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Histomorphometric evaluation of bone regeneration using autogenous bone and beta-tricalcium phosphate in diabetic rabbits

Histomorfometrijska analiza regeneracije kosti kod kunića sa dijabetesom melitusom posle primene autotransplantata kosti i beta-trikalcijum fosfata

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Abstract

Background/Aim. The mechanism of impaired bone healing in diabetes mellitus includes different tissue and cellular level activities due to micro- and macrovascular changes. As a chronic metabolic disease with vascular complications, diabetes affects a process of bone regeneration as well. The therapeutic approach in bone regeneration is based on the use of osteoinductive autogenous grafts as well as osteoconductive synthetic material, like a β-tricalcium phosphate. The aim of the study was to determine the quality and quantity of new bone formation after the use of autogenous bone and β-tricalcium phosphate in the model of calvarial critical-sized defect in rabbits with induced diabetes mellitus type I. **Methods.** The study included eight 4-month-old Chincilla rabbits with alloxan-induced diabetes mellitus type I. In all animals, there were surgically created two calvarial bilateral defects (diameter 12 mm), which were grafted with autogenous bone and β-tricalcium phosphate (n = 4) or served as unfilled controls (n = 4). After 4 weeks of healing, animals were sacrificed and calvarial bone blocks were taken for histologic and histomorphometric analysis. Beside descriptive histologic evaluation, the percentage of new bone formation, connective tissue and residual graft were calculated. All parameters were statistically evaluated by Friedman Test and post hock Wilcoxon Singed Ranks Test with a significance of $p < 0.05$. **Results.** Histology revealed active new bone formation peripherally with centrally located connective tissue, newly formed woven bone and well incorporated residual grafts in all treated defects. Control samples showed no bone bridging of defects. There was a significantly more new bone in autogeonous graft (53%) compared with β-tricalcium phosphate (30%), ($p < 0.030$) and control (7%), ($p < 0.000$) groups. A significant difference was also recorded between β-tricalcium phosphate and control groups ($p < 0.008$). **Conclusion.** In the present study on the rabbit grafting model with induced diabetes mellitus type I, the effective bone regeneration of critical bone defects was obtained using autogenous bone graft.

Key words:
rabbitis; diabetes mellitus; bone regeneration; transplantation, autologous; beta-tricalcium phosphate.

Apstrakt

Uvod/Cilj. Mehanizam otežanog zarastanja tkiva kod dijabetesa melitusa zasnovan je na različitim promenama funkcije na tkivnom i ćelijskom nivou, usled prisutnih mikro- i makrovaskularnih promena. Kao hronično metaboličko oboljenje sa vaskularnim komplikacijama, dijabetes melitus zahvata i proces koštane regeneracije. Terapijski postupci u okviru regeneracije kosti obuhvataju primenu autotransplantata sa oseoinduktivnim delovanjem i sintetskih osteokonduktivnih materijala, kao što je i β-trikalcijum fosfat. Cilj ovog rada bio je da se ispita kvantitet i kvalitet novoformiranog koštanog tkiva posle korišćenja autotransplantata kosti i β-trikalcijum fosfata, na modelu kritičnog defekta kalvarije kunića sa eksperimentalno izazvanim dijabetesom melitusom tipa I. **Metode**. U ovo istraživanje bilo je uključeno 8 kunića (soj Činičila), starosti 4 meseca, kod kojih je dijabetes melitus tipa I bio izazvan aloksanom. Kod svih životinja hirurški je urađen defekt kritične

