Immunohistochemical Evaluation of Osteocalcin Expression with Application of LIPUS During Relapse Phase of Orthodontic Therapy

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Abstract

There are many biomarkers (proteins) representing the biological modifications during bone resorption and formation phenomena, one of the most important proteins is the osteocalcin ,which is the most abundant non collagenous matrix protein in bone and dentine, that used as a clinical marker for bone turnover**.** Osteocalcin is expressed solely by highly differentiated osteoblasts and is incorporated into the bony matrix**,** and thought to play a role in the body's metabolic regulation and is pro-osteoblastic, or bone-building, by nature.

Low intensity pulsed ultrasound (LIPUS) is a medical technology used to enhance bone healing, bone growth, bone formation and maturation in different medical situations.

Material and method: The purpose of this study was to evaluate the effect of the LIPUS application on the osteocalcin expression and its role in accelerating the bone formation during relapse phase of orthodontic tooth movement. Fourteen male New Zealand rabbits were randomly divided into two groups: control and experimental (orthodontically treated(alone in the former) plus LIPUS therapy during relapse phase in the latter) group. ***Results:*** The result almost showed strong immunoreaction for osteocalcin expressed by dental cells and their progenitors in experimental group in the coronal, middle, and apical levels which play essential role in enhancing and accelerating the bone formation and regeneration via increasing the osteogenic cells activity that illustrates a benefit in orthodontic treatment.

Key words: Osteocalcin, LIPUS, relapse phase.

الخلاصة

هناك عدة مؤشرات بايولوجيه (بروتينات) تمثل التغيرات البايولجية خلال عملية هدم وبناء العظم . واحد من اهم البروتينات الاوستيوكالسين (الكالسين العظمي) والذي يعتبر اكثر انتشارا ذات المادة البينية اللاكالوجينية في العظم والعاج ,والذي يستخدم كمؤشر سريري لنمو العظم . الكالسين العظمي يفرز فريدا من خلايا عظمية بناءه ذات تمييز عالي والذي يغمر في المادة البينية العظمية ويعتقد انه يلعب دورا في تنظيم ايض الجسم وكمهيىء للخلايا العظمية البناءه او عملية بناء العظم , في الطبيعة .

موجات فوق الصوتية النبضيه ذات الشدة الواطئة هي تقنية طبية استخدمت لتحفيز التئام العظم ونمو العظم ,تكوين العظم ونضجه في اماكن طبية مختلفة .

المواد والطريقة: الغرض من هذه الدراسة كان لتقييم نتيجة تطبيق الموجات فوق الصوتية النبضية ذات الشدة الواطئة على تكوين الكالسين العظمي وبالتالي دوره في عملية تسريع تكوين العظم خلال مرحلة الانتكاسة اثناء تحريك السن تقويميا . اربعة عشر ارنب نيوزيلندي كانت قد قسمت عشوائيا الى مجموعتين: المجموعة المتحكم بها والتجريبية (عولجت تقويميا (وحده فقط في السابقه) مع الموجات الفوق الصوتية خلال مرحلة انتكاسة الحالة في المجموعه التاليه) .

النتائج : النتيجة كثيرا ماوضحت انه هناك تفاعل مناعي قوي للكالسين العظمي المفروز بواسطه الخلايا السنية والخلايا الاصلية لها في المجموعة التجريبية في المستويات العنقية والوسطية والقاعدية والذي يلعب دورا مميزا في عملية تحفيز وتسريع بناء العظم واعادة تكوينه من خلال زيادة نشاط الخلايا العظمية وهذا بدوره يجسد اهمية كبيره في العلاج التقويمي .

الكلمات المفتاحية : الكالسين العظمي,مرحلة انتكاسة الحالة ,الموجات فوق الصوتية النبضية ذات الشدة الواطئة .

Introduction

Ultrasound is a non-invasive therapeutic tool that is increasingly used to enhance bone fracture healing**(Parvizi and Vegari, 2005)** and soft tissue repair **(Uhlemann *et al.*, 2003**). LIPUS is an acoustic pulsed energy that demonstrated promising results in the dental field, as a form of non-invasive mechanical

stimulation. Several *in vitro* studies demonstrated anabolic effects of different LIPUS intensities upon cementoblasts (**Dalla-Bona *et al.*,2008)** periodontal ligament and bone cells **(Harle *et al.*,2001).** Clinical studies demonstrated the effectiveness of LIPUS at 30 mW/cm^2 intensity to accelerate periodontal wound healing and to increase cementum formation (**Ikai *etal.*,2008)**. Moreover, it accelerated healing of orthodontically induced root resorption (**El-Bialy *et al.*,2004),** and enhanced bone formation at osteodistraction sites **(Chan *et al*.,2006; El-Bialy *et al.*,2008).**

Osteocalcin is non collagenous bone matrix protein which plays a role in the body's metabolic regulation and is consider to be preosteoblastic or bone building protein secreted by osteoblast and also it seems to have a role in the early stages of bone formation, therefore it is often used as biomarker for bone formation process according to **Lee *et al*.,(2007).**

The purpose of the present research is to study the effect of the LIPUS on the bone formation during orthodontic relapse phase via studying the expression of the Osteocalcin.

