The Effects of Insulin and Strontium Ranelate on Guided Bone Regeneration in Diabetic Rats

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The aim of this study was to investigate the effect of insulin and strontium ranelate treatment on guided bone regeneration in diabetic rats. This study was carried out on 30 adult Wistar rats with an average weight of 250-300 grams. The animals underwent a unilateral osteotomy of the left proximal tibia followed by bone augmentation with collagenized porcine bone xenografts (Osteobiol® mp3, Tecnoss Dental s.r.l., Torino, Italy) and then were randomized into five groups: healthy (H), diabetes (D), diabetes with insuline (DI), diabetes with strontium ranelate (DS) and diabetes with insuline and strontium ranelate (DIS). Histomorphometric analysis was performed at the end of this study.

Keywords: bone graft, collagenized porcine xenografts, guided bone regeneration, experimental diabetes, insulin, strontium ranelate

The incidence of diabetes has grown worldwide, due to aging in population, high prevalence of obesity and lack of physical activity [1, 2]. Diabetes can be associated with cardiovascular, renal, eye diseases and also, it can be correlated with a decreased tissue regeneration capacity [3, 4]. One of the consequences and signs of diabetes is the reduced repairing and bone formation capacity [5].

Bone augmentation techniques or guided bone regeneration techniques (GBR), used in oral implantology are well known for improving the bone quantity [6]. These procedures have been frequently used by surgeons, due to efficiency and low risk. Although the relationship between diabetes and dental implants osseointegration has been analyzed, little information is known about the impact of diabetes on guided bone regeneration.

Studies that used animal models demonstrated that diabetes determines a reduced bone formation, which includes osteopenia and reduced healing capacity in fractures [7]. Recent studies that investigated the influence of diabetes on bone healing used experimental models of tibia and femur osteotomy [8, 9], and they proved that diabetes delays fracture healing and that insulin therapy reverses this effect [10]. The changes that appear in bone development in the augmented space, in diabetic patients is little known.

Nowadays, different types of biomaterials had been developed in biomedical industry and can be used in GBR techniques. These biomaterials are in a continuous development, increasing their biocompatibility and offering the best substrate for dental implants insertion.

The ideal biomaterial for GBR has to be biologic for the organism, which depends of its biocompatibility and the absence of toxicity [11]. These biomaterials can be synthetic (alloplastic graft), can be taken from an individual of the same specie (allogenic graft) or from other species (xenograft).

GBR with materials of porcine origin has been intense studied, due to the similar human genotype, and the results prove their osteoconductive effect [12]. The regeneration process starts with a phase of resorbtion of the inserted material, followed by bleeding, inflammation and, finally, bone formation [13].

Local inflammatory mediators, such as cytokines (IL-1, IL6, TNF-α) are produced by macrophages after phagocytosis and osteoclasts are recruited at the bone-implant interface [14].

Diabetes can influence bone turn-over and the quality of bone tissue, therefore it can influence bone regeneration [15]. The signs and symptoms of failure in bone augmentation apparently are not seen until late stages. Developing pharmacological strategies that can reduce progression of bone resorbtion is essential. Time is also a key factor of bone regeneration outcomes. Pharmaceutical agents can be used to improve bone quality [16, 17].

Strontium ranelate is an antiosteoporotic agent that can improve guided bone regeneration and dental implants osseointegration [13]. The benefits of strontium ranelate have been reported in different animal models: prevents bone loss using two mechanisms, maintain bone formation at a high level and inhibit bone resorption [18]. These *in vivo results* are correlated with *in vitro* data where it is shown that strontium ranelate reduced bone resorption with the help of osteoclasts, and augmented bone formation with the help of osteoblasts [19].

Moreover, strontium ranelate can improve bone biochemical and structural properties [20]. These data suggest that the antiosteoporotic agent might have the potential to improve bone structure and the process of bone regeneration.

Maxillary osteonecrosis has not been associated with strontium ranelate treatment, in comparison with bisphosphonates. Recently, it has been proven that there is a connection between femur fractures and bisphosphonates [21]. As a consequence, there is a justified demand of an alternative to bisphosphonates to improve bone quality in patients that have diseases that influence bone structure.