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veličine na kosti kalvarije (prečnika 12 mm), koji je popunjen autotransplantatom kosti i β-trikalcijum fosfatom (n = 4) ili je ostavljen da spontano zarasta kao kontrolni defekt (n = 4). Posle 4 nedelje, sve životinje su bile žrtvovane i koštani uzorci uzeti za histološku i histomorfometrijsku analizu. Pored deskriptivne histološke analize, urađena je i kvantitativna analiza novoformirane kosti, vezivnog tkiva i materijala za koštanu regeneraciju. Statistička analiza vršena je primenom Friedman-ovog testa i *post hock* Vilkoksonovog neparametrijskog testa sa stepenom značajnosti od $p < 0{,}05$. **Rezultati.** Histološka analiza uzoraka kosti pokazala je prisustvo novoformirane kosti na periferiji defekta, dok je u centralom delu bilo prisutno vezivno tkivo, nezrelo koštano tkivo i dobro sjedinjeni neresorbovani materijal za regeneraciju kosti. Kontrolni uzorci nisu pokazali koštano zarastanje defekata. Značajno više novoformirane kosti bilo je prisutno u defektima regenerisanim autotransplantatom (53%) u poređenju sa kontrolnim defektima (7%), ($p < 0{,}000$) i defektima popunjenim β-trikalcijum fosfatom (30%), ($p < 0{,}030$). Takođe, značajna razlika uočena je i između grupe sa β-trikalcijum fosfatom i kontrolnim koštanim defektom ($p < 0{,}008$). **Zaključak.** Primena autotransplantata kosti značajno povećava uspešnost regeneracije kritičnih defekata kosti kalvarije kunića sa dijabetesom melitusom tipa I.

Ključne reči:
zečevi; dijabetes melitus; kost, regeneracija; transplantacija, autologna; beta-trikalcijum fosfat.

Introduction

Diabetes mellitus (DM) is a chronic disease characterized with hyperglycemia which leads to complications of micro- and macrovascular diseases of various organs, including bone [1]. The process of bone regeneration is particularly affected in DM [2, 3]. Various animal studies showed impaired bone healing process in diabetic animals compared with non-diabetic controls [4]. There are multiple mechanism trough which diabetes may affect bone, including the expression of genes that regulate osteoblast differentiation and expression of growth factors that promote bone formation [5]. Hyperglycemic status in diabetes leads to an increase of bone resorption and a decrease of bone turnover [6]. Moreover, the delay in cell proliferation and the decrease of collagen metabolism, are direct consequences of diabetes that severely affects the tissue repair process [7, 8].

Poor blood supply and deficiency in bone marrow make rabbit calvaria the appropriate model for evaluation of bone repair and regeneration potential of different materials [9]. The rabbit calvaria model has been used extensively for the study of different bone substitutes in bone regeneration experiments because anatomical and physiological characteristics are sufficiently close to humans [10]. Bone substitute materials for regeneration of intraosseous defects should be osteoinductive, to stimulate osteogenesis, and osteoconductive, to provide a scaffold for bone deposition [11]. Autogenous bone graft remains the gold standard among bone reconstruction materials, since these requirements are adequately fulfilled. However, limited supply of bone and donor site morbidity are problematic [12]. Therefore, synthetic material, such as β-tricalcium phosphate (β-TCP), has been used in bone regeneration because its mineral composition resembles that of human bone, providing osteoconductive and biodegradable activity [13].

Currently, there is few information in the literature regarding the influence of DM on bone regeneration in the specific condition of the critical sized defect (CSD) healing [14–17]. CSD has been originally defined by Schmitz and Hollinger [9] as the smallest size intra-osseous wound in a particular bone and species that will not heal spontaneously by bone tissue, or less than 10% of bone regeneration should be observed during the life time of the animal. Recently modified by Cooper et al. [18], CSD has been also defined as the smallest size of a defect that does not heal spontaneously when left untreated for a certain period of time, except if bone regeneration therapy is used.

Since the appropriate model for the investigation of bone regeneration is still recognized by calvarial bone, especially related to bicortical type, it was of interest to evaluate success of bone regeneration in diabetic conditions. Therefore, the purpose of this interim study was to determine the quality and quantity of new bone formation after the use of autogenous bone and β-TCP in the model of calvarial-critical sized defect in rabbits with induced DM type I.