Materails and Methods

Animals and experimental protocol

Fourteen 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 adlibitum and standard laboratory rabbits pellets and green food.

According to the ultrasound application protocol, the rabbits were randomly divided into two groups:

Control group :- (n=7)Orthodontically treated only.

Experimental groups:-(n=7)Orthodontically treated plus LIPUS therapy during relapse movement.

Orthodontic appliance design and LIPUS 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 anaesthesia, induced by an intramuscular injection of a ketamine (50 mg/ml) at a dose of 50 mg/kg body weight and muscle relaxant Orbarcaine 2% at a dose of 5 mg/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 handmade 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 3 mm away from the incisal edge to allow for wear of the teeth and the lower border about 2 mm 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 receives a light continuous force of (50gm) according to **Proffit *et al.* (2007).** 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 22 days. Once the orthodontic appliances were removed and the relapse movement was started, the experimental group was received the LIPUS therapy of 50 mW/cm^2 intensity, 1 MHz frequency which was applied (20 min/day) for 4 weeks.

Histological preparation

Rabbits of control and experimental groups were sacrificed by injection of over dose of ketamine intramuscularly. The mandibles, include the central incisors, were dissected, the biopsies were taken by cutting the anterior segment of the mandible including the central incisors superiorly and the mandibular first premolars inferiorly, fixed in 10% neutral buffered formalin solution for 48 hours, and then decalcified by 10% formic acid for 30-35 days; they were checked regularly every 3 days with changing of the acid solution. The decalcified biopsies were washing thoroughly, dehydrated in a graded series of ethanol and embedded in paraffin. Blocks of paraffin wax were sectioning for immunohistological evaluation for expression of osteocalcin, using monoclonal antibody (osteocalcin) from Abcam company UK, the information and specification of the present monoclonal antibody was obtained from the data sheet, with detection kit system from Abcam Company England (ab64259). The paraffin blocks were adjusted to a microtome where cut at three levels 7600 μm apart: coronal, middle and apical. At each level, five histological sections per animal was prepared. Statistic analysis for the mean of positive cell that calculated from five histological sections per animal, in five microscopic fields, at 40x magnification was done using Chi square(X^2) and P value.

Results and Discussion

Immunohistochemical localization of Osteocalcin

1- The coronal level:

The labial, tension and compression sides as a whole of the coronal level of the control group expressed weak /moderate positive(+/++)osteocalcin immunoreactivity by osteoblast **(figure1),** while the labial, tension and compression sides of the

experimental group expressed osteocalcin immunoreactivity range from weak /moderate to strong(+/++/+++) by the osteoblast and extracellular matrix**(figure2)** .

2- The middle level

The labial, tension and compression sides as a whole of the middle level of the control group expressed weak /moderate positive(+/++) osteocalcin immunoreactivity by osteoblast. Localization of osteocalcin was shown by osteoprogenetor cells in haversian canal and osteoclast cells**(figure3),** while the labial, tension and compression sides of the experimental group expressed moderate to strong(++/+++) osteocalcin immunoreactivity by the osteoblast and osteoprogenetor cells in haversian canal of new bone**(figure4).**

3- The apical level

The labial, tension and compression sides as a whole of the apical level of the control group expressed weak /moderate positive(+/++) osteocalcin immunoreactivity by osteoblast. Localization of osteocalcin was shown by osteocyte cells**(figure5),**while the labial, tension and compression sides of the experimental group expressed osteocalcin immunoreactivity range from weak /moderate to strong(+/++/+++) by the osteoblast , extracellular matrix and osteocyte cells**(figure6).**

Figure1: Immune active view of compression side of coronal level of control group; show weak expression of osteocalcin by osteoblast cells(white arrow)of old bone area(OB). DAB stain x20.

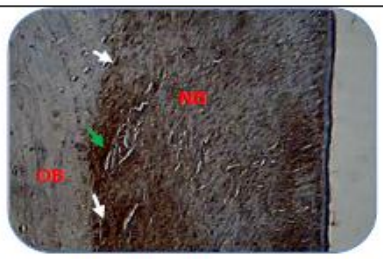

Figure2:Immune active view of compression side of coronal level of experimental group; show strong expression of osteocalcin by osteoblast cells(white arrow) in new bone area (NB),and extra cellular matrix (ECM-green arrow). DAB stain x20.

