Epidermal Growth Factor or Platelet Rich Plasma Combined With Induced Membrane Technique in the Treatment of Segmental Femur Defects: An Experimental Study

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Research article

Keywords: Epidermal Growth Factor (EGF), Platelet Rich Plasma (PRP), Masquelet technique, induced membrane, experimental study, femur

Posted Date: October 7th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-84460/v1

Version of Record: A version of this preprint was published on December 11th, 2020. See the published version at https://doi.org/10.1186/s13018-020-02142-2.

Abstract

Objective: Extensive bone defects remain a therapeutic challenge necessitating alternative surgical approaches with better outcomes. Can increase the effectiveness of PRP or EGF treatment in surgical treatment of large bone defects with masquelet technique?

Aim of this study examined potential therapeutic benefits of the Masquelet technique with induced membranes in combination with platelet rich plasma (PRP) or epidermal growth factor (EGF) in a rat model of segmental femur defect.

Methods: Three groups each consisting of 20 Sprague-Dawley rats were defined as follows: EGF group, PRP group, and control group. A femoral bone defect was created and filled with antibiotic embedded polymethyl metacrylate. Half of the animals in each group were sacrificed at week 6 and the pseudo-membranes formed were analyzed. In the remaining half, the cement was removed and the space was filled with autograft. After another six weeks, the structures formed were examined radiologically, histologically and biochemically.

Results: At week 6, both PRP and EGF groups had significantly higher membrane CD31, TGF and VEGF levels than controls. At week 12, when compared to controls, PRP and EGF groups had significantly higher membrane CD31 levels and PRP group had significantly higher membrane TGF levels. Regarding bone tissue levels, PRP and EGF groups had significantly higher VEGF levels and EGF group had significantly higher BMP levels. In addition, PRP and EGF groups had higher radiological scores than controls. However, the two experimental groups did not differ with respect to any parameter tested in this study.

Conclusion: Both PRP and EGF seems to be associated with histological, biochemical, and radiological improvements in experimental rat model of Masquelet technique, warranting in further clinical studies.

Introduction

Extensive bone defects due to traumatic injury remain a therapeutic challenge in terms of anatomical and functional outcomes. The traditional approach for restoration of bone defects requires extensive surgical intervention involving the use of bone grafting that is associated with donor-site complications and frequent occurrence of morbidity, with no guarantee of complete correction of the defect.[1] Thus, the search for alternative surgical approaches continues. Recently, Masquelet technique has been described as a two-stage treatment strategy for large bone defects that consists of a temporary cement spacer followed by bone grafting.[2] The technique allows reconstruction of extensive diaphysis defects even in the presence of previous radiation exposure or infections.

Platelet rich plasma (PRP) is a volume of fractionated plasma from patient's own blood containing platelet concentrate.[3] PRP contains several growth factors that play a major role in tissue repair mechanisms including but not limited to platelet derived growth factor, transforming growth factor-beta,

and vascular endothelial growth factor. As a result of its potential therapeutic effects, PRP has recently gained significant attention as a safe nonsurgical adjunctive treatment modality for osteoarthritis and musculoskeletal repair.[4] Despite the lack of definitive evidence for the therapeutic benefit of PRP in bone healing[5, 6], a multitude of recent experimental and clinical publications have suggested a potential utility.[7–13]

As compared to PRP however, published data on the use of epidermal growth factor (EGF) in orthopedic surgery is very scarce and most information relates to its potential therapeutic benefits in conditions other than bone healing such as wound healing, diabetic foot ulcers, or experimental dentistry.[14–18]

This study was undertaken as an initial experimental attempt to assess the feasibility and potential therapeutic benefits of the Masquelet technique with induced membranes in combination with PRP and EGF in a rat model of segmental femur defect. The assessments included comparison of tissue bone morphogenetic protein 2 (BPM-2), transforming growth factor-beta (TGF-beta), and vascular endothelial growth factor (VEGF) levels as markers of bone healing, osteoinduction, and angiogenesis; as well as histopathological and radiological examinations in experimental study groups.

