

Evaluation the effect of hyaluronic acid on bone healing process in rabbits (Immunohistochemical study for TGF- β)

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ABSTRACT

Background: Bone augmentation techniques are commonly employed in medical fields. This biomaterial system must be readily available, easily applicable by minimally-invasive technique and able to release an osteoinductive growth factor. Such a system will be able to engineer new bone formation locally at the site of injection. Hyaluronic acid has osteogenic potential that can be exploited not only for repairing bone defects but also for providing transplantable bone for the reconstruction of a variety of bone defects. The aims of this study were to evaluate the effects of Hyaluronic acid gel on bone healing by immunohistochemical estimation of transforming growth factor - beta 3 in experimental and control groups.

Materials and methods: Thirty two New Zealand male rabbits were used in this study. Two Intra bony holes were made for each rabbits on the right and left buccal side of the upper diastema. The right hole was filled with Hyaluronic acid gel (experimental one), while the left hole was left for normal healing (control one). The rabbits were randomly divided and sacrificed at 1, 2, 3 and 6 weeks post operatively. Immunohistochemical test for the expression of TGF- β 3 were performed on bone specimens of both control and experimental groups at all healing interval.

Results: Immunohistochemical examination of this study revealed that the hyaluronic acid treatment increased the positive expression of TGF- β 3 by osteoblasts, osteocytes and bone marrow stromal cells especially in 1 and 2 weeks intervals than that observed in control one.

Conclusions: The present study illustrated that the Hyaluronic acid was osteoconductive material that enhance osteogenesis and accelerated the bone healing process.

Key words: Bone augmentation, Osteoinductive, growth factor, Hyaluronic acid. (J Bagh Coll Dentistry 2015; 27(1):111-116).

INTRODUCTION

Bone defects in oral and craniofacial tissues are a clinical challenge and can be the result of trauma, tumor resection or congenital malformations ⁽¹⁾. The golden standard for reconstruction is autologous bone grafts, but bone may not always be readily available and donor-site morbidity might follow. Alternatives to autologous tissue are sought in the field of tissue engineering, where a range of biomaterials, searching to engineer the missing tissue ⁽²⁾.

Biomaterials can be divided into four different groups comprising inorganic materials, naturally-derived polymers, synthetic polymers and composite materials ⁽³⁾. Biomaterials of different origins as natural and synthetic ones have different mechanisms of host response. It should be stable, biocompatible; ideally osseointegrative and conductive, porous and similar to biological bone mechanically ⁽⁴⁾.

Hyaluronic acid is a naturally-derived polymers biomaterial. It is a major component of the extra cellular matrix (ECM) and present in nearly every mammalian tissue and fluid.

It plays a role in wound healing and it has been found in high concentrations in the early fracture callus, in lacunae surrounding hypertrophic chondrocyte in the growth plate and

in the cytoplasm of osteoprogenitor cells ⁽⁵⁾.

Hyaluronic acid (HA) has osteoconductive potential; it accelerates the bone regeneration by means of chemotaxis, proliferation and successive differentiation of mesenchymal cells. HA may act as biomaterial scaffold for other molecules, such as BMP-2 and TGF- β , used in guided bone regeneration techniques and tissue engineering research ⁽⁶⁾.

A number of growth factors have been shown to be expressed during different phases of experimental bone-healing. Among these growth factors is transforming growth factor-beta ⁽⁷⁾.

Transforming growth factor-beta, 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 ⁽⁸⁾.

