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SERUM BIOCHEMICAL CHANGES OF BONE REMODELING FOLLOWING RESIDRONATE- BIOGLASS SCAFFOLDS FOR THE RECONSTRUCTION OF EXPERIMENTALLY INDUCED MANDIBULAR DEFECTS IN DOG MODEL

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Abstract

Background: Bone tissue engineering has gained significant attention to restoring bone defects by using biodegradable scaffolds. **Purpose:** This study aimed to evaluate serum biochemical changes of bone remodeling following the use of hybrid scaffolds to reconstruct experimentally induced critical-sized mandibular defects in dogs. **Methods:** Hybrid scaffolds of chitosan:polyvinyl alcohol named, (CH) alone (no BG) and with bio-glass (BG) graft combined without/with anti-osteoporotic drug (Risedronate M) to form non-medicated and medicated (NM and M) forms were used. A critical size mandibular defect was created into the right mandible of twenty skeletally mature mongrel dogs. These dogs were divided into four equal groups: the 1st group; control (CH) (NM), the 2nd group; CH(M), the 3rd group; CH.BG (NM) and the last group, CH.BG (M). Clinical, radiographic, serum biochemical evaluation (alkaline phosphatase, calcium, phosphorus, calcitonin and osteocalcin) was evaluated at week interval till 12 weeks. **Results:** A non-significant decrease was found in the activity of osteocalcin in serum for (CH.BG) (M) group when compared to control group CH (NM). There is a highly noteworthy increase in serum calcium, alkaline phosphatase, calcitonin, and phosphorus levels in the (CH.BG) (M) group when compared to the control group CH (NM). **Conclusion:** Serum biochemical markers of bone healing provided a clinically useful tool to monitor early changes associated with bone healing before radiographic signs of bone healing become obvious. The addition of BG with risedronate drug M improved the degree of bone healing as presented.

INTRODUCTION

Bone is a highly specialized, metabolically active organ with dynamic properties of continuous remodeling to maintain skeletal integrity and strength. The bone remodeling cycle includes bone resorption and formation that both local and systemic regulators control to repair the microdamage in the bone matrix [1, 2]. Unlike other tissues, when subjected to

trauma, bone has a unique ability to self-regenerate to bone tissue resembling the original one [2]. However, bone has limited self-regenerative capacity when a large portion of bone is removed, such as severe trauma, tumor resection, osteonecrosis, or congenital bone disease. In large bone defects, known as critical-sized bone defects, the gap between the two fracture ends exceeds the bone's ability to self-regenerate; therefore, it necessitates the use of osteoinductive and osteoconductive materials to support bone healing [2-4].

Bone tissue engineering has gained great attention to restore bone defects by bridging the defect with a biodegradable porous material exhibiting bioactive properties known as scaffold [4]. Bioactive materials include the use of inorganic compounds such as bioceramics such as silicate glass. The main disadvantage of bioactive ceramics is their low fracture toughness; therefore, they are usually combined with biopolymers [5].

Chitosan is a natural polysaccharide polymer obtained from crustaceans exhibiting antibacterial, antifungal and mucoadhesive properties [6]. It has been used extensively in bone scaffolds; when applied locally in bone defects, it improves angiogenesis, osteogenesis by promoting osteoblastic proliferation and helping in mineralization of the bone matrix. However, its poor mechanical strength is the main limitation; therefore, it should be combined with other polymers to exhibit better mechanical stability [7].

Polyvinyl alcohol is a biocompatible bioactive compound that has been used in bone tissue engineering. It facilitates cell adhesion, proliferation and differentiation [8]. Therefore, it has been used in combination with bioactive compounds to mimic natural bone functionality and promote osteoconductivity [8, 9].

Risedronate is a bisphosphonate drug that has been reported to inhibit bone resorption by inhibiting osteoclastic activities [10]. Risedronate inhibits osteoclastic adhesion to the mineralized bone matrix and decreases the osteoclastic life span [10].

Although evaluation of bone healing is typically made through physical and radiographic examination, radiographic signs of bone healing are only conclusive at later stages of bone healing. Therefore, evaluation of early changes in serum biochemical markers of bone healing may provide a clinically useful tool to monitor early changes associated with bone healing before radiographic signs of bone healing become clearly obvious.

Biochemical markers of bone turnover include bone resorption and bone formation markers. Bone resorption markers are mediated by resorption of osteoclastic matrix (tartrate-resistant acid phosphatase) and degradation products of type I collagen in protein matrix (e.g hydroxyproline, telopeptides). Bone formation markers produced by osteoblasts including osteocalcin and bone-specific alkaline phosphatase [11].

Serum total alkaline phosphatase (ALP) is an indicator of osteoblastic activity with subsequent new bone formation, osteoid tissue formation and mineralization. Osteocalcin (OC) is a specific marker of osteoblastic function. Calcitonin is a hormone synthesized and released by the parafollicular cells of the thyroid gland and is released mainly in response to high serum calcium levels [12].

The aim of the present study was to study serum biochemical changes of bone remodeling following the use of risedronate-bioglass scaffolds for the reconstruction of experimentally induced critical-sized mandibular defects in dogs.

MATERIALS AND METHODS

Study Design

An *in vivo* study was designed to evaluate serum biochemical changes of bone remodeling following the use of different bone scaffolds on the healing of experimentally induced critical-sized mandibular bone defect in dogs. Preparation of 4 different combinations of chitosan/polyvinyl/bioglass/risedronate bone scaffolds was made. A critical-sized mandibular defect was induced into the right mandible of 20 dogs under the effect of general anesthesia. Dogs were randomly allocated into one of the 4 groups (5 dogs/group). The scaffold was press-fitted into the induced defect according to animal grouping. Serum biochemical changes were evaluated at weekly intervals till 12 weeks. Radiographic evaluation was made at 2-week interval till 12 weeks.

Preparation of Scaffolds

The four groups include; chitosan/polyvinyl alcohol non medicated control (CH)(NM), Chitosan/polyvinyl alcohol medicated by risedronate drug CH (M), Chitosan / polyvinyl alcohol/BG non medicated CH.BG (NM), and Chitosan/ polyvinyl alcohol /Bioglass/ medicated by risedronate CH.BG (M) scaffolds were prepared in the form of discs of 15 mm diameter to be press-fitted into the induced mandibular defect.

The scaffolds were prepared as previously mentioned in our work [13]. CH (from Sigma Aldrich, GmbH Co., Iceland) was dissolved in a 1% acetic acid solution for 2-3 hrs until the formation of a clear solution. Chitosan / polyvinyl alcohol/risedronate CH (M) scaffold was prepared by adding risedronate (donated from Al-Hikma Pharmaceutical and Chemical Industries Co., Egypt) (10 mg/g) to the CH solution and stirred for another 1 h at room temperature. Then, PVA (from Carl Roth GmbH Co., Germany) was gradually added to the above solution at 60 ± 5 °C with continuous stirring. Moreover, the glass content was added in percentage 10% to the previously prepared CH/PVA. Samples of a diameter of 15 mm were prepared and freeze dried in a freeze dryer (Christ freeze dryer,

ALPHA 2-4 LD plus, Germany) for 24 h to obtain dried scaffolds [13-16].

Characterization of Scaffolds

The porosity of the prepared scaffolds and morphology were examined using Scanning Electron Microscope (SEM Philips apparatus, USA, QUANTA 250 FEG instrument) after film coating of the scaffolds with gold.

Animals

Twenty skeletally mature male mongrel dogs weighing (mean ± SD) 23.9 ± 2.4 kg and aging (mean ± SD) 17.4 ± 2.1 months were used. The complete clinical and hematological examination was done for all dogs to exclude systemic and metabolic diseases. Dogs were quarantined for two weeks for acclimatization and routine de-worming. During the study, dogs were housed individually in separate cages, allowed free access to water and fed twice daily [13]. Study procedures were done at the Department of Surgery, Anesthesiology and Radiology- Faculty of Veterinary Medicine, Cairo University. The study procedures were followed and approved by the Cairo University Institutional Animal Care and Use Committee (CU-IACUC), approval # VetCU06202019050.