Methods

Experimental design

Eight, 4-month-old giant Chincilla rabbits (Chinchilla Chinchilla), weighing 3.5−4.0 kg, were assigned to receive alloxan in order to experimentally induce DM type I. Animal selection, housing conditions and surgical protocol were approved by the Ethical Committees of the Faculty of Veterinary Medicine and Faculty of Dental Medicine, University of Belgrade (Certification No. 36/17) and all experimental procedures were performed in accordance with the European Union regulations on the use of animals in scientific purposes. After the induction of diabetes, two circular bilateral defects (12 mm) were created on each rabbit calvarium. In 4 animals, bone defects were grafted with the following material: β-TCP (RTR® Septodont, France) and autogenous bone graft (AUTO), collected from the area of surgical site. The other 4 animals, with two bilateral defects, served as no-filled control group. The defects were analyzed 4 weeks postoperatively, after sacrificing the animals.

Induction of diabetes

During experiment, all animals were housed in separate cages with free access to food and water *ad libitum*. Diabetes mellitus type I was induced in the experimental group of rabbits with a single dose of alloxan (100 mg/kg, diluted in physiological saline solution) applied into the marginal ear vein. A solution of alloxan was prepared immediately prior to injection. To prevent severe hypoglycemia during the critical first 24 h after injection, animals were provided with 5% glucose in their drinking water. The blood glucose level was monitored three times a day. A week after the administration of alloxan, rabbits were monitored for the development of hyperglycaemia, measured by the level of glucose in the blood taken from the marginal ear vein, for the confirmation of hyperglycemia with glucose level greater than 11 mmol/L.

Surgical procedure

The surgical procedure was done under general anesthesia which was induced by an intramuscular injection of a combination of tiletiamine and zolazepam 15 mg/kg. The surgical site was shaved and the skin washed with 70% ethanol and povidone iodine. Local anesthesia (2% lidocaine with 1/100 000 epinephrine) was administered to control bleeding of the operating area. Sagittal incision at the midline of the calvaria was made through the skin and the periosteum, from the frontal bone to the occipital bone. A full thickness flap was elevated and surgical sites were exposed. Two standardized, circular, transosseus defects (12 mm in diameter) were made in the mid-portion of each parietal bone, using a stainless-steel trephine bur with an outer diameter of 12 mm, under copious irrigation with sterile saline solution. Care was taken to avoid injury to the dura. In 4 animals, one defect was filled with β-TCP and the other one with autogenous particulate bone collected from the surgical area. In the control group (4 animals) bone defects were naturally filled with blood clot. After the surgery, soft tissue was repositioned and sutured in layers with resorbable suture material (Vicryl, Ethicon, Somerville, NJ, USA). In a 3-day postoperative period, an antibiotic (oxitetracycline 15 mg/kg) and analgesic (buforphanol 0.6 mg/kg) were administered intramuscularly to prevent infection and control pain. Four weeks after the surgery rabbits were sacrificed by the lethal dose of pentobarbital sodium, 100 mg/kg.

Histological and histomorphometric evaluation

Block samples that included original surgical defect and surrounding tissue were removed after animals sacrifice. The sections were rinsed in sterile saline and fixed in 10% buffered formalin for 10 days. All specimens were then decalcified in 10% ethylendiamintetra acetic acid (EDTA) and dehydrated in a graded series of increasing ethanol concentrations and then embedded in paraffin. Longitudinal, 5-μm thick sections were cut through the center of the circular calvarial defects. Five sections that contained the central portion were selected from each block, and stained with Goldner's Trichrome.

Histomorphometry was carried out using a light microscope (Olympus BX-51; Olympus, Tokyo, Japan). Image acquisition and stage movement were controlled by the newCAST stereological software package (Visiopharm Isofarm Integrator System, ver. 2.12.1.0; Visiopharm; Denmark – VIS) running on a personal computer. Volume density estimation was used to determine the precentage of newly formed bone, connective tissue and residual graft material.

Statistical analysis

Statistical analysis was performed using the software program (SPSS 10.0, SPSS, Chicago, IL). Histomorphometric records were presented as mean ± SD values expressed in percentages. To compare the differences among the three investigated groups, Friedman Test and *post hoc* Wilcoxon Singed Ranks Test were used. A significance for analysis was set to $p < 0.05$.

Results

During the postoperative period, healing was uneventful for all animals. No animals had been lost. There were no signs of graft exposure, allergic reaction or grafted area infection. The total number of analyzed defects was four per group, with the exclusion of previously created defects in control group.

