ORIGINAL RESEARCH

# Comparison between Effect of Bisphosphonates, Concentrated Growth Factors or Combination on Rabbits' Tibial Bone Defects Healing: An Experimental Study

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## Abstract

**Aim:** This study was designed to evaluate the effect of bisphosphonates (BIS) or concentrated growth factors (CGF) or a combination of them on bone defect healing.

**Materials and methods:** Bone defects of 3-mm width and 6-mm depth were prepared in 24 rabbit tibias unilaterally, then randomly divided into the following four equal groups:

1. **Group I:** No treatment
2. **Group II:** Treated by BIS
3. **Group III:** Treated by CGF
4. **Group IV:** Treated by BIS + CGF

Animals were equally sacrificed at 4 weeks, and at 6 weeks then tibias were processed for hematoxylin and eosin (H&E) and Masson's trichrome (MTC) staining. The data were subjected to one-way analysis of variance (ANOVA) followed by *post hoc* Tukey test and unpaired Student's *t*-test.

**Results:** In group IV, the quality of newly formed bone was better than any other group with increased mineralization and decreased collagen, followed by group III, then group I, while group II showed the least favorable results. The statistical analysis showed a significant difference between groups.

**Conclusion:** Mixing BIS with CGF showed the best healing, and bone quality results, followed by CGF-treated group, then control, and finally, BIS-treated group.

**Clinical significance:** Using CGF as a scaffold and mixing it with BIS could help accelerate the healing of bone defects, reduce healing time, and minimize the risk of infection.

**Keywords:** Histochemical, Histological, Masson trichrome.

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## Introduction

Bone defects could be induced by a variety of diseases requiring surgical interventions or traumatic injuries which require constant remodeling of bone. Bone has the ability to rebuild its structure by special mechanisms through osteoclast and osteoblast activities.[1] The use of bone graft material may help to decrease the healing time of bone defects as it may have an osteoinductive (has factors that improve bone development and result in stem cells turning out to be active osteoblasts) or osteoconductive (acts as a framework for natural bone development) or osteogenesis (offers cells which will create bone comprising primitive mesenchymal stem cells, osteocytes, and osteoblast) effect depending on its type.[2]

Some materials used with bone grafts either increase the rate of osteoblast activity or decrease the rate of osteoclast activity or control the other cell activity in the manner of angiogenesis and decrease fibroblast activity.[3,4] Many materials are used to accelerate bone healing as platelet-rich fibrin (PRF), CGF, hyaluronic acid, and BIS with different outcomes.[5,6]

Bisphosphonates are a stable analog of pyrophosphate. Have a suppressing effect on the osteoclast, indirectly decreasing osteoblast activity. So classified as a bone resorption inhibitory drug rather than bone-forming agent.[7] Bisphosphonates therapy normalizes the turnover of the bone, reducing the number of remodeling of bone stress risers, restores the balance remodeling of the bone, prevents bone loss and deterioration of bone structures in osteoporosis patients, and reducing the risk of fracture.[8] Bisphosphonates represent the treatment of choice for several metabolic and oncological diseases affecting the skeletal system, such as osteoporosis, osteogenesis imperfect, Paget disease, fibrous dysplasia, and bone cancer.[9]

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**Conflict of interest:** None

Platelet gel is an autologous modification of fibrin glue, first described in 1997, it is prepared by collecting autologous blood and is composed of a combining of thrombin, calcium chloride, and platelet-rich plasma (PRP).[10] Platelet-rich plasma is an autologous source of many growth factors such as transforming growth factor β and platelet growth factor and that obtained by sequestering and concentrating platelets by gradient density centrifugation.[11] Platelet-rich plasma was discovered by J Choukroun in France using an innovative method that did not require anticoagulants or coagulation factors. Platelet-rich fibrin, which is called the second generation of platelet concentrate, has been shown more content of growth factors than PRP.[12]

Concentrated growth factors were first developed by Sacco in 2006 as a derivative of PRF.[12] Concentrated growth factors is a matrix of a fibrin-rich organic material that contains leukocytes, platelets, growth factors, and CD34+ stem cells that help in the processing of regeneration and also has immunological cells that are effective in regulating inflammation and minimizing the risk of infection.[13] Concentrated growth factors increase the success rate of bone grafting and implant therapy is now widely used to shorten the interval between bone graft placement and implant insertion. Many articles have been published on the application of CGF in the maxillofacial and dental fields.[14] So, this study was performed to compare the effect of using BIS or CGF or a combination of them on the bone healing process.

To our knowledge, no other previous studies have evaluated the effect of combining BIS with CGF on healing of bone defect.

## Materials and Methods

This study was conducted in Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Egypt. The study was performed for 5 months starting with bone defect preparation until statistical analysis in 2021. The study protocol was approved by the institutional ethical committee of the Faculty of Dentistry, Mansoura University, Egypt (No. A-16060421).

### Animal Selection

A total of 24 rabbits (New Zealand white adult males) with weighting (2–3 kg) were utilized in this study. All animals were kept in the same nutritional and environmental conditions. The housing of rabbits was in a room with a 12/12-hour light-dark cycle at a temperature of 22°C and 65–70% relative humidity. Animals were fed a commercial diet and water. The sample size was calculated using G*Power statistical software according to the similar previous studies.[15,16]

### Experimental Design and Sample Distribution

Rabbit well localized to a different group by using a simple randomization method. In each rabbit, two bone cavities were prepared in the right tibia. The rabbits were then randomly allocated into the following four groups:

1. Group I (positive control): The bone defects were prepared without any further treatment.
2. Group II: The bone defects were prepared and then treated with BIS.
3. Group III: The bone defects were prepared and then treated by CGF.
4. Group IV: The bone defects were prepared and then treated by a combination of BIS and CGF.

