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# OSTEOCALCIN AND OSTEONECTIN EXPRESSION AFTER DOUBLE-APPLICATION OF PLATELET-RICH PLASMA IN RABBITS

## *Tavşanlarda Çift Trombositten Zengin Plazma Uygulaması Sonrasında Osteokalsin ve Osteonektin Ekspresyonu*

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### *ABSTRACT*

***Purpose:*** *Platelet-rich plasma (PRP) is a novel method for transferring autogenous growth factors to the wound area. The aim of this study was to evaluate the efficacy of double-application of PRP (DA-PRP) on bone healing in rabbit cranial defects by examining osteonectin (ON) and osteocalcin (OC) expression.*
***Materials and Methods:*** *Twenty-eight rabbits, each with two surgically prepared calvarial bone defects, were included in this study and divided into six groups: The defects (N=56) were treated with either a single-application of PRP (SA-PRP) (n=10), a combination of SA-PRP and beta-tricalciumphosphate (SA-PRP+β-TCP) (n=10), only DA-PRP (n=8), both DA-PRP and beta-tricalciumphosphate (DA-PRP+β-TCP) (n=8), only beta-tricalciumphosphate (β-TCP) (n=10), or controls (n=10). The animals were sacrificed at 30th day postoperatively and samples were immunohistochemically examined for ON and OC expressions.*
***Results:*** *It was determined that DA-PRP did not significantly improve the ON and OC percentages achieved by SA-PRP or the controls. The three groups treated with β-TCP showed a higher percentage of ON than those treated without β-TCP ($p<0.05$). The β-TCP treated groups and SA-PRP group demonstrated higher OC percentage than DA-PRP and control groups ($p<0.05$).*
***Conclusion:*** *The present findings suggest that DA-PRP did not have a significant effect on the healing of non-critical size rabbit cranial bone defects.*

***Keywords:*** *Platelet-rich plasma; Osteonectin; Osteocalcin; Wound healing; Osteogenesis*

### *ÖZ*

***Amaç:*** *Trombositten-zengin plazma (TZP) otojen büyüme faktörlerini yara bölgesine taşıyan bir yöntemdir. Bu araştırmada çift TZP uygulamasının (Ç-TZP) tavşan kraniyal defektlerinde kemik iyileşmesi üzerine etkilerini osteonektin (ON) ve osteokalsin (OC) ekspresyonlarının değerlendirilerek incelenmesi amaçlandı.*
***Gereç ve Yöntem:*** *Her birinde cerrahi olarak oluşturulmuş iki adet kraniyal defekt olan yirmi sekiz tavşan çalışmaya dâhil edildi. Defektler (n=56) tek TZP uygulamasıyla (T-TZP)(n=10), tek TZP uygulaması ve beta-trikalsiyumfosfat kombinasyonuyla (T-TZP+β-TCP)(n=10), Ç-TZP ile (n=8) veya Ç-TZP ve β-TCP kombinasyonuyla (Ç-TZP+ β-TCP) (n=8) tedavi edilenler ve control grubu (n=10) olmak üzere altı gruba ayrıldı. Deney hayvanları 30. Günde sakrifiye edildiler ve örnekler immunohistokimyasal olarak ON ve OC için incelendi.*
***Bulgular:*** *Ç-TZP uygulamasının T-TZP ya da kontrol gruplarında elde edilen ON ve OC yüzdelerini anlamlı olarak arttırmadığı görüldü. β-TCP uygulanan üç grupta, β-TCP uygulanmayan gruplara göre daha yüksek ON yüzdeleri saptandı ($p<0.05$). β-TCP uygulanan gruplar ile T-TZP uygulanan grupta Ç-TZP ve kontrol grubuna göre daha yüksek OC yüzdeleri izlendi ($p<0.05$).*
***Sonuç:*** *Mevcut çalışmamızın sonuçları Ç-TZP'nin kritik boyutlu olmayan tavşan kraniyal defektlerinde iyileşme üzerine anlamlı etkileri olmadığını göstermiştir.*

***Anahtar kelimeler:*** *Trombositten zengin plazma; Osteokalsin; Osteonektin; Yara iyileşmesi; Osteogezis*

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**Introduction**

Previously, multiple growth factor combinations have been reported to be more effective on many cell types when compared to single growth factor applications. Autologous platelet-rich plasma (PRP) is a novel method for obtaining many autogenous growth factors and transferring them to the wound area. PRP application has been shown to increase local platelet concentration (1) and consequently, increases the local growth factor concentration (2) in the wound area. It has been suggested to have the potential to increase regeneration and accelerate wound healing, due to the various growth factors it consists, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), insulin-like growth factor (IGF), endothelial growth factor (EGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (1, 3-6). It is also known that autogenous PRP does not pose a risk of disease transmission or trigger immune reaction (5, 6).

