Effect of Osteon II Collagen with Hyaluronic Acid and Collagen Membrane on Bone Healing Process in Rabbits: A Radiographical Study

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ABSTRACT

Objective: To evaluate the effectiveness of local application a new injectable bone graft substitute osteon II collagen separately and with addition of hyaluronic acid and collagen membrane in surgically created bone defect in tibia of rabbits.

Materials and Methods: Twenty adult male rabbits were used in this study. Two bone defects were made in the tibia of each rabbit. The defects were filled with Osteon II/hyaluronic acid, Osteon II collagen with collagen membrane, Osteon II collagen alone and the other left unfilled as control. Specimens were collected in one, two and four weeks after surgery. The radiodensity and the amount of bone formation were evaluated radiographically.

Results: It was found that Osteon II collagen with Hyaluronic acid combination lead to significant increase in bone density and more amount of new bone formation (P≤ 0.05) in 2 and 4 weeks postoperatively than other groups. On the other hand Osteon II collagen with Collagen membrane showed better results than Osteon II collagen, and control groups.

Conclusions: It was concluded that topical application of Hyaluronic acid with bone substitute material will improve healing process of the bone represented with more new bone formation radiographically and densitometry compared with collagen membrane that added to bone graft material or with bone graft alone.

Key words: Bone regeneration, osteon II, Collagen membrane, Hyaluronic acid, Digital radiograph, CT scan

INTRODUCTION

Bone grafting therapy has become an integral part of dentistry; patients are becoming more aware of grafting as a treatment modality and expect better predictability, fit, function and esthetics. Today, with the introduction of advanced bone grafting techniques and the use of sophisticated bone replacement graft materials, it is possible to increase the volume, width, and height of bone in deficient areas to regenerate the tissues supporting (1). Osteon is one of alloplastic materials composed of hydroxyapatite 70% and beta-tricalcium phosphate 30% which are most close to major mineral components of human bone. Osteon is osteoconductive material that acts as bone growth scaffold. It has interconnected porosity structure which is similar to that of human cancellous bone (2). Hyaluronic acid (HA) has osteoconductive potential; it accelerates the bone regeneration by means of chemotaxis, proliferation and successive differentiation of mesenchyme cells. HA may act as biomaterial scaffold for other molecules, such as Bone Morphogenic Protein-2 (BMP-2) and Transformation Growth Factor-β (TGF-β) used in guided bone regeneration techniques and tissue engineering research (3). Resorbable collagen membranes (RCMs) are manufactured from allogeneic or xenogeneic sources to manage oral wounds such as extraction sockets, for sinus-lift procedures and repairs, and for periodontal or endodontic surgeries (4).

Barrier membranes are among the most widely studied scaffolds for tissue regeneration, including bone, and the choice of type of membrane depends largely on the required duration of membrane function (5) The purpose of the present study was designed to investigate and evaluate the effectiveness of a new injectable bone graft substitute osteon II collagen in bone healing process when used separately and to compare the effect of osteon II collagen when mixed with hyaluronic acid once or combined with collagen membranes in bone regeneration process. Rabbits were used as animal model in this study because of their short developmental period and their faster bone turnover the rabbit has faster skeletal change and bone turnover (significant intracortical, Haversian remodeling and it achieve skeletal maturity
(closure of epiphyseal plates) shortly after reaching complete sexual development at approximately 6 months (6). Also there is minimal literature between human and rabbit bone composition and bone density, some similarities are reported in the bone mineral density (7). There are many study conducted to investigate the sole effect of local application of hyaluronic acid, collagen membrane that added to the bone grafting material on bone regeneration process. According to our knowledge this is the first study that compare between these two materials (hyaluronic acid and collagen membrane) when adding to osteon II collagen to fill the bone defect. The bone density and the amount of new bone formation were determined by densitometric and radiographic analysis.

MATERIAL AND METHODS

Animal model and Surgical Procedure

The protocol of this study was approved by scientific Committee Department of oral and maxillofacial surgery college Dentistry of Mosul. The surgical part done in surgical department/Veterinary Collage of Duhok University. This study was carried out on local twenty male rabbits of (8–10 months) weighing 2000–3000 g was used as experimental animal. During the entire period of the experiment the rabbit was fed three times daily with greenery diet and tap water and general heath continuously monitored, the animal were housed in animal house of Veterinary Collage of Duhok University. Animals were quarantined for 7 days prior to the surgical procedure to check their general statement and ensure the absence of general infectious disease before the operation time, the animal legs were washed with soap and water before the surgical procedure and then the operation side was shaved, and disinfected with 10% povidin iodine.

