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# The Effect of Chitosan Extracted From Iraqi Freshwater Crab and Marine Shrimp on Bone Healing (Experimental Study on Sheep)

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**ABSTRACT**

**Aims:** The aims of this study is to evaluate the effect of chitosan prepared from Iraqi freshwater crabs and marine shrimps in accelerating bone healing in surgically created bone defects in sheep.

**Materials and Methods:** Five healthy male sheep were included in the experiment. Each sheep model was to serve as four observation subgroups where each limb in the same sheep represented a specific observation period and according to a pre-planned sacrifice schedule. In each limb, four monocortical defects were created, three filled with three chitosan samples of different sources and one defect left empty (negative control). Chitosan tissue responses in addition to its effect on accelerating bone healing were evaluated radio graphically and histopathologically at two, four, six and eight weeks post-surgical intervals.

**Results:** In this study, radio graphically there was a trend of greater radiopacity (represented by increase in mean gray scale) in all chitosan filled bone defects when comparing with negative control bone defects. Histopathologically, more osteoid tissue formation was observed at fourth, sixth and eighth postoperative periods in all chitosan filled bone defect in comparison with negative bone defects.

**Conclusion**: The chitosan was concluded to be a natural biocompatible biomaterial enhancing and accelerating bone formation.

**Key words**: Chitosan Extracted, Freshwater Crab, Marine Shrimp, Bone Healing

## INTRODUCTION

Bone defects, may develop following tumor removal, dental and bone lesion or due to periodontal tissue disease are serious challenges which need bone repair. The conventional methods of bone repair which commonly are used, such as autografts and allografts have their own problems and drawbacks. Autografts have limited materials availability and may result in donor site morbidity [1.].Allografts using may be more desirable in some cases, but the possible immune reaction and infection transmission limit their application. Today, naturally derived biomaterials have been attracting scientist's interest all over the world. Recently a special attention has been made toward using the materials which are derived from nature. Such materials would have some advantages over synthetic ones. Most notably, they have been shown to yield faster healing and expect to exhibit greater compatibility with bone[2,3.].

This study was an attempt for histological and radiographical evaluation of the effect of chitosan prepared from Iraqi freshwater crabs and marine shrimps in accelerating bone healing in surgically created bone defects in sheep.

## MATERIALS AND METHODS

**Experimental animals:**

A total number of 5 healthy male sheep with an average age (8-10months) and weighing at least (25-30 kilograms) were included in the study. They were examined by a veterinarian to rule out the presence of any disease, to check general health

and condition of the animal before the surgical procedures. The animals were housed at the animal housing unit in the College of Dentistry / Mosul University throughout the period of study until sacrification time. Their health and feedingwas regularly supervised by a vetenerian doctor.

**Surgical Procedure:**

Each sheep model was to serve as four observation subgroups where each limb in the same sheep represented a specific observation period and according to a pre-planned sacrifice schedule.

For general anesthesia (induction and maintenance), an intramuscularinjection of a mixture containing (10mg/ml/kg) Ketaminehydrochloride general anesthetic agent and (2mg/ml/kg) Xylazine sedative, analgesic solution was given [4]. A sufficient incision was made along the lateral longitudinal axis of bone of the limb including both skin and periosteum. A full-thickness flap was reflected by a periosteal elevator to expose the bony segment of each tibia or radius. For the creation of standardized bone defects, a trephine bur of 6 mm width and 4 mm depth level mounted on a straight angle handpiece was used. The trephine bur was positioned perpendicular to the long axis of bone surface and at a speed of 1000 revolutions per minute during the preparation of the defects. Four standard bone defects of 6 mm width and 4 mm depth, more than 6mm apart were made in each tibia or radius.

These bone defects are done under continuous irrigation with cold normal sterile 0.9% physiological saline. From a proximal to distal orientation, the first defect was gently packed with crab derived chitosan (Cr-chitosan) the second defect left to be filled with blood (negative control), the third defect filled with shrimp derived chitosan (Shr-chitosan) and the fourth filled with standard chitosan material brought from china (S-chitosan). At completion, the wound was inspected, and the flap was gently reapproximated and primary wound closure was performed using 3-0 non resorbable black silk suture. Intramuscular injection of antibiotic Ox tetracycline 20 mg /ml / 10 kg B.W and intramuscular injection of Diclofenac sodium 1ml/ 10 kg B.W per day were given for 5 days postoperatively [5,6].At the completion of planned surgery schedule for each sheep, all animals were slaughtered by a licensed butcher and the portion of limbwith the defects were separated from the bodyand placed in neutral buffered formalin 10%[7].