### Bisphosphonates Preparation

Bisphosphonate was prepared by dissolving 20-mg alendronate sodium (Fosamax) in 1 mL normal saline.[17] on a glass slap, then introduced by a spatula into the prepared socket.

### Concentrated Growth Factors Preparation

Whole blood was drawn from the marginal ear vein in a 10-mL tube containing no anticoagulant. The tube was immediately centrifuged using a program with the following characteristics: Acceleration at 2,700 rpm over 4 minutes, 2,400 rpm over 4 minutes, 2,700 rpm over 4 minutes, and 3,000 rpm over 3 minutes, and then subjected to a deceleration for 36 seconds till the end of the centrifugation. The three layers obtained were as follows: Lower RBCs, top platelet-poor plasma (PPP), and fibrin gel with concentrated growth membrane in-between. Initially, the PPP layer was discarded using a syringe. The middle membrane was pulled using forceps and separated from the lower layer through cutting using a scalpel.[18]

### Surgical Procedures

The rabbits were systemically anesthetized via intramuscular injections of diazepam (0.5 mg/kg), ketamine-HCl (20 mg/kg), and xylazine (25 mg/kg).[19] Besides, the surgical site was injected with local anesthetic 2% Mepivacaine HCL and 1:20,000 Levonordine (Alexandria, Egypt) in the proximal right and left tibia (Fig. 1A) to control the bleeding.[20]

At first, the hair covering the skin at the surgical site was shaved and scrubbed using disinfectant (povidine–iodine). After that, the rabbits were wrapped in sterile towels. At the surgical site, 2-cm inferior to the knee, 4-cm skin incisions were performed. The superficial fascia and deep fascia were incised. The tibial periosteum then was incised and reflected till reaching the bone (Figs 1B and C).

Two bony defects (3 mm and 6 mm for diameter and depth, respectively) were prepared under continuous rinsing saline utilizing a trephine bur of 3-mm diameter in the right tibia of each rabbit (Fig. 1D).[21] The size and depth of each defect were standardized through the utilization of trephine bur (of the same head size and same velocity) attached to a contra-angle which was fixed on a low-speed micro motor machine (strong, Saenshin, Koe) (Figs 1E and F).

After that, both bone defects were irrigated with saline by a 5-mL syringe for washing the debris within them and then dried by a sterile piece of gauze. The defects were filled either with BIS or CGF or by a mixture of BIS and a suitable amount of CGF.

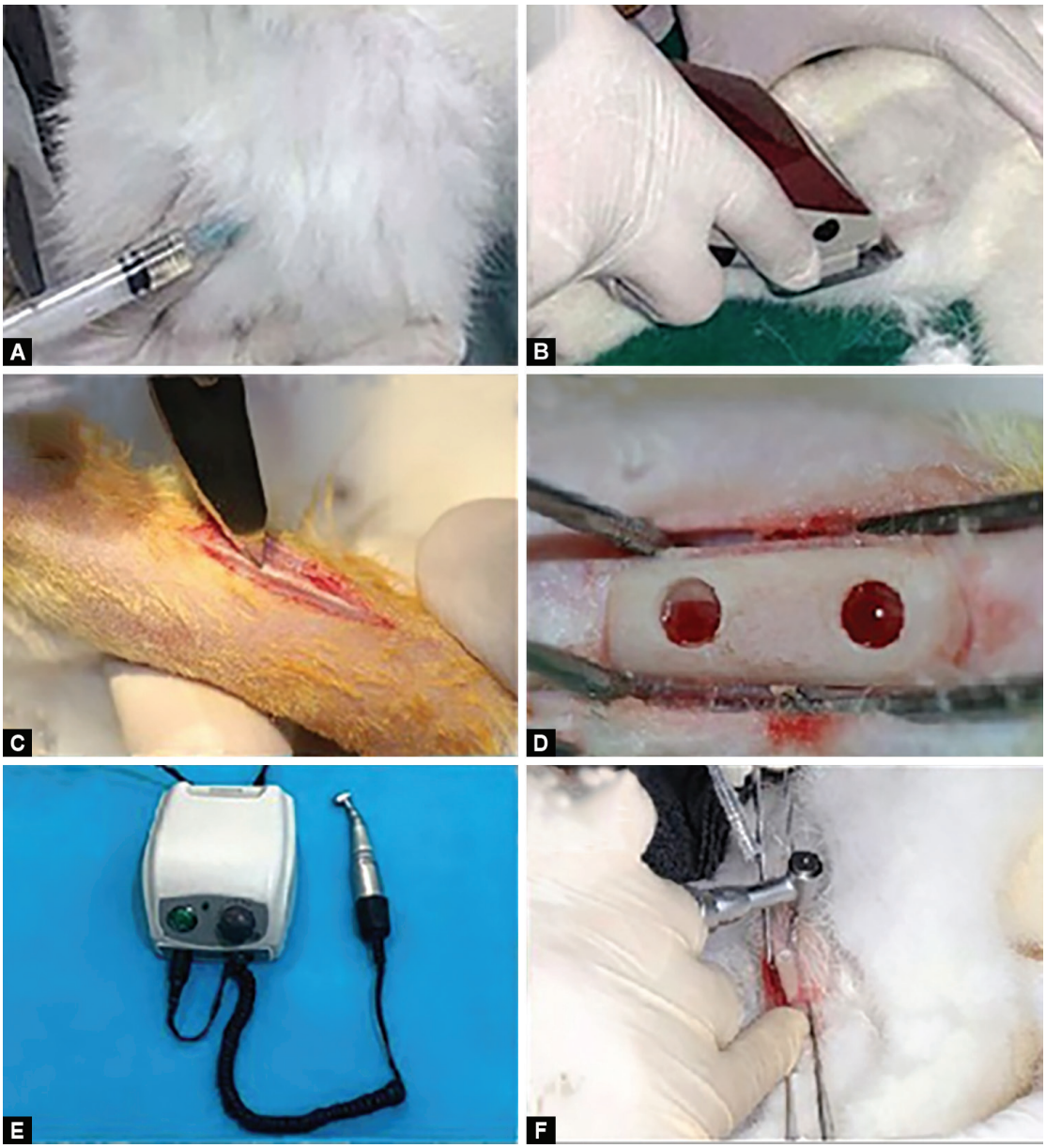
Wound edges were then approximated utilizing tissue forceps. The wound was sutured in layers using 3/0 resorbable (vicryl) string for the skin. The skin was scrubbed via iodine post-suturing.