Anesthetic Protocol

Dogs were premedicated using atropine sulphate 0.1% (Atropine Sulphate®, El Nasr pharm. Chem. Co. Egypt) at a dose of 0.05 mg/kg b.w. and xylazine HCl 2% (Xylaject®, ADWIA Co. Egypt) at a dose of 1mg/kg body weight through intramuscular injection. Anesthesia was induced using ketamine HCl 5% (Ketamine®, Rotex Medica, Germany) at a dose of 10 mg/kg body weight I.V. Anesthesia was maintained using thiopental sodium 2.5% (Anapental®: Sigma-Tec, Egypt) at a dose 25mg/ kg body weight.

Surgical Procedures

A critical-sized mandibular defect was created into the right mandible using an extra-oral approach. A 5-cm skin incision was made on the inferior border of the mandible. The periosteum was incised to expose the horizontal ramus of the mandible. Bone defect was created using a trephine bur of 12 mm diameter under continuous saline irrigation. The prepared defect was press-fitted based on animal grouping; control CH (NM), CH (M), CH.BG (NM) and CH.BG (M) each group of n=5.

Routine closure of the surgical wound including the subcutaneous tissue and skin was made using Vicryl sutures [17]. All surgical procedures were made by the same surgeon who was blind to animal grouping.

Biochemical Evaluation

Blood samples were collected from all dogs at the time of surgery (baseline) then at weekly intervals till 12 weeks. The collection of blood samples was made through the cephalic vein using sterile disposable syringes. Blood samples were centrifuged at 3000 rpm for 15 minutes, stored at -70 °C until further processing. The obtained sera were separated for the estimation of serum calcium (Ca), phosphorus level (P), alkaline phosphatase (ALP) activity, serum calcitonin and osteocalcin levels.

Serum Ca level was determined using colorimetric method (colorimetric kinetic method with ortho-cresolphthalein laboratories diagnostic kits, Egyptian Co. for Biotechnology, Egypt). Inorganic P was determined using colorimetric method (colorimetric kinetic phosphomolybdate laboratories diagnostic kits, Egyptian Co. for Biotechnology, Egypt). ALP activity was estimated using the enzymatic colorimetric method (DGKC, centronorm laboratories diagnostic kits, Egyptian Co. for Biotechnology, Egypt). Calcitonin and Osteocalcin levels were determined using enzyme immunoassay kit (Bioassay Technology Laboratory, Korean Biotech CO., LTD).

Radiographic Evaluation

Right lateral mandibular radiographs were obtained at 2-week interval till the end of the study at 12 weeks. Radiographic exposure parameters were set at 45 Kvp, 15 mA, and 1/10 Sec with FFD 75 cm.

Statistical Analysis

Serum biochemical parameters were tabulated for each group at examination time. Data were expressed as mean ± SEM. A one-way- ANOVA followed by post hoc test was used for statistical analysis. The *P* value was considered statistically significant if < 0.05. Data were analyzed using SPSS (SPSS 19, IBM, Chicago USA).

RESULTS

Characterization of Scaffold

The morphology for the fabricated scaffolds is presented in (Figure 1). It shows a spongy like structure of the scaffold (group 1) CH (NM) without any addition, Figure 1.a where its corresponding EDAX Figure 1.b presents the C and O elements only related to both types of polymer structure. The porosity of the structure ranged between 3- 20µm. Addition of the drug to the previous formula CH (M) (group 2) (Figure 1.c and its EDAX Figure 1.d show no change in the morphology of the scaffold where the drug looks homogenously distributed during preparation and the EDAX

show the same elemental components C and O referring to the polymer and drug composition.

However, addition of the nanobioactive glass (BG) with the drug CH.BG (M) (group 4) during the preparation of the scaffolds and its EDAX, Figure 1.e, and 1.f respectively, show white spots in the SEM micrographs and the Si, Na, Ca and P elements that appeared in the corresponding EDAX referring to the elements in BG. Therefore, the scaffold matrix appears in interconnected channels forming a porous structure suitable for bone growth, angiogenesis, and transport of nutrients.

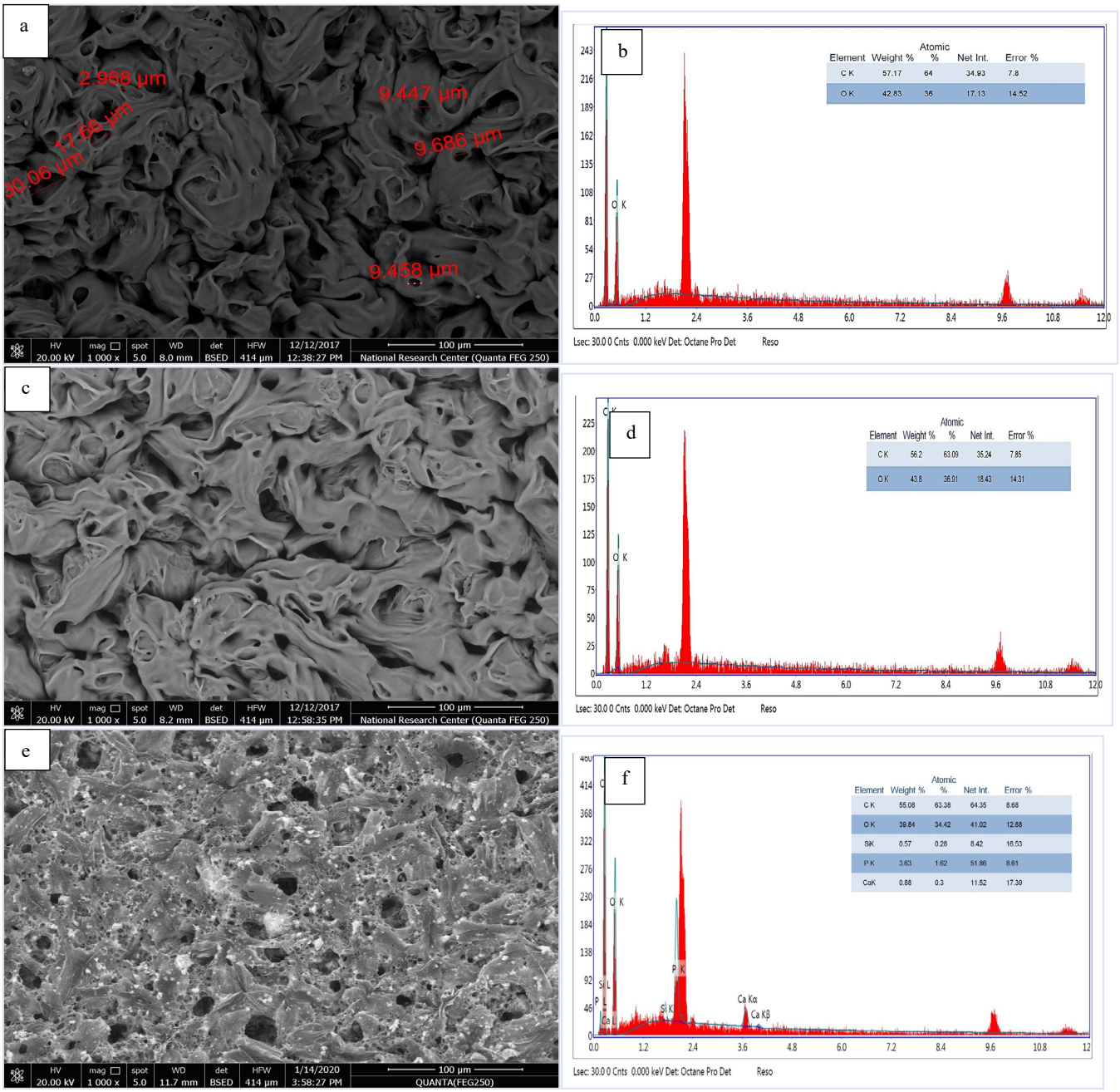

Figure 1: SEM micrographs for the scaffolds 1.a Group 1: CH (NM), 1.b EDAX of Group 1: (CH) (NM) shows C and O elements of both polymers, 1.c Group 2: CH (M), 1.d EDAX of Group 2: CH (M) shows C and O elements too, 1.e Group 4: CH.BG (M) and 1.f EDAX of Group 4: CH.BG. (M) shows Si Ca, P, Na, elements of BG besides C and O.