Materials And Methods

Design and experimental animals

A total of 60 male Sprague-Dawley rats weighing between 400 and 450 g were used for the experimental protocol. Three groups each consisting of 20 rats were defined as follows: EGF group, PRP group, and control group. Standard housing and feeding conditions were provided for the animals. The study protocol was approved by the Local Ethics Committee for Animal Experimentation, Kahramanmaras Sutcu Imam University with an approval date and no of 2014/01/05 and 17 April 2014, respectively.

A 5-mm bone defect was created and filled with antibiotic embedded polymethyl metacrylate followed by the fixation of the femur using external fixators. Figure 1 shown the defect model. Half of the animals in each group were sacrificed at week 6 and the pseudo-membranes formed were prepared for study analyses. In the remaining half of the groups, the cement was removed and the resultant space was filled with autograft obtained from the tail of each animal. After six weeks, the bony structures formed were examined radiologically, in addition to histological and biochemical assessments.

Procedures (first step)

Prior to surgery, each animal received a single 0.1 mg/kg intramuscular dose of cefazolin sodium (Cefozin, Bilim Ilac, Turkey) prophylactically. For surgery, anesthesia was provided with intramuscular ketamine HCl 200 mg/kg (Ketalar®Eczacibasi, Turkey) and xylazine 1 mg/kg (RompunR, Bayer, Turkey). Right hind legs were shaved and covered with sterile covers after disinfection with Betadine (polyvidone iodine solution). The skin and subcutaneous tissues were dissected in accordance with surgical

principles to expose the femur of rats. A 5-mm bony defect was created using a 1-mm drill tip and osteotome, then it was filled with antibiotic embedded polymethyl metacrylate (Cemex Genta ID Green TECRES® Italy). Femur was fixated using external fixators, and the skin was sutured using 4/ Ethilon nylon monofilament suture material.

Rats in the epidermal growth factor group received weekly local administration of epidermal growth factor at a dose of 25 µg/ml for three weeks (HEBERPROT-P® 75 µg, Hasbiotech Ilac San. ve Tic. A.S. Turkey). The blood obtained from the rats in the PRP group was used to prepare platelet rich plasma using a MAGELLAN® Autologous Platelet Separator System (Arteriocyte Medical Systems USA) that was administered with weekly volumes of 1 ml. Controls received weekly injections of 1 ml of physiological saline around the cement. Half of the rats were sacrificed at the end of 6 weeks using high dose pentobarbital anesthesia and the pseudo-membranes forming around the space filled with cement were extracted. Each membrane sample was divided into two halves, one being fixed in formaldehyde for histopathological assessments, and the other kept at -40 C for biochemical analyses. Three animals in the EGF and one in control group died and were therefore excluded from the analyses.

Procedures (second step)

In the remaining rats in study groups, the same procedures as described in step one were used to expose the femurs. The pseudo-membranes formed were carefully and longitudinally opened and autogenic bone grafts retrieved from the tail of each animal were placed into the resulting space. The operation site was sutured.

Six weeks after these procedures, antero-posterior and lateral x-rays and CT images were obtained for radiological assessments. The rats were sacrificed with high dose pentobarbital anesthesia, and the pseudo-membranes around the defect into which grafts were placed and bone tissue were removed and cut into two equal pieces, one being fixed in formaldehyde for histopathological assessments, and the other kept at -40 C for biochemical analyses. At this stage, 3, 4, and 2 animals died in PRP, EGF, and control groups, respectively, and were therefore excluded from the analyses.

Biochemical assessments

Rat tissue samples were placed into ice as soon as they were removed, blotted, and weighed. Homogenization for 15 to 20 minutes was performed in a homogenizer with ice boxes containing 1 g of tissue per 5 volume of cold 1.15% KCl (weight/volume). Then, the homogenate was centrifuged for 30 minutes at 14000 rpm at + 4 C, and the biochemical analyses were done on the supernatant. ELISA double-sandwich methodology was used for BMP-2, TGF-beta, and VEGF assessments.