Although there are numerous encouraging reports in favor of PRP (3, 7, 8), it still has many unknowns. It remains a question that how long PRP is effective. Regarding short life-span of platelets and platelet derived growth factors, PRP is believed to be effective predominantly at the early stages of the hard tissue healing (1, 9). Degradation of platelets and growth factor release were reported to occur at the first 3 to 5 days. Therefore, the growth factor activity is suggested to concentrate at the first 7 to 10 days (9). It was also suggested that direct effects of platelet derived growth factors start to disappear gradually after 5 to 6 days (1). It is an important question whether multiple applications of PRP may enhance the potential effects of PRP on wound healing. Nevertheless, there is no gold standard for the number of times it should be applied to the wound area. Most of the experimental (7, 8, 10) and clinical (1, 5, 6) studies which examine the effects of PRP on the healing of bony defects, have focused on the single-application of PRP to the wound area. Recently, promising results on soft tissue healing have been suggested by multiple-application of PRP (11-15). Crovetti *et al*. (11) reported that following topical treatment with multi-application of PRP, 9 out of 24 cutaneous chronic nonresponsive severe ulcers healed completely and eight healed by more than 50%. The authors reported that topical PRP hemo-therapy, an extension of hemocomponent use, has allowed them to increase and to improve the therapeutic approach to cutaneous wounds. Driver *et al*. (12) compared multi-application of PRP gel with a saline gel control as a topical dressing in the treatment of non-healing diabetic foot ulcers and reported that significantly more wounds were healed in the PRP gel treated group (81.3%) than in the control group (42.1%). Kon *et al*. (14) evaluated the efficacy of PRP in the treatment of articular cartilage degeneration of the knee after multi intra-articular PRP injections and preliminary results indicated that treatment with PRP injections was safe and had the potential to reduce pain, improve knee function and quality of life. Although numerous studies evaluated single application of PRP and/or graft-PRP combinations in the treatment of intrabony defects (1, 5-7, 10, 16), there is still lack of information on the efficacy of multi-application of PRP on osseous defects. Recently, we have demonstrated histomorphologically remarkable new bone formation in rabbit cranial bone defects treated with double-application PRP, with or without beta-tricalciumphosphate (β-TCP) (15). β-TCP is known for its interconnected system of micropores. Its calcium/phosphate (Ca/PO4) ratio is similar to that of natural bone and it resorbs approximately in 12 months in human intrabony defects. β-TCP has been widely used as a biologically safe osteoconductive alloplastic bone substitute (3, 17).

Osteonectin (ON) and osteocalcin (OC) are non-collagenous extracellular matrix proteins which are mostly synthesized by osteoblasts and secreted during the process of osteoblast differentiation and mineralization (10, 18). ON represents the most abundant non-collagenous protein in the mineralized bone matrix and is also referred to as the secreted protein acidic and rich in cysteine (SPARC) or basement membrane protein 40 (BM-40) (19). It has been suggested that ON binds selectively to both hydroxyapatite and collagen and links the bone mineral and collagen phases, probably initiating active mineralization in normal skeletal tissue (20). OC is mostly incorporated into the bone matrix where it is bound to hydroxyapatite and its serum concentration is a sensitive marker for bone formation which was shown to be correlated with histomorphometric indices of bone formation (18, 21, 22) . It indicates the mineralization process in bone formation performed by the calcification of the osteocytes in the collagen layer (10). OC contributes to the maintenance of recently formed new bone structures by supporting collagen fibril organization and by stabilizing hydroxyapatite molecules (23, 24). The purpose of this study was to

examine effects of double-application of PRP on the healing of intrabony defects. In order to evaluate the biology of bone formation after double-application of PRP, ON and OC expressions were examined during the bone healing in a rabbit cranial defect model.

