Immunohistochemical evaluation of vascular endothelial growth factor and transforming growth factor-beta on osseointegration of CpTi implant radiated by low level laser therapy

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ABSTRACT

Background: Dental implants provide a unique treatment modality for the replacement of lost dentition .This is accomplished by the insertion of relatively inert material (a biomaterial) into the soft and hard tissue of the jaws, there by providing support and retention for dental prostheses. Low level laser therapy (LLLT) is an effective tool used to prompt bone repair and modeling post surgery; this has referred to the biostimulation effect of LLLT. The aims of this study were to evaluate the immmunohistochemical expression of vascular endothelial growth factor (VEGF) and transforming growth factor -beta (TGF-β) in experimental and control groups with mechanical test.

Materials and Methods: Thirty two adult New Zealand white rabbits used, screw titanium implants inserted in the tibia. The right side is considered as experimental groups and the left side considered as control groups. Low power diode laser (GaAlAs) with wave length (904nm) and (5mW) power applied with the right screw implants. The sample divided into four groups, eight rabbits are sacrificed at four interval 4days, 1 weeks, 2weeks, and 6weeks respectively. immunohistochemical (VEGF&TGF-β), were done for each interval with mechanical test in 2 and 6 weeks .

Results: Immunohistochemical findings revealed high positive expression for VEGF and TGF-β in experimental implant in comparison to control one and the acceleration of bone formation and more rapid healing process in the screw implant with laser irradiation than in the control implant. Removal torque test showed dramatic increase with the presence of laser irradiation especially with advancing time.

Conclusion: This study was illustrated that the LLLT applications enhance bone formation and increase osseointegration.

Key words: Dental Implants, low level laser therapy, Biochemical bone marker. (J Bagh Coll Dentistry 2014; 26(2): 79-86).

الخلاصة

الخلفية: ان الغرسة السنية هي الطريقة المثلى لتعويض الأسنان المفقودة .يتم ذلك بإدخال مادة خاملة نسبيا (مادة حيوية) في الأنسجة الرخوة والعظمية للفكية مما يوفر إسنادا وتثبيتا للغرسة علاج الليزر المنخفض الطاقة اداة فعالة تستعمل لتحفيز اعادة بناء العظم بعد الجراحة ، وهذا ما يسمى بالتأثير المحفز لليزر المنخفض الطاقة. وكانت اهداف هذه الدراسة التقييم المناعي النسيجي الكيميائي لعامل نمو البطانة الدموية الوعائي (VEGF) وعامل تحول النمو (TGF-β) في مجاميع الاختبار والسيطرة مع الفحص الميكانيكي.

طريقة العمل والمواد المستخدمة: استخدم في هذه الدراسة 32 اثنان وثلاثون ارنب تحت التخدير العام وتم تعريض اشعة الليزر المنخفض الطاقة (GaAlAs) بطول موجة (904nm) وقوة (5mw) مع غرسة التيتانيوم في عظم الفخذ الايمن (مجموعة الاختبار) وادخال غرسة التيتانيوم فقط في الفخذ الايسر (مجموعة السيطرة).للحصول على النتائج اختبرت العينات كيميائيا نسيجيا مناعيا بعد (4 ايام ، اسبوع ، اسبوعان ، ستة اسابيع) بعد اجراء الزرع ، تم استخدام الاختبار النسيجي الكيميائي المناعي لاقتفاء ظهور مستقبلات عامل نمو البطانة الدموية الوعائي (VEGF) وعامل تحول النمو (TGF- β) على كافة الغرسات ولكل مراحل الالتئام مع الاختبار الميكانيكي في اسبوعان وستة اسابيع فقط.

النتائج: نتائج الفحص الكيميائي النسيج المناعي اظهرت مستوى ايجابي عالي الظهور في مجموعة الاختبار مقارنة بمجموعة السيطرة ، ان وجود اشعة الليزر المنخفض الطاقة يجعل عملية الالتئام وتكوين العظم اسرع من عملية الالتئام الفسلجية الطبيعية وفي نتائج الاختبار الميكانيكي بواسطة مقياس عزم التدوير كشفت النقاب عن زيادة في القوة الميكانيكية بوجود الليزر بازدياد الوقت.