MATERIALS AND METHODS

Thirty two New Zealand male rabbits, weighting (1.5– 2kg), aged (6-12) months were used in this study. Two Intra bony holes were made for each rabbits on the right and left buccal side of the upper diastema. The right hole was filled with 0.1ml Hyaluronic acid gel (experimental one). While the left hole was left for normal healing (control one). The sample divided into four groups, eight rabbits are sacrificed at four intervals 1 week, 2 weeks, 3 weeks and 6 weeks respectively. All tissue

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specimens, experimental and controls were fixed in 10% neutral formalin and processed in a routine paraffin blocks after complete decalcification of bone. Each paraffin-embedded specimen had serial sections were prepared as follows: 4 μ m thickness sections were mounted on clean glass slides for routine H&E staining procedure from each block of all studied sample. Other 4 sections of 4 μ m thickness were mounted on positively charged microscopic slides for immunohistochemical localization of TGF- β . The procedure of the IHC assay was carried out in accordance with the manufacturer instructions of Anti-TGF- β polyclonal antibody (ab15537) Abcam UK and Detection Kits System (ab80436) Abcam UK.

RESULTS

Expression of TGF- β findings

At 1 week duration

Control group

Immunohistochemical staining with TGF- β polyclonal antibody at one week duration showed weak positive expression in fat cells, fibroblast, progenitor cells and osteoblasts. Negative expression of TGF- β is seen in osteoid tissue **Figure (1)**.

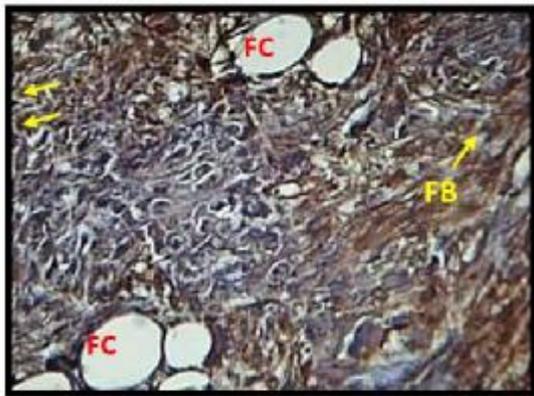


Figure 1: Positive localization of TGF- β in defect site of control group for 1 week duration in progenitor cells (arrows), Fat cells (FC) and fibroblast (FB). DAB stain with counter stain hematoxylin X20

Experimental group

Experimental group at 1 week duration labeled with TGF- β antibody shows positive expression of TGF- β in progenitor cells, osteoblasts, and osteocytes. Negative expression of TGF- β in bone spicules and osteoid tissue **Figure (2)**.

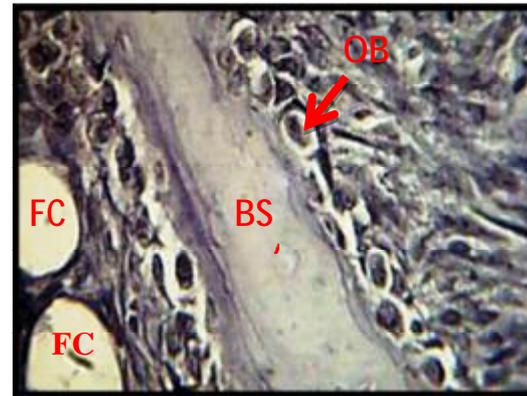


Figure 2: Immunohistochemical view of HA treated group at 1 week interval show positive expression of TGF- β in osteoblast (OB), fat cells (FC) and negative expression in bone spicules (BS). DAB stain with hematoxylin counter stain X40.

At 2 weeks duration

Control group

Control defect area at 2 weeks duration shows positive localization of TGF- β in fibrous connective tissue, osteoblasts, osteocytes, osteoclasts and negative expression of TGF- β in bone trabeculae **Figure (3)**.