**Materials and Methods**

This study protocol was approved by the ethical committee for animal experiments of Gazi University, Ankara, Turkey (G.Ü.ET-06.043). Twenty-eight healthy 6-month-old female New Zealand rabbits weighing between 3 kg and 4 kg were used in this study. Before the experiments, the general health of the rabbits was monitored for 10 days. The rabbits were kept in standard cages in an experimental animal room and were fed a standard laboratory chow and tap water.

*PRP Preparation*

A sterile disposable monovette system (Curasan, Pharma Gmbh AG, Lindigstrab, Germany) and compatible centrifuge machine (Heraeus Labofuge 300, Kendro Laboratory Products, D-37520 Osterrade, German) were used for preparations of PRP. Eight ml of peripheral blood was drawn from each animal by venipuncture and transferred into a red-marked monovette containing 0.5 ml citrate (10% trisodium citrate), approximately 30 min before the surgery. The monovettes were centrifuged at 2400 rpm for 10 min. A total of 4 mm of plasma, which consisted of the complete upper yellow layer and also the lower red layer's top 1-2 mm part, was transferred into a yellow-marked monovette. After the second centrifugation at 3600 rpm for 15 min, approximately 0.7 mm was plasma rich in platelets at bottom of the monovette, and the upper rest was plasma poor in platelets (4). Part of the platelet-poor plasma was collected and discarded. The remaining 0.7 ml of plasma at the bottom of the yellow-marked monovette was vortexed for 20s and final preparation of PRP transferred into an application injector.

*Surgical Procedure*

All surgical procedures were performed under aseptic conditions in an animal-operating suite at Gazi University. The rabbits were anaesthetized with an intramuscular dose of 35mg/kg ketamine (Ketanes, Alke, İstanbul, Turkey) and 5mg/kg xylazine (Rompun, Bayer, Leverkusen, Germany). The animals were placed in *sterna recumbency*. Their heads were shaved and the cutaneous surface was disinfected with a povidone iodine solution prior to surgery. The calvarial bone was exposed after incisions of the skin and periosteum, respectively. Two circular calvarial bone defects (0,5 mm thick x 10 mm inner diameter) were made in the parietal bone on each side of the median sagittal suture without crossing it, using a trephine bar on a slow-speed electric hand piece under physiologic saline irrigation without injuring the underlying duramater.

The rabbits were randomly divided into six groups and the cranial defects (n=56) were treated with single-application of PRP (SA-PRP) (n=10), SA-PRP and beta-tricalciumphosphate (β-TCP) graft (SA-PRP+TCP) (n=10), DA-PRP (n=8), DA-PRP and β-TCP (DA-PRP+TCP) (n=8), β-TCP (TCP) (n=10) or left untreated (control) (n=10). The study design is summarized in Figure 1.

*Immunohistochemistry*

Histopathologic and immunohistochemical procedures and evaluation were carried out at the Department of Oral Pathology, Faculty of Dentistry, Gazi University. Hard tissue samples were fixed with 10% buffered formalin for 24–72h and decalcified with 10% formic acid for 3wk. After washing with tap water, the samples were embedded in paraffin. Three sections with 4μm thickness were taken from the central region of each specimen to obtain maximum standardization of the cutting surface. All sections were deparaffinized at 56°C and by xylene, then they were incubated in 96% and absolute ethanol.

The streptavidin-biotin method was used for immunohistochemical detection of expressions of Osteonectin/SPARC (Monoclonal Antibody, Lot no: 010FD, Takara Bio Inc., Japan) and Osteocalcin (Monoclonal Antibody, Lot no: 009FD, Takara Bio inc, Japan) monoclonal antibodies. For this purpose, deparaffinized sections were microwave treated in 0.01 M sodium citrate buffer [2.64 g/l sodium citrate, pH 6.0] for 15 minutes at 360 W and 5 minutes at 600 W. After the sections were rinsed with phosphate-buffered saline [phosphate-buffered saline (PBS), pH 7.6], the endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 10 min.