### Postoperative Medication

After the surgery, the animals received antibiotics (150 mg/kg of Cefotaxime, Egyptian International Pharmaceutical Industries Co. E.I.P.I.Co., Egypt) injected every 12 hours for 5 days and analgesics (75 mg of Voltaren, Novartis Pharma S.A.E., Cairo, Egypt) every 8 hours for 2 days postoperatively.[22]

### Sacrifices of Animals

A total of 24 rabbits were randomly divided into four groups (6 rabbits per group). They were sacrificed by overdose of diethyl ether at 4 and 6 weeks after surgery, respectively, to dissect out right tibia immediately after scarification.




**Figs 1A to F:** Photomicrograph showing steps of bone defect preparation: (A) Intramuscular injection of systemic anesthesia; (B) Shaving the hair covering the skin at surgical site; (C) Superficial fascia, deep fascia, and tibial periosteum incision and reflected; (D) Two bony defects preparation in each tibia; (E) Low-speed micro motor machine; and (F) Contra-angle used in defect preparation

## Histological and Histochemical Evaluation

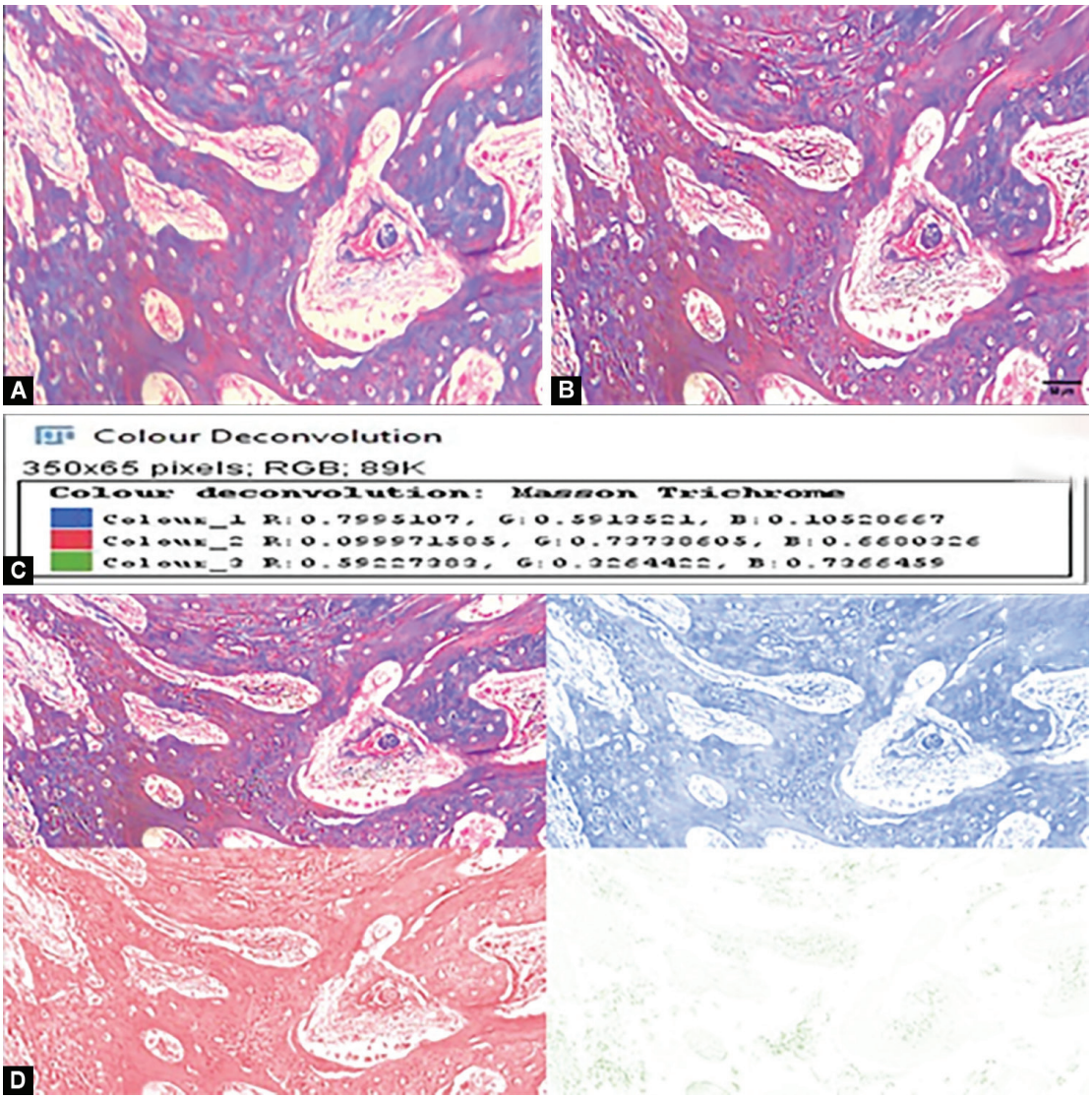
For postoperative assessment, 12 rabbits were sacrificed at each experimental period 4 and 6 weeks postoperatively using a high dose of diethyl ether. The right tibia from each animal was immediately removed, fixed with 10% formalin then demineralized with ethylenediaminetetra acetic acid (EDTA). The tibias were processed to paraffin block and prepared for examination histologically by H&E staining (Sigma–Aldrich, St. Louis, Missouri, United States) as a routine stain to evaluate the newly formed bone and histochemical evaluation by Masson's trichrome (Sigma–Aldrich, St. Louis, Missouri, United States) staining for detection of the amount of collagen fiber and degree of mineralization. The slide's examiners are unaware of the type of material that used in each slide.

