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Evaluation of the effects of platelet-rich fibrin on bone regeneration in diabetic rabbits

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ABSTRACT

Objectives: This study aimed to investigate the effect of platelet-rich fibrin on bone regeneration in critical size defects in the calvaria of diabetic rabbits.
Study design: In total, 40 male New Zealand rabbits, were divided into two groups a non-diabetic control group (Group A) and a diabetic experimental group (Group B). Two bicortical circular defects 15 mm in diameter were created in the parietal bone of each animal. Each group was further divided into four groups: subgroup E, the defect was left empty; subgroup PRF, the defects were filled only with PRF; subgroup AB, the defects were filled with autogenous bone; subgroup AB + PRF, the defects were filled with autogenous bone combined with PRF. The animals sacrificed at 4 weeks and 8 weeks. Bone formation was assessed by micro-computed tomography (micro-CT) scanning, histological and histomorphometric analysis.
Result: The total percent of new bone was the lowest in group A–E (6.77 ± 0.21 at 4 weeks, 11.01 ± 0.37 at 8 weeks) and highest in group A–AB + PRF (21.66 ± 0.91 at 4 weeks, 37.46 ± 1.25 at 8 weeks; $p < 0.05$). The mean percent of new bone was greatest in group B-AB + PRF at 4 and 8 weeks (16.87 ± 0.92, 29.59 ± 1.09, respectively) and lowest in group B–E (5.83 ± 0.09 at 4 weeks, 7.36 ± 1.02 at 8 weeks).
Conclusion: This study, despite its limitations, showed that PRF can be used safely and that PRF induced bone healing in diabetic rabbits.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterised by impaired metabolism of carbohydrates, lipids, and proteins. This disorder results insufficient secretion of insulin or from tissue resistance (Erdogan et al., 2014; Vieira et al., 2008; Margonar et al., 2003; Gay et al., 2014). There can also be problems with healing when surgery is performed in uncontrolled diabetic patients (Mariano et al., 2010). Various studies have shown that DM impairs bone-healing processes (Erdogan et al., 2014; Kotsovilis et al., 2006). Several factors contribute to wound healing deficiencies in diabetic patients, including decreased angiogenic responses, growth factor production, collagen accumulation, and changes in mineral metabolism (Erdogan et al., 2014; Vieira et al., 2008; Scully and Cawson, 2005). Diabetic cells produce inadequate levels of growth factors (Mariano et al., 2010; Galiano et al., 2004). Thus, using growth factors in wound sites to normalise the healing process is reasonable. The effects of growth factors on wound healing and bone healing in diabetic models have been evaluated. These studies demonstrated that wound and bone defects needed to be treated with higher levels of growth factors in DM patients than in controls (Mariano et al., 2010; Pietramaggiori et al., 2006).

Platelet-rich plasma (PRP) is an autologous concentrate of platelets suspended in plasma. It is a proven source of growth

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factors. PRP accelerates bone healing (Marx et al., 1998). However, its efficacy remains controversial because there are conflicting results. Platelet-rich fibrin (PRF), an autologous fibrin matrix, was developed in France by Choukroun et al. (Choukroun et al., 2001) It is a second-generation platelet concentrate and has been used widely (Choukroun et al., 2001; Dohan et al., 2006; Lee et al., 2012; Zhang et al., 2012) PRF contains numerous growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, and insulin-like growth factor (IGF). Theoretically, PRF accelerates early bone regeneration by angiogenesis, chemotaxis, mitosis, and stem-cell proliferation in the early phase of bone regeneration (Choukroun et al., 2001; Dohan et al., 2006; Lee et al., 2012; Zhang et al., 2012). There are various clinical and animal studies showing that PRF has a positive effect on bone healing and has some advantages over PRP (Pripatnanont et al., 2013; Acar et al., 2015).

In the present study, we evaluated the effects of PRF combined with autogenous bone (compared to PRF alone and autogenous bone alone) on bone regeneration in rabbits with diabetes.

2. Materials and methods

The experimental protocol of the present study was approved by the Ethics Committee on Animal Experimentation at Bulent Ecevit University.

2.1. Study design

In total, 40 male New Zealand rabbits, aged 4–6 months, weighing on average 3–4 kg, were divided into two groups (20 animals each): a non-diabetic control group (Group A) and a diabetic experimental group (Group B). Two bicortical circular defects 15 mm in diameter were created in the parietal bone of each animal. Each group was further divided into four groups: subgroup E, the defect was left empty; subgroup PRF, the defects were filled only with PRF; subgroup AB, the defects were filled with autogenous bone; subgroup AB + PRF, the defects were filled with autogenous bone combined with PRF.