**Radiographical evaluation of bone healing**

Conventional radiography was taken for each specimen using occlusal radiographic films (Kodak Ultra-speed Dental films / USA) with the following settings pre-adjusted:Cone to object distance (30 cm),Kilo voltage (Kv): 70,milliAmp: 8and Time of exposure: 0.8 seconds

For each occlusal film, a 1cm piece of stainless steel wire was placed nearby each specimen. This piece was placed on each of the occlusal films before exposure. The purpose was to aid in setting a scale of measurement when using software image analysis. After film processing, digital images (in JPG format) were obtained by using a high resolution scanner at 2400dpi resolution for all occlusal films in order to be subjected to a computerized software image analysis program (Image j software program / version 1.48v) and this was the objective modality for assessment.

## RESULTS

**Radiographical assessment of bone defect healing between groups**

**A- Two Weeks**

At two week intervals, Kruskal-Wallis test results (Chi-Square) for mean gray scale values showed no statistical significance among the four groups, table (1)and figure(1- A).

**Table (1) Mean gray scale value comparison among the four groups at two weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **-ve control** | 5 | 66.53 | 1.75 | 0.852 | 0.837 |
| **Crab source** | 5 | 65.96 | 2.26 | | |
| **shrimp source** | 5 | 67 | 0.85 | | |
| **+ve control** | 5 | 66.52 | 1.75 | | |

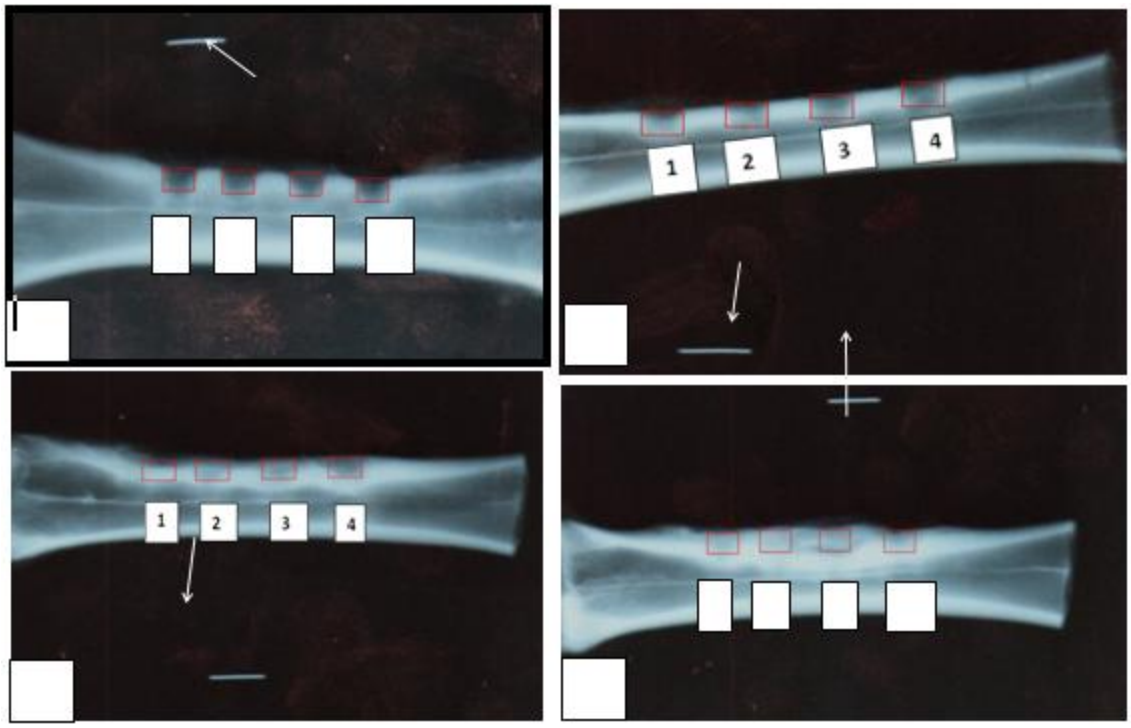


**Figure (1): radiographic picture of sheep tibia showing 10 mm stainless-steel wire (arrow). A: at two weeks interval post-Surgery, B: at four weeks interval post-Surgery, C: at six weeks interval post-Surgery and D: at eight weeks interval post-Surgery, : 1= represents the surgical bone defect filled with crab shell derived chitosan (Cr-chitosan); 2= represents the surgical bone defects left filled with blood (-ve control); 3= represents the surgical bone defect filled with shrimp shell derived chitosan (Shr-chitosan); 4= represent the surgical bone defects filled with standard or imported chitosan (+ve Control).**

**B- Four Week**

At four week intervals, Kruskal-Wallis test results (Chi-Square) for mean gray scale values showed a significant difference among the four groups, table (2) and figure (1- B).

**Table (2) Mean gray scale values comparison among the four groups at four weeks post – surgery interval.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **-ve control** | 5 | 115.51 | 4.24 | 10.749 | 0.013 |
| **Crab source** | 5 | 137.352.26 | 3.34 | | |
| **shrimp source** | 5 | 137.410.85 | 4.02 | | |
| **+ve control** | 5 | 137.311.75 | 3.89 | | |

In the Mann-Whitney test, the results showed a statistically significant difference for mean gray scale values between the negative control group at four week interval and values of the other three groups but no significant difference between crab source, shrimp source and positive groups at this interval, table (3).