## Computer-assisted Digital Image Analysis (Digital Morphometric Study)

Slides were photographed using ToupCam digital camera (model No.: XCAM1080PHA) installed on Olympus inverted microscope (CKX41SF, Japan), using a 200× objective. The resulting images were analyzed on an Intel core I7-based computer using Fiji ImageJ software (version 1.51r, NIH, Maryland, USA). For measuring the staining intensity, the color deconvolution plug-in was used. Five random fields from each slide were analyzed.

The software routine for staining intensity quantification (Fig. 2) is performed as follows:

- *Step 1:* Image acquiring from the camera using VideoTest-Morphology software (Russia) (Fig. 2A).




**Figs 2A to D:** Steps of digital image analysis

- *Step 2:* Enhancing color tone and contrast by using the auto enhancer function to reveal the target stain color (Fig. 2B).
- *Step 3:* The color deconvolution to separate collagen and calcium stains using a color deconvolution plug-in. Red, Blue, and Green (RGB) for collagen blue stain were (R: 0.7995107, G: 0.5913521, B: 0.10528667) and for calcium red stain were (R: 0.09997159, G: 0.73738605, B: 0.6680326) (Figs 2C and D).
- *Step 4:* The regions of interest were selected manually to represent all samples, and the staining intensity was measured as the "mean gray value" parameter. The average staining intensity for all measurements was calculated from five fields of view for each sample.
- *Step 5:* The measured data is exported to Excel sheets. In ImageJ, pixel density values for any color range from 0 to 255, where 0 represents the darkest shade and 255 represents the lightest shade of color.

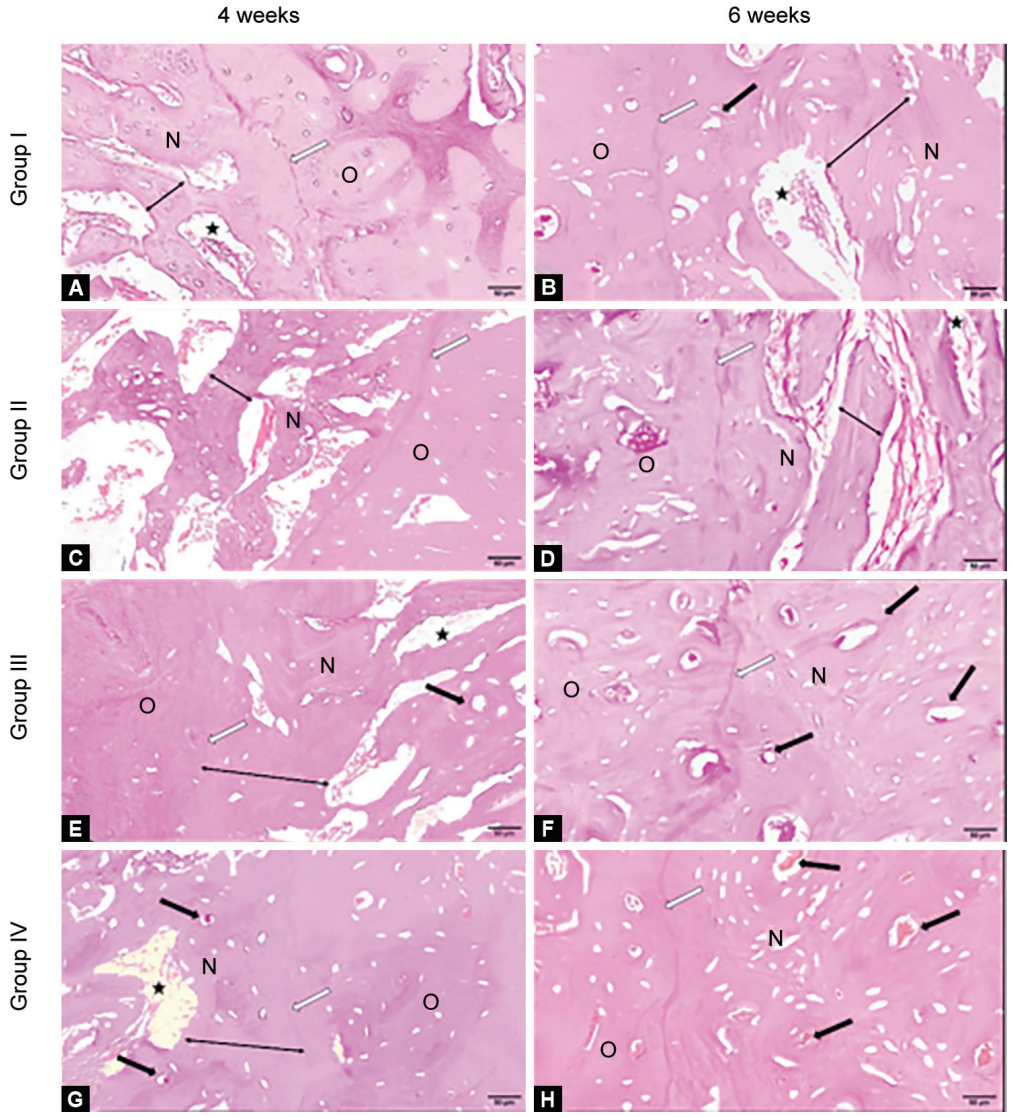
### Statistical Analysis

The data was tabulated and coded and then analyzed by using the computer program Statistical package for social science. Descriptive statistics and calculated in the form of Mean ± Standard deviation (SD). The Shapiro–Wilk test was used to assess the normality of the data. Significance of difference was tested using Student's *t*-test (unpaired) to compare the mean of two different sets of parametric (numerical) data and one-way ANOVA for the comparison of more than two sets of parametric (numerical) data followed by *post hoc* Tukey. A *p*-value below 0.05 is considered statistically significant.