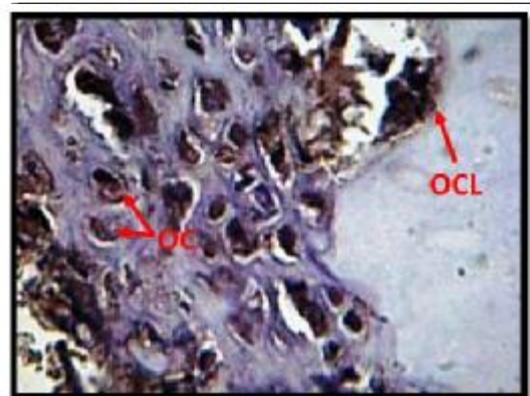


Figure 3: Immunohistochemical view for bone healing defect (control) at 2 weeks duration shows positive expression of TGF- β in osteocytes, osteoclasts (OCL). DAB stain with hematoxylin counter stain X40.

Experimental group

Immunohistochemical view of defect area treated with Hyaluronic acid at 2 week duration shows positive expression of TGF- β in osteoblasts, osteocytes, osteoclasts. Also positive localization of TGF- β in fibroblasts cell, endothelial cells and progenitor cells in bone marrow tissue **Figure (4)**.

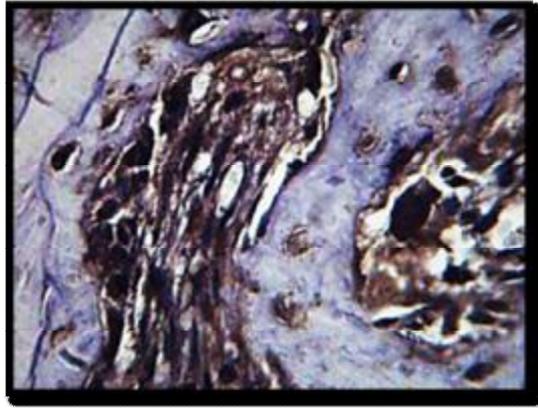


Figure 4: Immunohistochemical view of defect area treated with HA at 2 week duration shows positive expression of TGF- β in osteoblasts (OB), osteocytes (OC) and osteoclasts (OCL). DAB stain with hematoxylin counter stain X40.

At 3 weeks duration

Control group

Immunohistochemical view of control defect area at 3 weeks duration shows positive expression of TGF- β in progenitor cells, endothelial cells, osteoblasts, osteocytes, osteoclasts cells and negative expression of TGF- β in bone trabeculae **Figure (5)**.



Figure 5: Control defect area at 3 weeks duration shows positive expression of TGF- β in preosteocytes (arrows), osteocytes (OC) and osteoclasts (OCL). DAB stain with hematoxylin counter stain X40.

Experimental group

Immunohistochemical view of experimental defect area at 3 weeks duration shows positive expression of TGF- β in progenitor cells, osteoblasts, osteocytes, osteoclasts and negative expression of TGF- β in bone trabeculae **Figure (6)**.



Figure 6: View of defect area treated with Hyaluronic acid at 3 week duration shows positive expression of TGF- β in osteoclasts (OCL) and osteocytes (OC). DAB stain with hematoxylin counter stain X40.

At 6 weeks duration

Control group

Immunohistochemical view of control defect area at 6 weeks duration shows positive expression of TGF- β in bone marrow stromal cells in marrow tissue, osteoblasts, osteocytes and negative expression of TGF- β in bone trabeculae **Figure (7)**.



Figure 7: View of control defect area at 6 weeks duration shows positive immunohistochemical localization of TGF- β detected by BMSC in marrow tissue (arrows) and haversian canal (HC). DAB stain with hematoxylin counter stain X10.

Experimental group

Experimental defect area treated with Hyaluronic acid at 6 week duration labeled with TGF- β antibody shows positive expression of TGF- β in osteoblasts which lined the haversian canal and in osteocytes which embedded in osteon **Figure (8)**.