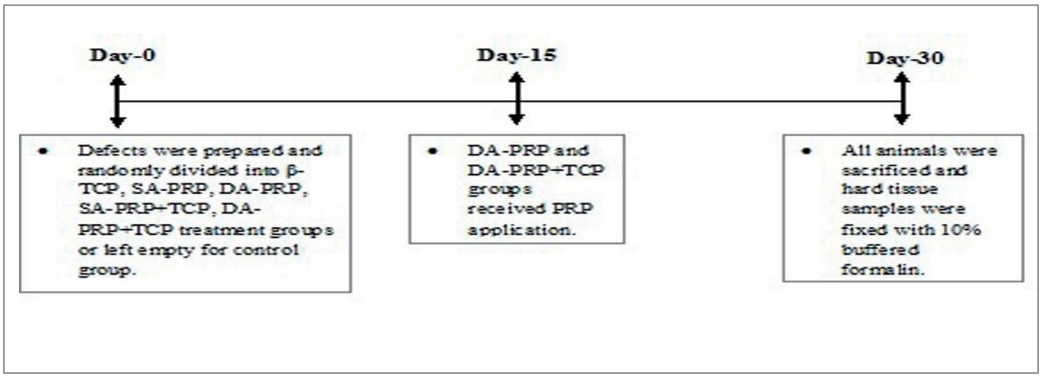


***Figure 1.*** *Study design.*

After incubation overnight at 4°C with antibodies in 1:200 dilution in PBS, a broad spectrum second antibody was applied for 20 minutes, followed by incubation in the HRP-streptavidin (Histostain Plus, Zymed Laboratories Inc., CA, USA) for 30 min. Then, diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen for the visualization of antibody expression. After counterstaining with Mayer's hematoxylin, the slides were dehydrated and mounted with mounting medium (Clearmount, Lot: 10364117, Zymed S.San Francisco, California, USA). Osteosarcoma was used as a positive control tissue for both antibodies. All histopathological evaluations were made with a Leica DM 4000 B light microscope (Leica Microsystems GmbH. Wetzlar, Germany).

Osteoblasts and osteocytes with brown cytoplasmic staining and brown extracellular staining on matrix on graft material, osteoid formation sites around graft material were considered positive (for osteonectin and osteocalcin), regardless of the intensity. All positive areas were evaluated for each section. The osteonectin and osteocalcin expression for each section was expressed as the percentage of positively stained areas per total defect area. Scoring of antibody reactivity was carried out using the Leica QWin Plus v3.3.1 image analyzer program (Leica Microsystems GmbH. Wetzlar, Germany).

*Statistical Analysis*

Statistical analysis was performed by statistical software (Statistical Package for Social Sciences (SPSS) 11.5, SPSS Inc., Chicago, IL, United States). Distribution of the continuous variables were determined by using the Shapiro Wilk test. Data were expressed as median and ($25^{th}$ – $75^{th}$) percentiles. Medians were compared by the Kruskal Wallis test. When the p-value of the Kruskal-Wallis test was statistically significant, multiple comparison test was used pairwise comparison. Spearman's correlation coefficient was used to determine the correlation between OC and ON data. p values less than 0.05 were considered as statistically significant.

**Results**

Histochemical results for ON % and OC % are summarized in Figures 2, 3, 4, 5, 6 and 7. Minimum-maximum values as well as $50^{th}$ (median), $25^{th}$ and $75^{th}$ percentiles are shown in Table 1. Mild foreign body reaction was noticeable in any groups. Mild inflammatory cell infiltration was found in the operated area. After 4 weeks, new bone formation and non-resorbed graft material were seen.

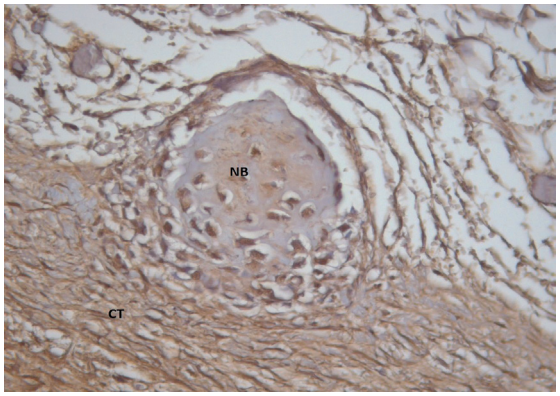


***Figure 2.*** *Histopathological section from SA – PRP group. Brown OC expression in osteocytes within new bone (NB) and connective tissue (CT) (Osteocalcin ABC X 200).*

