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RESEARCH ARTICLE

The effect of biocompatible carriers with vitamin D and platelet-rich plasma (PRP) on bone defects

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Abstract

Introduction: In oral and maxillofacial surgery, hard tissue augmentation is provided by materials that accelerate the healing, act as a template for reconstructing bony defects, and stimulate bone production and growth. This study investigated the effects of biocompatible carriers containing active vitamin D and platelet-rich plasma (PRP) on bone defects created in rat calvaria.

Material and Methods: This experimental animal study utilized fifty-two male Sprague Dawley rats divided into six groups and conducted histopathological, microtomographic, and biochemical comparisons by adding vitamin D, which has an accelerating effect on bone development. We examined the calvarial defects, sacrificing the rats in equal numbers (eight in each group and four to obtain PRP) at the first, second, fourth, and eighth weeks. The newly formed bone was assessed using histopathologic, microtomographic, and macroscopic methods as well as the biochemical analysis performed in the plasma samples. Expression of fibroblast growth factor 23 (FGF23), vitamin D receptor (VDR), and receptor activator of nuclear factor-kappa B (RANK), which determine bone formation, were investigated. The amounts of ossification, bone volume, and mineral densities were significantly higher in the fourth and eighth weeks as the biocompatible material was delivered with calcitriol.

Results: The histological and macroscopic examinations revealed that the defect area shrank and was colonized with new cells in the "calcitriol + chitosan + PRP" group. The expression of RANK, FGF23, and VDR antibodies was more intense in the "calcitriol + chitosan + PRP" group than in other groups of the experiment and controls.

Conclusion: Active vitamin D, PRP, and chitosan formulation positively contributed to the repair of bone defects and induced remarkable clinical improvement. This new delivery approach could be promising for healing bone defects following surgical operations in hard bony tissues.

Keywords: Calcitriol, calvaria, chitosan, platelet-rich plasma, PRP, experiment, animal, microtomography, Micro-CT

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Introduction

Calcium, phosphorus, fluoride, and vitamins A, C, and D are essential in bone development and metabolism. Vitamin D has a pivotal role in bone metabolism concerning matrix protein production and bone remodeling with osteoblasts and osteoclasts and affects the metabolism of parathormone/calcium[1]. Vitamin D, a steroid hormone, is the primary phosphate and calcium regulator and synergizes with parathyroid/calcitonin hormones. Active vitamin D [calcitriol; $1,25(OH)_2D_3$] increases the absorption rate of phosphorus and regulates bone resorption, affects the remodeling process by stimulating osteoblasts formation, and stimulates osteoclasts by increasing the level of osteoclast differentiation factor[2-4]. Bone defects in the oral and maxillofacial areas cause deterioration of the dental occlusion resulting in inadequate nutrition as well as functional and aesthetic problems, and atrophy of the related structures due to dysfunction[5-8]. Biomaterials are required to support bone formation in the early periods of bone repair, shorten the recovery period and treatment, correct dental occlusion, and rapidly restore function and aesthetics. For those reasons, bone defects should ideally heal quickly in oral and maxillofacial surgery. Animal studies have proven that calcitriol, chitosan, platelet-rich plasma (PRP), or their combinations are safe and reliable and do not cause any side effects such as toxicity, allergic reaction, or undesired effect[9-11]. PRP is used in musculoskeletal disorders, oral and maxillofacial, plastic, and car-

diovascular surgery. It increases the number of platelets and growth factors in the application area and contributes to coagulation, cell formation, angiogenesis, maturation, remodeling, and bone regeneration by activating stem cells and stimulating the repair mechanism[12-14]. Chitosan also has some advantages, such as the ability to resorb without creating any residue or toxic product, desired viscosity and consistency, easy application, and its antibacterial and adhesive properties[15-17].

Bone defects in the maxillofacial region cause occlusion deterioration, resulting in nutritional, functional, and aesthetic problems[5-8]. Biomaterials are required to support bone formation, shorten the repair, recovery, treat, correct the occlusion, replace function, and aesthetics[18,19]. We tested the local application of a mixture of calcitriol with PRP and chitosan as a vehicle to critical bone defects, where no force was applied, the defect area was kept stable with no movement, and the materials placed in the defect area had direct contact with the bone tissue. Our study was designed to investigate comparative histopathological, microtomographic, and biochemical aspects of the compensation of vitamin D deficiency, bone formation in the defect area, and its effects on the healing period after surgical procedures resulting in bone defects.