2.2. Induction of diabetes and experimental group

Before initiating the experimental protocol, each rabbit was weighed. Blood samples were collected from the ear vein and non-fasting blood glucose levels were measured using a Glucometer (Glucometer Accu-Chek, Roche, Mannheim, Germany) and commercially available Glucostix reagent strips.

For the induction of experimental diabetes, rabbits weighing 2.0–3.2 kg were sedated with 40 mg ketamine (Ketolar) administered intramuscularly. Alloxan monohydrate (Sigma Aldrich Chemical, St. Louis, MO, USA) was dissolved in sterile normal saline to achieve a concentration of 5% (w/v) and a single injection of alloxan monohydrate (150 mg/kg body weight) was administered via the ear vein following 16 h of fasting. To prevent hypoglycemia, 10 mL glucose 5% was injected intraperitoneally after the alloxan, and drinking water was supplemented with 10% glucose for the first 24 h after the alloxan injection. Then the animals were maintained on tap water and regular food *ad* libitum for 8 weeks. Three days later, blood samples were collected and glucose levels were determined to confirm the development of diabetes. Only rabbits with glucose concentrations of more than 200 mg/dL were used in further experiments. The blood sugar levels of rabbits were estimated using a Glucometer and Glucostix strips. Weight and blood glucose levels were determined on a weekly basis.

2.3. Platelet-rich fibrin preparation

Rabbits were sedated and autologous PRF was prepared using 8 mL autologous whole blood collected from the central ear artery of the rabbit. The whole blood without an anticoagulant was transferred to a 10 mL glass tube and centrifuged (3000 rpm, 10 min). At the end of the centrifugation there were three blood fractions: the upper serum layer, second buffy coat layer, and lower red blood cell (RBC) layer. The PRF clot was removed from the tube and separated from the RBCs using microsurgical scissors. The middle layer from the tube (Fig. 1) was used.

2.4. Surgical procedure

General anaesthesia was induced by intramuscular injection of a combination of 0.4 mL ketamine (Ketolar) and 0.3 mL xylazine (Rompun). The cranium was shaved and disinfected with povidone-iodine. Then a 6 cm anterior-posterior midline skin incision was made. The periosteum was removed completely from the parietal bone. Two bicortical square defects 15 mm in diameter were created in the parietal bone of each animal (Fig. 2).

Then subcutaneous tissue layers and skin were closed with Vicryl 3/0 and 3/0 silk sutures. After surgery, the rabbits received gentamycin 1 mg/1 kg (Genta) intramuscularly. Then, 10 animals from each group were sacrificed at 4 weeks; the remaining 10 from each group were sacrificed at 8 weeks. Calvarial specimens were fixed in 10% formalin and underwent micro computed tomography (micro-CT).

2.5. Micro-CT

The specimens were scanned using micro-CT (Skyscan 1174; Micro Photonics Inc., Allentown, PA, USA). Scanning was performed with a spatial resolution of 15 μm using 50 kV and 800 μA at a 0.7° rotation step for a total of 180°. All images were taken in three-dimensional reconstruction with the NRECON software, and then the collected data were evaluated with CTAn software; only the volume of mineralised new bone formation without graft materials was calculated.

2.6. Histological and histomorphometric analysis

All animals were sacrificed with pentobarbital sodium. Biopsies were removed *en bloc* using a surgical burr attached to a slow-

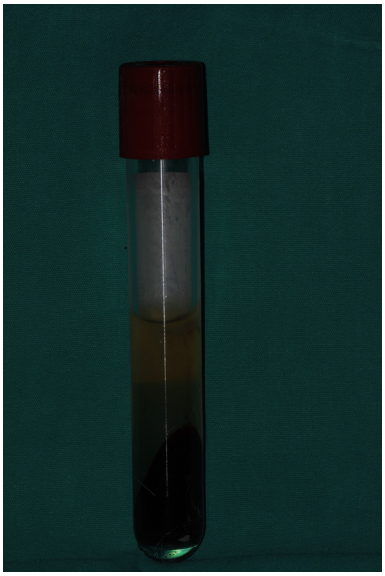

Fig. 1. Platelet rich fibrin.