Serum Biochemical Changes

Serum Calcium (mg/dl) Level

Serum calcium level demonstrated a significant increase in the calcium level in the CH(M) when compared to (CH)(NM) group at weeks 2,3,8,10,11,12. Also, there was a significant increase from 2 – 6 weeks then decreases from 7 – 12 weeks in (CH.BG)(NM) when compared to control (CH)(NM) group (P-value ≤ 0.05). There were significant differences in serum calcium level in the (CH.BG)(M) group when compared to the control group (CH)(NM) (P-value ≤ 0.05) as shown in (Table 1 & Figure 2).

Table 1: Serum calcium (mg/dl) level among the studied groups.

Parameters \ Groups	Group I Control CH (NM)	Group II CH(M)	Group III CH.BG(NM)	Group IV CH.BG(M)
Range	8.49 - 27.69	7.61 - 28.66	4.85 - 33.19	15.28 - 40.2
Pre-surgery	64.08 ± 0.617	65.09 ±1.27	97.53 ± 0.93	68.32 ± 0.454
Mean ± S.D (2-weeks)	49.73 ± 0.177	54.29 ±1.362*	90.02 ± 0.382*	66.38 ± 0.047NS
Mean ± S.D (3-weeks)	47.24 ± 0.055	49.6 ± 0.198 *	84.77 ± 0.891*	61.24 ± 0.529*
Mean ± S.D (4-weeks)	48.29 ± 0.241	50.51 ± 3.396NS	78.25 ± 0.781*	56.94 ± 0.554*
Mean ± S.D (5-weeks)	60.43 ± 0.162	62.71 ± 0.26NS	75.01 ± 0.515*	52.99 ± 0.169*
Mean ± S.D (6-weeks)	63.89 ± 0.299	64.65 ± 0.5NS	72.59 ± 0.146*	52.24 ± 0.05*
Mean ± S.D (7-weeks)	68.35 ± 0.751	68 ± 0.532NS	67.74 ± 1.235*	47.78 ± 0.613NS
Mean ± S.D (8-weeks)	74.43 ± 0.702	76.91 ± 1.2*	62.08 ± 0.261*	60.61 ± 0.153*
Mean ± S.D (9-weeks)	70.17 ± 7.421	71.25 ± 7.317NS	57.72 ± 1.108*	70.78 ± 1.565*
Mean ± S.D (10-weeks)	41.87 ± 0.808	42.21 ± 1.566*	49.07 ± 0.933*	88.41 ± 1.917*
Mean ± S.D (11-weeks)	32.37 ± 0.187	37.88 ± 0.877*	39.23 ± 1.713*	104.21 ± 1.434*
Mean ± S.D (12-weeks)	28.62 ± 1.016	30.42 ± 2.233*	20.78 ± 2.176*	115.87 ± 1.501*

P* ≤0.05 is significant when compared to group I , P^{NS} >0.05 is non-significant when compared to group I.

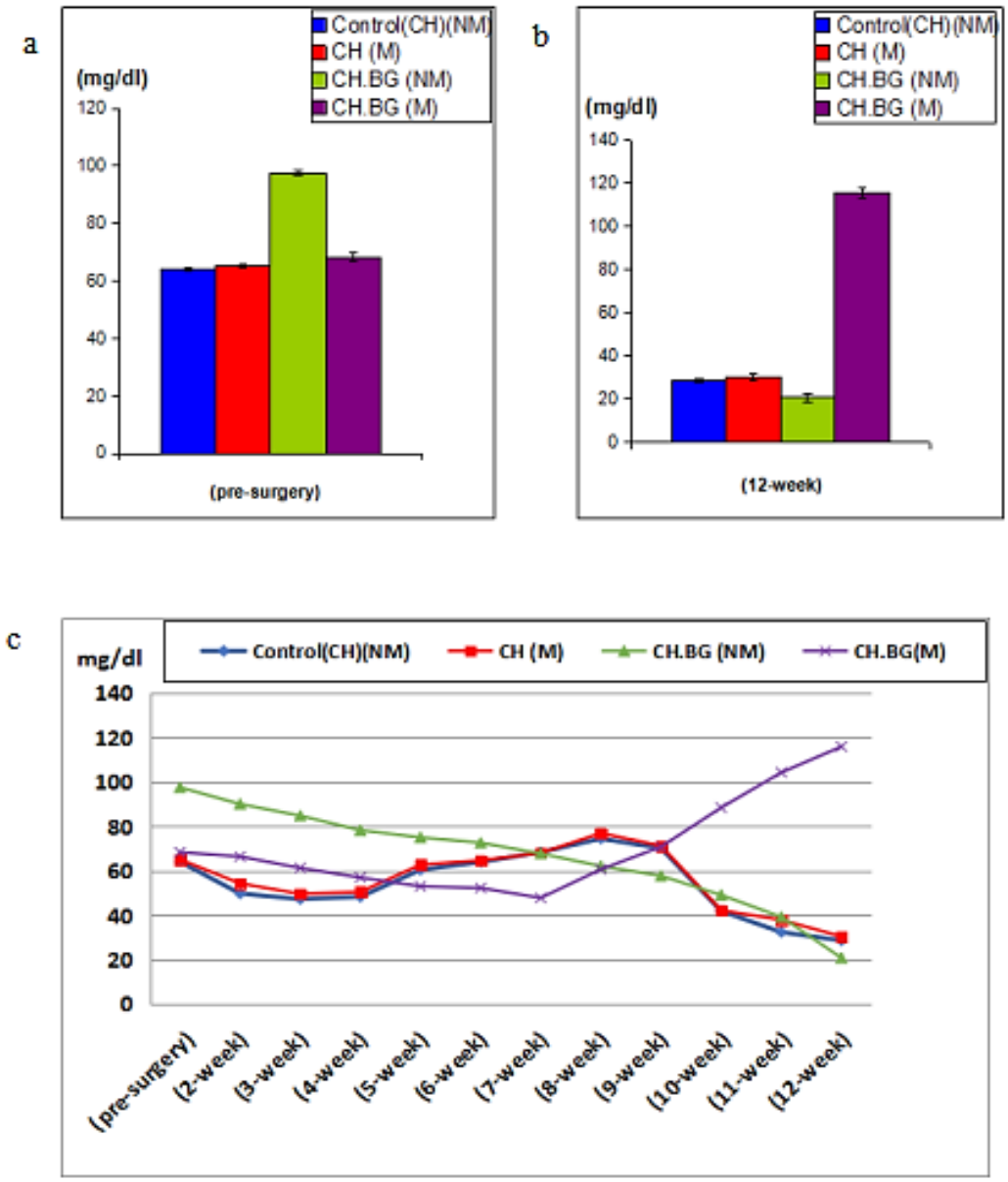

Figure 2: Mean ± SD. of dog serum calcium level in the different groups **a**: pre-surgery, **b**: 12 weeks, **c**: During experiment.

Serum Phosphorus (mg/dl) Level.

A significant increase in serum phosphorus activity in the (CH) (M) group was recorded among all weeks when compared to control group (CH) (NM). There was a significant increase in serum phosphorus activity in the (CH.BG) (NM) from 3 – 12 weeks when compared to the control group CH (NM). A significant decrease in serum phosphorus activity was recorded from 2-4 weeks then increase from 5 – 12 weeks in (CH.BG) (M) groups compared to the control group (CH) (NM) (P-value ≤ 0.05) as shown in (Table 2 & Figure 3).

Table 2: Serum phosphorus (mg/dl) level among the studied groups.