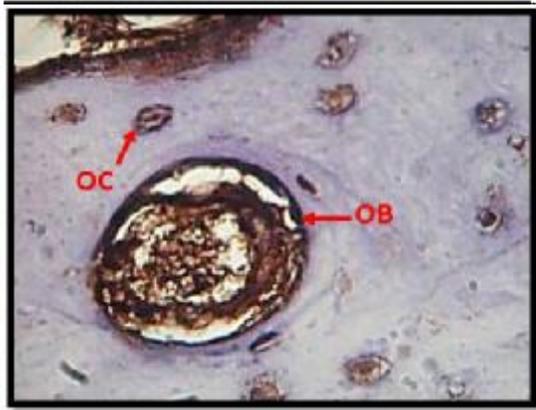


Figure 8: View of defect area treated with Hyaluronic acid at 6 week duration shows positive localization of TGF-β in osteocytes (OC) and in osteoblasts cell which lined the havercian canals. DAB with hematoxylin counter stain X40.

Table 1: Groups' comparison for Positive Stromal Cell expressed TGF-β in each duration

| Variables | Duration | Groups' Comparisons d.f. = 7 | |
|----------------------------------|----------|---------------------------------|------------|
| | | t-test | p-value |
| Bone Marrow Stromal Cells (BMSC) | 1 week | -22.12 | 0.000 (HS) |
| | 2 weeks | -13.53 | 0.000 (HS) |
| | 3 weeks | -11.97 | 0.000 (HS) |
| | 6 weeks | -9.26 | 0.000 (HS) |

2- Bone cells

The highest mean value for positive expression for TGF-β by osteoblasts and osteocytes were seen in 2 weeks duration for HA treated group .While the highest mean value for positive expression of osteoclast was seen in the 2 weeks for control group **Figures (10,11,12).**

Immunohistochemical scoring for expression of cells for TGF – beta
1-Bone marrow stromal cells (BMSC)

There was decrease in positively stained BMSC score mean values for TGF – beta with the time for both groups with the increase in mean values for HA treated group than that of the control one in all intervals, as shown in **Figures (9).**

According to the T-test (table 1) which illustrates highly significant differences between experimental and control groups in positive expression BMSC for TGF-β in all intervals.

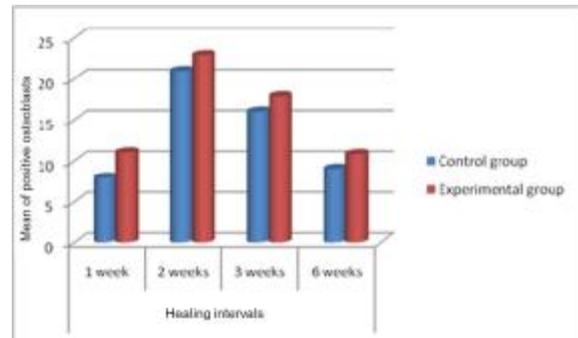


Figure 10: Comparison the Mean of positive osteoblasts for TGF-β with time of healing for control and experimental groups.

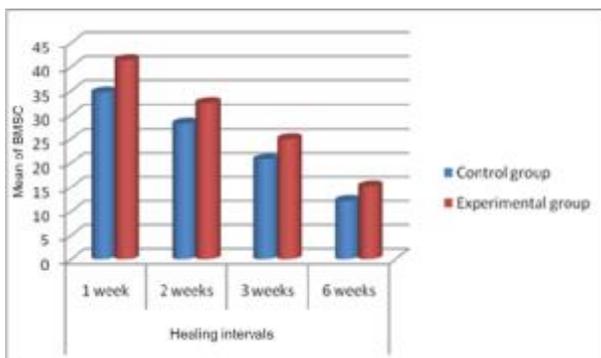


Figure 9: Comparison the Mean of positive (BMSC) expressed TGF-β with time of healing for both groups.