الاستنتاج: هذه الدراسة اكدت بان تطبيق اشعة الليزر المنخفض الطاقة كانت مادة محفزة للعظم اذ سرعت عملية التكوين العظمي حول غرسة التيتانيوم اكثر من عملية الالتئام الفسلجية الطبيعية .

الكلمات الرئيسية : الغرسة السنية ، الليزر المنخفض الطاقة ، علامات العظم الكيميائية.

INTRODUCTION

Dental implants are biocompatible screw like titanium objects that are surgically placed into the mandible or maxilla to replace missing teeth. The mechanism by which an implant is biomechanically accepted by the jaw bone is called osseointegration

The clinical long-term success of the implants depends on the osseointegration and the adhesion of the soft tissues and epithelium to the titanium surfaces of the implant [3].

Titanium is the most wide spread metal for orthopedic implants intended for bone integration. It represents high fatigue strength and comparatively low modulus of elasticity, respect to other metals, so it is able to support loads and distribute them to bone, limiting stress shielding. Besides titanium is characterized by a thin natural oxide layer on the surface that limits ion release and reactivity, making the surface almost inert and biocompatible [4].

Several treatments have been proposed to improve and accelerate bone formation onto implant surface, among which low-level laser therapy (LLLT) [5]. LLLT Known as cold laser, soft laser, biostimulation, or photobiomodulation, It basically exposes cell or tissue to laser or low-level red or near-infrared (IR) light generated from light-emitting diode. LLLT stimulates or controls cellular function to minimize the extinction of cell or tissue, accelerates the healing

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of fractures, fast recovery from the damage of soft tissue, nerve, bone, and cartilage, and relieves acute and chronic pain and inflammation [6].

Transforming growth factor-beta (TGF-β), the largest source of which is bone, has been implicated in osteoblast proliferation and differentiation and is expressed at high levels during bone growth and development with an adequate blood supply [7].

Vascular endothelial growth factor (VEGF), which is secreted by many cells including osteoblasts and osteoblast-like cells, plays an important role for adequate angiogenesis and may be intimately related to bone development and fracture healing because both intramembranous and endochondral ossifications are associated with capillary development.. These two proteins are associated with osteogenesis during bone growth, development, and healing; but they do not stimulate stem cells or bone progenitor cells to generate to be osteoblasts as directly as bone morphogenetic proteins (BMPs). However, these proteins have efficacy on not only cell migration and propagation but also on angiogenesis indispensable for bone formation [7].

MATERIALS AND METHODS

Thirty- two adult New Zealand white rabbits male weighing 2-3kg were used in this study, screw titanium implants inserted in the tibia under general anesthesia. The right side is considered as experimental groups and the left side considered as control groups. Low power diode laser (GaAlAs) with wave length (904nm) and (5mW)power applied with the right screw implants. The sample divided into four groups, eight rabbits are sacrificed at four interval 4days, 1 weeks, 2weeks, and 6weeks respectively. immunohistochemical (VEGF&TGF-β) were done for each interval with mechanical test in 2 and 6 weeks.

RESULTS

Expression of VEGF findings

At 4 days duration

Control group

Immunohistochemical findings of implant site shows positive expression of VEGF in bone marrow stromal cell Figure (1).