Methods

In this experimental animal study, we utilized fifty-two male Sprague Dawley rats (10-12 weeks of age and 250-350 g) supplied by the Department of Science of Experimental Animals of the Istanbul University Aziz Sancar Institute of Experimental Medicine. The experiments were performed in the Facility of Experimental Animals of the Faculty of Pharmacy of Istanbul University. We housed rats in polystyrene cages of up to four animals in rooms equipped with temperature control (22 ± 2 °C) and humidity (55 ± 5 %) with the standard 12h light /12h darkness period. Tap water and standard laboratory animal chow were provided *ad libitum*. We conducted all experiments according to the approved guidelines for animal experimental procedures by the Istanbul University Local Ethics Committee of Animal Experiments (IUHADYEK, approval No: 466879/2017).

According to the assumption that "type-1 error probability" (significance level) is 0.001 and the test's power is at least 95 % (maximum 20 % of Type-2 error), the number of subjects was calculated at 48 animals for the use of one-way ANOVA method for the comparison of six groups in the G power statistical program. We utilized four experimental animals for PRP preparations, which were selected randomly.

We used fifty-two animals in total, 48 divided into the six experimental groups (eight for each treatment group), and the remaining four experimental animals were used in the third, fourth, and sixth groups to obtain PRP. There were no inclusion and/or exclusion criteria for the experimental animals used in each group.

Initially, we prepared separate gels containing chitosan (5 %) and poloxamer (1 %), then chitosan-poloxamer 407 gels containing calcitriol and/or PRP. According to the pre-formulation studies, a 3:1 mixture of gels prepared with chitosan (5 %) and Poloxamer 407 (15 %) was considered suitable; thus, this ratio gel mixture was used. Chitosan (5 %, w/v) was prepared by dispersing one g of chitosan (chitosan medium molecular weight, Aldrich, Taufkirchen, Germany) with 20 ml of distilled water and gelled by adding 10 % acetic acid solution dropwise to these dispersions, resulting in a pH between 4-5.

In group one, no material was added. In group two, chitosan gel was applied. In group three, 0.050 g PRP was applied, and then gels containing calcitriol [EP/USP (C0044–B00127)] and calcitriol-PRP were prepared and administered. In group four, chitosan-poloxamer gels containing PRP were administered. Preparation of chitosan-pluronic gels containing PRP included 0.025 g of chitosan (5 %)-poloxamer 407 (15 %) gel mixture taken into the vial and added 0.025 g of PRP at a ratio of 1:1 and mixed with vortex for two minutes. In group five, chitosan-poloxamer gels containing calcitriol were administered. The formulation of gels prepared to administer to each subject included 0.05 g of chitosan (5 %)-poloxamer 407 (15 %) gel mixture weighed into Eppendorf tubes and addition to this mixture with a micropipette of calcitriol dissolved (150 µg) in 30 µl of ethanol, and the mixture was vortexed. In group six, chitosan-poloxamer gels containing PRP and calcitriol were administered. Preparation of gels containing calcitriol and PRP included 0.025 g of chitosan (5 %)-poloxamer 407 (15 %) gel mixture and 0.025 g of PRP prepared for administration to each subject, weighed into Eppendorf tubes and mixed by the vortex. Calcitriol dissolved (150 µg) in 30 µl of ethanol was added to this mixture and vortexed.

Before the surgical procedure, 2 mg/kg lidocaine (Jetokain®, ADEKA, Samsun, Turkey) was injected subcutaneously into the surgical area. The area was shaved and cleaned with polyvinylpyrrolidone. Ketamine 50 mg/kg (Ketalar® flacon, Zentiva Sağlık Ürünleri San. Ve Tic. A.Ş. Kırklareli, Turkey) and xylazine 10 mg/kg (Rompun®, Bayer, Leverkusen, Germany) were administered intraperitoneally (IP) to provide general anesthesia. If necessary, ketamine (10 mg/kg, IP)(Pfizer, New York, USA) was used to maintain anesthesia during the experiment. We made a sagittal incision in the midline of the cranium, allowing for the mobilization of a full-thickness flap. We created a five mm diameter and approximately 1-1.5 mm thickness bone defect on the right side of the midsagittal suture by creating a bone incision with a five mm outer diameter trephine steel bur (Meisinger® 229 RA L/040, Centennial, Colorado, USA), using copious continuous irrigation with isotonic saline solution to avoid any dura mater damage. We added the materials mentioned in detail in the group description above and filled the entire calvarial defect area. Following the completion of the surgical procedure, the wound surfaces were primarily closed with absorbable sutures (Doğsan®, Trabzon, Turkey). The wound surfaces were wiped with an antiseptic solution, and 50 mg/kg cefazolin (Pharma-

Vision, Istanbul, Turkey) was administered IP to animals to prevent postoperative infection. Likewise, 2 mg/kg Tramadol (Abdi İbrahim İlaç San. ve Tic. A.S, Istanbul, Turkey) was administered IP to rats to eliminate severe postoperative pain. Drug administration was initiated after surgery and given twice daily for three days. Animals were weighed before the surgery for bone defect creation and until the sacrification.