Histopathological assessments

The samples were fixed for 24 hours in 10% buffered formaldehyde and then were embedded. After obtaining appropriate cross-sections from paraffin blocks, they were stained with hematoxylin and eosin (HE) and were examined under light microscope (Olympus-BX53). In order to perform a histochemical assessment of the neovascularization of the pseudo-membrane, granulation tissue was identified in HE

stained cross sections. Based on its high specificity and sensitivity as an endothelial marker, CD31 (platelet endothelial cell adhesion molecule - PECAM1) was chosen as a marker of endothelial proliferation and neovascularization, and was applied immunohistochemically to the areas of granulation identified with HE staining. Cross-sections of 3-micron thickness were stained with CD31 using a “LEICA BENCHMARK XT” immunohistochemistry device and were assessed using light microscope with quantification of vascular structures (capillaries as well as immature vascular structures) in each 1 mm^2 at 40x magnification power. (Fig. 2)

Radiological assessments

In each rat, antero-posterior and lateral x-rays as well as computed tomography images (Toshiba Alexon 16 multi-slice) were obtained (Fig. 3). A radiologist blinded to the study procedures used a CT version of Lane-Sandhu scoring system for image scoring as follows: 0, no callus, clear fracture line; 1, 25% callus tissue, the fracture line can be clearly observed; 2, 50% callus tissue, blurred fracture line; 3, 75% callus tissue, fracture line barely visible; 4, 100% callus tissue, fracture line cannot be seen.[19]

Statistical analyses

Statistical Package for Social Sciences (SPSS) version 21 was used for the analysis of data. Normality was tested using Shaphiro-Wilk test and graphical methods. Kruskal-Wallis test or Analysis of Variance (ANOVA) was used to test intergroup differences; and built-in post hoc test for Kruskal-Wallis or Tukey HSD was used for pairwise comparisons, respectively. A p value smaller than 0.05 was considered the indication for statistical significance.

Results

Table 1 shows the comparison of the groups with respect to study assessments, at week 6 and week 12.

Table 1
Comparison of the groups with regard to histological, biochemical and radiological study assessments

	Group 1 PRP	Group 2 EGF	Group 3 Controls	p
Assessments at week 6 (mean ± SD)				
Histological CD31 assessment	143.7 ± 8.2	151.1 ± 9.2	125.2 ± 13.3	< 0.001
Membrane TGF level	246.4 ± 54.3	257.7 ± 55.2	141.8 ± 20.8	< 0.001
Membrane VEGF level	842.0 ± 98.5	754.8 ± 118.7	430.5 ± 92.2	< 0.001
Assessments at week 12 (mean ± SD)				
Histological CD31 assessment	137.3 ± 17.3	138.1 ± 14.0	99.6 ± 25.5	0.003
Membrane TGF level	397.4 ± 44.4	379.3 ± 63.3	326.4 ± 42.0	0.032
Membrane VEGF level	318.0 ± 95.2	273.0 ± 72.1	228.9 ± 44.9	0.086
Bone VEGF level	192.2 ± 6.7	198.1 ± 5.7	176.3 ± 7.2	0.003
Bone BMP-2 level	191.9 ± 8.4	237.6 ± 37.3	117.1 ± 43.0	0.012
Lane score (median, range)				
AP	4 (3–4)	3 (2–4)	1 (1–2)	0.015
Lateral	4 (2–4)	3.5 (2–4)	1 (1–2)	0.026
BT	3 (2–4)	3 (2–3)	1 (1–2)	0.026
3D	3.5 (2–4)	4 (3–4)	1.5 (1–3)	0.044

Membrane tissue assessments at week 6

At week 6, PRP group had significantly higher membrane CD31 ($p = 0.002$), TGF ($p < 0.001$) and VEGF ($p < 0.001$) levels than controls. Similarly, EGF group had significantly higher membrane CD31, TGF and VEGF levels than controls ($p < 0.001$ for all three comparisons). However, the two groups did not differ with regard to any of these three parameters.

Biochemical, histological and radiological assessments at week 12

At week 12, PRP ($p < 0.014$) and EGF ($p < 0.008$) groups had significantly higher membrane CD31 levels than controls, with no significant difference between the former two groups. PRP group had significantly higher membrane TGF levels than controls ($p = 0.032$); however, no other pairwise difference reached significance.

Regarding bone tissue levels, PRP ($p < 0.019$) and EGF ($p < 0.003$) groups had significantly higher VEGF levels than controls, without any significant difference between the former two groups. On the other hand, EGF group had significantly higher bone BMP levels than controls ($p = 0.010$), with no other significant pairwise differences.