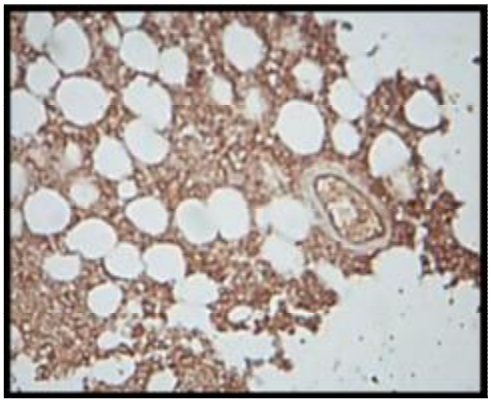

Figure 1: Positive expression of VEGF in bone marrow stromal cell of implant in rabbit tibia (control) for 4 days duration DAB stain with hematoxylin counter stain X100

Experimental group

Immunohischemical localization of VEGF in rabbit tibia shows strong positive expression of VEGF in bone marrow stromal cell with the formation of primitive osteoid tissue Figure (2).

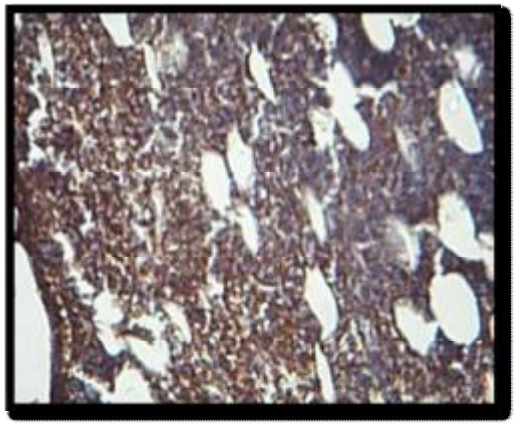

Figure 2: Immunohistochemical view for positive expression of VEGF in bone marrow stromal cell (BMSC) in bone marrow of implant in rabbit tibia treated with laser irradiation for 4 days duration DAB stain with hematoxylin counter stain X100

At 1 week duration

Control group

Woven bone are formed at implant site with weak positive expression of VEGF in progenitor cell, extracellular matrix , blood vessel and osteoid tissue ,fat cell shows negative expression of VEGF. DAB stain with hematoxylin counter stain X200. Figure (3).

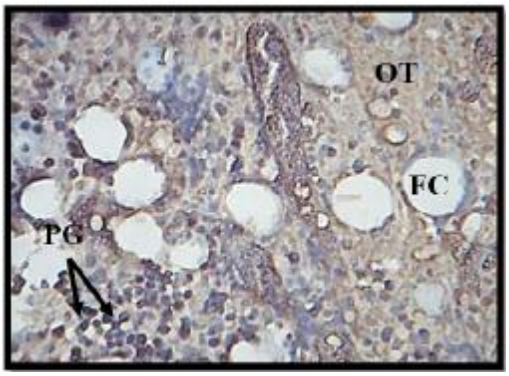

Figure 3: View for positive expression of VEGF in osteoid tissue (OT), progenitor cell (PG), fat cell (FC) DAB stain with hematoxylin counter stain X200

Experimental group

Positive immunohistochemical localization of VEGF is viewed in large area of newly formed woven bone, endothelial cell of blood vessel, osteoblast cell and active osteoprogenitor cell. Figure (4).

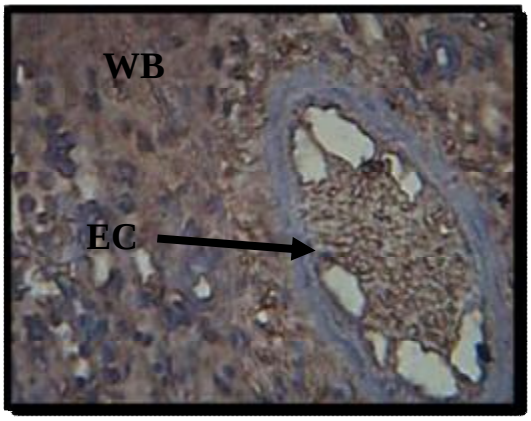

Figure 4: positive immunohistochemical localization of VEGF in implant site of rabbit tibia for one week duration treated with laser irradiation shows positive expression o f VEGF in woven bone (WB), and endothelial cell (EC) DAB stain with hematoxylin counter stain X400

At 2 week duration

Control group

Micrscopical evaluation of bone section at implant site shows osteoid tissue formation that is positively expressed by VEGF Figure (5).