Parameters \ Groups	Group I Control CH (NM)	Group II CH(M)	Group III CH.BG(NM)	Group IV CH.BG(M)
Range	6 - 18.63	6.05 - 19.5	5.9 - 19.48	5 - 19.35
Pre-surgery	18.54 ± 0.158	18.96 ± 0.252	18.31 ± 0.195	15.13 ± 0.04
Mean ± S.D (2-weeks)	20.49 ± 0.036	22.84 ± 1.18^{NS}	22.85 ± 1.237^{NS}	16.19 ± 0.223*
Mean ± S.D (3-weeks)	26.29 ± 0.055	30.44 ± 0.603*	33.42 ± 0.468*	21.74 ± 0.306*
Mean ± S.D (4-weeks)	32.08 ± 0.2	35.77 ± 0.109*	36.52 ± 0.29*	27.9 ± 0.838*
Mean ± S.D (5-weeks)	33.79 ± 0.165	39.36 ± 0.474*	39.13 ± 0.293*	35.53 ± 0.856 *
Mean ± S.D (6-weeks)	37.59 ± 0.23	42.76 ± 0.273*	41.85 ± 0.266*	39.36 ± 0.317*
Mean ± S.D (7-weeks)	40 ± 0.126	44.59 ± 0.145*	44.36 ± 0.148*	41.88 ± 0.412*
Mean ± S.D (8-weeks)	41.72 ± 0.335	46.62 ± 0.255*	47.2 ± 0.146*	44.54 ± 0.325*
Mean ± S.D (9-weeks)	42.63 ± 0.075	49.23 ± 0.177*	49.41 ± 1.108*	47.2 ± 0.208*
Mean ± S.D (10-weeks)	45.89 ± 0.813	52.26 ± 0.627*	52.64 ± 0.536*	49.65 ± 0.285*
Mean ± S.D (11-weeks)	49.38 ± 0.315	54.7 ± 0.688*	54.94 ± 0.352*	53.68 ± 0.38*
Mean ± S.D (12-weeks)	53.14 ± 0.83	57.49 ± 0.298*	57.63 ± 0.248*	56.14 ± 0.55*

P * ≤0.05 is significant when compared to group I , P^{NS} >0.05 is non-significant when compared to group I.

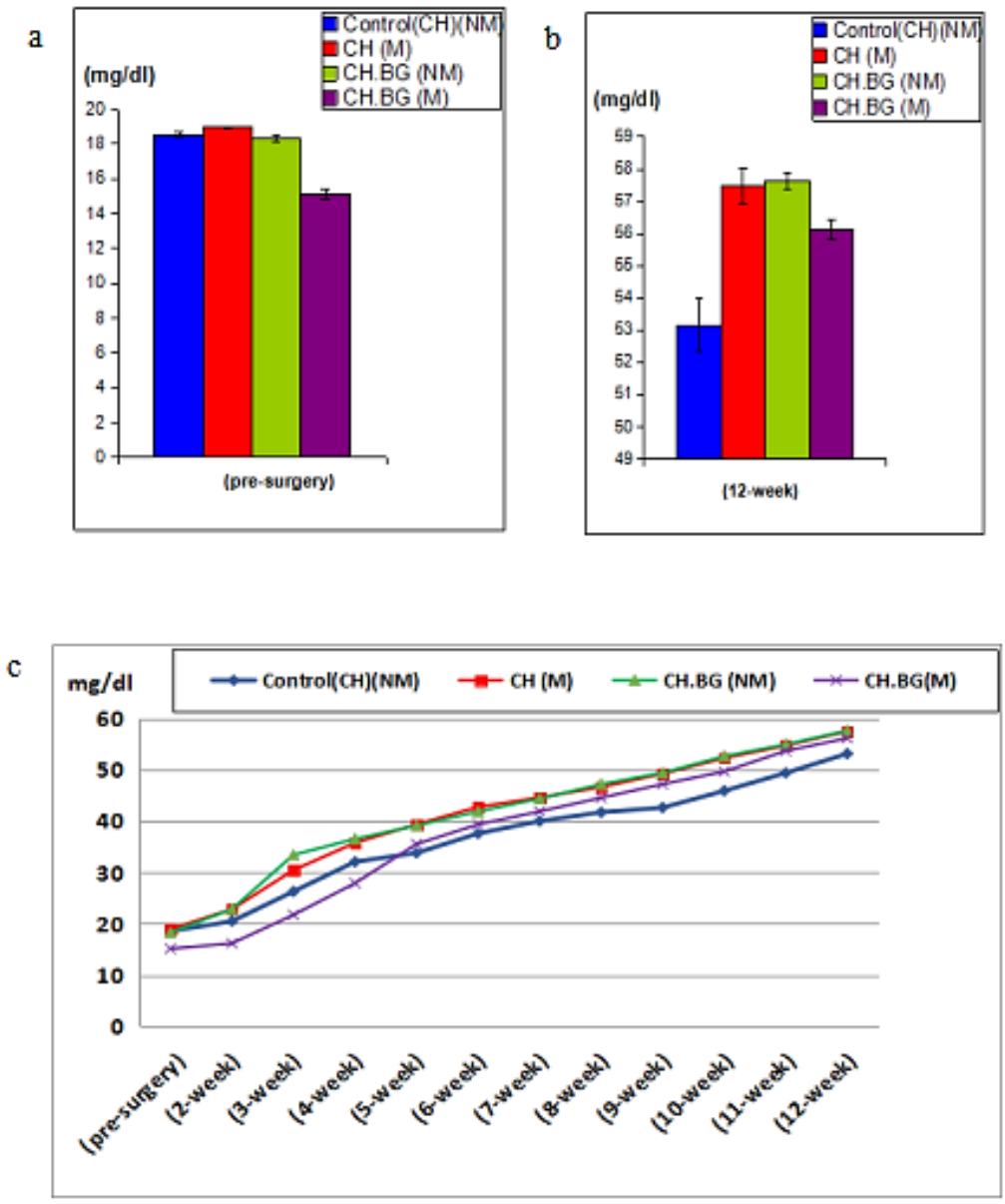

Figure 3: Mean ± SD. of dog serum phosphorus level in the different groups **a**: pre-surgery, **b**: 12 weeks, **c**: During experiment.

Serum Alkaline Phosphatase (U/l) Activity.

There was a significant increase in serum alkaline phosphatase activity in (CH) (M) group when compared to control group (CH) (NM) from 7 to 11 weeks. At the same time, there were non-significant differences in serum ALP in (CH.BG) (NM) when compared to the control group (CH) (NM) (P-value > 0.05). There was a significant difference in serum alkaline phosphatase activity in (CH.BG) (M) group when compared to the control group (CH) (NM) from 3 – 11 weeks ($P \leq 0.05$) as shown in (Table 3 & Figure 4).

Table 3: Serum Alkaline Phosphatase (U/l) activity among the studied groups.

Parameters \ Groups	Group I Control CH (NM)	Group II CH(M)	Group III CH.BG(NM)	Group IV CH.BG(M)
Range	114 - 939	110 – 982	123 – 951	150 - 981
Pre-surgery	1118 ± 446.292	837 ± 152.04	512 ± 23.45	2068 ± 393.81
Mean ± S.D (2-weeks)	2518 ± 36.909	1119 ± 490.502^{NS}	472 ± 34.501^{NS}	2401 ± 16.502^{NS}
Mean ± S.D (3-weeks)	417 ± 16.643	847 ± 4.932^{NS}	2337 ± 234.412^{NS}	1827 ± 144.647*
Mean ± S.D (4-weeks)	821 ± 9.073	957 ± 7.549^{NS}	2057 ± 20.84^{NS}	715 ± 29.022*
Mean ± S.D (5-weeks)	934 ± 18.175	1051 ± 18.009^{NS}	1420 ± 127.962^{NS}	621 ± 76.544*
Mean ± S.D (6-weeks)	1064 ± 6.806	1240 ± 39.399^{NS}	1085 ± 14.153^{NS}	998 ± 3.055*
Mean ± S.D (7-weeks)	1214 ± 34.21	1605 ± 24.062*	1039 ± 4.725^{NS}	1061 ± 14.047*
Mean ± S.D (8-weeks)	1487 ± 26.576	1873 ± 23.245*	819 ± 4.582^{NS}	1450 ± 78.398*
Mean ± S.D (9-weeks)	1771 ± 36.678	2092 ± 28.183*	790 ± 3.055^{NS}	2195 ± 143.597*
Mean ± S.D (10-weeks)	1974 ± 20.663	2592 ± 54.671*	726 ± 8^{NS}	2844 ± 43.278*
Mean ± S.D (11-weeks)	1744 ± 377.971	2906 ± 16.653*	641 ± 12.096^{NS}	1620 ± 165.447*
Mean ± S.D (12-weeks)	2133 ± 351.66	1267 ± 173.96^{NS}	536 ± 20.98^{NS}	1823 ± 372.36^{NS}

P * ≤0.05 is significant when compared to group I , P^{NS} >0.05 is non-significant when compared to group I.