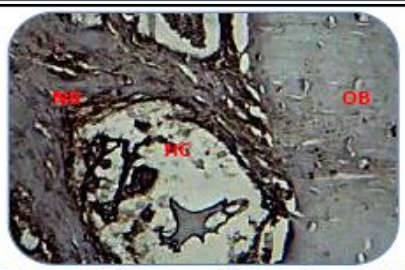

Figure3:immune active magnifying view of labial side of middle level of control group;show moderate positive expression of osteocalcin by osteoprogeneter cells in the haversian canal(HC.)of NB. DAB stain x40.

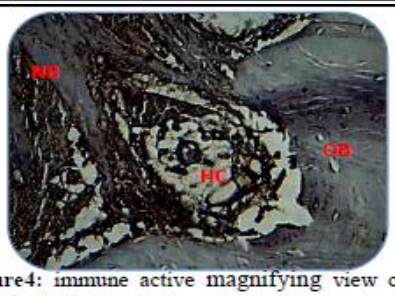

Figure4: immune active magnifying view of labial side of middle level of experimental group;show strong positive expression of osteocalcin by osteoprogeneter cells in the haversian canal (HC.)of NB.DAB stain x40.

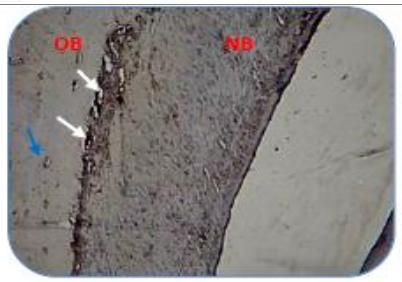

Figure5:immune active view of tension side of the apical level of control group;show moderate positive expression of osteocalcin by osteoblast (white arrow) and osteocyte (blue arrow). DAB stain x20.

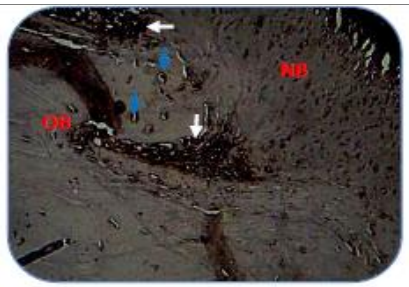

Figure6:immune active view of tension side of the apical level of experimental group ;show moderate positive expression of osteocalcin by osteoblast(white arrow)and osteocyte(blue arrow)cells .DAB stain x20.

Statistic analysis for positive cells expressed osteocalcin:

The nonparametric statistic (Chi square(X^2)) for the positive bone cells expressed Osteocalcin at the three sides of the coronal and apical level in the experimental and control groups, shows significant difference ($P \leq 0.05$) tables (1 and 3), while at the middle level was highly significant value ($P < 0.01$) table 2.

Table (1): Immunohistochemical score and percentage of osteocalcin expression at ***the coronal level***

Levels		Coronal level				Total
Groups		- ve	+ ve			
			Weak (+)	Moderate (++)	Strong (+++)	
Control	N	-	6	1	-	7
	%	-	85.7%	14.3%	-	100.0 %
Experimental	N	-	1	3	3	7
	%	-	14.3%	42.85%	42.85%	100.0 %
X^2		9.168				
p-value		0.01				

Table (2): Immunohistochemical score and percentage of osteocalcin expression at ***the middle level***

Levels		Middle level				Total
Groups		- ve	+ ve			
			Weak (+)	Moderate (++)	Strong (+++)	
Control	N	-	6	1	-	7
	%	-	85.7%	14.3%	-	100.0 %
Experimental	N	-	-	2	5	7
	%	-	-	28.6%	71.4%	100.0 %
X^2		15.589				
p-value		0.000				

Table (3) : Immunohistochemical score and percentage of osteocalcin expression at ***the apical level***

Levels		Apical level				Total
Groups		- ve	+ ve			
			Weak (+)	Moderate (++)	Strong (+++)	
Control	N	-	6	1	-	7
	%	-	85.7 %	14.3%	-	100.0 %
Experimental	N	-	1	4	2	7
	%	-	14.3 %	57.1%	28.6%	100.0 %
X^2		8.662				
p-value		0.01				

The immunohistochemical examination and analysis of the osteocalcin expressions was done due to fact of the possible role of the osteocalcin in body's metabolic regulation and is consider to be pre-osteoblastic or bone building protein secreted by osteoblast and also it seems to have a role in the early stages of bone formation, therefore it is often used as biomarker for bone formation process according to **Lee *et al*.,(2007),** so that for accurate assessment of the effect of LIPUS on the bone formation in the present study, we are depending on the analysis of the osteocalcin expressions at different levels and sides between experimental and control groups.