## Results

### Hematoxylin and Eosin (H&E) Histological Examination Results (Fig. 3)

The microscopic examination of the histological slides in group I after 4 weeks showed the formation of new, thin bone trabeculae extending along the border of the defect with osteocytic lacunae scattered within them and surrounding many active vascular bone marrow cavities, while after 6 weeks it showed the formation of thicker bone trabeculae containing a number of small osteons and in between large areas of immature woven bone containing




**Figs 3A to H:** Hematoxylin and eosin group I after 4 weeks (A); after 6 weeks (B); Group II: After 4 weeks (C) and after 6 weeks (D); Group III: After 4 weeks (E) and after 6 weeks (F); Group IV: After 4 weeks (G) and after 6 weeks (H). At 200× magnification. N, new bone; O, old bone; Black arrow: Newly formed osteons; Double-headed arrow: Thickness of bone trabeculae; White arrow: Reversal line; *Bone marrow spaces

osteocytic lacunae with many active vascular bone marrow cavities containing large inflammatory cells, the osseous fusion between old and new bone was found to be more harmonious when compared with 4-weeks subgroup (Figs 3A and B).

Group II showed the formation of new bone with thinner bone trabeculae containing scattered osteocytic lacunae radiating from old bone with wider bone marrow spaces compared to the control group after 4 weeks, while after 6 weeks it showed the formation of larger areas of new bone compared to 4 weeks, but with less thickness bone trabeculae containing fewer osteons and wider bone marrow spaces compared with a control group of the same period. Bone trabeculae contained large areas of immature bone with scattered osteocytic lacunae with the osseous fusion between old and new bone (Figs 3C and D).

Group III after 4 weeks showed formation of new bone with thicker, well-arranged bone trabeculae containing newly formed osteons and in between large areas of immature woven bone and narrower bone marrow spaces compared to groups I and II. While after 6 weeks, the bone almost filled bone defect with thicker, well-arranged bone trabeculae containing numerous osteons with narrow areas of immature woven bone in between containing scattered osteocytic lacunae compared to groups I and II of same period. A complete and more harmonious osseous fusion was observed between old and new bone (Figs 3E and F).

Group IV after 4 weeks showed the formation of new bone with large, better-arranged bone trabeculae radiating from old bone almost filling the defect with narrower bone marrow spaces. Bone trabeculae contained larger osteons with osteocytes arranged circumferentially around the Haversian canal and in between smaller areas of woven bone containing scattered osteocytic lacunae. While after 6 weeks, it showed the formation of thick coalesced new bone filling the defect with a large number of well-formed osteons, and in between smaller areas of immature bone containing osteocytic lacunae, a complete harmonious osseous

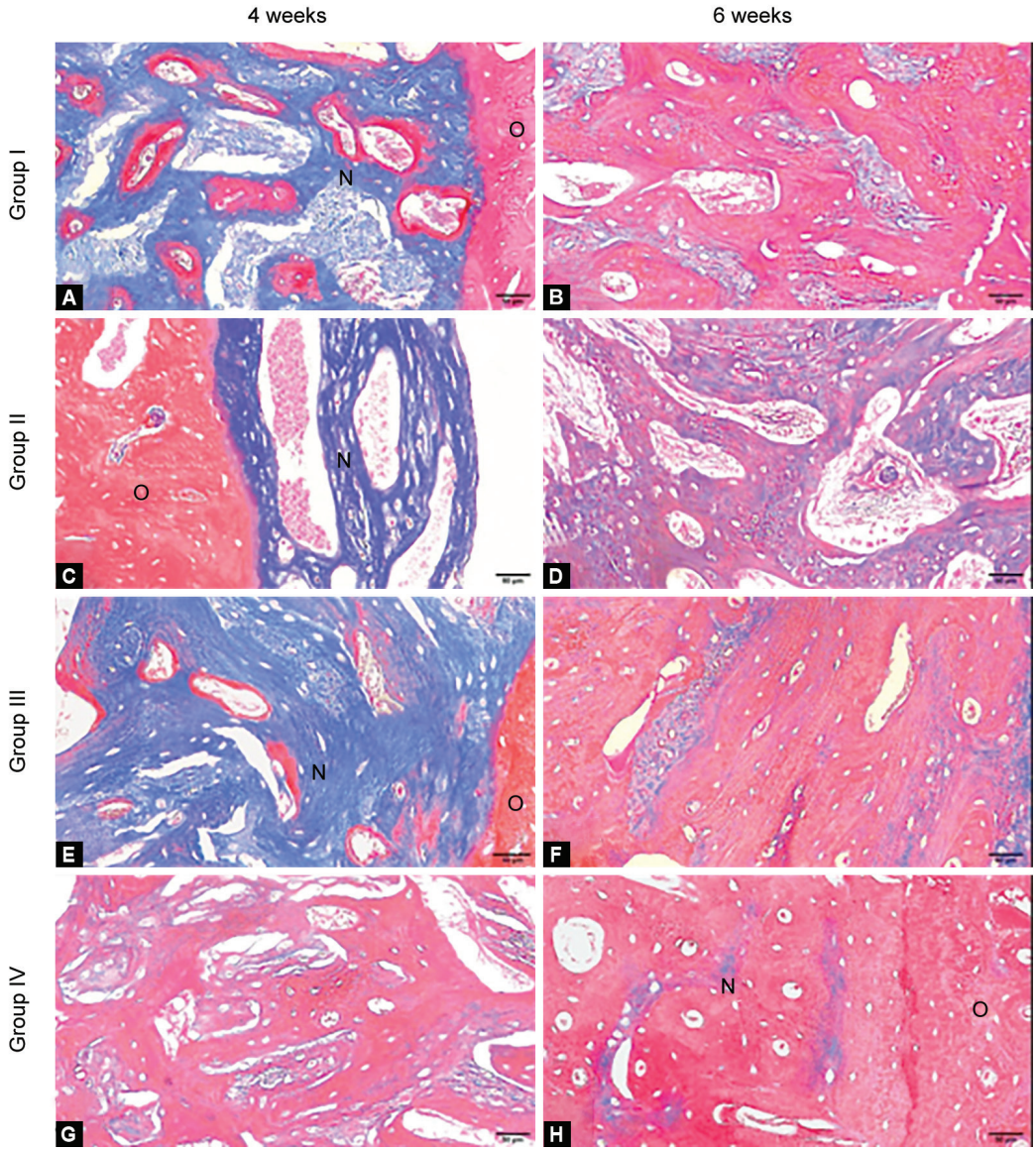
fusion was observed between old and new bone and the quantity and the quality of the formed bone was better than other groups at the same time points (Figs 3G and H).