Blood samples were taken from the orbital vein under light anesthesia with isofluorane (Isofluran Volatil®Eczacibasi, Istanbul, Turkey) 3.5 %, which was prepared by mixing with air using a vaporizer (SN-487-0T; Shinano Co., Ltd., Tokyo, Japan), for 2 min before the surgery for bone defect creation and before the sacrification.

Scanning of the specimens fixed in the falcon tube was performed on the SkyScan 1174v2 microtomography device (SKyScan, Kontich, Belgium) using a 0.5 mm aluminum filter at 50 kVp voltage, 800 µA current, and 40 W power. The raw images were reconstructed with NRecon Ver. 1.6.10.2 (Micro Photonics Inc., Allentown, Pennsylvania, USA) program and horizontal sections were obtained in bitmap (BMP) format. Bone mineral density analyses were performed using calcium densities 0.25 g/mm^3 and 0.75 g/mm^3 CaHA calibration bar (phantom). Sections obtained after reconstruction were transferred to the CTAN Ver. 1.16.4.1+ (Comprehensive TEX Archive Network, Heidelberg, Germany). The region of interest was drawn semi-automatically.

Bone samples were decalcified in 5 % formic acid for two days. The tissues were fixed in 10 % buffered formaldehyde, passed through the increasing ethanol concentrations, and incubated in toluene (Sigma-Aldrich, St. Louis, USA) after dehydration. After three times incubation, the samples were placed for one hour in pure paraffin in an incubator (Heraeus, Hanau, Germany) at 56 °C and embedded in paraffin blocks. We took 2.5 µm thick sections from the paraffin blocks with a microtome and placed them on positively charged slides. Background staining was made with Mayer hematoxylin. Sections were histologically examined using a light microscope (Olympus®, BX40F4, Tokyo, Japan) and evaluated in terms of the quantity of staining and reaction intensity of the area using a 10-lens. The reaction density was directly evaluated by a single investigator and was scored as no reaction (-), weak (+), medium (++), and strong reaction (+++).

Data are presented as frequency and percentage (%) for qualitative and mean ± standard deviation for quantitative variables. We utilized one-way analysis of variance (ANOVA) to compare the means of more than two groups in normally distributed variables, and if a significant difference was found, the post-hoc Dunnet and Bonferroni tests were used for pairwise comparisons of subgroups. In experimental setups containing three or more cross-sections in dependent samples, the significance of the observed differences was checked by using the repeated measures ANOVA method. In cases where there was a significant difference, pairwise comparisons were also tested using the post-hoc Bonferroni or least significant difference (LSD tests to interpret the differences between the sections. The level of significance was set at p =0.001 in interpretations. The IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA) was used for all biostatistical analyses.

Results

The wound surfaces were followed macroscopically, and no inflammatory reaction, necrotic tissue formation, side effects, toxicity, or body reaction was observed.

Bone mineral density (BMD) in the eighth week was significant compared to the fourth week in the “calcitriol + chitosan + PRP” and “calcitriol + chitosan” groups ($p < 0.001$). BMD values of all groups were higher than the control group. In the eighth week, the BMD value of the “calcitriol + chitosan + PRP” group was higher than all groups ($p < 0.001$) (Figure 1).

The bone volume (BV) values of the “calcitriol + chitosan” group in the fourth and eighth weeks were higher than in the “calcitriol + chitosan + PRP” group ($p < 0.001$) (Figure 2).

The percentage of ossification in the fourth and eighth **weeks** was statistically significantly higher in the” calcitriol + chitosan” group than the “calcitriol + chitosan + PRP” group ($p < 0.001$). The percentage of ossification of the “chitosan + PRP” group was higher in the eighth week than the “PRP” and “chitosan” groups ($p < 0.001$) (Figure 3).

Hematoxylin-eosin staining shows ossification areas developed in the fourth and eighth weeks in the “PRP”, “calcitriol + chitosan”, and “calcitriol + chitosan + PRP” groups. No bone tissue development was observed in other groups (Figure 4).

Immunohistochemical evaluation

In receptor activator of nuclear factor-kappa B (RANK) reactivity, 75-90 % quantity, and vigorous intensity were detected in the first week, and a gradual decrease was observed in the eighth week with 10 % quantity and weak intensity. In the PRP group and the groups with PRP added, the quantity of the RANK reaction was 50 %, and moderate intensity was observed in the eighth week. In the “calcitriol-chitosan-PRP” group, 75 % quantity and vigorous intensity were seen in all weeks. Fibroblast growth factor 23 (FGF23) reactivity in the groups to which PRP was added, 90 % quantity, and vigorous intensity were observed in the first week. In the following weeks, there was a reaction of 50 % quantity and moderate intensity. When compared with the control group, there was a decrease in the quantity and intensity of the FGF23 reaction as the weeks progressed in the other groups. Compared to the control group, especially in the “PRP” and PRP added groups, 75-90 % quantity, and vigorous intensity were observed in vitamin D receptor (VDR) reactivity from the first week. A gradual decrease in both staining quantity and intensity was observed to-