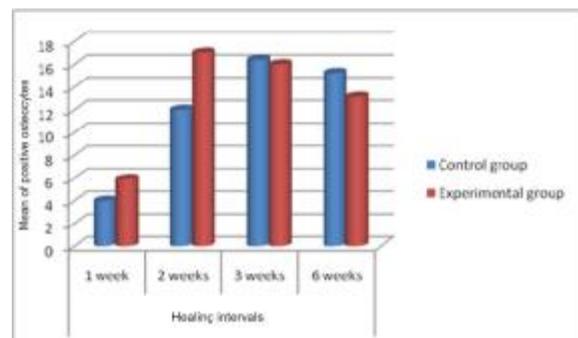


Figure 11: Comparison the Mean of positive osteocytes for TGF-β with time of healing for control and experimental groups.

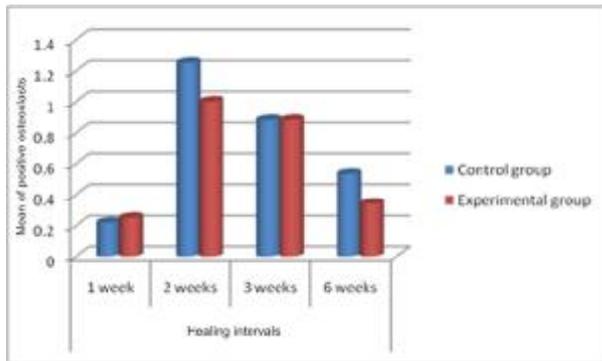


Figure 12: Comparison the Mean of positive osteoclasts with time of healing for control and experimental groups.

According to t-test (table 2), showed highly significant differences between both groups in all interval in the number of positive expression of osteoblasts for TGF-β. The positive osteocytes number showed significant difference between control and experimental groups at 3 weeks interval and high significant difference between both groups in other periods. While the results showed none- significant differences between control and experimental groups for positive expression of osteoclasts for TGF-β in all intervals.

Table 2: Groups' comparison for Positive Bone cells expressed TGF-β in each duration

| Variables | Duration | Groups' Comparisons d.f. = 7 | |
|-------------|----------|---------------------------------|------------|
| | | t-test | p-value |
| Osteoblasts | 1 week | -22.56 | 0.000 (HS) |
| | 2 weeks | -21.36 | 0.000 (HS) |
| | 3 weeks | -14.54 | 0.000 (HS) |
| | 6 weeks | -24.59 | 0.000 (HS) |
| Osteocytes | 1 week | -24.18 | 0.000 (HS) |
| | 2 weeks | -29.57 | 0.000 (HS) |
| | 3 weeks | 2.50 | 0.041 (S) |
| | 6 weeks | 9.75 | 0.000 (HS) |
| Osteoclasts | 1 week | -0.36 | 0.732 (NS) |
| | 2 weeks | 0.89 | 0.401 (NS) |
| | 3 weeks | 0 | 1 (NS) |
| | 6 weeks | 1.53 | 0.170 (NS) |

DISCUSSION

TGF-β regulates a broad range of biological processes, including cell proliferation, cell survival, cell differentiation, cell migration, and production of extracellular matrix (ECM) (9,10). The combined actions of these cellular responses mediate the global effects of TGF-β on immune responses, angiogenesis, wound healing, development, and bone formation (11).

The cell types in which TGF-beta could be detected immunohistochemically varied with time: first inflammatory cells, then cells in late hypertrophy and calcifying cartilage, then osteoblasts and bone marrow granulocytes stained for TGF-beta. TGF-β is expressed from the very early stages throughout roles as a potent chemotactic factor of mesenchymal progenitor cells and macrophages to the wound healing site, induction of their subsequent proliferation and stimulates production of the extracellular collagenous matrix by osteoblasts (12).

The current results showed that BMSC cells had a highly positive expression for TGF-β polyclonal antibody at early stage of bone healing (1 and 2 weeks), and the expression decreased in late stage (3 and 6 weeks) in both groups. This result was in agreement with Jaafar (13). TGF-β has a positive function in the early differentiation stage of osteoprogenitor cells but it has an inhibition effect on differentiation in the terminal stage (14).