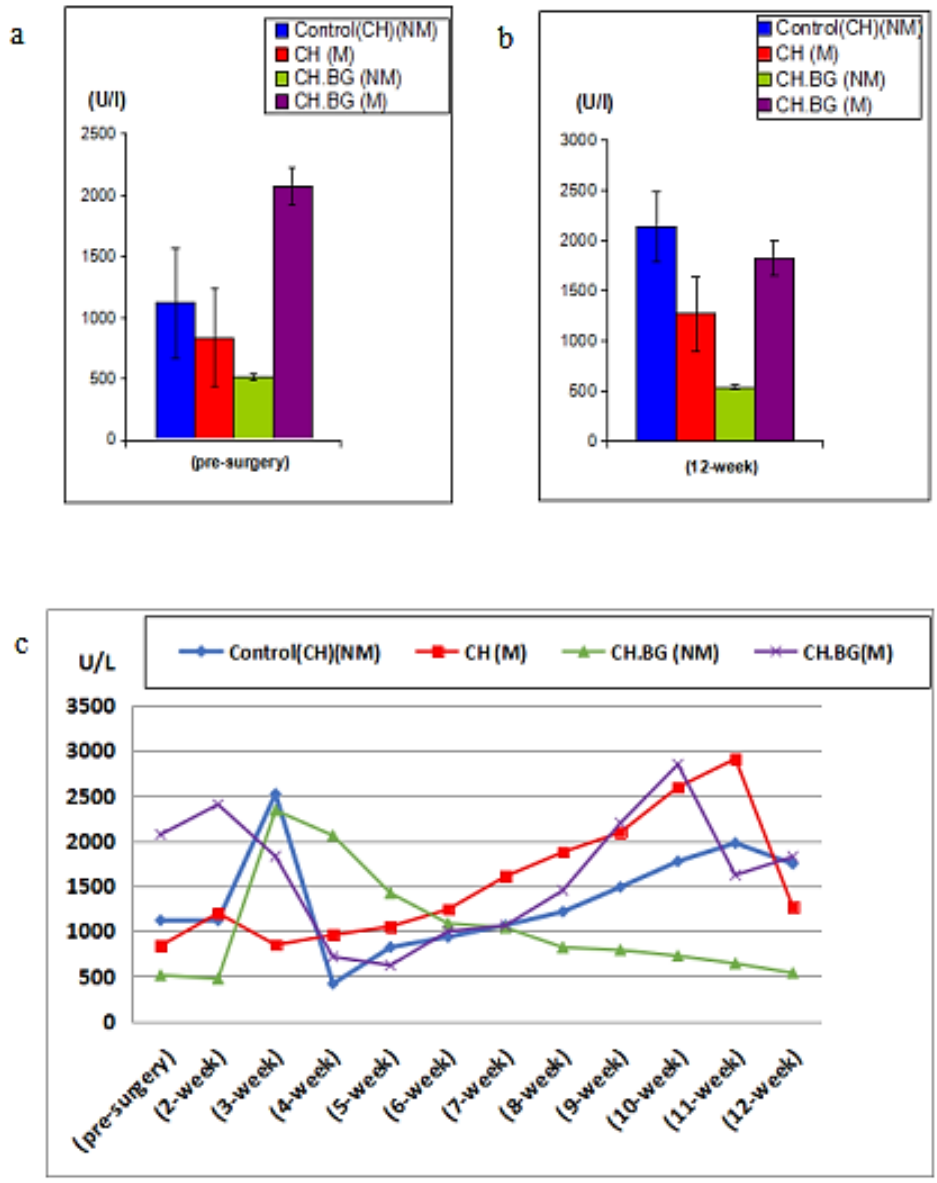

Figure 4: Mean ± SD. of dog serum Alkaline phosphatase level in the different groups **a**: pre-surgery, **b**: 12 weeks, **c**: During experiment.

Serum Calcitonin (ng/L) Level.

There was a significant decrease in serum calcitonin activity in the (CH)(M) group when compared to control group (CH)(NM) from 2-11 weeks (P-value $\leq$ 0.05), while (CH.BG)(NM) groups showed significant decrease in serum calcitonin activity during weeks 2,3,5,6,7,9,10,11 when compared to control group (CH)(NM). There was a significant decrease in serum calcitonin activity in the (CH.BG)(M) when compared to the control group (CH)(NM) during 2,4,6,8,9,10,11,12 weeks (P-value $\leq$ 0.05) as shown in (Table 4 & Figure 5).

Table 4: Serum calcitonin (ng/L) level among the studied group.

Parameters \ Groups	Group I Control CH (NM)	Group II CH(M)	Group III CH.BG(NM)	Group IV CH.BG(M)
Range	0.186 - 2.529	1.329 - 2.695	0.982 - 2.774	0.87 - 1.288
Pre-surgery	2.878 ± 0.063	3.996 ± 0.003	3.153 ± 0.09	3.9 ± 0.03
Mean ± S.D (2-weeks)	7.237 ± 0.019	5.883 ± 0.089*	6.27 ± 0.071*	3.338 ± 0.022*
Mean ± S.D (3-weeks)	7.502 ± 0.041	3.679 ± 0.007*	6.682 ± 0.027*	3.631 ± 0.311 ^{NS}
Mean ± S.D (4-weeks)	7.384 ± 0.01	6.781 ± 0.023*	4.541 ± 0.518^{NS}	3.292 ± 0.084*
Mean ± S.D (5-weeks)	7.331 ± 0.007	5.514 ± 0.064*	4.766 ± 0.33*	3.765 ± 0.021^{NS}
Mean ± S.D (6-weeks)	7.243 ± 0.023	5.741 ± 0.019*	5.821 ± 0.038*	3.346 ± 0.09*
Mean ± S.D (7-weeks)	7.038 ± 0.022	6.491 ± 0.006*	5.553 ± 0.028*	3.797 ± 0.022^{NS}
Mean ± S.D (8-weeks)	5.724 ± 0.016	5.413 ± 0.003*	4.148 ± 0.346^{NS}	3.082 ± 0.161*
Mean ± S.D (9-weeks)	5.105 ± 0.014	5.511 ± 0.006*	6.026 ± 0.205*	2.66 ± 0.075*
Mean ± S.D (10-weeks)	4.992 ± 0.008	6.087 ± 0.026*	6.125 ± 0.126*	3.61 ± 0.055*
Mean ± S.D (11-weeks)	4.866 ± 0.0193	6.448 ± 0.0116*	6.946 ± 0.076*	3.085 ± 0.053*
Mean ± S.D (12-weeks)	4.259 ± 0.174	7.597 ± 0.142*	7.621 ± 0.205*	3.146 ± 0.067*

P * ≤0.05 is significant when compared to group I , P^{NS} >0.05 is non-significant when compared to group I.