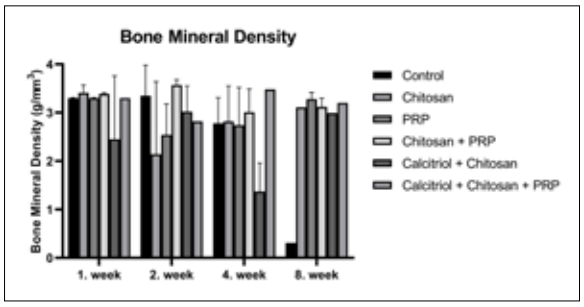


Figure 1: Effect of the biocompatible materials on bone mineral density (BMD). The values of the "calcitriol + chitosan + PRP" group in the fourth and eighth weeks were higher than the "calcitriol + chitosan" group ($p < 0.001$). All comparisons of BMDs in the first and second weeks are nonsignificant ($p > 0.001$). Although harder bone area was detected macroscopically in the PRP group than the control and chitosan groups, this difference was remarkable at eighth weeks ($p < 0.001$).
PRP: platelet-rich plasma.

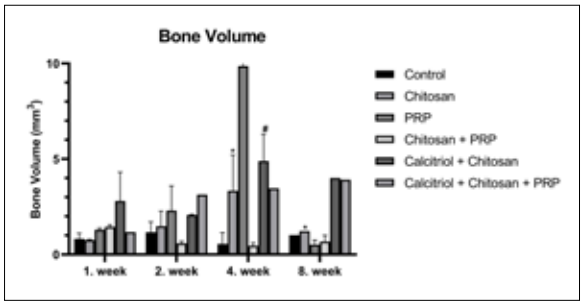


Figure 2: Effect of the biocompatible materials on bone volume (BV). The increase in BV from the first to the eighth weeks was statistically significant in the "calcitriol + chitosan + PRP" group ($p < 0.001$). The BV values of the "chitosan" group were higher in the fourth and eighth weeks than the control group ($p < 0.001$). The comparison in the first and second weeks in the control, "chitosan", "PRP", and "Chitosan + PRP" groups are statistically nonsignificant ($p < 0.001$).
PRP: platelet-rich plasma, *: "control" group vs "chitosan" group comparison, #: "chitosan" vs "calcitriol + chitosan" group comparison.

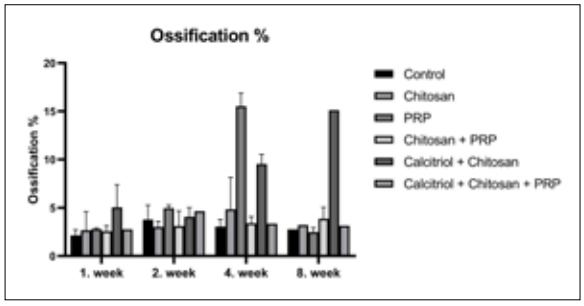


Figure 3: Effect of the biocompatible materials on the percentage of ossification. The percentage of ossification in the fourth and eighth weeks was significantly higher in the "calcitriol + chitosan" group compared to the "calcitriol + chitosan + PRP" group ($p < 0.001$). It is statistically significant that the ossification percentage of the "chitosan + PRP" group is higher than the "PRP" and "chitosan" groups in the eighth week ($p < 0.001$).
PRP: platelet-rich plasma.

wards the eighth week (Figure 5).

Discussion

Increasing people's lifespan may cause environmental exposure to chemical substances and prolonged exposure times, genetic transfers, genetically corrupted foods, viral-bacterial contaminations, bad habits, and living conditions that provoke the emergence of various diseases, such as cancer and disruption of metabolic activities. In almost every disease, a low vitamin D level can lead to delays and complications of wound and bone tissue healing after maxillofacial surgical procedures[20-22]. In the current study, the effects of vitamin D and various biomaterials and pharmaceutical mixtures on the healing of critical bone defects were examined on Sprague Dawley rats.