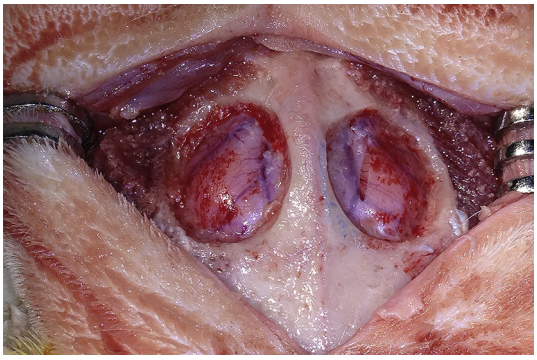

Fig. 2. Rabbit calvarium showing bilateral 15-mm critical-sized defect.

speed electrical hand-piece and preserved in formaldehyde. The specimens were decalcified by 10% formic acid and then embedded in paraffin. Serial transverse sections (5 μm) were obtained and stained with haematoxylin and eosin (H&E) for analysis by light microscopy. Histological analysis was performed by a single examiner who was also blinded to the identity of the samples. Connective tissue, granulation tissue, bone formation, and the presence of grafted material were evaluated.

In the histomorphometric analyses, images from six different areas of each histological slide were obtained at ×200 using a motorised light microscope (Leica DM-4000B; Leica Microsystems, Wetzlar, Germany). The areas of new bone, graft material, and soft tissue were quantified in square micrometres with an image analysis program (Leica Q-Win Plus V3.5.1; Leica Microsystems, Heerbrugg, Switzerland). The amount of new bone formation was calculated as the percentage of new bone and graft area to the total defect area.

2.6.1. Statistical analysis

The paired Student t test (paired observations) was carried out for samples (histomorphometric data and the microscopic computed tomography (micro-CT) values) from same animal. One-way analysis of variance (One-way ANOVA) and Bonferroni post-hoc Tukey's (unpaired observations) was carried out for percentage and volume of new bone area. All tests were performed using statistical software (SPSS Inc., version 16.0, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Animals

All animals tolerated surgery well and survived the post-surgical period. No wound dehiscence or wound infections or abscess formation were observed at any surgical site.

3.2. Micro-CT and histomorphometric findings

Percentage of new bone is summarised in Table 1. The volumes of new bone (mm3) are shown in Table 2.

3.3. Non-diabetic groups (Group A and subgroups)

There was a correlation between the micro-CT and histomorphometric analyses. The total percent of new bone was the lowest in group A–E (6.77 ± 0.21 at 4 weeks, 11.01 ± 0.37 at 8 weeks) and highest in group A–AB + PRF (21.66 ± 0.91 at 4 weeks, 37.46 ± 1.25 at 8 weeks; $p < 0.05$). The highest volumetric values of new bone were observed in the PRF + autogenous bone groups (Fig. 3) (262.62 ± 21.27 at 4 weeks, 452.56 ± 18.13 at 8 weeks), and the lowest values were found in the empty defect groups (109.40 ± 5.69 at 4 weeks, 140.05 ± 5.60 at 8 weeks; $P < 0.05$). There was a difference between the autogenous bone group and PRF + autogenous bone group in terms of the percentage and volume of new bone ($P < 0.05$), while no difference was observed between the empty and PRF groups ($P > 0.05$) at 4 and 8 weeks.

3.4. Diabetic groups (Group B and subgroups)

The mean percent of new bone was greatest in group B-AB + PRF at 4 and 8 weeks (16.87 ± 0.92, 29.59 ± 1.09, respectively) and lowest in group B–E (5.83 ± 0.09 at 4 weeks, 7.36 ± 1.02 at 8 weeks). However, no difference was observed between the PRF group and empty group (6.32 ± 0.65 at 4 weeks, 8.47 ± 0.95 at 8 weeks; $P > 0.05$). The highest volumetric values of new bone were observed for the PRF + autogenous bone group (198.87 ± 13.12 at 4 weeks, 330.44 ± 20.83 at 8 weeks) (Fig. 3), and the lowest values were found in the empty defect group (103.77 ± 3.68 at 4 weeks, 119.37 ± 4.45 at 8 weeks; $p < 0.05$) (Fig. 4). There was a difference between the autogenous bone and PRF + autogenous bone groups in terms of the percentage and volume of new bone at 4 and 8 weeks ($P < 0.05$).

The mean total new bone percentage was lower in the diabetic groups than in the non-diabetic control groups for all time points (except the PRF groups at 4 weeks), and the differences were statistically significant ($P < 0.05$). The mean volumes of new bone were lower in the diabetic groups than in the non-diabetic groups at all time points (except empty and PRF groups at 4 weeks), and the differences were statistically significant ($P < 0.05$).

3.5. Histological findings

Diabetic and non-diabetic groups were shared similar histological features; histomorphometric analyses related to these groups were seen in Table 1.