Descriptive histology evaluation

Four weeks after the surgery, defects filled with β-TCP exhibited residual graft particles in the middle part of bone samples, mostly surrounded by the connective tissue. Newly formed bone was restricted to areas close to the margins of the surgical defect (Figure 1). The bony islands of new bone formation were found inside the porosity of β-TCP in a close proximity to the connective tissue and β-TCP. It was surrounded by a small number of osteoblasts and it was irregular woven type of bone (Figure 2).

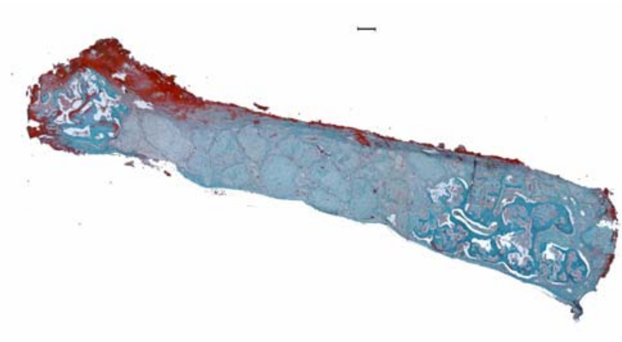

Fig. 1 – Photomicrograph of bone sample in its entirety obtained after 4 weeks of regeneration with beta-tricalcium phosphate (β-TCP). The sample contains new bone formation, residual graft and connective tissue (Goldner's Trichrome staining, bar – 400 μm, ×25 magnification).

Defects filled with autogenous graft showed areas of newly formed bone with thin immature trabeculae and wide intratrabecular spaces with collagen fibers (Figure 3). Autogenous grafts were well incorporated in new bone. The newly formed bone was a woven type. Part of autogenous graft fragments were recognized by the absence of osteocytes

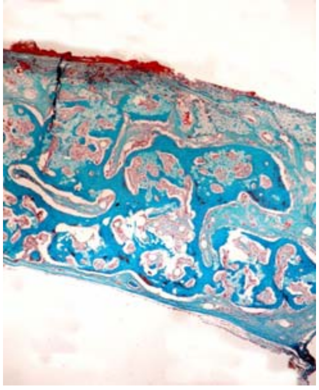

Fig. 2 − Histologic specimen from beta-tricalcium phosphate (β-TCP) grafted calvarial defects showing areas of woven bone with lining osteoblasts and small amounts of marrow spaces. The residual β-TCP is mostly incorporated inside woven and marrow bone (Goldner's Trichrome staining, bar – 200 µm, ×40 magnification).

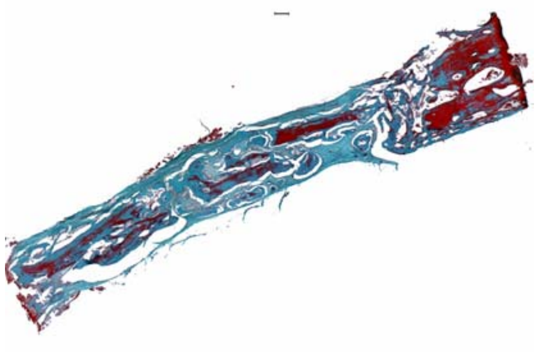

Fig. 3 − Photomicrograph of bone sample in its entirety obtained after 4 weeks of regeneration with autogenous bone graft. The sample contains newly formed mineralized and marrow bone, particulate bone graft and connective tissue (Goldner's Trichrome staining, bar – 400 µm, ×25 magnification).

in lacunas. Osteoblasts were detected on the surface of new bone (Figure 4).

In the control group, minimal amounts of new bone tissue were formed at the defect margins while no bone bridging was seen (Figure 5). The greater parts of the defects were filled with thin fibrous connective tissue layers with newly formed bone islands in the middle of bone defects (Figure 6).