A mixture containing (10mg/kg) ketamine hydrochloride general anesthetic agent (Gracure pharaceuticals Ltd.,Bhwadi,India) and (2mg/kg) xylazine sedative (Xyla - Interchemie, Holland) and atropine 2mg/kg solution (pharmaceuticals Ltd.,Bhwadi ,India) were given intramuscularly to achieved general anesthetization. Complete anesthesia was obtained within 10 minutes, this dose kept the rabbit anesthetized for about half hour. Local anesthesia, 2% lidocaine HCL with epinephrine 1:80.000 local anesthetic agent was administered by infiltration at the surgical site prior the incision for hemostasis. The animal was placed in lateral position during the procedure. A 5cm skin incision was made along the longitudinal axis of lateral aspect of each tibia, fascia was dissected and full-thickness flap was reflected to expose the under lining bone of each tibia under aseptic condition (Fig.1).

![Figure (1) The study materials: Application of the materials, two bone defect D1 control group,D2 hyaluronic acid mixed with osteon II collagen, on the right tibia, D3 defect osteon II collagen with collagen membrane, osteon II collagen alone D4, on the left tibia.](image)

After exposure and reflection of soft tissue, two monocortical holes (cylindrical in shape) on each tibia was performed with trephine bur of 3mm in width (Dentium Company- Made in Korea), and 4mm depth until reach the marrow space
under constant normal saline irrigation on a slow speed surgical hand piece at 1500 rpm to prevent thermal bone necrosis (SAESHIN X-Cube Implant surgery motor,South Korea). The distance which were left between each hole about 1cm. Then the bone cavities were carefully washed with sterile normal saline to eliminate bone debris before being filled with the materials. The right tibia had 2 defect :the first one serve as control which left empty, the second one filled with (0.2 ml of hyaluronic acid gel(manufactured by Bio Polymer, Germany) and bone graft material osteon II collagen, manufactured by Dentium Company (GENOSS Seoul, Korea.), the mixture was left untouched for 5 minutes to achieve homogeneity. The mixture was loaded in each defect and gently pressed. The second operation in the left tibia had 2 bone defects also; one of them filled with osteon II collagen alone, another one had osteon II collagen covered with collagen membrane (Resorbable Membrane made in Korea). At completion of material placement, the flap was gently approximated and primary wound closure was performed, using 3-0 non resorbable black silk suture (Huaian A. M. instrument.,China), which was to be removed 10 days post – operatively. Finally, topical antibiotic was applied at the wounds site and dressed. The animals were given oxytetracycline hydrochloride injectable solution 50mg/Kg (Chongqing Fangtong Animal pharmaceutical, China). The animals were sacrificed at 1, 2, and 4 weeks postoperatively.

Radiological analysis

The operated tibias were dissected sub peristeal to allow direct observation of newly formed bone; and fixed in buffered 10% formalin solution.

A. Computer Tomography Scan assessment:

To measure the bone density all specimens were subjected to CT scan radiographical examination in Wan- hospital (CT scan Siemens AG2002computer tomography, Siemens TR. 1, D91301, Germany Dicum print4 system). All images were scan with 32 slices multidetector CT dental protocol axial slices to assess the amount of bone density. Computed tomography (CT) scans, increment thickness of 0.6 mm, and multi-planar coronal, sagittal reconstructions. Three-dimensional changes in the bone were evaluated by measuring the largest diameter (mm) in the anterior–posterior direction, medial –lateral direction and depth on each scan. The anterior–posterior size was measured at the level of the sub choral bone plate on the sagittal CT reconstruction. The image from CT scan was loaded to work station (Siemens AG 2002, T R .1, Da130, Germany) with work place software (Synco CT). The cross section images of tibia were transferred and reformed in three planes (axial, coronal and sagittal) through multi planer reconstruction, drawing three points inside the defect and take the mean for it. The anterior–posterior size was measured at the level of the sub choral bone plate on the sagittal CT reconstruction. The radiographic computer tomography was measured (3D) for each defect after make three point, two in the lateral and one medium position inside the defect then take the mean for each defect. The results are expressed as the mean ± standard deviation (SD) for each defect was performed.

B. Digital dental x-ray:

The radiographic examination was carried out in digital radiography which confirmed by correct positioning of the specimen in lateral radiograph. A digital radiograph was taken to all specimen using portable dental x-ray system and digital sensor (Rextar X, made in Japan), attached to it calibration stainless steel wire of 10 mm in length. The tibia of the rabbit was placed in contact with the sensor and the lateral border of the tibia is parallel to the sensor and the distance between the end of the long cone and the sensor was focused to 30 cm) and the cone was kept perpendicular to the sensor all the time. Using the following parameters 8 mA, 63Kv, and exposure time 0.12 sec. and the read out starts automatically, the image was displayed gradually on the computer screen, when the read out was completed; the newly read image was stored.