Neither inflammatory reaction nor secondary infection findings were observed at any surgical specimen. Fibrous connective tissue was mainly observed in empty and PRF groups. Centripetal

Table 1
The percentage of new bone area from histomorphometric analysis in defects.

GROUP	NON-DIABETIC		DIABETIC	
	4 weeks	8 weeks	4 weeks	8 weeks
Empty defect	6.77 ± 0.21	11.01 ± 0.37	5.83 ± 0.09	7.36 ± 1.02
PRF	7.05 ± 0.52	11.65 ± 0.77	6.32 ± 0.65	8.47 ± 0.95
Autogenous bone	19.46 ± 0.91*	32.49 ± 2.83*	13.62 ± 1.50*	19.86 ± 1.68*
PRF + autogenous bone	21.66 ± 0.91*	37.46 ± 1.25*	16.87 ± 0.92*	29.59 ± 1.09*

Data are expressed as the mean ± standard deviation.
*Statistically significant difference between groups ($P < 0.05$).

Table 2
Results of the micro-computerized tomography measurements (mm^3).

GROUP	NON-DIABETIC		DIABETIC	
	4 weeks	8 weeks	4 weeks	8 weeks
Empty defect	109.40 ± 5.69	140.05 ± 5.60	103.77 ± 3.68	119.37 ± 4.45
PRF	115.60 ± 6.32	149.73 ± 10.03	108.50 ± 4.40	123.66 ± 5.21
Autogenous bone	196.92 ± 13.75*	343.70 ± 20.54*	156.54 ± 10.24*	252.06 ± 7.02*
PRF + autogenous bone	262.62 ± 21.27*	452.56 ± 18.13*	198.87 ± 13.12*	330.44 ± 20.83*

Data are expressed as the mean ± standard deviation.
*Statistically significant difference between groups ($P < 0.05$).