Lane scores at week 12

PRP group had significantly higher AP ($p = 0.013$), lateral ($p = 0.040$) and CT scores ($p = 0.040$) than controls, whereas no other pairwise difference was evident regarding these parameters. On the other hand, EGF group had significantly higher 3D scores than controls ($p = 0.047$), representing the only significant pairwise difference for this parameter.

Discussion

This study examining the effect of two therapeutic approaches, i.e. PRP and EGF, on bone healing when used as an adjunct to Masquelet technique for the management extensive bone defects has provided promising results in terms of a potential therapeutic benefit. Accordingly, both PRP and EGF were associated with improved biochemical and histological outcomes, both at 6- and 12-week assessment time-points, as reflected by higher membrane/bone tissue CD31, TGF, and VEGF levels in PRP and EGF groups. In addition, these adjunctive treatments to the Masquelet technique resulted in radiological improvements in AP lateral x-ray and CT scores (for PRP) and 3D scores (EGF). Of these two approaches, PRP has previously been subject to extensive research regarding its therapeutic utility in orthopedic procedures, while literature data on EGF is rather scarce. Furthermore, to the best of our knowledge, no previous studies have examined these two agents in combination with the Masquelet technique in such an experimental setting.

PRP has been generally reviewed as a “a promising therapeutic approach for future regenerative treatments” [20] and a great majority of the preclinical studies on the treatment of bone defects support the use of PRP.[5] Specifically, positive results have been reported in rabbit ulnar defects [21], rat femoral fractures [11], as well as in rabbit tibia shaft fractures.[22] In humans, clinical reports on healing rate of long bone non-union fractures have also indicated positive outcomes in terms of cure rate, healing duration, and limb shortening.[8, 9] Despite these initial promising results, it has been underscored that the evidence to support the routine use of this intervention in clinical practice is currently insufficient.[10]

In contrast with PRP, research on the possible therapeutic effects of epidermal growth factor (EGF) in tissue healing is mainly limited to the field of experimental dentistry[15, 17], chronic diabetic foot ulcers [14] or other types of soft tissue pathologies including chronic radiation ulcers.[18] On the other hand, EGFR receptors are known to play a role in endochondral bone formation [23], justifying research assessing its potential effects in bone healing. For instance, loss of epidermal growth factor receptor (EGFR) activity was found to alter the development of the growth plate, impair endochondrial ossification, and retard the growth in a mice model.[23] Furthermore, administration of EGF in liposomes resulted in a faster recovery

of injured alveoli of rats after the extraction of the maxillary second molar teeth, providing protective effects against early absorption and degradation.[17] In a case report exploring the potential benefits of recombinant epidermal growth factor in a patient with radiation induced chronic wound, successful healing within 16 weeks was reported after the failure of flap surgery and conventional treatments given over a 3 year period.[18] Similar enhancement of wound healing leading to thicker epidermal and dermal layers was also reported with the use of decellularized scaffolds loaded with epidermal growth factor in an experimental model of wound healing.[16] Overall, our results are in concordance with the above-mentioned findings as reflected by improved biochemical, histopathological, as well as radiological outcomes in rats receiving additional EGF treatment as a part of Masquelet technique for segmental bone defects.

Production of growth factors (VEGF, TGF beta 1) as well as osteoinductive factors (BMP-2) have been previously documented in the induced membranes in a rabbit model of Masquelet technique.[24] Furthermore, platelets are known to contain alpha-granules that are rich in a number of growth factors including platelet-derived growth factor, transforming growth factor-beta, insulin-like growth factor, vascular endothelial growth factor and epidermal growth factor, which are of major significance in tissue repair mechanisms.[20]

Thus, our results corroborate previous studies and provide further evidence for the first time that PRP and EGF have the potential to be used as an adjunct for accelerated healing in Masquelet technique for the management extensive bone defects. However, the small sample size is certainly a limitation of our study. Further studies may confirm our findings for each of these agents and may possibly explore the utility of the combined use of these two agents for potential synergistic therapeutic effects.

Conclusions

Masquelet technique is an effective surgical method that can be applied easily in the restoration of large bone defects. In Masqulet technique, psudomembrane formation is the main factor in the restoration of bone defect. The addition of EGF or PRP to the masqulet technique has been shown to increase vascularization in the pseudomembrane.