**Table (3) Mann- Whitney test comparison among the four groups at four weeks post – surgery.**

| Groups | Value | Sig. |
|---|---|---|
| **-ve control vs. crab source** | 0.000 | 0.009 |
| **-ve control vs. shrimp source** | 0.000 | 0.009 |
| **-ve control vs. +ve control** | 0.000 | 0.009 |

| | | |
|---|---|---|
| **Crab source vs. shrimp source** | 11.000 | 0.754 |
| **Crab source vs. +ve source** | 12.000 | 0.917 |
| **shrimp source vs. +ve source** | 12.000 | 0.917 |

**C- Six Weeks**

At six week post-surgery intervals, Kruskal-Wallis test results (Chi-Square) for mean gray scale values showed no statistical significance between the four group defects table (4) and Figure(1-C).

**Table (4)** Meangray scale value comparison among four groups at six weeks post – surgery.

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **-ve control** | 5 | 142.95 | 1.58 | 6.754 | 0.080 |
| **Crab source** | 5 | 145.20 | 1.86 | | |
| **shrimp source** | 5 | 145.22 | 1.25 | | |
| **+ve control** | 5 | 145.76 | 1.72 | | |

**D- Eight Weeks**

At eight week post-surgery intervals, Kruskal-Wallis test results (Chi-Square) for mean gray scale values of surgical bone defects showed no statistical significance difference between the groups table (5) and figure (1-D).

**Table (5) Mean gray scale valuescomparison among four groups at eight weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **-ve control** | 5 | 143.16 | 1.99 | 6.112 | 0.106 |
| **Crab source** | 5 | 145.69 | 2.55 | | |
| **shrimp source** | 5 | 145.73 | 1.26 | | |
| **+ve control** | 5 | 146.76 | 2.09 | | |

**Histopathological Finding Assessment of Surgical Bone Defects Healing Between Groups.**

The histopathological examination was performed by two histopathologists and the researcher in a blind manner. Results and grading of each microscopical finding or criteria depended (inflammation, granulation tissue formation, fibrous tissue formation and presence of osteoid tissue) were recorded for each slide.

**A- Two Weeks Period**

At the end of the second week post-surgery, Kruskal-Wallis test analysis (Chi-Square) to compare among the four groups in concerning the inflammation and granulation tissue formation means score values showed highly statistical significance difference among the four groups,table(6) and Table (8). Concerning fibrous and osteoid tissue formation, no significant difference was detected among the four groups table (7). By using Mann-Whitney test to compare between each two groups, the results showed significant difference in inflammation and granulation tissue means score values between negative control group when compared with other three groups table (10), table (11) and figure(2) .

**Table (6) Inflammation means score values comparison among the four groups at two weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **--ve control** | 5 | 1.54 | 0.22 | 9.684 | 0.021 |
| **Crab source** | 5 | 2.26 | 0.48 | | |
| **shrimp source** | 5 | 2.32 | 0.46 | | |
| **+ve control** | 5 | 2.26 | 0.43 | | |

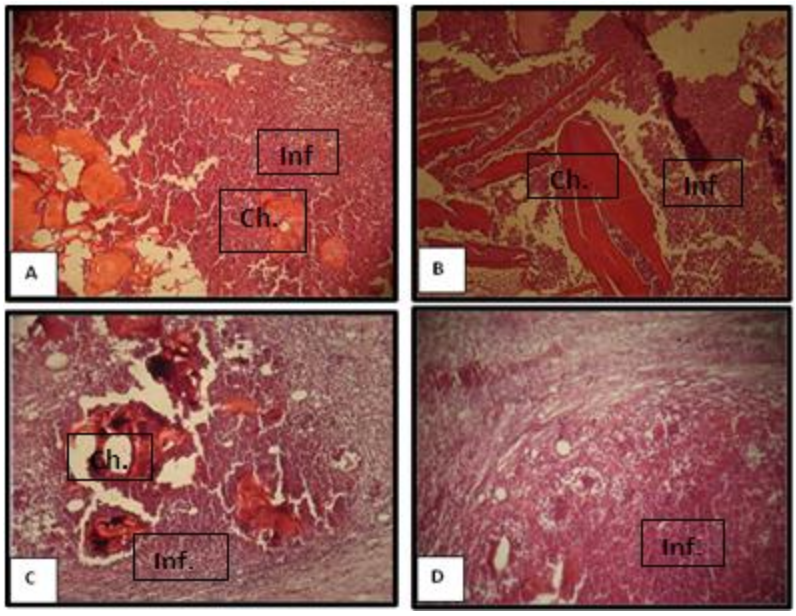


**Figure (2): Microphotographs of histological sections taken from the surgical bone defects after two weeks interval. A= positive control, B= shrimp source chitosan, C= crab source chitosan, D= negative control. The four sections have shown sever inflammatory reaction (Inf.)with erosion and degradation of chitosan particles (Ch.) (H&E,Magnification=10X).**