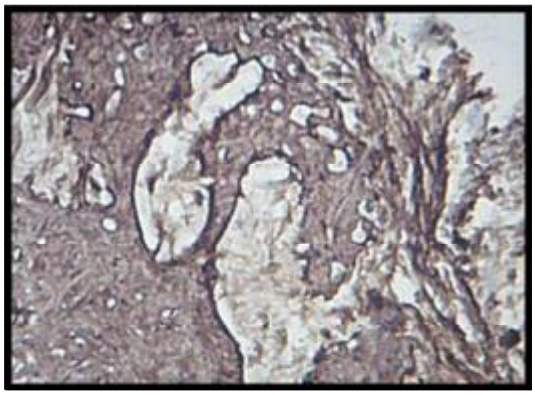

Figure 5: Microphotograph of bone section in rabbit tibia after 2 weeks of implantation illustrates positive immunohistochemical localization of VEGF by osteoid tissue (OT) DAB stain with hematoxylin counter stain

Experimental group

Bone section in rabbit tibia shows positive expression of VEGF in bone trabeculae in which osteocyte are embedded and formative osteoblast are seen rimming bone surface Figure (6).

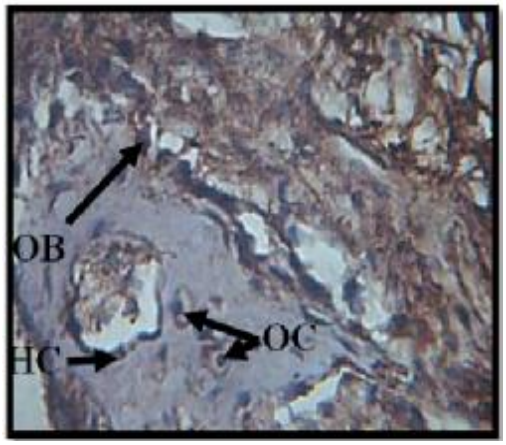

Figure 6: Magnifying view showing bone trabeculae with numerous osteocyte (OC), osteoblast on the surface of bone (OB) and havarisan canal(HC),all are positively stained DAB stain with counter hematoxylin X400.

At 6 week duration

Control group

Micrphotograph view in rabbit tibia shows positively stained immature bone formation with numerous osteocyte that are irregularly scattered in the bone Figure (7).

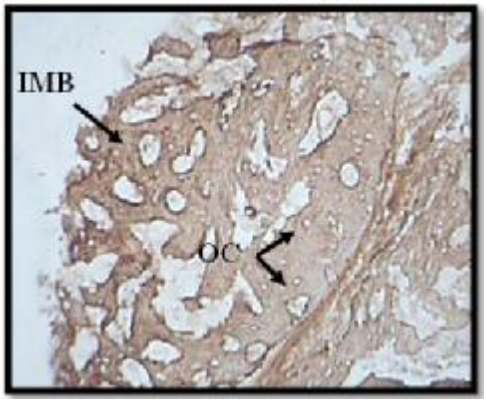

Figure 7: Positive immunohistochemical localization of VEGF expressed by immature bone (IMB) and osteocyte cell (OC) in rabbit tibia of 6 weeks duration (control) DAB stain with counter hematoxylin stain X100.

Experimental group

Bone section at implant site shows positive localization of VEGF in mature bone , bone trabeculae appear with lamellated bone , osteocyte are arranged regularly inside bone matrix , and blood vessel within bone trabeculae Figure (8).