Histomorphometry

Histomorphometric analysis is summarized in Table 1. The percentage of newly formed bone was significantly higher in the AUTO and β-TCP group than in the control group. A significant difference in new bone formation was detected between the AUTO and β-TCP. In the control group, the percentage of connective tissue was significantly higher comparing to the AUTO and β-TCP group. Analysis of regenerated tissue inside the treated bone defects showed significantly more new bone and grafts *vs* connective tissue in the AUTO, while this difference was seen only for new bone *vs* connective tissue in the β-TCP group (Table 1).

Discussion

Generally, the bone repair process is particularly affected in diabetic individuals. In the field of the effective bone

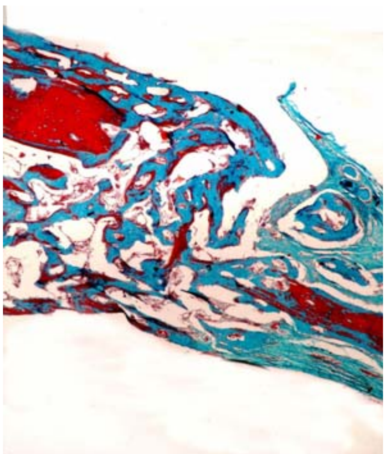

Fig. 4 − Histologic specimen from autogenous bone grafted calvarial defects showed trabecular connectivity of new bone formation associated with residual particle of autogenous graft through entire bone defect. The bone was woven type which surrounded marrow spaces and residual grafts (Goldner's Trichrome staining, bar – 200 µm, ×40 magnification).

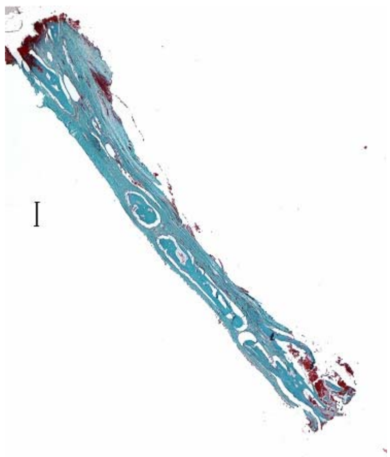

Fig. 5 − Photomicrograph of bone sample in its entirety obtained after 4 weeks of healing of unfilled control defect. The sample contains newly formed bone at the defect margin, while connective tissue filled central part of specimen (Goldner's Trichrome staining, bar – 400 µm, ×25 magnification).

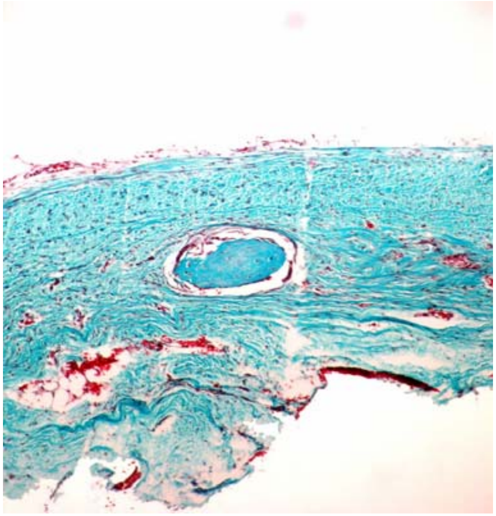

Fig. 6 − Histologic specimen of unfilled calvarial defects represents areas of fibrovascular connective tissue and reduced islands of new bone formation (Goldner's Trichrome staining, bar – 200 µm, ×40 magnification).

Table 1

Histomorphometric results (%) of critical sized defect (CSD) healing in diabetic rabbits

Spacimen	Auto	β-TCP	Control	*p*
New bone	53.15 ± 10.82	30.15 ± 5.71	7.32 ± 8.40	0.030[a] 0.008[b] 0.000[c]
Connective tissue	14.41 ± 7.24	22.39 ± 11.57	92.68 ± 5.63	0.000[b] 0.000[c]
Graft 32.44 ± 9.17	32.44 ± 9.17	47.46 ± 6.92	0	ns
p	0.004[d] 0.021[e]	0.042[e]	0.000[d]	