**Table (7) Mann-Whitney test values of inflammation mean score in surgical bone defects at two weeks post – surgery interval.**

| Groups | Value | Sig. |
|---|---|---|
| **-ve control vs. crab source** | 1.500 | 0.017 |
| **-ve control vs. shrimp source** | 1.500 | 0.016 |
| **-ve control vs. +ve control** | 0.000 | 0.007 |
| **Crab source vs. shrimp source** | 11.000 | 0.736 |
| **Crab source vs. +ve control** | 12.000 | 0.913 |
| **shrimp source vs. +ve control** | 10.000 | 0.584 |

**Table (8) Granulation tissue formation means score values comparison among the four groups at two weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **--ve control** | 5 | 1.76 | 0.13 | 11.819 | 0.008 |
| **Crab source** | 5 | 2.72 | 0.38 | | |
| **shrimp source** | 5 | 2.72 | 0.38 | | |
| **+ve control** | 5 | 2.66 | 00.48 | | |

**Table (9) Mann-Whitney test values of granulation tissue formation mean score in surgical bone defects at two weeks post – surgery interval.**

| Groups | Value | Sig. |
|---|---|---|
| **-ve control vs. crab source** | 0.000 | 0.006 |
| **-ve control vs. shrimp source** | 0.000 | 0.006 |
| **-ve control vs. +ve control** | 0.000 | 0.009 |

| | | |
|---|---|---|
| **Crab source vs. shrimp source** | 12.500 | 1.000 |
| **Crab source vs. +ve control** | 11.500 | 0.811 |
| **shrimp source vs. +ve control** | 11.500 | 0.811 |

**Table (10) Fibrous tissue formation means score values comparison among the four groups at two weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **--ve control** | 5 | 0.84 | 0.22 | 0.144 | 0.986 |
| **Crab source** | 5 | 0.76 | 0.13 | | |
| **shrimp source** | 5 | 0.74 | 0.29 | | |
| **+ve control** | 5 | 0.74 | 0.29 | | |

**Table (11) Osteoid tissue formation means score values comparison among the four groups at two weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **--ve control** | 5 | 0.00 | 0.00 | 0.00 | 1.00 |
| **Crab source** | 5 | 0.00 | 0.00 | | |
| **shrimp source** | 5 | 0.00 | 0.00 | | |
| **+ve control** | 5 | 0.00 | 0.00 | | |

**B- Four Weeks Period**

At the end of the four weeks post-surgery interval, Kruskal-Wallis test analysis (Chi-Square) for granulation tissue and osteoid tissue formation means score values showed highly statistical significance difference among the four groups table (13) and table (16) and figure (3) . By using Mann-whitney test to compare between each two groups, the results showed significant difference in granulation and osteoid tissue means score values between negative control group when compared with other three groups, table (14) and table (17).

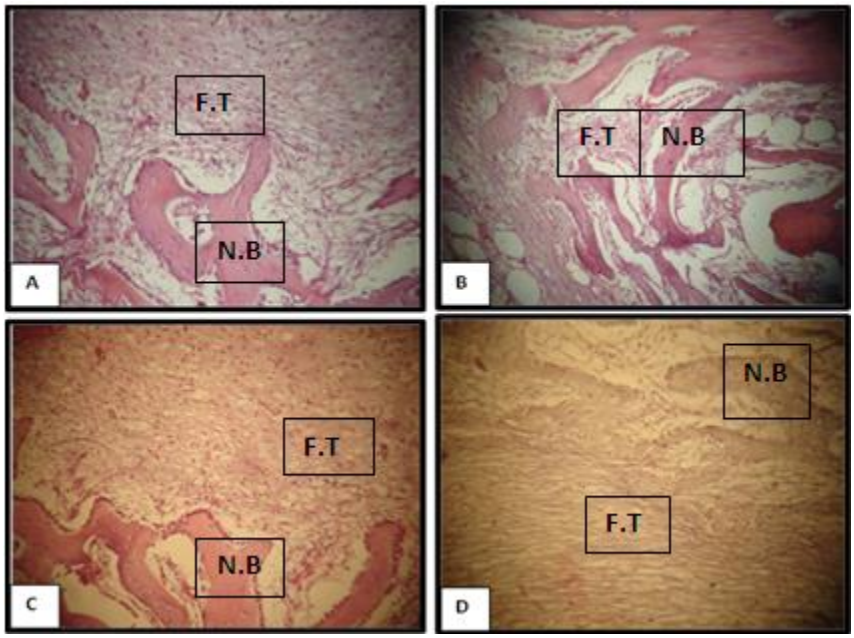


**Figure (3): Microphotographs of histological sections taken from the surgical bone defects after four weeks interval. A= positive control, B= shrimp source chitosan, C= crab source chitosan, D= negative control. The four sections have shown fibrous tissue formation (F.T) in addition to new bone formation (N.B). (H&E, Magnification=10X).**

Concerning the inflammation and fibrous tissue formation, no significant difference was detected among the four groups at four weeks post-surgery intervals table (12) and table (15).