## Masson's Trichrome Histochemical Staining Results (Fig. 4)

The microscopic examination of the histological slides in group I after 4 weeks presented a large amount of collagen fiber marked by the blue coloration (152.47 ± 8.04) surrounding newly formed small osteons showing signs of mineralization around them marked by the appearance of red coloration (201.4 ± 7.84), while after 6 weeks it showed formation large areas of more mature bone trabeculae, more arranged and highly mineralized marked by an increase in red coloration (140.58 ± 17.84) with a less amount of collagen fiber marked by the decrease in blue coloration (200.86 ± 18.68) (Figs 4A and B).

Group II presented a larger amount of collagen fiber marked by intense blue coloration (107.06 ± 9.83) and less mineralization marked by minimal red coloration (226.41 ± 4.98) after 4 weeks. While after 6 weeks, it showed the formation of larger bone trabeculae containing small osteons showing fewer signs of mineralization around them marked by red coloration (175.78 ± 24.60) mixed with a bluer coloration of immature collagen fibers (183.63 ± 13.46) compared to group I (Figs 4C and D).

In group III after 4 weeks, the amount of collagen fiber was larger marked by the increased blue coloration (127.10 ± 5.50) with more mineralization marked by the appearance of greater areas of red coloration (173.37 ± 38.61) compared to groups I and II. After 6 weeks, more mature bone trabeculae with a greater number of osteons that were more arranged and highly mineralized marked by large areas of red coloration (110.81 ± 5.78) and a less amount of




**Figs 4A to H:** Masson's trichrome group I, after 4 weeks (A) and after 6 weeks (B); Group II after 4 weeks (C) and after 6 weeks (D); Group III after 4 weeks (E) and after 6 weeks (F); Group IV after 4 weeks (G) and after 6 weeks (H). All photomicrographs were captured at 200× magnification. N, new bone; O, old bone

**Table 1:** One-way ANOVA followed by *post hoc* Tukey test for comparison of mean of collagen stain between groups I–IV within 4- and 6-week subgroups

| | *Group I* | *Group II* | *Group III* | *Group IV* | *p-value* |
|---|---|---|---|---|---|
| 4 weeks | 152.47 ± 8.04 | 107.06 ± 9.83 | 127.10 ± 5.50 | 187.31 ± 16.48 | <0.001* |
| *Post hoc* | | P1 = <0.001* | P1 = 0.009* | P1 = 0.001* | |
| | | | P2 = 0.04* | P2 = <0.001* | |
| | | | | P3 = <0.001* | |
| 6 weeks | 200.86 ± 18.68 | 183.63 ± 13.46 | 230.08 ± 6.58 | 245.63 ± 4.06 | <0.001* |
| *Post hoc* | | P1 = 0.15 | P1 = 0.006* | P1 = <0.001* | |
| | | | P2 = <0.001* | P2 = <0.001* | |
| | | | | P3 = 0.26 | |

Data expressed as mean ± SD. SD, standard deviation; P, probability; P1, significance vs group I; P2, significance vs group II; P3, significance vs group III; *Significant when $p$ <0.05

collagen fiber marked by the small areas of blue coloration (230.08 ± 6.58) was formed compared to groups I and II (Figs 4E and F).

In group IV after 4 weeks, a large area of more mature mineralized bone trabeculae was formed marked by more red coloration (78.22 ± 3.70) with a smaller amount of immature collagen fibers marked by less blue coloration (187.31 ± 16.48) when compared with group III in the same period. After 6 weeks, a larger area of more mature bone with more condensed, highly mineralized osteons marked by increased red coloration (59.81 ± 7.86) with a minimal amount of immature collagen fibers marked by decreased blue coloration (245.63 ± 4.06) compared to other groups (Figs 4G and H).