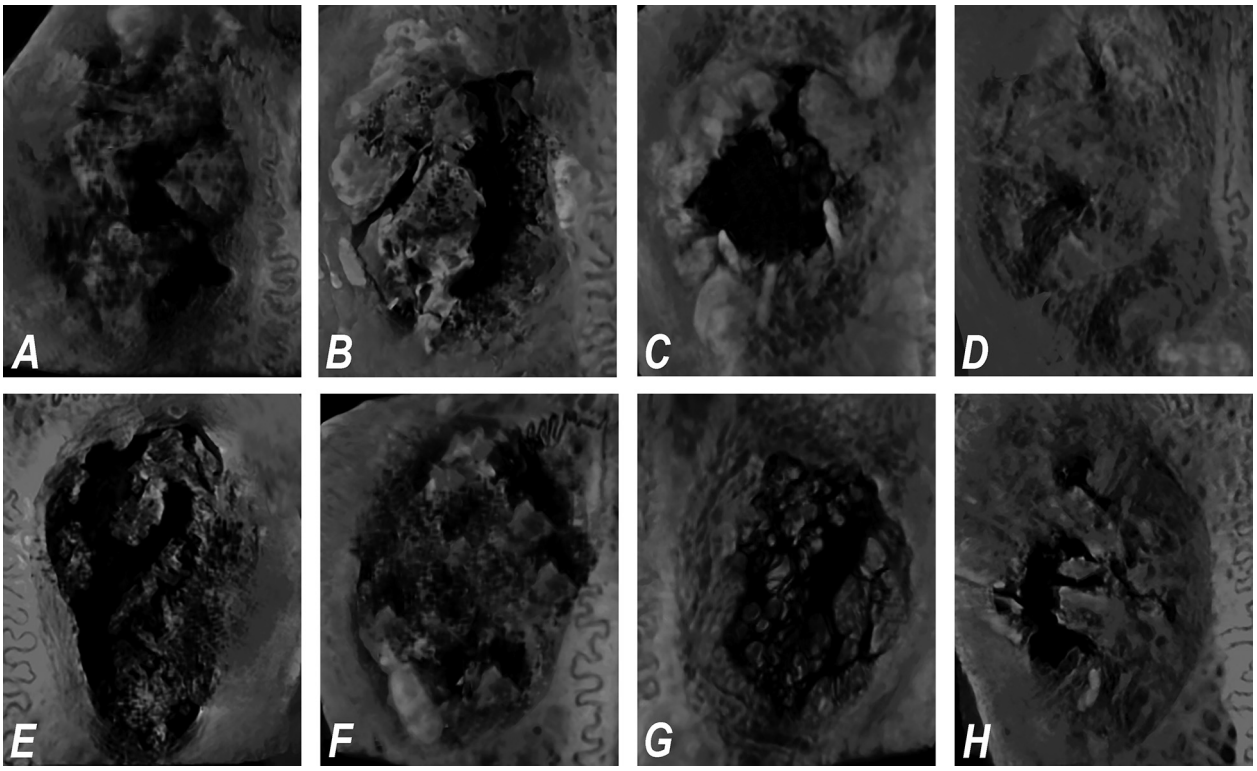

Fig. 3. Three-dimensional images of new bone formation. A. Group A–AB on 4th week. B. Group A–AB on 8th week. C. Group A– AB/PRF on on 4th week. D. Group A– AB/PRF on on 8th week. E. Group B-AB on 4th week. F. Group B-AB on 8th week. G. Group B-AB/PRF on 4th week. H. Group B-AB/PRF on 8th week.

(from the periphery toward the center of defects new bone formation) was seen in all groups. No new bone formation was detected at the centre of the defect area in empty and PRF groups. Although new bone formation was no significant difference between empty and PRF groups; more cellular collagenated connective tissue stroma was seen in PRF group. The lowest new bone formation was found in empty group (group B–E) (5.83 ± 0.09 at 4 weeks; 7.36 ± 1.02 at 8 weeks) (Fig. 5), however no difference was observed from PRF group (6.32 ± 0.65 at 4 weeks; 8.47 ± 0.95 at 8 weeks) ($P > 0.05$).

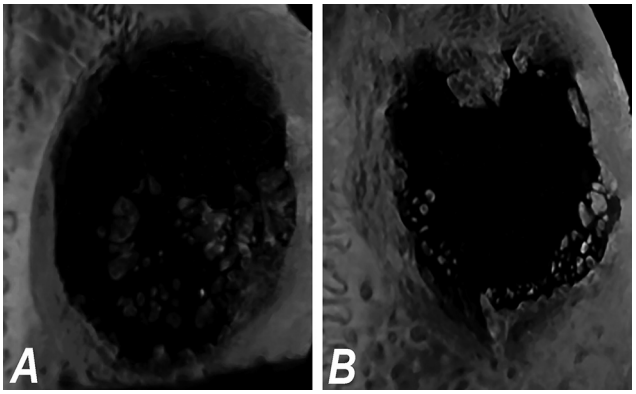

Fig. 4. A. Three-dimensional images of group B–E on 4th week. B. Three-dimensional images of group B–E on 8th week.

In graft groups, new bone formation around the graft particles was observed. The bone-grafted groups differed from non-grafted groups in term new bone formation areas. Unlike the non-diabetic graft groups, some graft particles were resorbed and osteoclasts were observed around the graft particles in diabetic graft groups (Figs. 6 and 7).

4. Discussion

Various animal species have been used to evaluate new bone formation. In particular, rats and rabbits are used frequently because they are cheaper to purchase and their care is relatively easy. Rabbits were used in the present study because blood volume in rats is not sufficient for the preparation of PRF. Rabbit calvarial critical-size defects are a selective experimental model for bone regeneration. Different defect sizes have been used in previous