Measurement the amount of bone formation:

The radiographic image was transferred to the Image J 1.47v software (National Institute of Health, USA), which enable us to quantify the amount of bone formation. Then by selecting a rectangular area of pre- defined size (12mm2 ) to define original size of bone defect to assess the newly formed bone (Fig.2-A). The region of defect was delineated through drawing lines of 4 mm in length which represent the upper and lower border of defect and these lines are joined with two vertical lines of 3mm in height which represent the side wall boundaries of the defect. Then start to estimate the opacification area through using a polygonal shaped symbol in selector tool of the main windows of image J program to demonstrate the periphery of radio dense area within the defect cavity that representing ossification zone of newly formed bone. The image were calibrated first to get a reading in millimeters by measuring the length of reference wire, so the reference distance was defined before using the function of area measure from analyze tool in the program(Fig.2-B). The measurements were performed three times in each defect in different days to minimize inter-observer measurement error and the mean values of all reading for each defect were obtained.
Figure (2-A): Demonstrate the area of bone formation using ImageJ software used for digital assessment of healing area which is displayed in the "result" window in mm.

Figure (2-B): Demonstrate the area of bone formation using ImageJ software used for digital assessment of healing area which is displayed in the "Result" window in mm.

The average value of bone formation was measured by two examiners who didn’t know to which group the animals belonged to the estimation of new bone formation was applied by using an equation as follow:

\[
\text{Area of new bone formation} = \text{Total defect area} - \text{non formed bone area} \\
\text{Percentage of bone formation} = \frac{\text{Area of new bone formation}}{\text{Total defect area}} \times 100
\]

All the increased area of opacification from the external and internal surface of original defect size was measured. The mean of value were submitted to, one-way analysis of variance (ANOVA, followed by Duncan test to identify significant differences among groups and P value \( \leq 0.05 \) were taken to be significant.
RESULTS

Radiological Assessment of bone regeneration by Computer Tomography (Measurements of bone Density):

The mean value of bone density of bone defects, standard deviation, and p values in all experimental groups at one, two and four weeks intervals are summarized in (Table 1). The analysis of bone density for all test sites utilized by CT scan demonstrated at each period intervals showed a significant increase in both experimental groups compared with control group along the periods of the study (chart 1).

Table (1) Mean and P value radiographic finding of bone density evaluation by CT scan of regenerated area in all groups’ radio graphical using ANOVA and Duncan test.

<table>
<thead>
<tr>
<th>HEALING PERIOD GROUPS</th>
<th>1WEEK</th>
<th>2WEEKS</th>
<th>4WEEKS</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 Control group</td>
<td>A 74.8±15.98</td>
<td>A 115.60±31.16</td>
<td>A 338.60±125.26</td>
<td>.001</td>
</tr>
<tr>
<td>D2 Osteon with hyaluronic acid</td>
<td>104.2±44.36</td>
<td>BC 215.6 ±46.35</td>
<td>B 705.20±258.08</td>
<td>.000</td>
</tr>
<tr>
<td>D3 Osteon with Collagen group</td>
<td>113.4±44.105</td>
<td>C 292.0±48.54</td>
<td>AB 603.0±188.85</td>
<td>.000</td>
</tr>
<tr>
<td>D4 Osteon group</td>
<td>A 124.2±60.28</td>
<td>B 184.2±89.62</td>
<td>A 375.4±159.51</td>
<td>.001</td>
</tr>
<tr>
<td>P value</td>
<td>.327</td>
<td>.000</td>
<td>.020</td>
<td></td>
</tr>
</tbody>
</table>

Mean significant at p≤ 0. 05
Capital letters refer to comparison among treatments (Vertically). Small letters mean comparison between periods of healing. (Horizontally)

Chart (1): Mean of CT Scan Radiographical finding using Duncan Test ; D1- Control group. D2- Osteon with hyaluronic acid group.D3-Osteon with collagen membrane group.D4-Osteon group.
Evaluation of bone density at one week postoperatively: Fig. (3-A)

At the end of one week, the mean of CT scan density of new bone was no significantly deference between groups only slight superiority in the treatment groups (p value=.327).