Experimental studies have the advantages of ensuring standardization, repeating experiments as necessary, being independent of time and place, with accessible follow-up periods, and examining the necessary analyses pathologically and histologically. The necessity of performing a biopsy in order to examine the effects of the methods applied in the experiments on the tissues, the requirement of sacrificing the tissues before obtaining them, the ability to collect samples such as blood and saliva throughout the experimental process, the fact that rodent models in tissue engineering are more beneficial due to their genetic similarity to the human organism, the metabolic effects of the expected results. The fact that it can be observed more quickly due to its speed and contributes to the creation of new methods for experiments constitutes the decision to conduct an experimental study. The information obtained after the experiments guides later applications on humans and reduces morbidity[23-24]. The current study examined the effects of locally adding calcitriol and biomaterials to heal bone defects. It has been shown in the literature that PRP and chitosan contribute positively to new bone formation both macroscopically and at the cellular level, as well as microtomographically[25-26]. In our groups with PRP, we observed more cellular area, intense and more staining in immunohistochemical staining than the control and chitosan groups. However, the BV, BMD, and percentage of ossification values encountered in the first and second weeks were not statistically significant. The values were higher in the fourth to the eighth weeks in the PRP-containing formulations than in the groups without PRP. Early elimination and release of PRP cause the inability to reach the t growth factors and insufficient activation for chemotaxis, which are apparent explanations[27-28].

When the "chitosan", "chitosan + PRP", and "PRP" groups were compared to each other with the chitosan variable, the ossification values at the first, second, and fourth weeks were higher in the groups with PRP. In the "PRP" group in the first, second, and fourth weeks, while in the eighth week, the ossification value was higher in the "chitosan + PRP" group. Nagata et al evaluated the histological effects of a mixture of autogenous bone

