Niida*et al.*(1999)**whoreported that osteoclasts disappeared after injections of mice with anti-VEGF antibody, demonstrating that endogenously produced VEGF is responsible for the appearance of osteoclasts .

2.Acceleration of tooth movement and osteoclastogenesis on the pressure site in an experimental tooth movement model by low-energy laser irradiation (LELI), which stimulated the RANK/RANKL system and /macrophage colony-stimulating factor system**(Yoshida*et al.*,2009).**

The distribution of osteoclasts and their precursors in the periodontal ligament (PDL) of teeth was initially characterized by immunohistochemical expression analyses of markers of osteoclast differentiation represented by M-CSF**(Brooks*et al.*,2011).**

3.Low-level laser irradiation accelerates the bone remodeling process by stimulating osteoblastic and osteoclastic cell proliferation and function during orthodontic tooth movement**(Seifi&Vahid-Dastjerdi*et al.*,2009).**

4.Both vascular endothelial growth factor(VEGF) and macrophage colonystimulating factor (M-CSF) can induce osteoclast recruitment. Thus, VEGF and M-CSF are considered to be closely involved in the bone remodeling processvia osteoclastic bone resorption**(Kaku*et al.*,2008).**

3Immunohistochemical Finding ForAlkaline phosphatase(ALP)

Bone remodeling that occurs during orthodontic tooth movement is a biologic process involving an acute inflammatory response in periodontal tissues. A sequence characterized by periods of activation, resorption, reversal, and formation has been recently described as occurring in both tension and compression tooth sites during orthodontic tooth movement , ALP activity reflects the biologic activity in the periodontium during orthodontic movement and therefore it aids as a diagnostic tool for monitoring orthodontic tooth movement in clinical practice**(Perinetti*et al.*,2002).**

In the present at the 1st week and 3rd week the results show that combination group records a high expression of ALP, followed by laser and VEGF groups ,and the cells that expressed positive DAB stain for ALP include bone (osteoblast,osteocyte) and fibroblast cell in periodontal ligament.These cells involved in processes leading to mineral formation in tissues like bone**.** These results are in agreement with findings of**Ingliset *al.*(2016)**who reported that dynamic vasculature is a prerequisite for bone formation where the interaction of bone cells and endothelial cells which expressed ALP is essential for both the development and the healing process of bone.Other study that mimic our findings showed that stimulation of mesenchymal stem cells (MSCs) with inflammatory cytokines promotes osteogenesis through a paracrine mediator. MSCs were pre-stimulated with the inflammatory factors IFN-β and TNF-β. After pre-stimulation, the MSC secretion levels of VEGF increased and the expression levels of the osteoblast differentiation markers ALP, Collagen I were significantly elevated**(Lic *et al.*,2016) .**

Joensuu*et al.*(2015) demonstrated that the in vitro ALP activity and mineralization of human BM-MSCs is more efficient in the presence of exogenously added VEGF and further enhances the stimulatory effect. These results are coincide with our findings by using of exogenous VEGF as in separated group and in combination group .

Madhu*et al.*(2014) reported that combined action of exogenous VEGF and BMP on MSCs enhances osteoblastic differentiation of MSCs and increases their bone forming ability.

In the present study the result illustrates at the end of 2nd week ,a high expression of ALP by laser groupfollowed by combination group**,** this result is in agreement withresults of **Stein *et al.*(2005**) **and Asia *et al.*(2014**) who reported that application of low level LL light can enhance osteoblast cell proliferation and osteogenic differentiation with enhancement of ALP activitywhen the cells are cultured for a relatively long time.

Wu *et al.*(2013) suggested the potential use of LLLT in clinical applications for periodontal tissue regeneration. They showed an increment in potential osteogenic capacity, as it stimulated ALP activity, calcium deposition, and osteogenic gene expression.

Petri *et al.*(2010)indicated that LLLT modulates cell responses in a complex way stimulating osteoblastic differentiation, which suggests possible benefits on implant osseointegration with influence of ALP activity.

In conclusion;
In recent research projects, the effect of laser therapy was tested regarding the stimulatory effect on bone remodeling with the potential to influence the tooth movement rate as related to photo-biological responses of oral tissue after application of laser**(Seifi&Vahid-Dastjerdi2015).**

There are three effects that commonly occur asa result of tissue exposure to laser photons**(Torri&Weber, *2013*).** They are:

Primary effects of photoreception are a result of the interactionof photons and cell mitochondria which capture, direct, andtransducer photon energy to chemical energy used to regulatecellular activity.

Secondary effects occur in the same cell in which photonsproduced the primary effects and are induced by these primaryeffects. Secondary effects include cell proliferation, protein synthesis,degranulation, growth factor secretion, myofibroblastcontraction and neurotransmitter modifica-

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tion—depending onthe cell type and its sensitivity.

Tertiary effects are the indirect responses of distant cells tochanges in other cells that have interacted directly with photons.They are the least predictable because they are dependenton both variable environmental factors and intercellular interactions. They are, however, the most clinically significant. Tertiaryeffects include all the systemic effects of phototherapy. Primary,secondary, and tertiary events summate to produce phototherapeuticactivity.

Orthodontic tooth movement occurs in the presence of a mechanical stimuli sequenced by remodeling of the alveolar bone and periodontal ligament (PDL). Bone remodeling is a process of both bone resorption on the pressure site and bone formation on the tension site**(Baloul, 2015).**Orthodontic tooth movement can be controlled by the size of the applied force and the biological responses from the PDL .The force applied on the teeth will cause changes in the microenvironment around the PDL due to alterations of blood flow**(McCormack *et al*.,2014)**, leading to the secretion of different inflammatory mediators such as cytokines, growth factors, neurotransmitters, colony-stimulating factors, and arachidonic acid metabolites**(Kumar *et al*.,2015).** As a result of these secretions, remodeling of the bone occurs .The most important growth factor is **Vascular endothelial growth factor (VEGF)**,this protein is a member of the PDGF/VEGF growth factor family and encodes a protein that is often found as a disulfide linked homodimer. This protein is a glycosylated mitogen that specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis.VEGFis involved in tissue neo-formation that is strictly correlated with the presence of blood vessels**(Wu. *et al*.,2009).**

During orthodontic tooth movement, compressive forces induce angiogenesis of periodontal ligament together with the role of mediator of the VEGF. The localization of VEGF was analyzed in many in vivo researches and illustrated an increment in its expression in periodontal tissue during experimental tooth movement**(Salomão *et al*.,2014).** Therefore, VEGF exerts a fundamental role in remodeling periodontal ligament and is also involved in bone resorption and formation.

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