Histopathological and biochemical healing displayed by the addition of EGF or PRP to the Masquelet technique in the restoration of large bone defects also provided a radiologically significant improvement.

Both PRP and EGF were associated with improved histological, biochemical, and radiological improvements in this experimental rat model of Masquelet technique. These promising observations may be confirmed in further clinical studies involving humans.

Declarations

Ethics approval and consent to participate: The study protocol was approved by the Local Ethics Committee for Animal Experimentation, Kahramanmaras Sutcu Imam University with an approval date and no of 2014/01/05 and 17 April 2014, respectively.

Consent for publication: All authors have agreed to publish this content in your journal.

Availability of data and materials: The data and materials of patients participating in this study are available to us and will be provided by us upon request.

Competing interests: The authors declare that there is no conflict of interest.

Funding: This study was supported by the research projects management department of Kahramanmaraş Sütçü Imam University.

Author Contribution Statement: Ökkeş BİLAL, Duran TOPAK and Mustafa KINAŞ constructed the idea or hypothesis for research; Ökkeş BİLAL and Duran TOPAK took the responsibility in drafting of the whole the manuscript; Ökkeş BİLAL and Betül KIZILDAĞ reviewed the article before submission not only for spelling and grammar but also for its intellectual content; Ergül Belge KURUTAŞ and Abdulkadir Yasir BAHAR tooks responsibility in drafting of the project and ethic paper; Ökkeş BİLAL ,Duran TOPAK and Betül KIZILDAĞ tooks the responsibility in the execution of the experiments, data management, and reporting. All authors reviewed and approved the

Animal and human rights statement: All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

None of the authors received any type of financial support that could be considered

potential conflict of interest regarding the manuscript or its submission.

Acknowledgement: Not applicable.