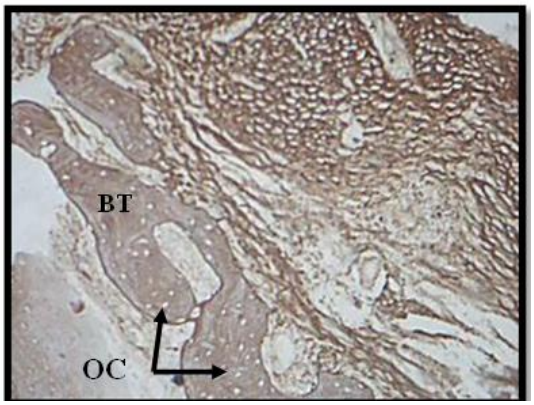

Figure 8: View of mature bone showing bone trabeculae (BT) and osteocyte cell (OC) with regular arrangement in rabbit tibia of 6 weeks duration treated with laser irradiation DAB stain with counter hematoxylin stain X 200.

Expression of TGF-β findings

<u>At 4 days duration</u>

Control group

Immunohistochemical view revealed primitive new bone formation in which future bone is formed as embryonic type.This bone is characterized by presence of progenitor cells that are scattered randomly which shows positive expression of TGF-β Figure (9).

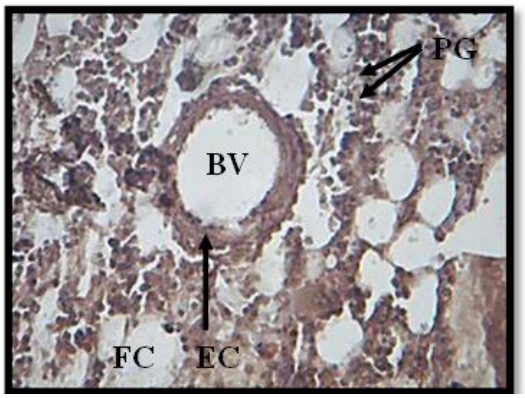

Figure 9: magnifying view shows positive localization of TGF- β in progenitor cell (PG),fat cell (FC) and endothelial cell(EC) of blood vessel DAB stain with hematoxylin counter stain X400

Experimental group

Positive localization of TGF-β expressed by large number of active mitotic progenitor cells , endothelial cells of blood vessel ,fat cell with surrounding extracellular matrix in bone marrow Figure (10).

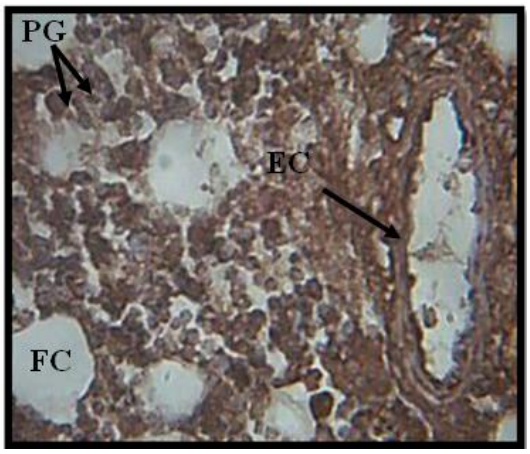

Figure 10: Positive expression of TGF-β in progenitor cell (PG) ,fat cell (FC),endothelial cell (EC) and in ground substance of bone marrow in implant site treated with laser irradiation for 4 days duration DAB stain with hematoxylin counter stain X400

<u>At 1 week duration</u>

Control group

Implant site of 1 week duration shows positive expression of TGF-β in osteoid tissue, fat cell, endothelial cell, progenitor cell with extracellular matrix Figure (11).

Experimental group

Osteoid tissue shows positive localization of TGF-β in osteoid tissue,fatcell,endothelial cell and in progenitor cell,all are irregularly arranged within primitive bone formed at implant site Figure (12).