## Statistical Analysis Results

One-way ANOVA followed by a *post hoc* Tukey test for comparison of the mean of collagen (blue stain) showed that in 4-weeks subgroups, group II showed the highest amount of collagen fibers with a mean of (107.06 ± 9.83), followed by group III (127.10 ± 5.50), then group I (152.47 ± 8.04). In contrast, group IV showed the least amount (187.31 ± 16.48). The *post hoc* Tukey test showed a significant difference between all groups (<0.05). In 6-weeks subgroups, group II showed the highest amount of collagen fibers with a mean of (183.63 ± 13.46) followed by group I (200.86 ± 18.68), then group III (230.08 ± 6.58), while group IV showed the least amount (245.63 ± 4.06). The *post hoc* Tukey test showed a significant difference between all groups (<0.05), except group II showed a non-significant increase (P1 = 0.15) compared to group I, while group IV showed a non-significant decrease (P3 = 0.26) compared to group III (Table 1). Student's *t*-test for comparison of the mean of collagen stain between 4- and 6-weeks subgroups showed a significant decrease within all groups (Table 2).

One-way ANOVA followed by a *post hoc* Tukey test for comparison of the mean of calcium (red stain) showed that in 4-weeks subgroups group II showed the slightest degree of mineralization with a mean of (226.41 ± 4.98), followed by group I (201.4 ± 7.84), then group III (173.37 ± 38.61), while group IV showed the highest degree with a mean of (78.22 ± 3.70). The *post hoc* Tukey test showed a significant difference between all groups (<0.05) except group II showed a non-significant decrease compared to groups I and III showed a non-significant increase compared to group I. In 6-weeks subgroups, group II showed the least degree of mineralization with a mean of (175.78 ± 24.60), followed by group I (140.58 ± 17.84), then group III (110.81 ± 5.78), while group IV showed the highest degree with a mean of (59.81±7.86). The *post hoc* Tukey test showed a significant difference between all groups (<0.05)

**Table 2:** Student's *t*-test for comparison of mean of collagen stain between 4 weeks and 6 weeks within groups I–IV

| | *4 weeks* | *6 weeks* | *p-value* |
|---|---|---|---|
| Group I | 152.47 ± 8.04 | 200.86 ± 18.68 | 0.001* |
| Group II | 107.06 ± 9.83 | 183.63 ± 13.46 | <0.001* |
| Group III | 127.10 ± 5.50 | 230.08 ± 6.58 | <0.001* |
| Group IV | 187.31 ± 16.48 | 245.63 ± 4.06 | <0.001* |

Data expressed as mean ± SD. SD, standard deviation; P, probability; *Significant when $p$ <0.05

(Table 3). Student's *t*-test for comparison of mean of Ca red stain between 4- and 6-weeks subgroups showed a significant increase within all groups (Table 4).

# Discussion

Bone defects in oral and maxilla-facial surgery procedures are a fundamental problem in treating diseases. It could result from the surgical evacuation of cysts, deep seat impacted teeth, birth defects, injury, malignancies, huge post-extraction sockets, atrophy, dental disease, getting older[23] and a variety of other bone diseases (e.g., osteoporosis or periodontal disease) that requires a long period to heal and fill.[24] Many trials have been made over the years to compensate for lost bone and stimulate healing with variable degrees of success in regenerating bone.[25]

Bisphosphonate is a drug that has been documented to restrain bone resorption by means of inactivating the osteoclasts.[26] Concentrated growth factors plays a significant role in enhancing bone healing as it contains numerous autogenous growth factors that play a fundamental role in the movements, differentiation, and multiplications of osteoblast cells as well as extracellular matrix synthesis.[27] So, this study was performed to assess the effect of each BIS or CGF or using a combination of them in bone and healing.

Rabbits were chosen as an animal model for this study because they are easier to study and manage and have a shorter vital cycle. Moreover, they have relatively lower costs to buy and house[28] faster skeletal and bones turnover in contrast to bigger animal models.[29]

In this study, the histological slides in group I after 4 weeks showed formations of new, thin bone trabeculae containing many active vascular bone marrow cavities. While after 6 weeks, it formed new thicker bone trabeculae containing a number of small osteons and in between large areas of immature woven bone containing osteocytic lacunae. These results were consistent with the studies

**Table 3:** One-way ANOVA followed by *post hoc* Tukey for comparison of mean of calcium red stain between groups I–IV within 4- and 6 weeks subgroups

| | *Group I* | *Group II* | *Group III* | *Group IV* | *p-value* |
|---|---|---|---|---|---|
| 4 weeks | 201.4 ± 7.84 | 226.41 ± 4.98 | 173.37 ± 38.61 | 78.22 ± 3.70 | <0.001* |
| *Post hoc* | | P1 = 0.23 | P1 = 0.15<br>P2 = 0.003* | P1 = <0.001*<br>P2 = <0.001*<br>P3 = <0.001* | |
| 6 weeks | 140.58 ± 17.84 | 175.78 ± 24.60 | 110.81 ± 5.78 | 59.81 ± 7.86 | <0.001* |
| *Post hoc* | | P1 = 0.01* | P1 = 0.04*<br>P2 = <0.001* | P1 = <0.001*<br>P2 = <0.001*<br>P3 = 0.001* | |

Data expressed as mean ± SD. SD, standard deviation; P, probability; P1, significance vs group I; P2, significance vs group II; P3, significance vs group III; *Significant when $p$ <0.05

**Table 4:** Student's *t*-test for comparison of mean of calcium stains between 4 and 6 weeks within groups I–IV

| | *4 weeks* | *6 weeks* | *p-value* |
|---|---|---|---|
| Group I | 201.4 ± 7.84 | 140.58 ± 17.84 | <0.001* |
| Group II | 226.41 ± 4.98 | 175.78 ± 24.60 | 0.002* |
| Group III | 173.37 ± 38.61 | 110.81 ± 5.78 | 0.007* |
| Group IV | 78.22 ± 3.70 | 59.81 ± 7.86 | 0.001* |

Data expressed as mean ± SD. SD, standard deviation; P, probability; *Significance when $p$ <0.05

conducted by Khalil et al.[30] and Salih et al.[31] who reported the formation of lamellar bone containing wide Haversian canal lined by active osteoblast, with moderate thickness trabecular bone at fourth week postoperation. Tebyanian et al.[32] also found that after 4 weeks, immature bone generation was detected, followed by new bone formation after 8 weeks.