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Figures

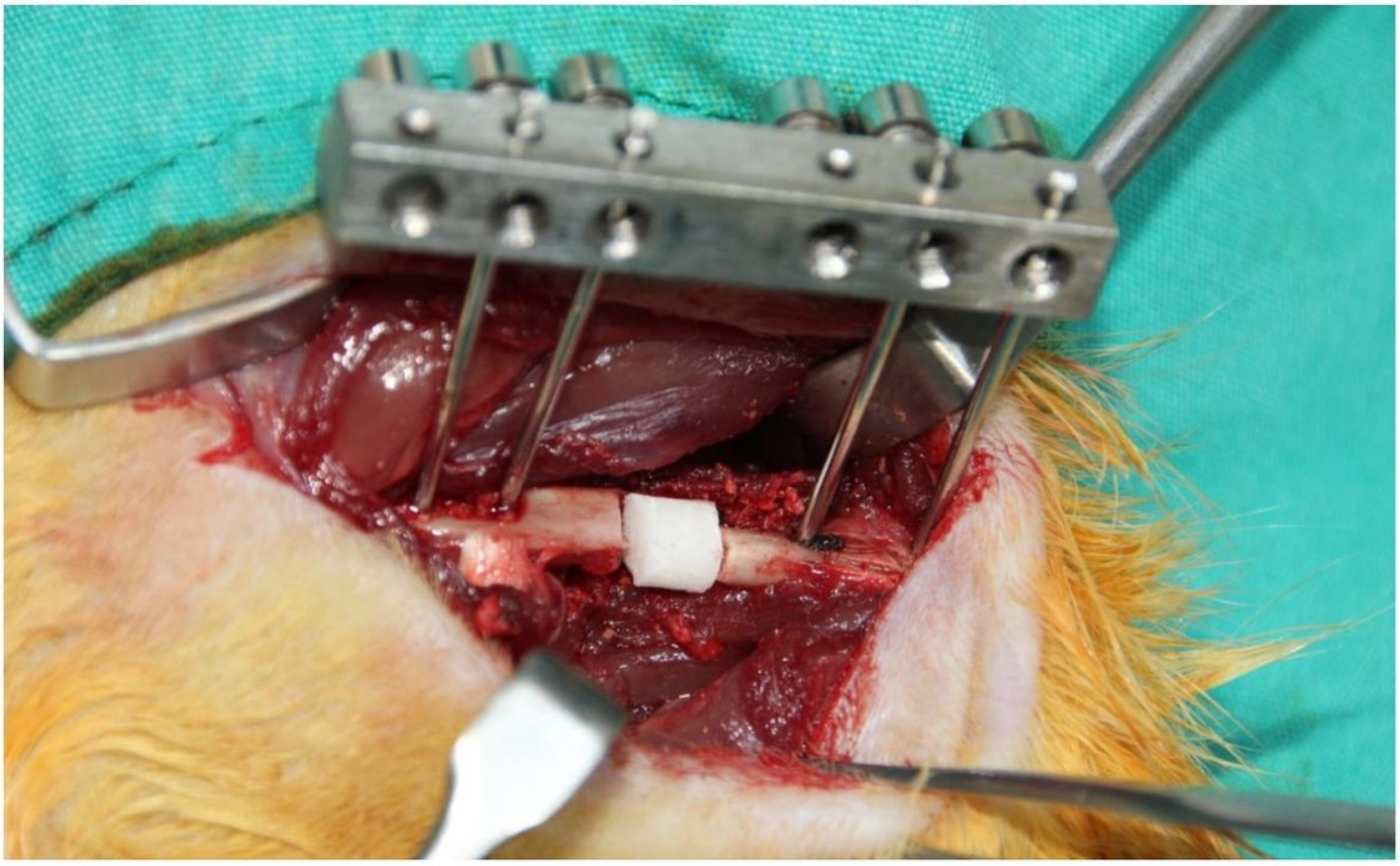

Figure 1

Segment creation and spacer application with antibiotic-embedded cement in rat femur (blue arrow)

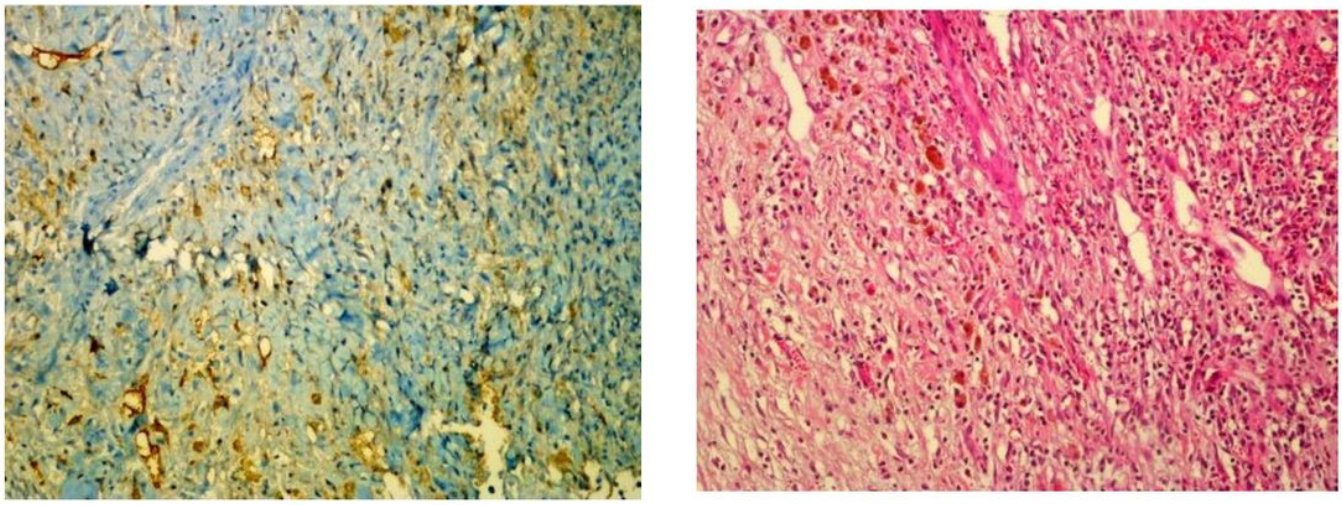

Figure 2

Pathological images of fracture healing in experimental cases a: Microscopic image of prepatlar stained with CD 31 at 200 magnification (brown areas indicate endothelial cells stained with CD 31). b: Microscopic image of prepatlar stained with hematoxylin eosin at 200 magnification (Areas of inflammatory granulation tissue was showen)