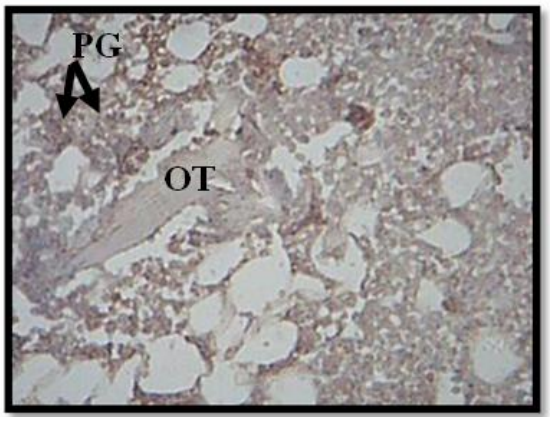

Figure 12: View of implant site at one week duration treated with laser irradiation shows positive expression of TGF- β in osteoid tissue (OT), and progenitor cell (PG) DAB stain with hematoxylin counter stain X200.

At 2 weeks duration

Control group

Microscopic evaluation of the bone section related to implant shows bone thread with bone trabeculae that are negatively stained enclosing area of woven bone Figure (13).

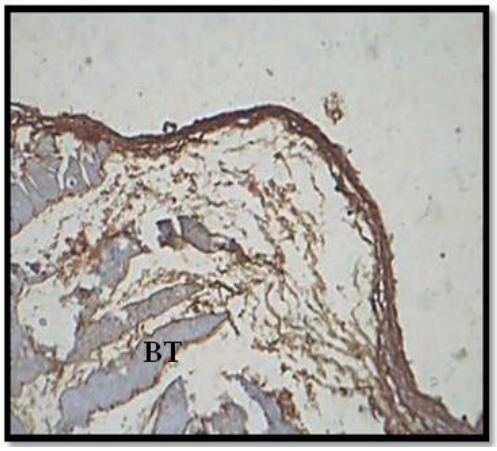

Figure 13: Immunohistochemical localization of TGF-B in bone trabeculae (BT) of thread region in implant site of 2 weeks duration (control) DAB stain with hematoxylin counter stain X100

Experimental group

View of rabbit tibia with implant shows positive localization of TGF-β, in osteoid tissue, bone trabeculae, osteocyte cell and in marrow tissue Figure (14).

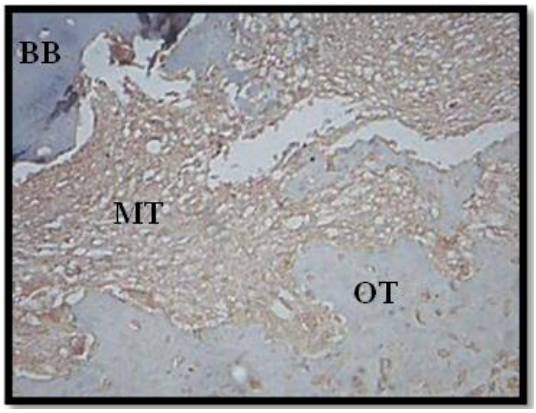

Figure 14: positive immunohisto chemical localization of TGF- β in osteoid tissue (OT) and in marrow tissue (MT) in implant site treated with laser irradiation for 2weeks duration while basal bone (BB) is negatively stain DAB stain with hematoxylin X200.

At 6 week duration

Control group

Positive localization of TGF-β in immature bone deposited at implant site in marrow tissue Figure (15).

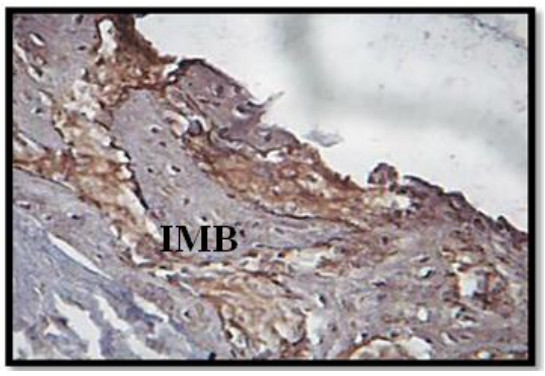

Figure 15: Immunohistochemical localization of TGF-β in immature bone (IMB) at implant site (control) after 6 weeks of implantation. DAB stain with hematoxylin counter stain X200

Experimental group

Positive expression of TGF-β in mature bone deposited at implant site, it shows havarsian bone, marrow tissue, osteocyte cell embeded in bone matrix , reversal line that separated old bone from new bone Figure (16).