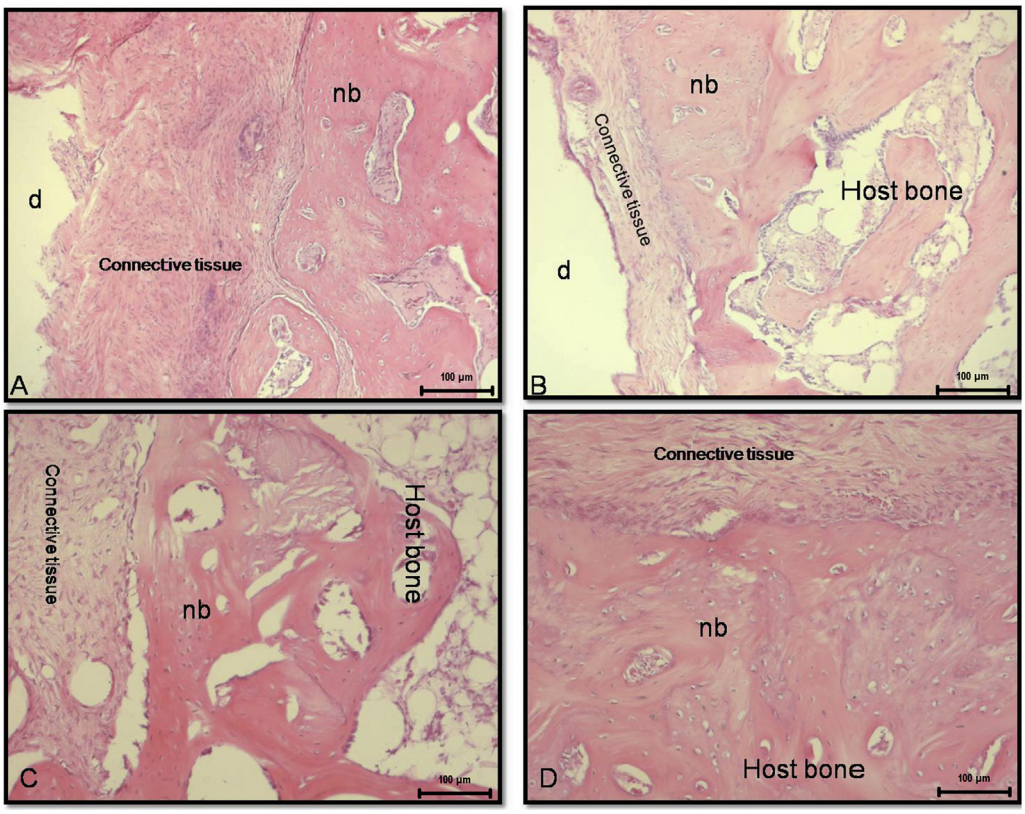

Fig. 5. Histological view of defect region. Connective tissue (CT), defect (d), new bone (nb) (haematoxylin-eosin, original magnification ×200). A. Group A–E on 4th week. B. Group A–PRF on 4th week. C. Group B–E on 4th week. D. Group B-PRF on on 4th week.

studies. We used circular defects of 15 mm diameter. This size is commonly used in bone regeneration studies in rabbit calvaria (Delgado-Ruiz et al., 2014; Findikcioglu et al., 2009).

The effects of diabetes have been studied in relation to oral and maxillofacial surgery for many years (Mealey, 2006; Cozen, 1972). Diabetes is associated with bone resorption, poor osseous healing, and impaired bone regeneration (Mealey, 2006; Cozen, 1972). Clinical and experimental studies have shown that osteoblastic activity decreases in type I diabetes mellitus (Erdogan et al., 2014; Vieira et al., 2008; Sohn et al., 2010). Thus, healing in diabetic patients is a problem with major and minor surgeries. Some studies have evaluated the effects of bone substitute materials on bone healing in diabetic animals (Mariano et al., 2010; Ezirganlı et al., 2014; Lee et al., 2013). Ezirganli et al. (Ezirganlı et al., 2014). used local simvastatin in diabetic rats with critical-size defects and conducted radiographic and histomorphometric assessments. They reported that the application of local simvastatin induced bone healing in diabetic rats. Lee et al. (Lee et al., 2013) used titanium domes to achieve new bone formation in diabetic rats. Mariano et al. (Mariano et al., 2010) used PRP to treat calvarial defects in diabetic rats. They applied PRP locally in one group and filled a fibrin clot in the control group. The animals were sacrificed 1 month postoperatively and histological and histomorphometric analyses were performed. They reported that the defects filled with PRP enhanced bone healing. To the best of our knowledge, the present study is the first to evaluate the effects of PRF on bone healing in diabetic animals with critical-size defects. The specimens were evaluated histomorphometrically, histologically, and radiologically.

PRF second-generation platelet concentrate was developed by Chukroun (Choukroun et al., 2001) and has advantages over platelet-rich plasma (PRP) (Choukroun et al., 2001; Acar et al., 2015) PRF is a biomaterial that slowly releases growth factors. It has been hypothesised that PRF may protect growth factors from proteolysis (Dohan et al., 2006; Lee et al., 2012; Zhang et al., 2012). Thus, growth factors may keep their activity over relatively longer periods and stimulate bone regeneration effectively. Beside this the influence of fibrin in PRF promotes bone healing and supports adhesion. Bagot D'arc et al. (Bagot d'Arc et al., 2004) performed seventy two mastoid reconstruction with composite of biphasic ceramic granules and fibrin sealant. They reported that fibrin sealant maked bone reconstruction possible in apposition and promotes bone healing. PRF has been reported to enhance the regeneration of osseous and soft tissues in oral and maxillofacial surgery (Oliveira et al., 2015; Baslarli et al., 2015).