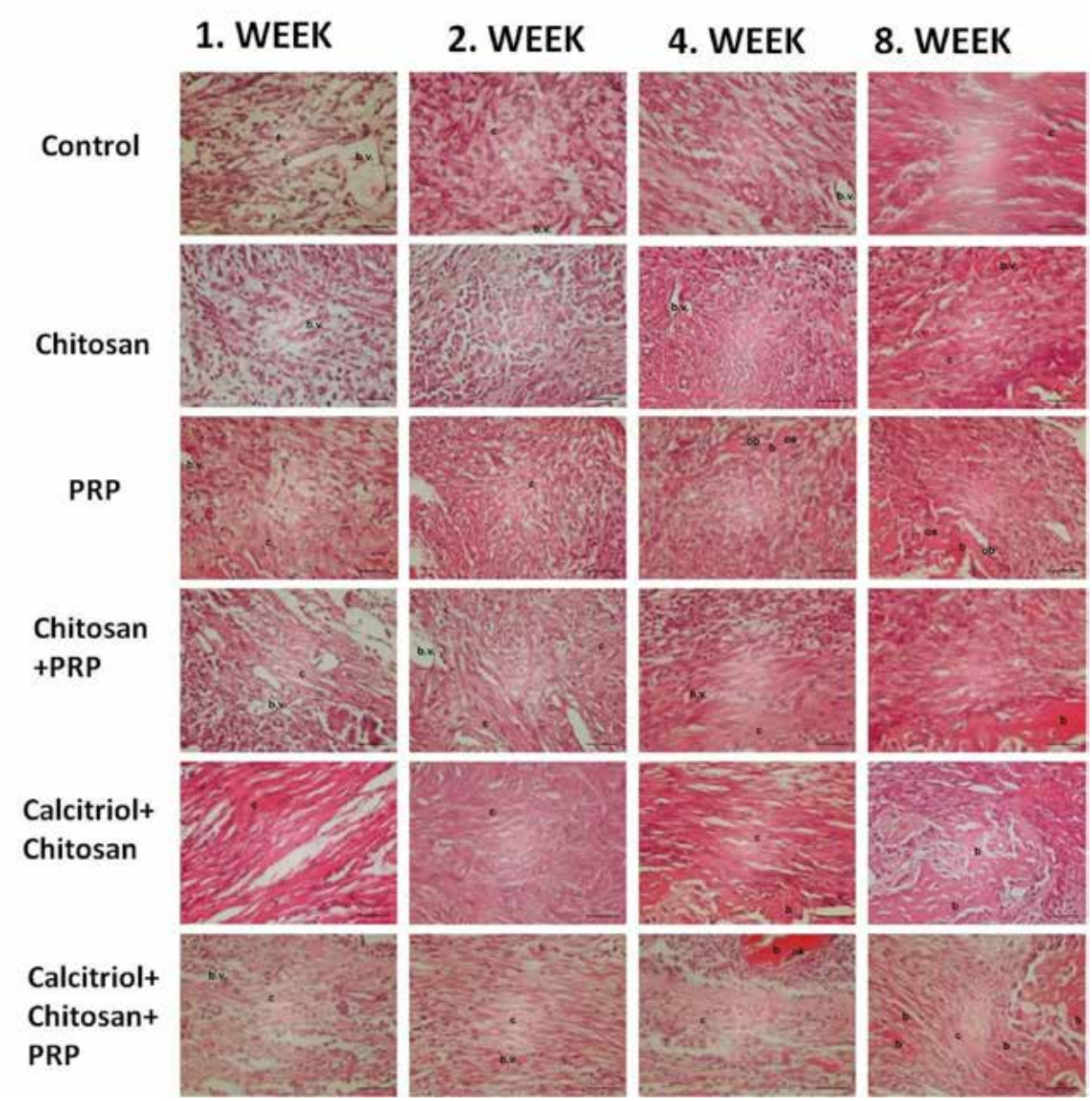
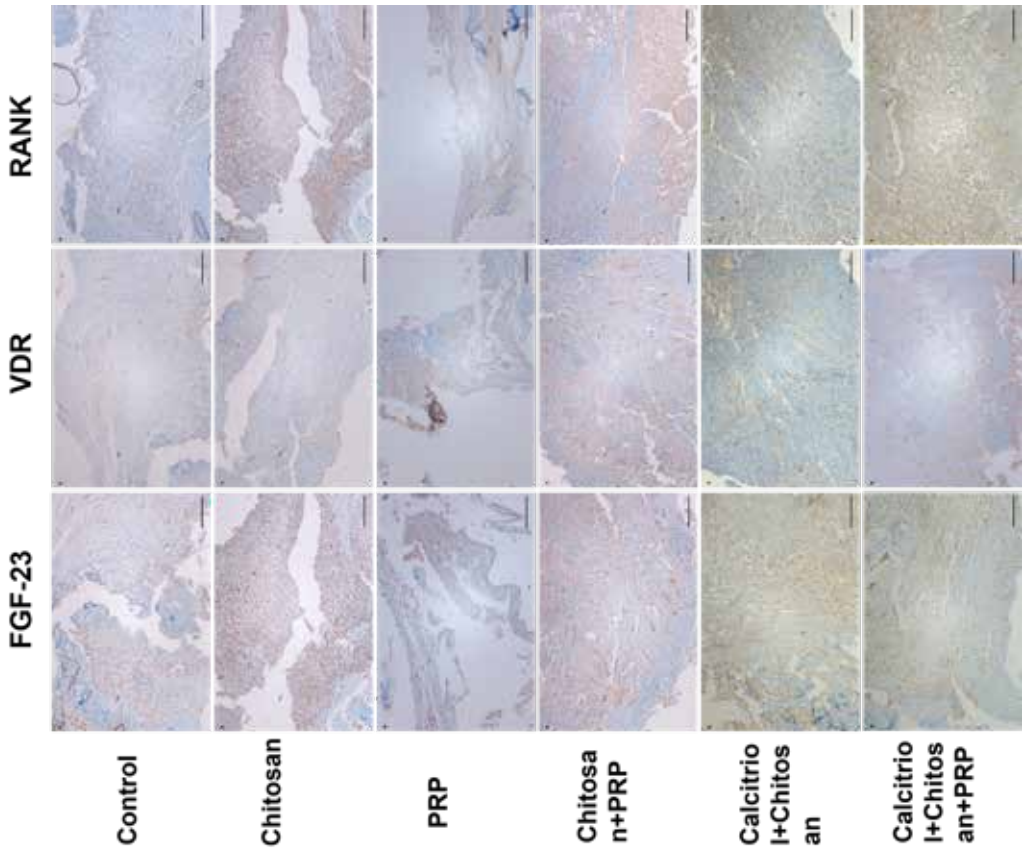
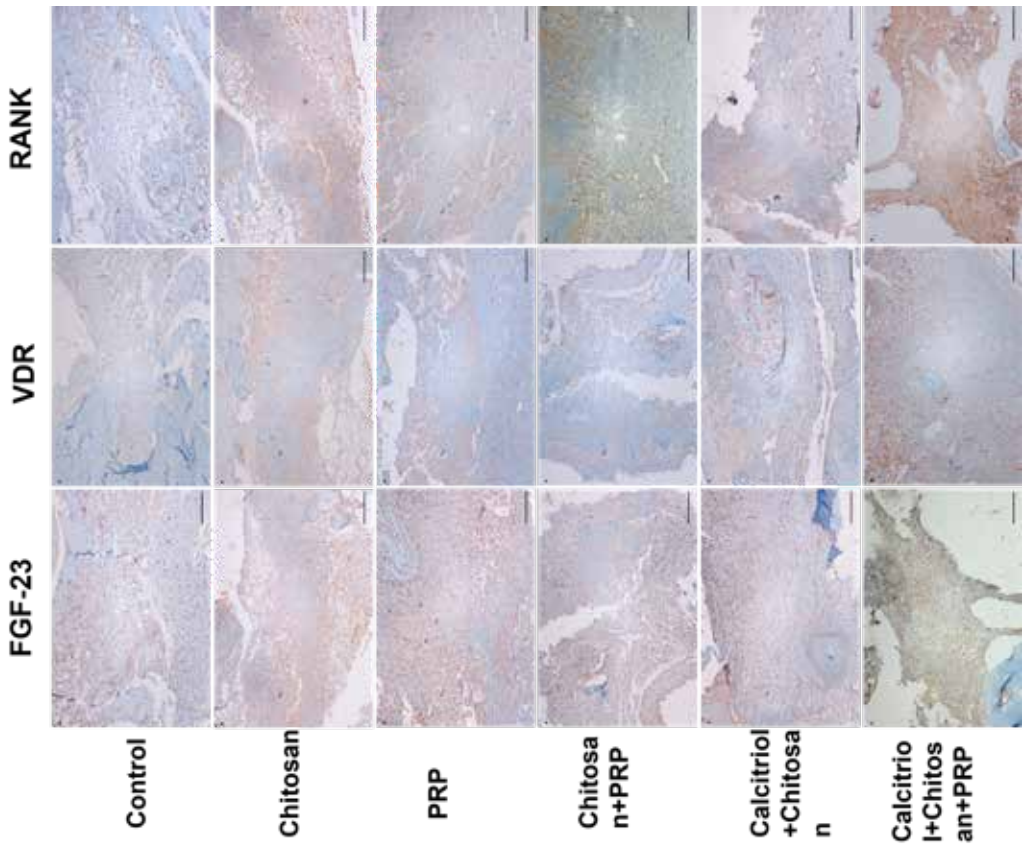