**Table (12) Inflammation means score values comparison among the four groups at four weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| --ve control | 5 | 0.94 | 0.32 | 6,823 | 0.078 |
| Crab source | 5 | 0.40 | 0.30 | | |
| shrimp source | 5 | 0.40 | 0.30 | | |
| +ve control | 5 | 0.38 | 0.37 | | |

**Table (13) Granulation tissue formation means score values comparison among the four groups at four weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| --ve control | 5 | 1.68 | 0.25 | 11.400 | 0.01 |
| Crab source | 5 | 0.68 | 0.25 | | |
| shrimp source | 5 | 0.68 | 0.25 | | |
| +ve control | 5 | 0.66 | 0.35 | | |

**Table (14) Mann-Whitney test values of granulation tissue formation mean score in surgical bone defects at four weeks post – surgery interval.**

| Groups | Value | Sig. |
|---|---|---|
| -ve control vs. crab source | 0.000 | 0.007 |
| -ve control vs. shrimp source | 0.000 | 0.007 |
| -ve control vs. +ve control | 0.000 | 0.008 |
| Crab source vs. shrimp source | 12.500 | 1.000 |
| Crab source vs. +ve control | 12.500 | 1.000 |
| shrimp source vs. +ve control | 12.500 | 1.000 |

**Table (15) Fibrous tissue formation means score values comparison among the four groups at four weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| --ve control | 5 | 1.94 | 0.13 | 2.117 | 0.549 |
| Crab source | 5 | 2.26 | 0.43 | | |
| shrimp source | 5 | 2.20 | 0.49 | | |
| +ve control | 5 | 2.34 | 0.61 | | |

**Table (16) Osteoid tissue formation means score values comparison among the four groups at four weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| --ve control | 5 | 0.12 | 0.16 | 13.274 | 0.004 |
| Crab source | 5 | 0.76 | 0.13 | | |
| shrimp source | 5 | 0.82 | 0.16 | | |
| +ve control | 5 | 0.76 | 0.13 | | |

**Table (17) Mann-Whitney test values of osteoid tissue formation mean score in surgical bone defects at four weeks post – surgery interval.**

| Groups | Value | Sig. |
|---|---|---|
| **-ve control vs. crab source** | 0.000 | 0.007 |
| **-ve control vs. shrimp source** | 0.000 | 0.007 |
| **-ve control vs. +ve control** | 0.000 | 0.008 |
| **Crab source vs. shrimp source** | 12.500 | 1.000 |
| **Crab source vs. +ve control** | 12.500 | 1.000 |
| **shrimp source vs. +ve control** | 12.500 | 1.000 |

**C- Six Weeks Period**

At the end of the six weeks post- surgery interval, Kruskal-Wallis test analysis (Chi-Square) to compare among the four groups in concerning inflammation, granulation tissue and fibrous tissue formation means score values showed no statistical significance difference table (18), table (19) and table(20).

Concerning the osteoid tissue formation, significant difference was detected among the four groups table (21). By using Mann-whitney test to compare between each two groups, the results showed only significant difference between osteoid tissue formation means score values in negative control group when compared with other three groups table (22) and figure(4) .

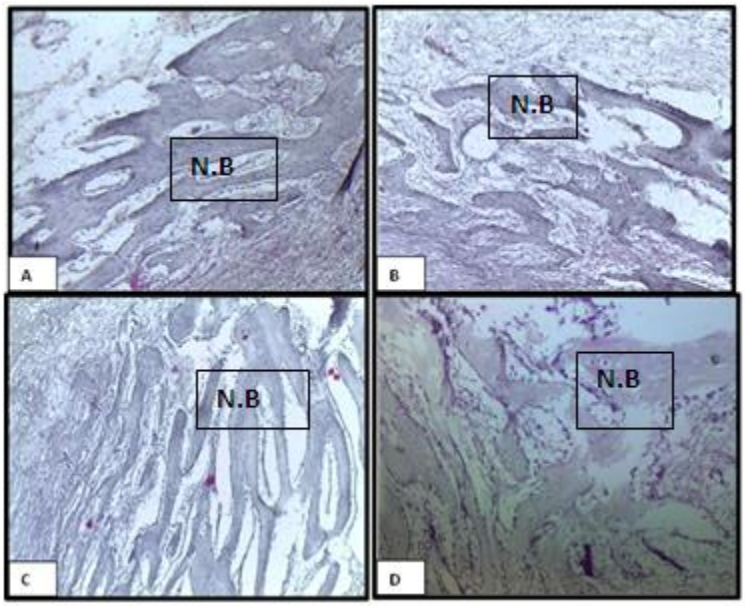


**Figure (4): Microphotographs of histological sections taken from the surgical bone defects after six weeks interval. A= positive control, B= shrimp source chitosan, C= crab source chitosan, D= negative control. Sections have shown Progressive new bone formation (N.B). (H&E, Magnification=10X).**