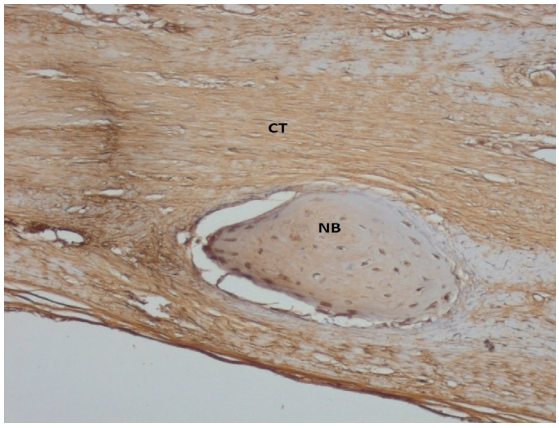


***Figure 3.*** *Histopathological section from SA – PRP group. ON expression in osteocytes within new bone (NB) is more prominent than connective tissue (CT) expression (Osteonectin ABC X 200).*

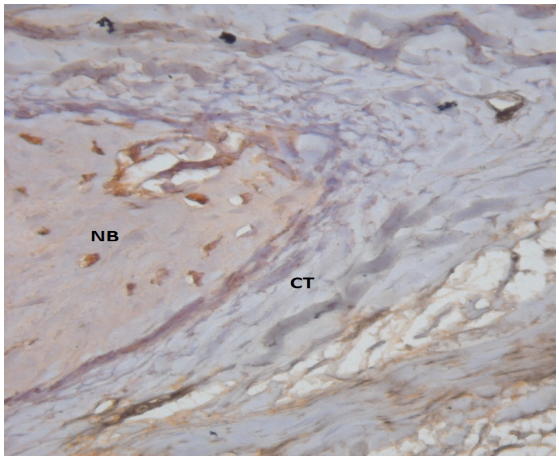


***Figure 4.*** *Histopathological section from DA – PRP group. OC expression in connective tissue (CT) is weak and scarce whereas expression in osteocytes within new bone (NB) is intense (Osteocalcin ABC X 200).*

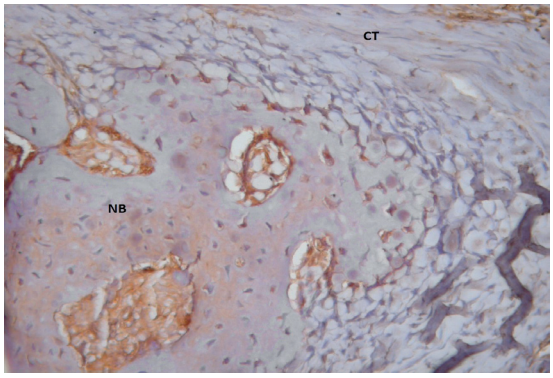


***Figure 5.*** *Histopathological section from DA – PRP group. Brown ON expression in osteocytes within new bone (NB) and connective tissue (CT) (Osteonectin ABC X 200).*

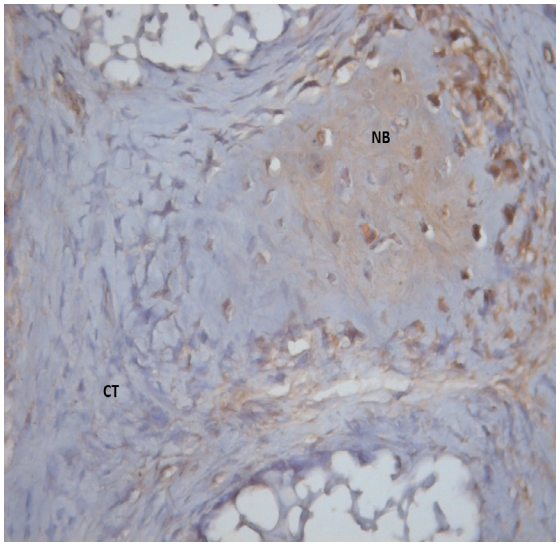


***Figure 6.*** *Histopathological section from DA–PRP +β-TCP group. OC expression in osteocytes of new bone (NB), connective tissue (CT) and at the periphery of graft material (G) (Osteocalcin ABC X 200).*