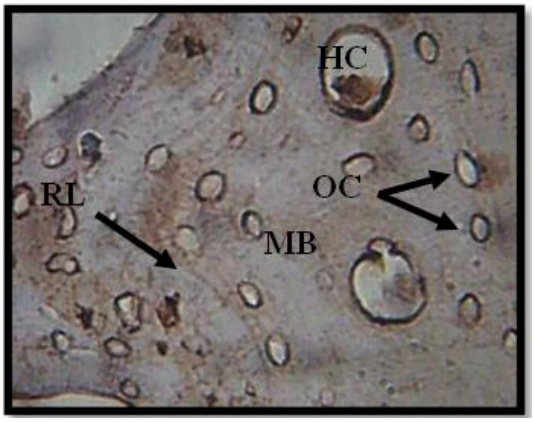

Figure 16: View of bone thread in implant site after 6 weeks duration treated with laser irradiation shows mature bone (MB), positive localization of TGF- β in osteocyte (OC), havarsial canal (HC) and reversalLine (RL). DAB stain with hematoxylin counter stain X400.

Mechanical testing

Figure (17) shows the summary statistics of the removal torque value of CPTi implants (control and experimental) after two and six weeks of implantation times, the torque value needed to remove all the implant was higher at six weeks healing period for both control and experimental groups.

After 6 weeks of implantation there was an obvious increase in the means values of the torque force that were needed to unscrew the implants.

The mean torque values for the implants control group was (22.75 n. cm) the highest torque mean value was obtained with implants treated with laser irradiation (25.75 n.cm).

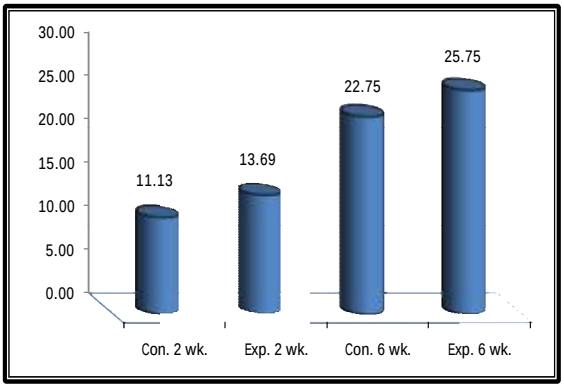

Figure 17: Bar chart for mean values of the studied torque removal test parameter for the two independent groups (study and control) at the two periods of times

Bone marrow stromal cells (BMSC)

From the obvious findings we can noticed that, there was decrease in BMSC score mean values of positively stained cells for both VEGF and TGF – beta, during the 4 days, 1,2 and 6 weeks of healing intervals concerning control group while the experimented group a slight increase in VEGF score at 2and 6 week period, whereas the mean values of scores of TGF beta showed decrease in 2 and 6 weeks of healing intervals, as shown in Figures (18, 19).

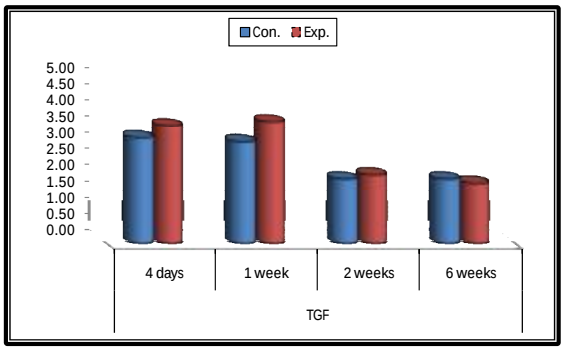

Figure 18: Cluster bar chart for mean values of (BMSC Outcomes) distributed among different of the studied sources of variations at TGF Marker