**Table (18) Inflammation means score values comparison among the four groups at six weeks post – surgery**.

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **--ve control** | 5 | 0.62 | 0.19 | 6.854 | 0.077 |
| **Crab source** | 5 | 0.26 | 0.29 | | |
| **shrimp source** | 5 | 0.32 | 0.25 | | |
| **+ve control** | 5 | 0.24 | 0.13 | | |

**Table (19) Granulation tissueformation means score values comparison among the four groups at six weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **--ve control** | 5 | 0.54 | 0.13 | 7.226 | 0.065 |
| **Crab source** | 5 | 0.18 | 0.16 | | |
| **shrimp source** | 5 | 0.18 | 0.16 | | |
| **+ve control** | 5 | 0.20 | 0.31 | | |

**Table (20) Fibrous tissueformation means score values comparison among the four groups at six weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **--ve control** | 5 | 2.34 | 0.61 | 0.722 | 0.868 |
| **Crab source** | 5 | 2.52 | 0.45 | | |
| **shrimp source** | 5 | 2.46 | 0.55 | | |
| **+ve control** | 5 | 2.52 | 0.45 | | |

**Table (21) Osteoid tissueformation means score values comparison among the four groups at six weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **--ve control** | 5 | 1.88 | 0.16 | 10.224 | 0.017 |
| **Crab source** | 5 | 2.58 | 0.38 | | |
| **shrimp source** | 5 | 2.52 | 0.45 | | |
| **+ve control** | 5 | 2.32 | 0.41 | | |

**Table (22) Mann-Whitney test values of osteoid tissue formation mean score in surgical bone defects at six weeks post – surgery interval**.

| Groups | Value | Sig. |
|---|---|---|
| **-ve control vs. crab source** | 0.000 | 0.007 |
| **-ve control vs. shrimp source** | 1.500 | 0.017 |
| **-ve control vs. +ve control** | 3.000 | 0.033 |
| **Crab source vs. shrimp source** | 11.000 | 0.792 |
| **Crab source vs. +ve control** | 7.000 | 0.212 |
| **shrimp source vs. +ve control** | 9.000 | 0.439 |

**D- Eight Weeks Period**

At the end of the eight weeks post- surgery interval, Kruskal-Wallis test analysis (Chi-Square) to compare among the four groups in concerning inflammation, granulation tissue and fibrous tissue formation means score values showed no statistical significance difference table (23), table (24) and table (25).

Concerning the osteoid tissue formation, significant difference was detected among the four groups, table (26) and figure (5). By using Mann-Whitney test to compare between each two groups, the results showed significant difference between osteoid tissue means score values in negative control group when compared with other three groups table (27).

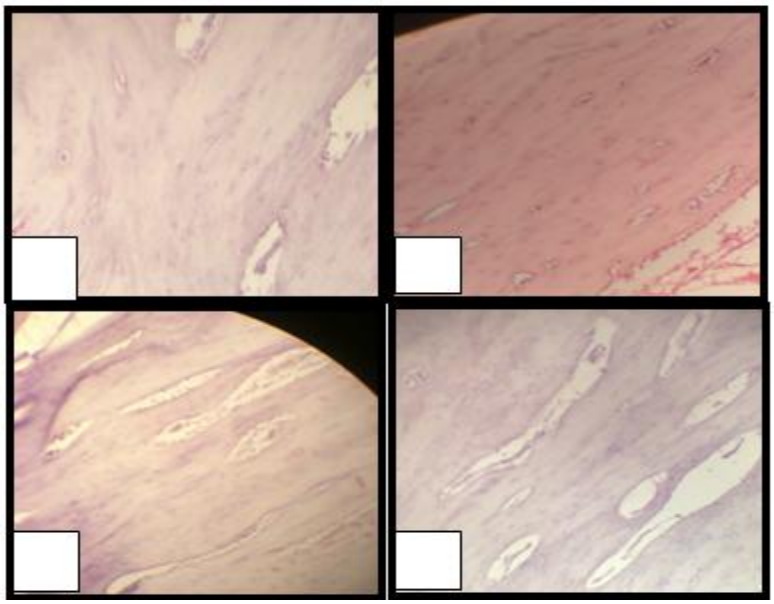

**Figure (5): Microphotographs of histological sections taken from the surgical bone defects after eight weeks interval. A= positive control, B= shrimp source chitosan, C= crab source chitosan, D= negative control.Sections have shown complete bone formation(H&E, Magnification=10X).**