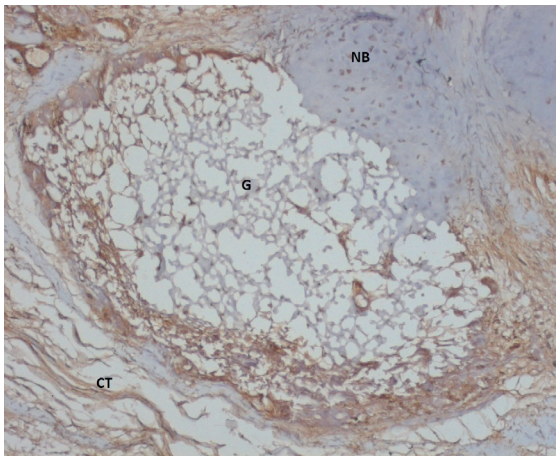


***Figure 7.*** *Histopathological section from DA–PRP +β-TCP group. ON expression in graft material (G) and the osteocytes of the new bone (NB) formed at the periphery of graft material and also in the connective tissue (CT) (Osteonectin ABC X 200).*

A considerable number of ON and OC immunopositive osteoblasts were observed on the surface of newly-formed bone. ON immunoexpression was noted in cells adjacent to β-TCP particles showing strong pattern and also in the newly formed osteoid of the defect area. Although immunoreactivities of both proteins were remarkable in rimming osteoblast, pre-existing bone trabeculae and active osteocytes embedded in newly formed bone were also stained

positive. The highest ON % was calculated in the defects of the DA-PRP+ β-TCP group, followed by the β-TCP, SA-PRP+ β-TCP, DA-PRP, SA-PRP and control groups, respectively (Table 1). The groups treated with b-TCP showed statistically more ON% than groups treated without β-TCP ($p<0.05$). The highest OC % expression was seen in the defects of the DA-PRP+TCP group (Table 1). The three b-TCP treated groups and the SA-PRP group revealed significantly more OC % than the DA-PRP and control groups ($p<0.05$). There were no significant differences detected between the DA-PRP+ β-TCP and control groups.

**Discussion**

The purpose of this study was to evaluate the expression of two immunohistochemical markers of bone formation, the osteonectin and osteocalcin, during bone healing in a rabbit cranial defect model in order to examine the effects of double-application of PRP and to compare it with controls and single-application of PRP. For this purpose, we created two circular bone defects 10 mm in diameter on each rabbit calvaria. Previously, the critical size for rabbit calvarial bone defects was described as 15 mm in diameter. However, it is also known that creating multiple critical size bone defects on rabbit calvaria is not always possible due to the small size of rabbit calvaria (16). Recently, healing of rabbit cranial bone defects with 6 mm, 9 mm and 15 mm diameters were compared, and it was concluded that the diameters of the defects had no influence on new bone formation in the defect area (25). In our study, we examined ON and OC, which are non-collagenous extracellular matrix proteins often used for examining the bone formation process in defect areas. As an advantage of immunohistochemistry, we were able to show the sources of ON and OC as well as quantitatively analyze their expressions. ON has been confirmed to be an early and effective marker of bone formation, whereas OC has been suggested as a marker for the late phase of bone formation (10). It has been stated that before calcification of the bone matrix, in the osteogenic phase, ON is expressed strongly and it regulates collagen fibril diameter (26, 27). On the other hand, the OC, produced by mature osteoblasts during mineralization, has been considered to be one of the latest of expression markers in mature osteoblasts (28, 29). It was shown that bone turnover is initiated by the appearance of OC, and it functions as an inhibitor of bone formation and a stimulator of bone mineral maturation *in vivo* (29, 30). Also, it has been reported that OC plays a role in delaying nucleation, regulation of osteoclasts and preventing excessive crystal growth *in vitro* (27, 31). Considering ON expression levels, DA-PRP did not differ significantly from control and SA-PRP within the limits of this study. Remarkably, OC% levels of the DA-PRP group appeared very similar to those of the control group. In the literature, there are some promising reports regarding multiple-application of PRP via injection (13, 14) or topically (11, 12) for skin or articular joint defect treatments. However, in this study, the addition of DA-PRP alone via injection did not seem to have a significant influence on early or later stages of bone formation. Moreover, the SA-PRP group showed significantly higher OC % after 30 days compared to the DA-PRP and control groups, which also indicates that a single-application of PRP seems to be better at supporting later phases of bone formation. It has been suggested that OC is released when osteoblast proliferation is completed and the mineralization phase of the newly formed bone matrix begins (32).