Some authors have used PRF in critical-size defects alone to evaluate bone healing. Lee et al. (Lee et al., 2012) used PRF in peri-implant defects in rabbit tibias. They repaired one group of defects with PRF and one group of defects was left empty. Bone healing was evaluated histologically at 8 weeks. They reported that peri-implant defects were repaired successfully when they used PRF alone (Lee et al., 2012). Pripatnanont et al. (Pripatnanont et al., 2013) also used PRF in critical-size defects. They reported that the PRF showed significantly higher bone growth than did empty defects at 8 weeks. However, we did not see any significant bone at 8 weeks, histologically or radiologically, in the healthy or diabetic groups of the present study. PRF application alone was not beneficial for hard tissue.

Regeneration of osseous and soft tissue may be stimulated with PRF in healthy models. PRF has been used with various bone substitutes (Oliveira et al., 2015; Baslarli et al., 2015; Nacopoulos

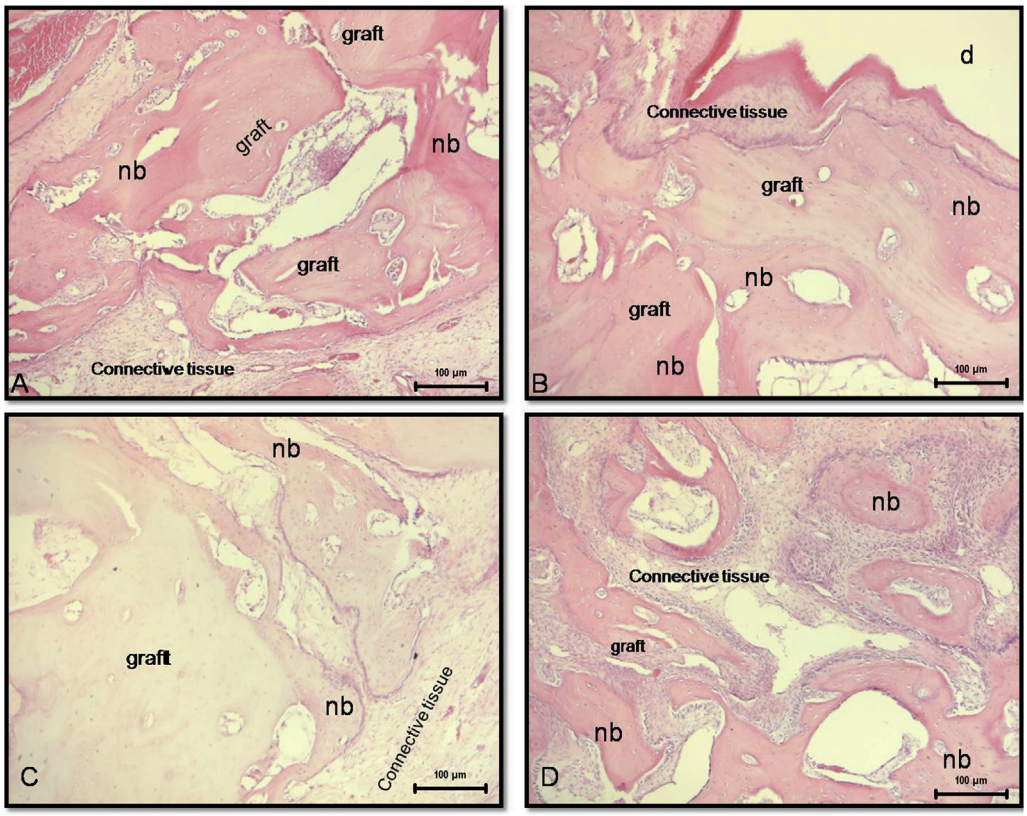

Fig. 6. Histological view of defect region. Connective tissue (CT), defect (d), autogenous graft (Graft), new bone (nb) (haematoxylin-eosin, original magnification ×200). A. Group B-AB on 4th week. B. Group B-AB/PRF on 4th week C. Group A–AB on 4th week. D. Group A– AB/PRF on on 4th week.