Figure 4: Histopathological evaluation of the biocompatible materials. Histology images (Hematoxylin-Eosin staining, x 40; scale bar: 50µm) showing ossification areas developed in the fourth and eighth week in the "PRP", "calcitriol + chitosan", and "calcitriol + chitosan + PRP" groups. No bone tissue was observed in the other groups.
PRP: platelet-rich plasma, c: collagen, bv.: blood vessel, b: bone, os: osteocyte, ob: osteoblast, ok: osteoclast.

grafts and PRP on five mm critical-size defects in the calvarium of rats euthanized at thirty days post-operative. They concluded that the defect was almost completely filled with the newly formed bone. PRP promotes chemotaxis, cell proliferation and attracts growth factors[29]. We believe it does not contain sufficient activation for target growth factors and chemotaxis processes due to its early elimination and early release. That is the reason for the lower bone volume and less ossification percentage in the "chitosan + PRP" group at first and eighth weeks period compared to the "chitosan" group and less bone volume in the "calcitriol + chitosan + PRP" group compared to the "calcitriol + chitosan" group at first, fourth, and eighth weeks period[30-34]. The formation of connective tissue with fibroblast cells, which started as the first step of ossification in the "chitosan + PRP" and the "PRP" groups, started with staining of equal intensity in the same area with the FGF23. In the subsequent, second, fourth, and eighth weeks, more intense staining was observed in the "chitosan + PRP" group, and a decrease in area and severity was observed towards the eighth week in both groups. The high staining and density of the detected RANK antibody can be explained by the return of the connective tissue function of FGF23 to bone for-


FGF-23
VDR
RANK
Control
Chitosan
PRP
Chitosan+PRP
Calcitriol+Chitosan
Calcitriol+Chitosan+PRP


FGF-23
VDR
RANK
Control
Chitosan
PRP
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Calcitriol+Chitosan
Calcitriol+Chitosan+PRP