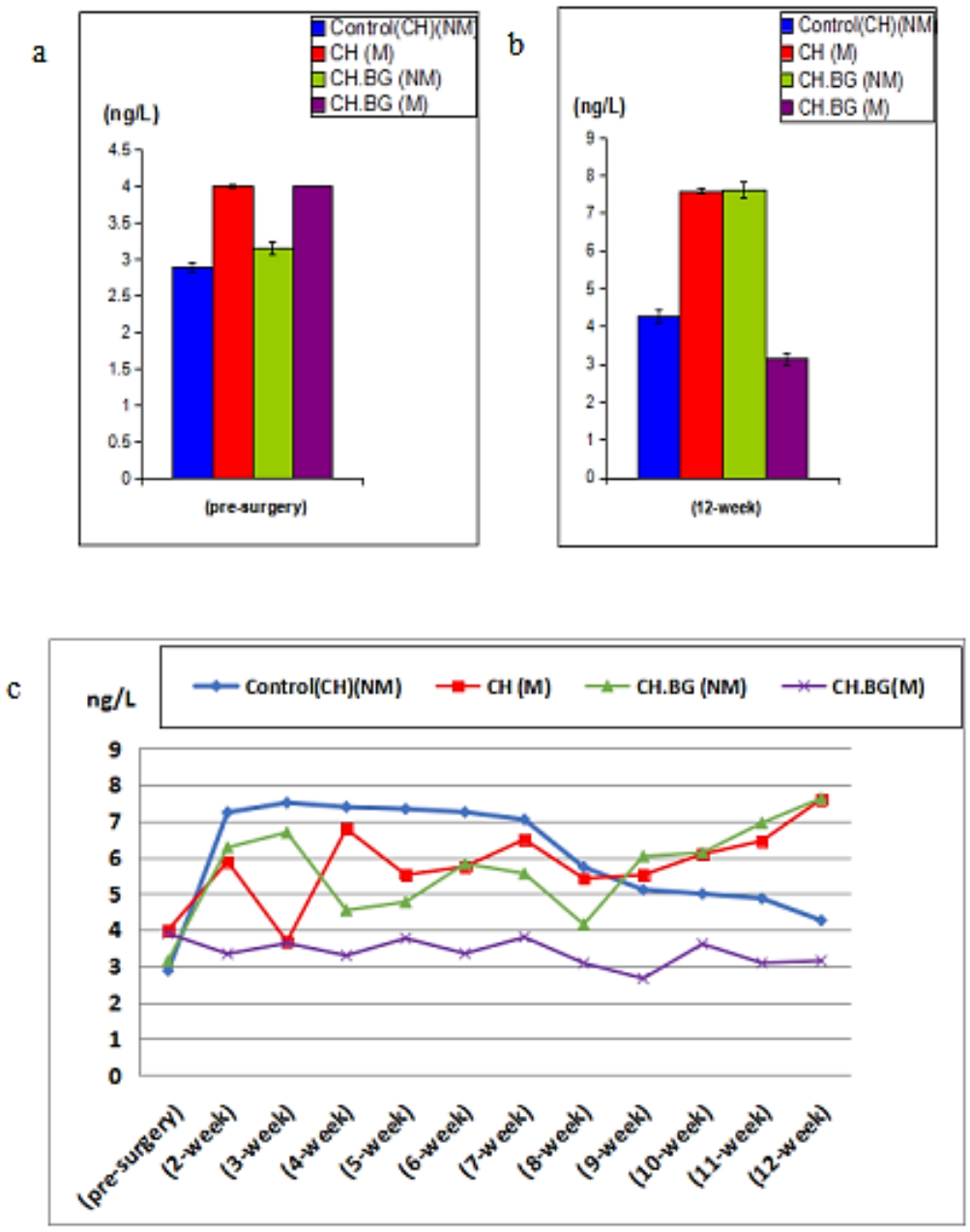

Figure 5: Mean ± SD. of dog serum calcitonin level in the different groups a: pre-surgery, b: 12 weeks, c: During experiment.

Serum Osteocalcin (ng/ml) Level.

There was a non-significant decrease in serum OC level in the (CH)(M) group when compared to control group (CH)(NM) only at 3 weeks (*P*-value > 0.05), while there was a significant differences in serum OC level in the (CH.BG)(NM) group when compared to control group CH(NM) among all weeks. There was a significant decrease in serum OC level in the (CH.BG) (M) when compared to control group (CH) (NM) as shown in (Table 5 & Figure 6).

Table 5: Serum osteocalcin (ng/ml) level among the studied groups.

Parameters \ Groups	Group I Control CH (NM)	Group II CH(M)	Group III CH.BG(NM)	Group IV CH.BG(M)
Range	0.039 - 2.513	0.958 - 2.155	0.927 - 2.345	0.654 - 1.324
Pre-surgery	2.39 ± 0.253	2.885 ± 0.004	2.883 ± 0.035	3.56 ± 0.125
Mean ± S.D (2-weeks)	7.5 ± 0.012	6.382 ± 0.005*	5.459 ± 0.185*	2.814 ± 0.016*
Mean ± S.D (3-weeks)	7.349 ± 0.025	2.886 ± 0.015^{NS}	6.108 ± 0.043*	2.629 ± 0.02*
Mean ± S.D (4-weeks)	6.524 ± 0.01	5.896 ± 0.004*	5.726 ± 0.055*	2.525 ± 0.021*
Mean ± S.D (5-weeks)	6.156 ± 0.09	4.203 ± 0.006*	2.518 ± 0.025*	2.446 ± 0.084*
Mean ± S.D (6-weeks)	6.074 ± 0.08	3.961 ± 0.006*	5.545 ± 0.095*	2.49 ± 0.069*
Mean ± S.D (7-weeks)	6.065 ± 0.078	5.427 ± 0.007*	6.316 ± 0.076*	2.734 ± 0.033*
Mean ± S.D (8-weeks)	5.553 ± 0.062	5.556 ± 0.006*	4.996 ± 0.011*	2.456 ± 0.064*
Mean ± S.D (9-weeks)	5.222 ± 0.059	5.669 ± 0.008*	5.46 ± 0.054*	2.373 ± 0.094*
Mean ± S.D (10-weeks)	5.332 ± 0.056	6.037 ± 0.01*	5.846 ± 0.042*	2.434 ± 0.04*
Mean ± S.D (11-weeks)	5.797 ± 0.056	5.281 ± 0.071*	6.738 ± 0.078*	2.244 ± 0.101*
Mean ± S.D (12-weeks)	5.32 ± 0.031	5.078 ± 0.402*	6.178 ± 0.254*	2.24 ± 0.041*

P * ≤0.05 is significant when compared to group I , P^{NS} >0.05 is non-significant when compared to group I.