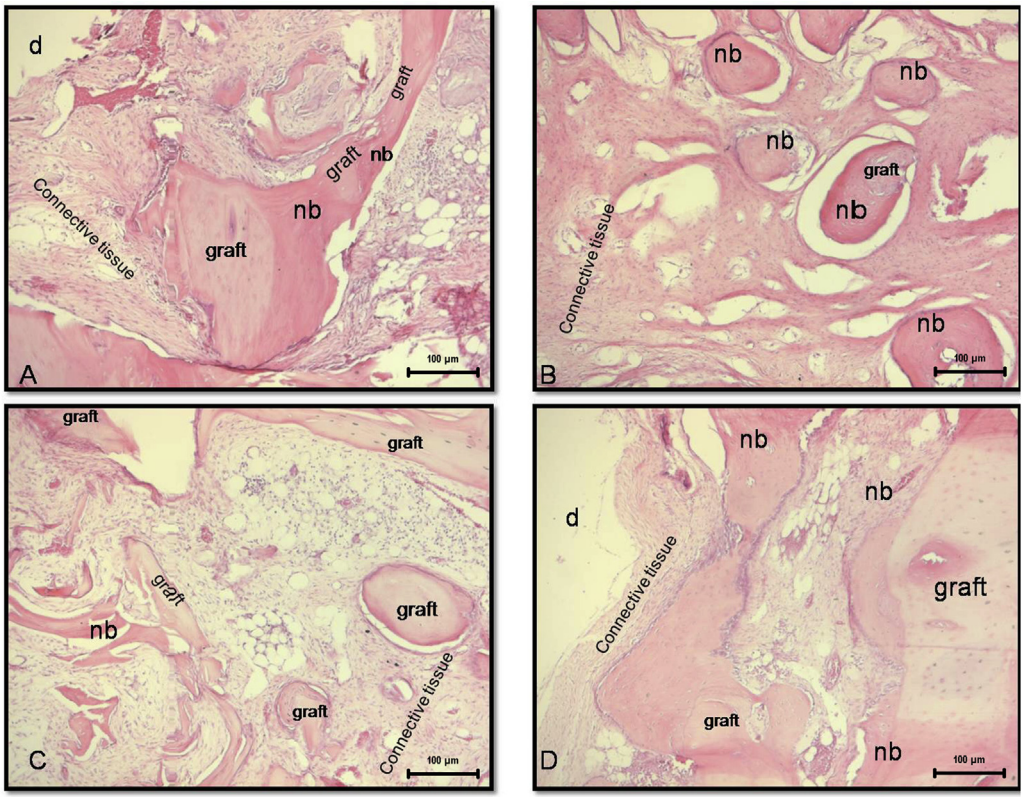

Fig. 7. Histological view of defect region. Connective tissue (CT), defect (d) autogenous graft (Graft), new bone (nb) (haematoxylin-eosin, original magnification ×200). A. Group B–AB on 8th week. B. Group B–AB/PRF on 8th week C. Group A–AB on 8th week. D. Group A– AB/PRF on on 8th week.

et al., 2014; Yoon et al., 2014). In efforts to improve the healing of large bone defects, autologous bone grafting is accepted as the gold standard. Autologous bone grafts possess optimal osteoinductive, osteoconductive, and osteogenic properties that are required for an ideal graft. Currently, there are no viable heterologous or synthetic bone substitutes (Durmuşlar et al., 2014). A mixture of growth factors and autogenous bone seems to be the best bone substitute material. Various studies have used PRF with autogenous bone (Lee et al., 2007, 2008). However, to the best of our knowledge, the synergistic effects of PRF and autogenous bone in a diabetic model have not been reported before. In the present study, we focused on PRF in combination with autogenous bone in diabetic animals. Pripatnanont et al. (Pripatnanont et al., 2013). used PRF with autogenous bone in rabbit cranial defects. They reported more bone formation in the PRF with autogenous bone group versus the autogenous bone group at week 8. Similarly, we found more new bone formation in the PRF with autogenous bone group compared to the autogenous bone group in healthy and diabetic animals at 4^{th} and 8^{th} weeks. However, the mean total new bone percentage was lower in the diabetic groups than in the non-diabetic control groups for all time points (except the PRF groups at 4 weeks), and the differences were statistically significant ($P < 0.05$). In particular, the healthy autogenous group had significantly more new bone formation than the diabetic autogenous group (group A–AB vs. group B-AB). This indicates that the application of autogenous bone alone was not successful for bone healing in diabetic rabbits. When we compared the PRF plus autogenous bone group to the autogenous bone alone group at 4 and 8 weeks in diabetic rabbits, new bone formation was higher in the former group.

Bone healing in diabetic patients is slower than in healthy individuals. Moreover, the risk of infection is higher (Alkan et al., 2002). The present study showed that PRF stimulated bone regeneration in diabetic and healthy rabbits. More bone formation was seen in the PRF and autogenous bone groups in diabetic and control animals. This may be due to the release of growth factors from PRF. Further studies are needed to more fully characterise the effects of PRF on diabetic animals.

5. Conclusions

The mixture of PRF and autogenous bone in calvarial defects in diabetic rabbits induced new bone formation. This study, despite its limitations, showed that PRF can be used safely and that PRF induced bone healing in diabetic rabbits. In addition, micro-CT can be used for sensitive measurements in bone-regeneration studies.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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