At one week duration both groups shows positive localization of TGF-beta in numerous active mesenchymal stromal cells within marrow tissue. Also bone defect area showed positively stained formative cells that are irregularly arranged within primitive osteoid tissue and in fibroblasts but HA-treated group shows additional positive TGF-beta expression in some osteoblasts which lined bone spicules than that of control one.

After 2 and 3 weeks interval, TGF-beta expression was illustrated in osteoblasts which lined the bone trabeculea and in osteocytes for both groups. At 6 weeks interval, control and experimental groups showed moderate positive TGF-beta expression in osteoblasts lined the haversian canals and osteocyte cells in new bone. These findings agreed with others (15,16).

TGF-β is released by platelets into the bone defect hematoma, and then synthesized by osteoblasts and chondrocytes throughout the healing process (17). This explains the increase of TGF-β concentrations within the first 2 weeks after bone defect in the present study. This increase may partly be attributable to the absorption of cytokines from the fracture site into the circulation.

Finally the present study illustrated that the Hyaluronic acid was osteoconductive material that enhance osteogenesis and accelerated bone healing process by promoting cell adhesion and osteoblast differentiation.

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الخلاصة

الخلفية: إن تقنية تعويض العظام باستخدام نظام بدائل عظمية أصبحت شائعة الاستخدام في الكثير من المجالات الطبية. وهذه البدائل يجب ان تكون متوفرة، سهلة التطبيق بأقل ضرر نسيجي ممكن ولها القدرة على اطلاق عامل التحفيز العظمي و قدرة على تكوين عظام جديدة موضعيا في موقع الحقن. حمض الهيالورونيك يتميز بخاصية تكوين العظام التي يمكن استخدامها ليس فقط لإصلاح عيوب العظام ولكن أيضا لتوفير غرس عظمية تستخدم لإعادة بناء أنواع مختلفة من عيوب العظام. وكان هدف هذه الدراسة هو تقييم تأثير حمض الهيالورونيك على التئام العظم من خلال دراسة شفاء العظام باستخدام التقييم المناعي النسيجي الكيميائي لعامل نمو (TGF- β 3) في مجاميع الاختبار والسيطرة.

طريقة العمل والمواد المستخدمة: تم استخدام اثنان وثلاثون من ذكر الأرانب النيوزلندي الناضج. تم عمل ثقبين لكل أرنب على الجانب الأيمن والأيسر من الفك العلوي. الثقب الأيمن ملء بهلام حمض الهيالورونيك لكي يستخدم كعينة تجريبية و ترك الثقب الأيسر لكي يشفى تلقائيا ويستخدم كعينة مقارنة. تم تقسيم الارانب عشوائيا الى أربع فترات حسب وقت القتل (اسبوع، اسبوعان، ثلاثة أسابيع و ستة أسابيع) بعد اجراء العملية وتم استخدام فحوصات نسيجية مناعية كيميائية لاقتفاء ظهور مستقبلات عامل تحول النمو (TGF- β 3) لجميع الحيوانات ولكل مراحل الالتئام.

النتائج: اظهرت نتائج الفحص الكيميائي النسيجي المناعي ان العلاج باستخدام حمض الهيالورونيك يزيد من مستوى التعبير الايجابي لعامل النمو المحول TGF- β 3 على الظهور في الخلايا البانية للعظم والخلايا المكونة للعظم وخلايا نسيج نخاع العظم خاصة في الاسبوع الأول والثاني من فترات شفاء العظم مقارنة بتلك التي لوحظت في مجموعة المقارنة.

الاستنتاج: اظهرت هذه الدراسة ان حمض الهيالورونيك هو مادة تظهر خاصية توصيل عظمي ولها القدرة على تعزيز تكوين العظم وتسريع عملية الشفاء العظم أكثر من عملية الشفاء الفسيولوجية الطبيعية.

الكلمات الرئيسية: تعويض العظام، عامل التحفيز العظمي، عامل النمو، حمض الهيالورونيك.