Values were given as mean ± SD. Statistical significance between groups (Friedman Test, *post-hoc* Wilcoxon Singed Ranks Test): [a]AUTO *vs* β−TCP; [b]β−TCP *vs* the control group; [c]AUTO *vs* the control group. Statistical significance inside the groups (Friedman Test, *post-hoc* Wilcoxon Singed Ranks Test): [d]new bone *vs* connective tissue; [e]connective tissue *vs* graft. β−TCP – beta tricalciumphosphate; Auto – autogenous bone graft.

healing, the success rate of bone regeneration should be analyzed after the use of different therapeutic approaches to improve the process of bone healing in DM. In the present study, we assessed the effectiveness of autogenous bone graft and synthetic osteoconductive bone substitute β-TCP in bone regeneration using critical-sized 12-mm defects in the calvarium of diabetic rabbits.

Histomorphometric analysis of this study showed that the treatment of critical bone defects in DM using the AUTO and β-TCP elicited more new bone formation compared to the control groups, which normally healed spontaneously with connective tissue. However, the percentage of newly formed bone was higher in the AUTO group than in the β-TCP group, probably due to osteogenesis that was taking place in the AUTO. This result is consistent with the results of Esteves et al.[16], who showed that the bone repair of surgical defects filled with bone autografts occurred earlier than that of surgical defects filled with blood clot in both control and diabetic groups. It is likely that such result is due to osteinduction effect of autogenous graft with increased regenerative potential of different growth factors and their cellular activity. In accordance with that, Mariano et al.[17] showed that the use of platelet-rich plasma in bone regeneration, as a method which express a high concentration of growth factors, significantly increased the quantity and quality of bone healing in calvarial critical-sized defects of diabetic rats.

Beside positive histomorphometric evidence of regenerative therapy in diabetic condition, histological view illustrated that the newly formed bone was well incorporated into the both autogenous bone particles and β-TCP material, suggesting the mechanisms of bone regeneration based on its osteoconductive property. This finding is consistent with the previously published data [19–21] indicating that β-TCP behaves as an osteoconductive material, which acts as a scaffold for the cell in-growth, growth factor production inside the material and subsequent increased in bone formation. Furthermore, Murai et al.[13] reported that osteoblasts and osteoid formation were present on the surfaces of β-TCP particles what was also seen in the presented histologic analysis. However, it was observed that the major part of the healing process came from the periosteal and the defect edges in the treated and untreated defects, which agrees with the previously published data in healthy animal models [22, 23]. That observation may provide evidence for the regenerative potential in the diabetic bone, which occurred using the same mechanism of healing in healthy and DM, beginning from the margin of rest bone. Nevertheless, the amount of regenerated bone in DM may be dependent on the different proliferation rate of varying types of cells affected by DM, local trauma and the size of bone defects. Moreover, data obtained from the study of Retzepi et al. [24] demonstrated, that *de novo* alveolar bone formation can be achieved in experimentally induced DM with application of the guided bone regeneration (GBR) technique, the major strategy conducted to improve bone healing.

Concerning the fact that DM may impair the process of bone regeneration, probable related to changes in bone metabolism [25], it is interesting to note that the amount of connective tissue in the AUTO and β-TCP-treated bone defects did not exceed the quantity of connective tissue expected during bone healing in healthy individuals, especially in the early phase of healing, what was the scope of this study. In relation to this evidence, other authors have reported similar amount of connective tissue in healthy rabbits when bone defects were treated with autogenous bone grafts (6-mm calvarial defects) [23] or in unfilled defects (6-mm control tibia defects) [26]. Apparently, DM could not have any influence in the quantity of regenerated tissue, but have changed the quality of newly formed bone in the time-related manner. To support that evidence, Vieira et al.[14] showed that bone repair was slower in the diabetic group than in the control and diabetic-polytetrafluoroethylene (PTFE) membrane treated groups.

Conclusion

In the present study on the rabbit grafting model, the effective bone regeneration of critical bone defects was significantly obtained by the use of autogenous bone grafts. Further studies, which would include healthy individuals and different healing intervals, could probably clarify the mechanisms of bone healing and differences between autogenous bone grafts and other bone substitutes.

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