Bisphosphonates-treated group showed the formation of thinner bone trabeculae radiating from old bone and wider bone marrow spaces compared to the control group at both time periods, which was consistent with the study presented by Gao et al.[33] who stated that BIS suppressed preosteoclast by releasing platelet-derived growth factor-BB (PDGF-BB), which harmed angiogenesis and osteogenesis. According to Alidadi et al.[34] even though confirmation some studies that the efficient application of BIS, there was the possibility of delayed or impaired bone healing. Barton et al.[35] also reported that non-union or mal union after BIS administration resulted from an inhibition of bone resorption by BIS.

Moreover, Lechner et al.[36] reported that BP administration like zoledronate in the long-term was associated with some side effects such as osteonecrosis of the jaw and atypical femoral fracture. However, Kwak et al.[37] reported that local application of bisphosphonate promoted new bone formation by inhibiting osteoclast formation, thus inhibiting bone resorption.

In the CGF-treated group, histological slides showed the formation of new bone with thicker, well-arranged bone trabeculae almost filling the defect containing newly formed osteons and in between narrow areas of immature woven bone and narrow bone marrow spaces compared to control and BIS groups of the same time periods which was consistent with the research presented by Kim et al.[38] who stated that when CGF was applied, it had a better result than empty defects. Also, the study conducted by Borsani et al.[39] showed that CGF significantly increased osteoblast proliferation and differentiation, protecting it against the harmful effect of BIS.

However, these results were not consistent with the findings of Wang et al.[40] who reported that no sufficient amount of formation of new bone tissue was observed in the bone defect area that was filled by CGF alone, and there was no significant difference between CGF and the control group which was attributed to the possibility that the differentiation and proliferation of osteoblasts needed complex microenvironment that regulated by different types of growth factors and pathways and although the CGF action takes longer than that of other blood extracts, it was still relatively short in the long process of osteogenesis.

In BIS + CGF-treated group showed the formation of new, well-arranged bone, filling the defect with narrower bone marrow spaces and containing larger osteons with osteocytes arranged circumferentially around the Haversian canal. The quality and quantity of the newly formed bone trabeculae were found to be the best of all groups in both subgroups. However, to our knowledge, no other previous studies have evaluated the effect of combining CGF with BIS on bone defect healing. Hence, the results of this study could not be correlated with any previous research.

Nevertheless, a previous study conducted by Lei et al.[41] reported no significant difference in growth factors level between CGF and PRF, even though CGF has more abundant growth factors because of its special centrifugation process. Masuki et al.[42] also reported that both A-PRF and CGF extracts contained similar amounts of growth factors capable of stimulating periosteal cell proliferation, suggesting that A-PRF and CGF preparations both functions similarly as a reservoir to deliver certain growth factors at the site of application.

So, the results of this study were found to be comparable to the findings of Li et al.[43] who confirmed the synergistic effect of applying PRF with BIS in bone regeneration. However, their study also raised the concerns that adding PRF with bisphosphonate might have potential risks of induced osteonecrosis of the jaw and impairment of new bone formation because of added BIS as Abdik et al.[44] reported.

Masson trichrome staining results showed fewer collagen fibers and more mineralization in group I compared to group II which might be explained by the fact stated by Alidadi et al.[34] who reported that BIS delayed healing in bone defects, so the formed bone was more immature compared with that of control group marked by an increase in blue coloration reflecting collagen content and decrease in red coloration reflecting mineralization. Also, Koyama et al.[45] reported that the presence of BIS significantly decreased mineralization than in the control group. Chavarry et al.[46]

also reported that BIS-treated group showed increased collagen accumulation in a rabbit dental extraction study.

In this study, group III showed a greater amount of bone with more collagen and more mineralization than each group I and II that agreed with Mijiritsky et al.[47] and Masuki et al.[42] who supported the beneficial effect of using CGF in bone regeneration. Fang et al.[48] reported that the CGF was rich in growth factors, including transforming growth factor beta 1 (TGF-β1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor I (IGF-I). Also, TGF-β1 stimulated the chemotaxis and mitosis of osteoblasts and guided the mass synthesis of type I collagen fiber and fibronectin, which could explain the increased amount of collagen fibers and enhanced mineralization.

In this study, group IV showed the least amount of collagen and the highest degree of mineralization of all other groups, which was consistent with H&E staining results that showed that the quality of newly formed bone was better than any other group proved by this increase in mineralization and decrease in collagen content. This was comparable to the results of the studies by Kanoriya et al.[49] and Wanikar et al.,[50] who reported that PRF + BIS treated defects showed better clinical and radiographic outcomes as radiographic examination showed a higher degree of bone defect fill when compared to PRF alone. On the other hand, in the study conducted by Tiwari et al.,[51] they stated that there was no significant difference between using BIS with PRF and using PRF alone.

This study was conducted on rabbits which have some differences from humans in age, diet, anatomical and physiologic conditions. These limitations should be considered and need further investigation before being conducted on humans. We recommend studying the effect of material with different concentrations to choose the best dose that can be applied.

## Conclusion

With the limitation of this study, it could be concluded that using a combination of BPH and CGF had both osteoconductive and osteoinductive effects inducing the best healing and osteogenic results, followed by using CGF, then control, and finally using BPH. Using BPH and CGF could be a promising strategy in the bone healing process.

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