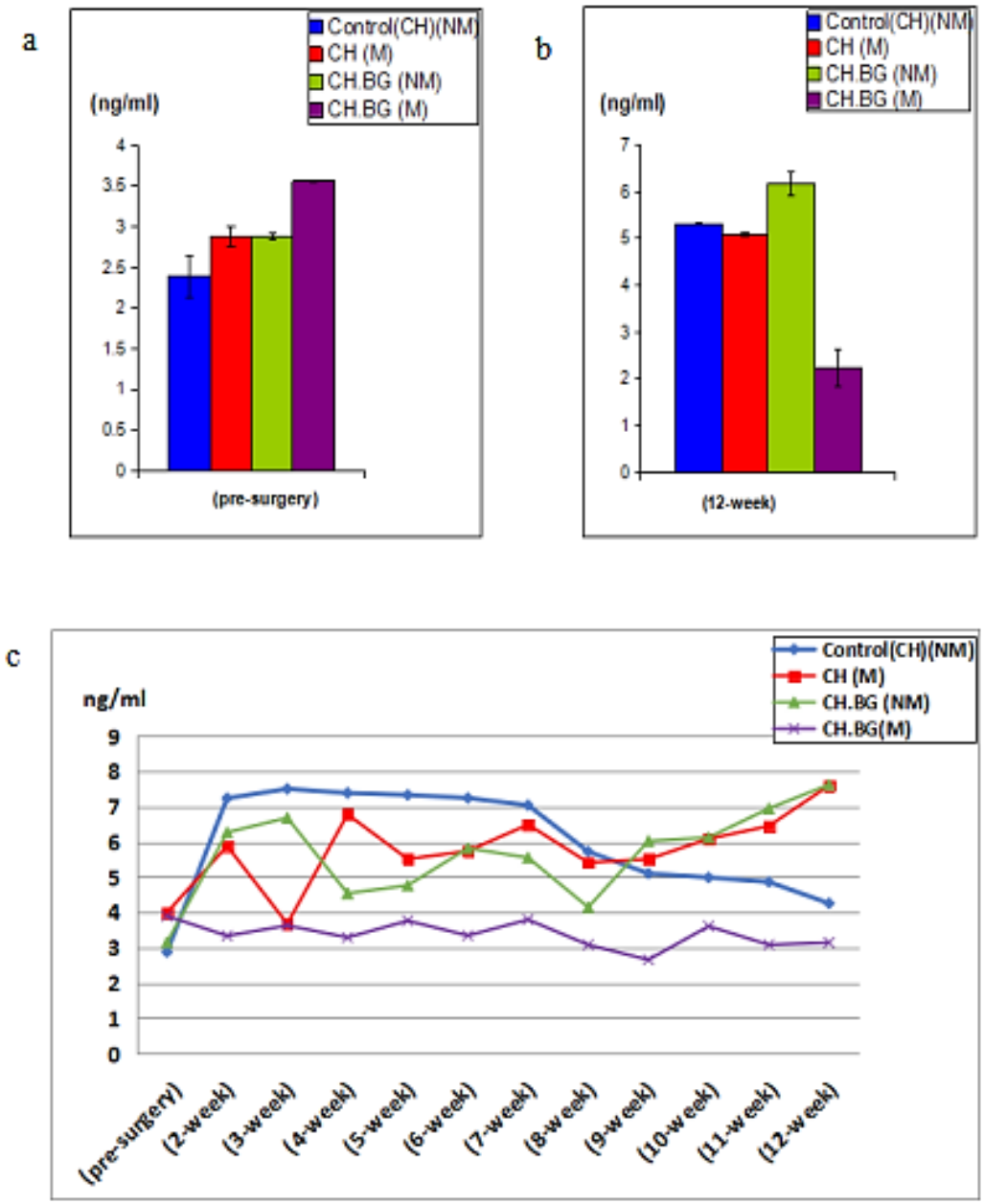

Figure 6: Mean ± SD. of dog serum osteocalcin level in the different groups a: pre-surgery, b: 12 weeks, c: During experiment.

Radiographic Evaluation

In all groups, lateral mandibular radiographs obtained during the first three weeks demonstrated a clearly defined mandibular defect with a smooth, well-defined margin. At the end of the study, defects of (CH)(M) group showed irregular ill-defined margin (Figure 7.b). In CH.BG (NM) group, bone defects demonstrated a gradual increase in bone density occupying the entire defect (Figures 7.c). In CH.BG (M) group, most of the induced defects were completely obliterated by the newly formed bone (Figure 7.d).

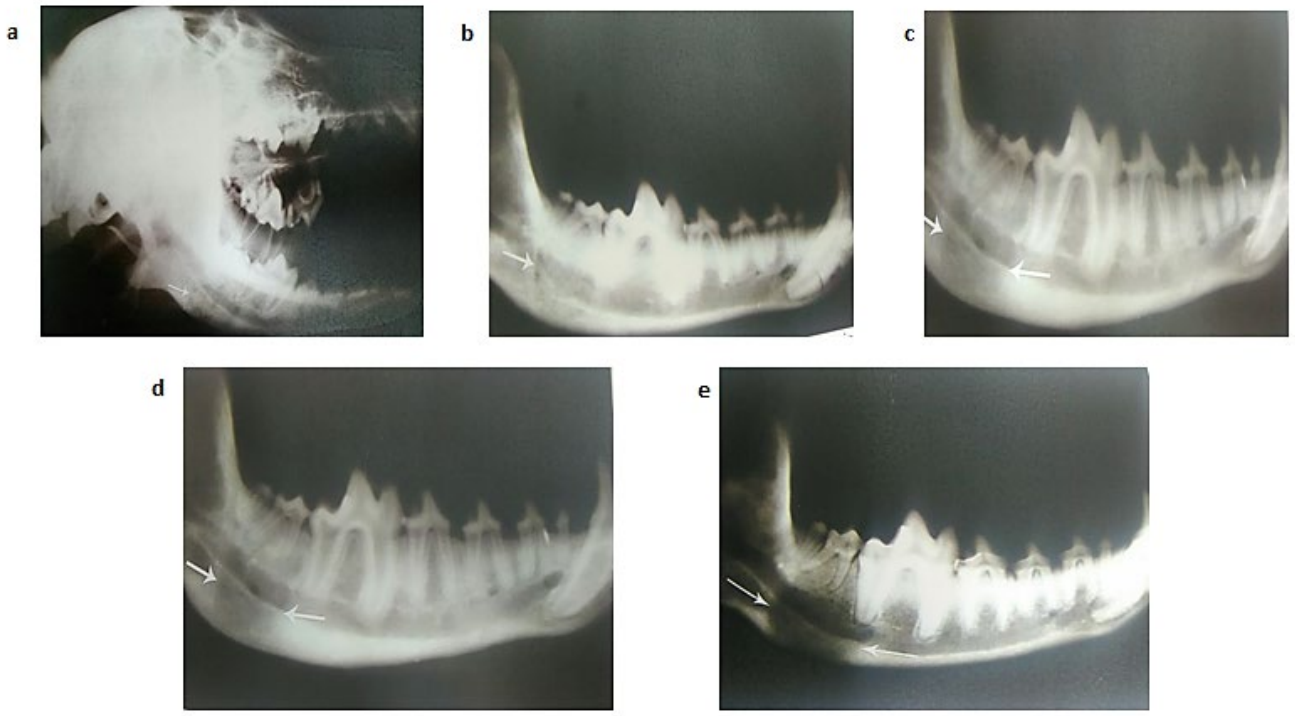

Figure 7: a: A lateral oblique radiographic picture showing the defect in the right side of the mandible after being obliterated by the newly formed bone after 3 months (radio-obaque circle). (Arrow), **b**: A contact radiographic picture showing the defect in the right side of the mandible obliterated with the newly formed bone after 3 months. Notice, the homogeneity of the radiodensity of the bone of the mandible with the newly formed bone. Gp1 (CH.BG) (M), **c:** A contact radiographic picture showing the defect in the right side of the mandible 3 months post-operatively. Notice, the defect still radiolucent with some degree of bone formation. Gp 2 (CH)(M), **d:** A contact radiographic picture showing the defect in the right side of the mandible. Notice, the faint radio-opacity of the defect. Gp 3 (CH.BG) (NM), **e:** A contact radiographic picture showing the defect in the right side of the mandible 3 months post operatively. The defect appears clearly as well-defined radiolucency with a radio-opaque surrounding normal bone. (Arrow), Gp 4 (CH)(NM).

DISCUSSION

The present study demonstrated that serum biochemical markers might provide a clinically useful tool to monitor early changes in bone formation before radiographic signs of bone healing. The study also demonstrated that bioactive glass combined with chitosan and risedronate provided optimum tissue guiding for the regeneration of surgically created mandibular defects in a dog model.

Bone healing is a local process with major effects on systemic minerals, vitamins, hormones, enzyme homeostasis. Away from the radiological changes of bone callus formation as it is time-consuming, serum biochemical markers are considered a clinically valuable tool for monitoring early healing. The close interrelationship of bone resorption and bone formation necessitated the need of biochemical markers that evaluate both processes [18].

Chitosan scaffolds have the potential to induce bone regeneration and can be degraded by time after implantation. The highly porous chitosan scaffolds with well-interconnected open pores could enhance bone formation. Furthermore, chitosan is potentially an effective template for repairing chondral and osseous defects [7, 19].