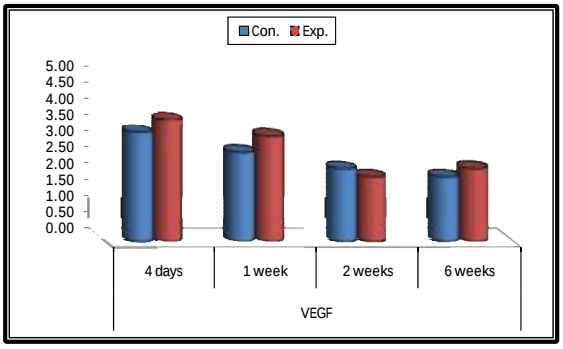

Figure 19: Cluster bar chart for mean values of (BMSC Outcomes) distributed among different of the studied sources of variations at VEGF Marker

Bone cell

The results of bone cells in present study are illustrated in Figure (20). The majority of the bone cells parameter was reported at the VEGF and TGF beta during healing periods, had been decreased down stair sequentially by the times periods passed of the studied trials. In addition to that, the comparisons significant among different periods of times after treatment reported anon significant differences at P>0.05 due to different markers, and the same statistical results were obtained due to comparisons significant among different periods of times in each groups (control and experimental)at p> 0.05.

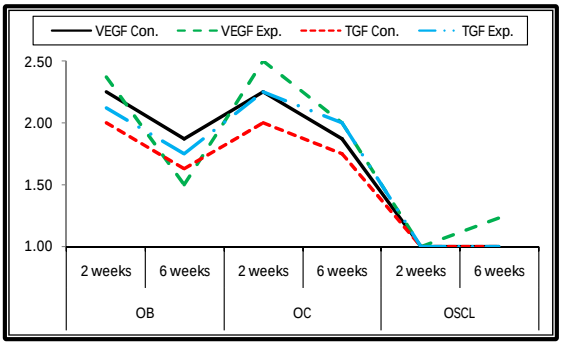

Figure 20: Multiple Line chart of the mean values for (Bone Outcomes) distributed among different of the studied sources of variations at VEGF and TGF Markers

DISCUSSION

All animals tolerated the implantation well, no sign of cross infection, tissue reaction or any other negative clinical indications like mobility of the implants were noted around the implants site.All implants were stable during healing periods in the sense that they could not be removed with manual force without the aid of the torque gage instrument as observed from the results of [8].

The increased removal torque values for lased group comparing with control group indicates that low- energy laser therapy(in the dose given in this study) affects osseointegration formation and bone maturation around the implant positively .This result was in agreement with [9-11]

The present result based on application of LLLT with implant, LLLT creates a number of environmental conditions that appear to accelerate the healing of bone [12], LLLT-related effects include stimulation of blood flow, recruitment and activation of osteoblasts, osteosynthesis, a decrease in osteoclastic activity and anti-inflammatory action [13] could also be considered as factors that stimulate biomaterial osseointegration.

VEGF induces the proliferation, differentiation and migration of vascular endothelial cells and enhances their survival by preventing their apoptosis; it also increases the permeability of the capillaries [14]. VEGF works in both processes of endochondral ossification and intramembranous ossification acts as an essential mediator during these processes. It is involved not only in bone angiogenesis, but also in various aspects of bone development, including chondrocyte differentiation, osteoblast differentiation and osteoclast recruitment [15]. Therefore our results record positive expression of VEGF in experimental group at 6 weeks duration. TGF-β increases bone formation mainly by recruiting osteoblast progenitors and stimulating their proliferation, thus expanding the pool of committed osteoblasts, as well as by promoting the early stages of differentiation (bone matrix production). On the other hand, it blocks later phases of differentiation and mineralization [16,17] .TGF-β increases the pool of osteoprogenitors both by inducing chemotaxis and proliferation[18].

The present study suggests for beneficial use of LLLT in practice of dentistry implantation or in other branch related to osseointegration process.

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