The result of the osteocalcin expressions in the control group at different levels (coronal, middle and apical) in the present study is low positively range from the more weakly to less moderately expressions, these results can be explained separately as follow:

1-The positively expression in the control groups is indicated of bone formation process which is generally considered to be a normal results, as during the relapse movement there will be a alveolar bone remodeling ,which according to **Yoshida *et al.*, (1999)** is consider to be one of the main causes of relapse, this remodeling includes bone apposition in the tension sides and bone resorption in the compression sides of the relapse direction, so this new bone apposition causing this positively expression of osteocalcin.

2-The low positively expressions (weakly positive expression),which was the mostly expressions in the control group, may be due to little numbers of the osteoblast cells that secreted the osteocalcin in this group according to **Hannon and Eastell,(2006**) and **Lee *et al*.,(2007).**

Generally, this result is in agreement with **Oztürk *et al*., (2011)** who found that the osteocalcin immunoreactivity in the active osteoblast cells in the control group was minimal/moderate and significantly less than those in the experimental group, while the osteocalcin expressions in the experimental group at different levels (coronal, middle and apical) in the present study was high positively expression range from more moderately/strongly to less weakly expressions, these results can be explained separately as follow:

1- The positively expression in the experimental group is indicated of bone formation process that occurs due to the effect of LIPUS therapy, and also it may be due to presence of specific interaction and correlation of osteocalcin with alkaline phosphatase which is responsible in mineralization stage of the bone according to **Mayr-Wohlfart *et al.*, (2001).**The ability of LIPUS to speed up bone formation according to **Dyson** and **Brookes,(1983); Parvizi *et al.*, (1997)**; and **Leung *et al.*, (2004)** was due to the non-thermal effect of ultrasound (its physical pulses) that can lead to the acousting streaming which will lead to direct mechanical effects on the cell membrane by which it has the capacity to alter cell membrane permeability to ions that leading to increase the activity of the cells, causing the cells to absorb as much calcium as they can in order to activate protein kinase A, which may lead

to the differentiation of osteoblasts, that will stimulate to synthesize extracellular matrix.

Despite of that the exact underlying biophysical mechanism of action of ultrasound during bone formation is not known, but a number of studies have investigated and explained the potential cellular processes that influenced by LIPUS and it was found that the LIPUS, in vitro investigations, able to induce cell proliferation, collagen/non-collagenous protein (NCP) production, bone formation, and angiogenesis according to **Doan *et al*., (1999)**, then it was found that the LIPUS has been shown to influence directly in a number of cells associated with the repair process, including osteoblasts according to **Warden *et at*., (2001)** while according to **Chen *et al*.,(2003)** it was determined that LIPUS can stimulate the differentiation in osteoblast precursor cells and induces the expression of growth factors, proteins within osteoblasts, chondrocytes and fibroblasts, and also in vivo investigations, it was found that the LIPUS induces a direct anabolic reaction of osteogenic cells, leading to bone matrix formation according to **Naruse *et al*.,(2000**).

According to these studies, it is clear that, the LIPUS has direct stimulatory effect on the osteogenesis reactions, cells proliferations and cells differentiation which all lead to increase both the number and activity of the osteoblast cells, which in turn lead to increase in bone matrix formation, that in turn explained the causes that made the numbers of the osteoblast cells and in turn osteocalcin expressions in the present study higher significantly in the experimental group than the control group.

2-The high positively expression (moderately/strongly positive expression) of osteocalcin, which was the mostly expressions in the experimental group, may be related to the stimulatory effect of the LIPUS on the bone marrow stem cells that will respond to it by the proliferations and the differentiations of the osteogenic cells(osteoblasts) with elevated the levels of the IGF mRNAs, osteocalcin, and bone sialoprotein mRNAs., according to **Naruse *et al*.,(2000**) and **Warden *et al*., (2001)**.

Generally this result is disagree with **Oztürk *et al*.,(2011)**, who found that the osteocalcin immunoreactivity in the active osteoblast cells in the treated group was purely strong, this disagreement may be stem from the difference in the type of bone accelerated therapy as we used LIPUS, whereas they use drug administration (bisphosphonate).

Conclusion

LIPUS therapy has been demonstrated to elevate the level of the bone matrix proteins, specially the production of Osteocalcin; these effects play essential role in enhancing and accelerating the bone formation and regeneration via increasing the osteogenic cells activity that illustrates a benefit in orthodontic treatment.

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