Figure (3) - CT Scan Radiograph: A - one week D1,D2,D3,D4. All groups showed no radiographical bone healing; B - Two weeks D1,D2,D3,D4 showed more bone healing in D2,D3 more than D1,D2; C - four weeks D1,D2,D3,D4, superiority in bone density in D2,D3 more than D1,D2; R (radiographic taken to demonstrate the area of bone density)
Evaluation of bone density at two weeks postoperatively: Fig. (3-B)

At the end of two weeks, the density of bone defects in CT scan, bone healing was superior in all groups, and exhibited bone density in D3 Osteon II collagen with Collagen groups, was (292.0±48.54) and D2 Osteon II collagen with Hyaluronic acid groups(215.6±46.35) and D4 groups Osteon II group (184.20±89.63), significant difference (p value= 0.000)

Evaluation of bone density at four weeks postoperatively: Fig. (3-C)

The CT scan examination of healed bone defects site demonstrated significant progression bone density in D2 Osteon II with Hyaluronic acid groups was (705.20 ±258.08) and D3 Osteon II with Collagen groups was (603.0± 188.85) and Osteon II collagen group (375.4±159.51). Bone healing was exhibited a more advanced phase of bone formation in groups Osteon II with hyaluronic acid group and Osteon II collagen with collagen membrane group, treated defects. (pvalue=.020).

Computer Tomography (CT) imaging of bone regeneration:

The restoration of bone at the defect was observed using X-ray- CT scan detailing the progressive bone regeneration illustrated in Figure (3) the control group shows no signs of bridging ossification at one week thus healing of the defect was representative of incomplete healing. The defects were clearly detectable tile four weeks although bone regeneration seems to be proceeding. The effectiveness of Osteon II collagen with Hyaluronic acid and osteon II collagen with collagen membrane showed at two weeks a thin layer of bone developing at the defects were barely detectable from surrounding normal structure at four weeks although the defects seemed complete healing and significant difference in all groups was observation.

Radiographic evaluation of bone formation (DIGITAL):

Radiographic evaluation for groups through different period: Table (2), Chart (2).

Table (2) Mean & p-value of Image J radiographical finding for bone Formation Using ANOVA and Duncan Test.

<table>
<thead>
<tr>
<th>Healing period</th>
<th>Groups</th>
<th>1week</th>
<th>2weeks</th>
<th>4weeks</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1:control</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>22.69±29.07</td>
<td>28.48±7.561</td>
<td>70.42±13.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D2: Osteon II with HA</td>
<td>A</td>
<td>C</td>
<td>B</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>30.44±15.60</td>
<td>54.96±9.53</td>
<td>95.46±12.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D3:Osteon II With collagen</td>
<td>A</td>
<td>BC</td>
<td>AB</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>34.44±15.60</td>
<td>45.34±7.44</td>
<td>86.12±10.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D4: Osteon II</td>
<td>A</td>
<td>AB</td>
<td>A</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>25.69±20.33</td>
<td>36.56±7.32</td>
<td>76.86±10.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>.106</td>
<td>.001</td>
<td>0.022</td>
<td></td>
</tr>
</tbody>
</table>

Mean significant at p≤0.05
Capital letters refer to comparison among treatment (vertically).
Small letter refer to comparison among period (Horizontally).
Chart (2) Mean of Digital Radiograph using Duncan Test D1: Control group; D2: Osteon II with hyaluronic group; D3: Osteon II with collagen membrane group; D4: Osteon II group

All groups showed Radiographic evaluation through different period postoperatively there was new bone formation and significant statistical difference found between them (p value=.000).

Radiographic evaluation at one week: Fig. (4)
Figure (4) Digital radiograph: show new bone formation at difference period: 1, 2, and 4 weeks. D1: Control group; D2: Osteon II with hyaluronic group; D3: Osteon II with collagen membrane group; D4: Osteon II group.

When a radiological comparison was made between groups through 1 week postoperatively there were no significant statistical difference found between them at one week. (p value=.106). Radiographic evaluation of bone healing was superior in (D3) Osteon with collagen groups (34.44±15.60) and D2 Osteon II with Hyaluronic groups (30.44±15.60).

Radiographic evaluation at two weeks: Fig. (4)

Radiographic evaluation of bone healing was superior in (D2) Osteon II with Hyaluronic acid (54.96±9.53) and bone formation in D3 Osteon II with collagen (45.34±7.44) while Osteon II alone group 36.56±7.32; p value = .001.

Radiographic evaluation at four weeks: Fig. (4)

Radiographic evaluation of bone healing was superior in (D2) Osteon II with Hyaluronic acid (95.46 ±12.30) then (D3) Osteon II with collagen was (86.12±10.92), Osteon II alone group was (76.86±10.630). There were statistically differences in the results of bone formation through the detection periods of evaluation. P value =.022.