**Table (23) Inflammation means score values comparison among the four groups at eight weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| --ve control | 5 | 0.46 | 0.22 | 7.558 | 0.056 |
| Crab source | 5 | 0.12 | 0.16 | | |
| shrimp source | 5 | 0.12 | 0.16 | | |
| +ve control | 5 | 0.12 | 0.16 | | |

**Table (24) Granulation tissue formation means score values comparison among the four groups at eight weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| --ve control | 5 | 0.36 | 0.134 | 7.77 | 0.054 |
| Crab source | 5 | 0.12 | 0.164 | | |
| shrimp source | 5 | 0.06 | 0.134 | | |
| +ve control | 5 | 0.12 | 0.164 | | |

**Table (25) Fibrous tissue formation means score values comparison among the four groups at eight weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| --ve control | 5 | 2.40 | 0.548 | 0.924 | 0.475 |
| Crab source | 5 | 2.46 | 0.508 | | |
| shrimp source | 5 | 2.52 | 0.455 | | |
| +ve control | 5 | 2.46 | 0.508 | | |

**Table (26) Osteoid tissue formation means score values comparison among the four groups at eight weeks post – surgery.**

| Group | No. | Mean | ±SD | Chi-Square | Sig. |
|---|---|---|---|---|---|
| **Crab source** | 5 | 2.58 | 0.383 | | |
| **shrimp source** | 5 | 2.72 | 0.383 | | |
| **+ve control** | 5 | 2.66 | 0.477 | | |

**Table (27) Mann-Whitney test values of osteoid tissue formation mean score in surgical bone defects at eight weeks post – surgery interval.**

| Groups | Value | Sig. |
|---|---|---|
| **-ve control vs. crab source** | 1.500 | 0.016 |
| **-ve control vs. shrimp source** | 1.000 | 0.013 |
| **-ve control vs. +ve control** | 3.000 | 0.037 |
| **Crab source vs. shrimp source** | 10.000 | 0.549 |
| **Crab source vs. +ve control** | 11.500 | 0.817 |
| **shrimp source vs. +ve control** | 11.500 | 0.811 |

## DISCUSSION

Ideal bone substitutes should display osteogenic, osteoinductive, and osteoconductive properties. They should be degraded and gradually replaced by newly formed bone [8]. Polymers of natural origin are attractive options, mainly due to their similarities with the extracellular matrix (ECM) as well as chemical versatility and biological performance[9].Chitosan has been reported as a potential natural polymer material for promoting bone regeneration because of its apparent osteoconductive and bio-degradable properties[10]. The availability of the raw material of chitin in Iraq in addition to biological properties gave us the impetus for chitosan preparation and using it as bone substitute material.

Clinical healing of all animals progressed uneventfully, no deaths occurred in all animals throughout all periods in this study and no change in the living habit or loss of appetite. This could be interpreted by the provision of comprehensive care, submission of extremely sterile surgical procedures, the daily cleaning and disinfection of animal house, good nourishment and the reasonable weather during postoperative care[11].

### Radiographical Evaluation Using ImageJ Program

In this study, the conventional radiography was used to assess acceleration of bone healing. The advantage of conventional film-screen is the high resolution compared with computed radiography[12].Unfortunately, conventional radiography (which is considered the most widely used auxiliary examination in dentistry) presents some limitations due to low sensibility and high inter-examiner disagreement [13,14] . For that reason, radiographs were converted into digital images and their gray scale values used to clarify and analyze healing of created bone defects. No adverse reactions or complications were found radiographically in bone defects during the postsurgical intervals.

ImageJprogramisa public, domain, Java-based imageprocessing program developed at the National Institutes of Health(NIH), which calculates area and pixel value statistics for user-defined selections[15]. This program was used in the present study for radiographic assessment since it fulfills most of routine image processing and analysis requirements. Meantime the large number of automated image segmentation algorithms will enable the operator to pick the most suitable one, which is considered a significant advantage[16,15].ImageJ calculates area as a number of pixels; also it measures density in pixels depending on the gray tone difference in pixel value statistics for user-defined selections[17].

For mean gray values, it is known that the higher the gray value of a pixel, the higher the absorbance of X-rays for the given pixel is. As X- rays get absorbed more, the pixel has a higher gray scale value in the 0-255 scale (where 0 is for black while 255 for white) meaning increased radio-opaqueness due to bone formation or presence of bone like substances. Any surgical bone defects with no bone graft at the immediate post-surgical period will usually show complete radiolucency

which changes with time into opacity of different density as a result of healing and this is indicated by an increase in mean gray scale values[18].By this way, bone healing process was evaluated and monitored in this present study.

For mean gray scale values, no significant difference was obvious between the four group defects at the two week post-surgical intervals with low mean gray scale values and no evident bone formation. Chitosan appeared homogenous and radiolucent in immediate postoperative radiography [19.]. For visibility of bone changes, the bone mineralization should be at least 40 percent; otherwise, it is not visible in the radiograph[20].