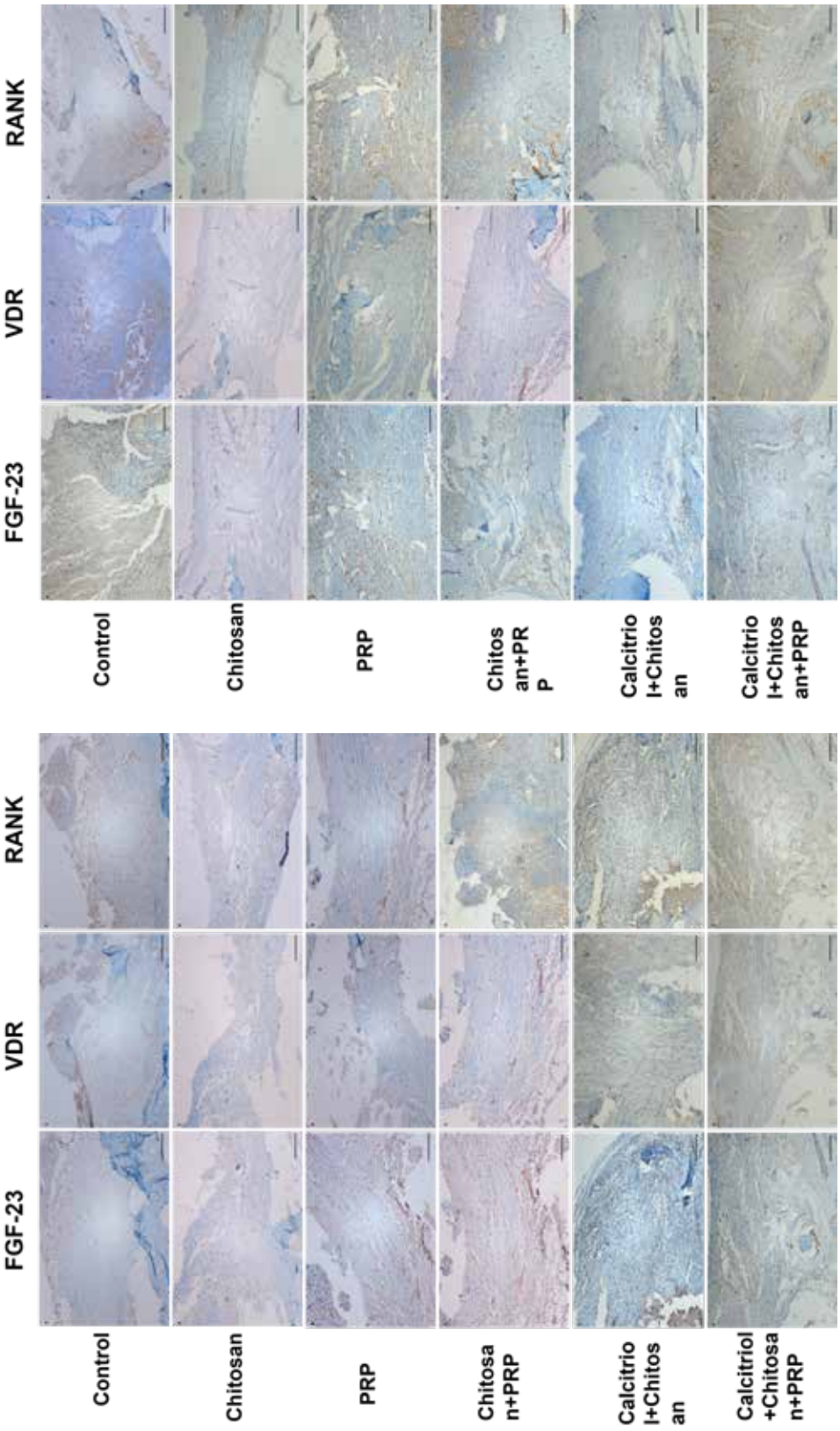


Figure 5: Immunohistochemical images (Immunohistochemical staining, x 40; scale bar: 50μm) of the calvarial bone tissue from first to eighth week. Immunohistochemical reactivity of fibroblast growth factor 23, vitamin D receptor, and receptor activator of nuclear factor-kappa B seen at the different [a) first, b) second, c) fourth, d) eighth] weeks of the experiment.
PRP: platelet-rich plasma, *: new ossification areas, ft: fibrous tissue kappa B., FGF23: fibroblast growth factor 23, VDR: vitamin D receptor, RANK: receptor activator of nuclear factor.

mation-destruction metabolism and promote early bone formation.

When examined immunohistochemically, more staining area and intensity are seen in the groups containing PRP, regarding VDR and FGF23 than in the groups without PRP, but macroscopically the smaller defect area, the observation of cells in histological evaluation and the more remarkable healing of the defect area are in favor of the "calcitriol + chitosan + PRP" group. Since connective tissue proliferation is observed in the first weeks and the presence of vitamin D receptors in the area, we assume that these connective tissue areas indicate new bone tissue that will form in the ensuing weeks.

The use of calcitriol with PRP accelerates wound and bone healing. Using calcitriol combined with PRP and chitosan has a healing effect on bone defects from the fourth week. The bone healing was the best in the "calcitriol + chitosan + PRP" group from the beginning of the fourth week. All methods employed in this study and macroscopically confirmed the increment in bone formation. Our clinical observations also confirm a study investigating the influence of calcitriol on osteoinduction following local administration into mandibular bone defects in rats[34]. Calcitriol showed strong potential in inhibiting osteoclastogenesis and promoting osteogenic differentiation at first and second weeks. In fourth and eight weeks, more mineralized bone and uniform collagen structure were formed.

Unlike the control group, in the group where the defect area was filled with PRP, bone volume values in the first, second, and fourth weeks and bone mineral density values in the eighth week were measured higher than the control group, and in the group where the defect area was filled with PRP, bone mineral density values at the first, fourth, and eighth weeks were measured higher than the control group; these were statistically significant.

We acknowledge that the results and effects of employing PRP obtained from a blood sample of the same organism may be more appropriate, and this effect can be augmented by adding various graft materials, especially autogenous grafts targeting bone formation, to this mixture. This condition can be envisioned as the limit of this study. Supporting bone formation in the early period reduces the healing duration in repairing maxillofacial defects and complements the loss of function and aesthetics as soon as possible.

Conflict of interest statement

The authors declare no competing interests.

Acknowledgments

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