DISCUSSION

Treatment of large bone defects represents a great challenge, as bone regeneration is required in large quantity and may be beyond the potential for self-healing. Large bone defects include segmental or large cortical defects created by trauma, infection, tumor resection, aseptic loosening around implants and skeletal abnormalities. In this study used new bone graft material Osteon II collagen which is a newly developed alloplastic material containing 70% HA and 30% b-TCP which are quite close to major mineral components of the human bone. The addition of collagen with bone graft material to increase the osteoconductivity of Osteon II.

No evidence of post-operative infection was noticed in this study. This may be due to the application of sterile surgical procedure and the use of antibiotic during postoperative care in the form of intramuscular injection and topical antibiotic spray and the high concentration of high molecular weight of HA has the greatest bacteriostatic effect particularly on aggregatibacter actinomy cetemcomitans Prevotella and Staphylococcus aureus strains commonly found in oral gingival lesions and periodontal wounds. Clinical application of HA gels as surgical therapy may reduce the bacterial contamination of surgical wound site, thereby, lessening the risk of post-surgical infection and promoting more predictable bone generation.

The use of Collagen membranes which was designed in this study is to prevented the apical migration of epithelium and supported new connective tissue attachment and tissue regeneration. The additional use of bone-grafting materials within the membrane to fill the defect should also be evaluated, aiming to 'mimic' or even accelerate the normal process of bone formation. The rabbit has faster skeletal change and bone turnover so it choses as animal model for this study. According to the scope of the present study it is the first time to evaluate the effectiveness of locally application hyaluronic acid and collagen membrane with Osteon II collagen and compare between them on healing processes of the bone.
In the present experimental investigations were focused on material that used for accelerating bone regeneration and maturation of bone to shorten the treatment period and improve the quality of bone mass. Groups using deminestometric analysis by CT scan indicates more maturation of the regenerated bone in tested groups, as confirmed by increased bone density over all time points. Observation and assessment of callus density is facilitated because the holes of bone defect are quickly distinguishable on the images obtained by CT scan clearer than using DEXA. Although the radiographic evaluation showed that the Osteon II collagen with hyaluronic acid groups have greater osteogenic potency than Osteon II collagen with collagen membrane group; these two groups have greater osteogenic potency than Osteon II collagen alone and control groups. These result was agree with (Aslan et al., 2006) which demonstrated that the Osteon II collagen with hyaluronic acid groups have superiority bone healing histologically. (15).

At the first week postoperatively no significant difference between the four experimental groups, although Osteon II collagen with hyaluronic acid groups, Osteon II collagen with collagen membrane groups enhance bone formation that correlated to increase bone healing. At the end of the second week postoperatively, there were significant increased bone formation among experimental groups. At the end of four weeks the newly formation bone in Osteon II collagen with hyaluronic acid showed more radiodense and greater amount of bone formation than other experimental groups. The result of this study was agreed with Kim (16) that demonstrated the important of osteoconductive and osteoinduction processes which are actively ongoing. The explanation of Hyalonect and grafting that significantly enhance the healing process when used alone or together related to increase morphogenesis and tissue healing during bone regeneration (8). The study done by Aslan et al. (15) were compared the effects of autologous bone grafting with or without HA in a rabbit tibia defect model they reported that HA requires an osteoconductive scaffold to be effective. The present study showed that Hyalonect or grafting significantly speeds the healing process with better early radiological bone healing in combination group than bone grafting alone, especially in the short term (16). At the end of four weeks the result showed more bone formation in Osteon II collagen with collagen membrane due to the action of collagen membrane preventing the non osteogenic cells from proliferation in the site where bone formation is wanted to grow (17). Resorbable collagen membranes are frequently used as wound dressings because they act as a scaffold, promote platelet aggregation, stabilize clots, and attract fibroblasts, facilitating wound healing; therefore often used for GBR (17). Collagen membrane act as scaffolds for bone deposition in guided bone regeneration (GBR) facilitating wound healing (16).

CONCLUSION

The use of osteon II collagen with hyaluronic acid seemed to produce appositive effect on radiographical assessment of amount of bone formation at second and fourth weeks of the study. A good correlation between osteon II collagen with hyaluronic acid group compare with Osteon II collagen with collagen membrane group radiographically found superiority of Osteon II collagen with hyaluronic acid. The bone density of regenerated bone is greater in Hyaluronic acid with Osteon II collagen and in collagen membrane with Osteon II collagen compared to control groups and Osteon II collagen groups along period of study.

REFERENCES