At the fourth week post-surgical interval, a significant difference was evident in mean gray values between negative control defect group and crab source, shrimp source and positive groups with higher values in the latter three groups (137.35, 137.41, and 137.31 respectively) compared to negative control defects (115.51).The difference in mean gray scale values reflects the differences in the opacity recognized and thus different rates of bone formation of the bone defects were obtained. The difference in mean gray scale values may indicate that bone formation has already began in defects stimulated by chitosan biomaterial in faster rate than that in negative control surgical bone defects in which healing seemed to be also occurring, but in slower rate.

At the sixth and eighth week, mean gray scale values showed no significant difference between all four groups but the mean gray values were higher than the values recorded in the second and fourth postoperative intervals which reflect that the bone formation and bone density proceeded uneventfully and without any complications. In this study, there was a trend of greater radiopacity (represented by increase in mean gray scale) in all chitosan filled bone defects when comparing with negative control bone defects. This result can be explained by the characteristics of chitosan, which is an osteoinductive material that stimulates the release of growth factors, differentiation and cell aggregation in the wound, and thus promoting and accelerating the regeneration of bone tissue[21,22] , proposed that chitosan can increase mineralization by upregulating the associated genes as a mechanism for the osteogenesis of this substance.

**Histopathological evaluation**

Histological evaluation of the tissue adjacent to implanted materials has been the most commonly used method of evaluating the biocompatibility and tissue responses to biomaterials [23].

At the end of the second week post-surgery**,** the crab source chitosan , shrimp source chitosan and positive control bone defects showed moderate to severe degree of inflammatory response with mean values were (2.26, 2.32 and 2.26) respectively. The negative control defect revealed mild to moderate inflammation with mean value = 1.74 with significant difference between the four groups. This mean chitosan induces an acute inflammatory response which characterized by migration of neutrophils to the implant site and this finding was similar with that reported by Hutmacher *et al.*, (1998) and Li *et al.*, (2009[24,25]but was different from other researchers who reported no inflammatory significant difference between chitosan and control groups[26].This difference in result with other researcher could be due to the variation in DDA of chitosan, and the form of chitosan used in our study which was unmodified chitosan powder whereas other studies used it as compounds with some other materials like hydroxyapatite and calcium sulfate. Chitosan with low DDA induced high degree of inflammatory response when compared with high DDA chitosan [27,28] . This inflammatory response subsided in time and associated with early breakdown and erosion of chitosan particles and this could be due to lysosomal enzymes of these inflammatory cells and this chitosan particles degradation was also documented by other researchers[27,29].

Chitosan promotes the migration of inflammatory cells which are capable of production and secretion of a large group of pro-inflammatory products and growth factors at a very early phase of healing. Chitosan enhances the functions of inflammatory cells such as polymorph nuclear leukocytes (PMN) (phagocytosis, production of osteopontin and leukotrieneB4), macrophages (phagocytosis, production of interleukin (IL-1), transforming growth factor β 1and platelet-derived growth factor). As a result, chitosan promotes granulation tissue formation and organization and in turn help in healing of large openwounds in animals[30,31]. In this study, there was significant difference in granulation tissue formation between chitosan treated bone defects ( freshwater crab derived chitosan, marine shrimp derived chitosan and imported chitosan filled defects) and negative control group (left filled with blood only) but nowoven bone formation was seen in all groups at two weeks intervals post operatively. With time progressing, new bone formation started at a rapid rate in chitosan filled bone defects in comparing with blood filled bone defect (negative control group)

At four weeks period. Early signs of osteoid tissue formation were observed, with the high mean score in freshwater crab derived chitosan, marine shrimp derived chitosan and imported chitosan filled defects ( 0.76, 0.82, 0.76 respectively). Chitosan reported to promote the differentiation of mesenchymal stem cells into osteoblasts and facilitated the formation of bone in vitro[32]and also had osteogenenic activity on artificially made bone defects in animal models ( in vivo) [33,34].

The more attractive and encouraging feature at the end of six weeks postoperative interval was the appearance of profuse quantity of osteoid tissue in all three chitosan filled bone defects (mean = 2.58, 2.52, 2.32) with a highly significant difference over negative control bone defect. This tendency of increased new bone formation with the increase time of chitosan implantation agreed with finding of Al-sarrag and Yassen, (2008)[35].

With the end of eight weeks interim, high significant difference was noticed in relation to osteoid tissue formation between the negative control bone defects (mean = 2.00) and chitosan filled bone defects (mean= 2.58, 2.72, 2.66) in which more organized mature lamellar bone was noticed . No remnants of chitosan particles were noticed owing to complete degradation of the material. These results suggest that this material acts simply as a biofunctional filler material and may play a more active role in osteogenesis[33, 34,36,37,38.].

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