Although there are conflicting reports about the effectiveness of PRP when it is applied alone (6, 11, 16); our findings supports that a single-application of PRP may have a possible effect on late phases of bone formation, including bone mineralization and maturation. In the literature, there are conflicting reports about the additive effects of a single PRP application in addition to β-TCP. Some authors reported that PRP had significantly improved the results achieved by β-TCP alone (7, 8, 33), whereas others claimed that PRP added no additional benefits (34-36). Interestingly, all the groups in this study treated with b-TCP revealed significantly higher ON percentages than the control, SA-PRP or DA-PRP groups. In other words, double- or single-application of PRP did not significantly enhance ON expression unless the PRP is applied in conjunction with β-TCP. In agreement with a previous report stating that β-TCP contributed significantly to early bone formation (37), the current results suggest that b-TCP has a significant potential to promote osteoblastic activity at the early phases of bone formation. The difference between SA-PRP group and the β-TCP treated groups were insignificant, but these groups revealed significantly higher OC expressions than the control and double-application PRP groups.

***Table 1.*** *Immunohistochemical staining results, median and 25th -75th percentile values are given for ON% and OC %.*

| Groups | ON % Median ($25^{th}$ -$75^{th}$ Percentile) | OC % Median ($25^{th}$ -$75^{th}$ Percentile) |
|---|---|---|
| **Control** | 15.9 (8.1-29.2) | 7.6 (2.4-20.0) |
| **SA - PRP** | 27.7 (23.1-41.9) | 45.4 (39.5-61.9)* |
| **DA – PRP** | 30.1 (3.4-45.2) | 8.7 (6.5-15.5)† |
| **TCP** | 51.4 (41.1-60.3)*,†,‡ | 36.9 (33.6-42.6)*,‡ |
| **SA–PRP +β-TCP** | 44.5 (43.6-56.6)*,†,‡ | 50.9 (30.0-63.7)*,‡ |
| **DA–PRP +β-TCP** | 58.0 (52.1-67.9)*, †, ‡ | 53.1 (49.9-56.7)*,‡ |

** Compared to Control group p<0.05, † Compared to SA-PRP group p<0.05, ‡ Compared to DA-PRP group p<0.05.*

Overall, in accordance with previous reports (7, 8, 15, 33-35), we have found that the groups treated with β-TCP, with or without single or double PRP application, had a better success rate in terms of bone defect healing. In particular, double-application of PRP did not significantly improve the results achieved by β-TCP, single-application of PRP or control groups. Multiple-application of PRP is an attractive new topic for many researchers aiming to overcome the limitations of PRP. Encouraging results are being reported in favor of multiple-application of PRP for face and neck rejuvenation, scar attenuation, treatment of chronic diabetic ulcers and degenerative lesions of articular cartilage of the knee (11-14). Earlier, we had reported that new bone formation was histomorphologically remarkable in double-application PRP treated groups, but statistical analyses of the histomorphometric data revealed no significant difference (15). Histomorphological observations revealed that newly-formed bone trabeculae in double-application PRP groups were also more mature and lamellar than both the control and single-application PRP groups, either with or without TCP (15).

Above all, the control group presented a limited maturation of bone. The histomorphological analyses revealed a remarkable increase in new bone formation with the addition of a double-application of PRP (15).In this study, the highest ON and OC expression was detected in the DA-PRP+ β-TCP group among all the study groups; however, these values were significantly different only from the control group, but not from the other β-TCP applied groups. The results indicated that in the DA-PRP+ β-TCP group, the early stage of new bone formation still continues and mineralization is also initiated for the newly-formed bone matrix. We still think that a double-application of PRP may have the potential to affect bone formation, even though our current data does not support that hypothesis. The relatively small sample size of this study, consisting of 8 to 10 defects in each group, may have contributed to the lack of statistical significance between the different groups. Nonetheless, more evidence is required for the better understanding of the potential of double-application of PRP in the treatment of intrabony defects.

## Conclusion

Double-application of PRP did not have additional benefits on the levels of ON and OC expression during bone healing in a rabbit cranial defect model. Moreover, our findings revealed that the presence of β-TCP has affected ON and OC expressions, which suggested a potential of β-TCP for supporting early bone healing. The results of further *in vivo* studies conducted with both shorter and longer evaluation periods are needed to clarify the additional effects of double-application of PRP.

**Source of funding**

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**Conflict of interest**

None declared

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