The present study clarified that combining bioglass and chitosan as a composite scaffold had great osteogenic potential, as demonstrated by new bone inside the induced defect during radiographic examination. This newly formed bone got connected with the defect margins when the mature bone was seen at later stages of the study. These results were compatible with the previous study of Jebahi *et al*., [20].

Significant differences were observed in the calcium levels among (CH.BG) (NM) and (CH.BG) (M) groups when compared to (CH)(NM) group. While (CH) (M) group showed significant difference in the first and last two weeks of the study when compared to (CH)(NM) group. Our results agreed with previous reports demonstrated by Uma Rani and Ganesh, [21], which stated that mean serum calcium values showed a significant rise up at 2 weeks followed by a gradual decrease then reaching generally at 60 days the postoperative period. The serum calcium level in all dogs fluctuated within a normal physiological range due to severe trauma with comminuted and unstable fractures. The initial decrease in serum calcium was probably due to its increased urinary excretion after the traumatic bone injury as explained by

Kumar *et al*. [22].

The current serum phosphorus level showed significant increase in (CH) (M) and (CH.BG) (NM) groups when compared to control group (CH) (NM). While a significant decrease in serum phosphorus level was recorded in the first five weeks, followed by an increase in (CH.BG) (M) group compared to the control group (CH) (NM). These results agreed with that showed by Singh *et al*., [23] who stated that serum phosphorous mean values showed no significant variation postoperatively and the values were within the normal range. In the present study, there was a significant increase in serum phosphorus activity in the (CH.BG)(M) group when compared to control group (CH)(NM), which agreed with that showed by Siemens, [24] who stated increased phosphorous levels on 30th postoperative day and Nagaraj *et al*., [25] noticed a significant increase in phosphorous levels up to 15th postoperative day.

In the current study, there was a significant increase in serum alkaline phosphatase activity in (CH) (M) group when compared to control group (CH) (NM). While there was a non-significant difference in serum ALP activity in (CH.BG) (NM) when compared to control group (CH) (NM). Also, there was a significant difference in serum alkaline phosphatase activity in (CH.BG) (M) group when compared to the control group (CH) (NM) among study weeks. The present results agreed with Guyton, [26], Maiti *et al*., [27] and Uma Rani and Ganesh, [21] who stated that the serum alkaline phosphatase values significantly increased from preoperative day to 14th day and thereafter the levels decreased reaching normal by 60th day. The increase in serum ALP could be attributed to increased chondroblast proliferation for subsequent bone formation during fractured bone repair. Also increased ALP activities may be from the periosteum of destructed bone which is a rich source of ALP. Also, these results agreed with that showed by Singh *et al*., [23] who stated that reported increased serum ALP activity throughout the study period was attributed to the muscle, skin trauma and early stage of bone repair.

Our study showed significant difference in serum calcitonin level in (CH)(M) and (CH.BG) (NM) groups when compared to control group (CH)(NM). Also, (CH.BG) (M) group showed significant difference in serum calcitonin level among most weeks when compared to (CH)(NM) group. These findings disagreed with that reported by Dogan *et al*., [28] who stated that calcitonin proved to enhance osseous healing of the experimental cavities in the early stages. However, there was no significant difference of osteogenic activity of calcitonin on the healing of osseous defects at the end of weeks 3 and 6. In contrast to the previous, calcitonin administration in distractive osteogenesis in rabbits revealed a failure to enhance regenerated bone mineralization rate and tendency during bone lengthening.

The current study agreed with that reported by Kaskani *et al*., [29] who found that calcitonin influenced the increase in bone mineral density which is concurrent with proliferative and maturation phases of bone healing. When local mesenchymal cells begin to differentiate into fibroblasts and osteoblasts, they secrete a collagenous matrix and contribute to its mineralization. This explanation was provided by Dogan *et al*., [28] who reported that calcitonin was capable of stimulating osteoblasts proliferation in experimental animals.

Osteocalcin is a special marker of bone turnover in humans and rodents [30-32]. In the present study, There was a significant difference in serum OC level in the (CH)(M) and (CH.BG)(NM) groups when compared to control group (CH)(NM) among weeks. Also, there was a significant decrease in serum OC level in the (CH.BG) (M) when compared to control group (CH) (NM). This agreed with that reported by Obrant *et al*., [33] who demonstrated a progressive increase in osteocalcin concentration from the day after fracture to more than 2 months later. Also, the present study agreed with that reported by Nyman *et al.* [34], who observed a considerable increase in osteocalcin level by the 6th week in groups, followed by reduced concentrations in the group of normally healing fractures and persistence of high activities, although with insignificant differences between groups. Also, Herrmann *et al*., [35] showed that osteocalcin could be an earlier marker as it remains unchanged after the 4th week, whereas in normally healing fractures, it is significantly elevated.

On radiographic evaluation, the defect appears clearly as well-defined radiolucency with a radio-opaque surrounding normal bone in the control group Gp1 (CH)(NM). The control group showed a radiolucent area of the defect with less accentuated margins. This reflects the osteogenic potential of the graft material (overstimulation and osteoconduction) as well as the biocompatibility of the material to the bone without any adverse immune reactions or inflammatory reactions that might result in bone resorption which could be represented as a radiolucent area in the radiographs, Notice in Gp 2 (CH)(M), the defect still radiolucent with some degree of bone formation rather than the control animal. the right mandible showed a marked increase in radiodensity. The defect could be located with difficulty as it is radiodensity was almost as that of the surrounding normal bone.

The faint radio-opacity of the defect in Gp 3 (CH.BG)(NM) rather than the control animal. The right side of the mandible showed the poorly defined radiolucent area with some discrete radio-opacities seen inside the defect resembling bony spicules and loss of continuity of the component. Notice, the homogeneity of the radiodensity of the bone of the mandible with the newly formed bone in Gp 4 (CH.BG)(M) rather than the control animal. The right mandible defect after 3 months showed the margins of the defect couldn't be demarcated and bone density was greater indicating maturation and remodeling of the newly formed lamellar bone. This could be correlated and relevant to biochemical results.

Limitations of the present study may include the absence of a quantitative radiographic evaluation of bone healing. Future studies should be directed towards evaluating bone healing using cone-beam computed tomography with a quantitative radiographic evaluation of the reparative tissue. Also, studies should be directed towards the application of chitosan/polyvinyl alcohol/residronate scaffolds to treat bone defects in naturally occurring animal models of bone defects to simulate clinical sittings before being applied to human practice.

CONCLUSION

In conclusion, the prepared scaffolds are highly porous with interconnected pore morphology. The porosity ranged to few microns that are appropriate for angiogenesis with cell growth. The BG incorporated with the drug during preparation are well mixed and physically coexisted with the composite CH.PVA structure.

The study highlighted the usefulness of serum biochemical markers as a clinically useful tool to monitor early changes in bone formation before radiographic signs of bone healing. The study also demonstrated that bioactive glass in combination with chitosan and residronate provided optimum tissue guiding for regeneration of surgically created mandibular defects in dog model. Such combination provided accelerated bone healing compared to other groups with reducing the time required for optimum healing.

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DECLARATIONS

Ethics Approval and Consent to Participate

The authors follow the rules of ethics in using animals, Institutional Animal Care and Use Committee (IACUC) has approved the above referenced Animal Use Protocol (AUP) with VetCU06202019050.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

All authors conceived and designed the study. Asmaa A. M. Rady, performed the biochemical analysis, wrote the first draft of the manuscript. Mohamed A. Abdel-Hamid and Elham A. Hassan performed the surgical procedures, collected blood samples and performed radiographic evaluation. Soha M. Hamdy, Marwa G. A. Hegazy, Shadia A. Fathy and Heba A. Ewida analyzed biochemical data, performed the statistical analysis and interpreted the biochemical analysis. Amany A. Mostafa, a major contributor in conceiving and designing the study, preparing and characterizing bone scaffolds, revising the manuscript.

All authors critically revised the manuscript for important intellectual contents and approved the final version.

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