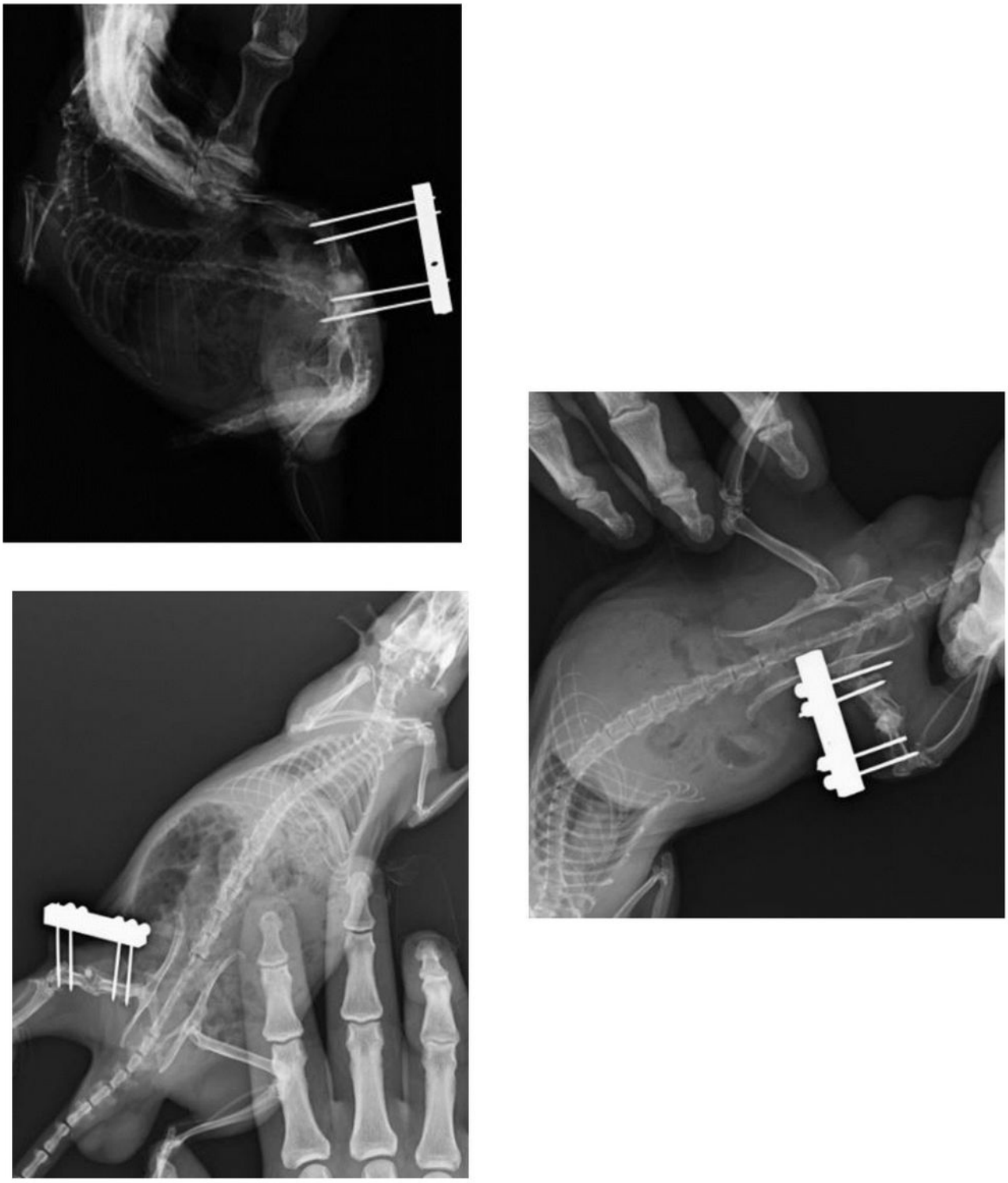

Figure 3

Radiological image of experimental cases a: Radiographic image of the case in the control group after 6 weeks b: Radiographic image of the case in the PRP group after 6 weeks c: Radiographic image of the case in the EGF group after 6 weeks