The purpose of this study was to evaluate the effects of insulin and strontium ranelate on guided bone regeneration in diabetic rats.

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Experimental part

Methods and materials

Study design, ethics, and diabetes induction

Thirty Wistar male rats, with the medium weight 350 – 400 g, were acclimatized to the study conditions for a period of 14 days before the surgery. The animals were housed individually at 25°C. They were fed with a laboratory diet containing 15% casein, 0.8% phosphorus, 1% calcium and 5% fat throughout the experimental period. Demineralized water was available ad libitum.

The procedures were performed without stress and pain for the animals, and their sacrifice was performed under anesthesia. The protocol of this study was approved by the Local Ethics Committee. All the experimental procedures used in this study were according to the international ethical laws.

The subjects were divided in 5 group : Group H, with healthy subjects; Group D, with experimentally induced diabetes; Group DI, with experimentally induced diabetes treated with insulin, daily; Group DS, with experimentally induced diabetes, treated with strontium ranelate 5 days/ week; Group DIS, with experimentally induced diabetes treated with insulin, daily and with strontium ranelate 5 days/week.

Diabetes is obtained by intraperitoneally administration of spretozotocin *(Sigma-Aldrich, Dorset, UK)* 40 mg/kg dissolved in sodium citrate 10 mM (*p*H = 4.5) at a dose of 40 mg/kg of body weight. The subjects were diagnosed with diabetes, if the values of glycemia were over 200 mg/ dL.

The subjects that had their general status altered were sacrificed along the study, the rest were sacrificed at 12 weeks after streptozotocin administration. Before inducing diabetes, blood was taken from tail vein to evaluate serum glucose concentration. During this study, the weight and serum glucose concentration were monitored periodically.

Surgical procedures

The surgical procedures were performed 7 days following diabetes induction. Animals were anesthetized by intramuscular injection of ketamine 40 mg/kg and pentobarbital solution 20 mg/kg. An incision was made to gain access to proximal metaphysis of the left tibia. Subsequently, muscular-periosteal flaps were elevated and the proximal metaphysis of the left tibia were exposed. A 1 mm diameter hole was drilled, in which we applied cortical - lamellar bone *(Osteobiol® mp3, Tecnoss Dental s.r.l., Torino, Italy)* and a collagen membrane (*OsteoBiol® Lamina; Tecnoss Dental s.r.l., Torino, Italy).* The bone graft is a mixture of cortical – lamellar bone (90%), of porcine origin (600-1000µ granulometry) combined with a collagen gel (10%) (*OsteoBiol® Gel 0, Tecnoss Dental s.r.l., Torino, Italy).*

The skin was sutured using a 5-0 absorbable suture (*Vicryl 5-0; Ethicon GmbH, Norderstedt, Germany).* Postoperative analgesia was ensured by butorphanol (0.05 mg/kg).

Insulin and strontium ranelate therapy

The subjects from group DI and DIS received by subcutaneous injection, insulin at a rate of 1 IU/day, 12 weeks. Group H, D, DS received instead of insulin a similar dose of sterile saline solution.

For a period of 12 weeks following surgery, subjects from group DS and DIS were treated with strontium ranelate (*Osseor®, Les Laboratoires Servier Industrie, France*) by gavage at a dose 625 mg/kg, 5 days/week. This dose leads to a serum strontium ranelate concentration close to the human exposure after therapeutic dose of 2g/day [22]. Group H, D, DI received 0.5% carboxymethylcellulose aqueous solution by gavage, 5 days/week with volumes corresponding to those administered in the strontium ranelate treated group.

Sample processing

After 12 weeks, the animals were euthanized by abdominal injection of ketamine 40 mg/kg and pentobarbital solution 20 mg/kg. When we observed the absence of vital signs the animals were dissected for left tibia harvesting.

The bone specimens were fixed in 10% neutral buffered formalin (24-48h) and then decalcified in Bouin solution (72%). After tissue processing, the specimens were embedded into parafin blocks *(Leica TP1020, Leica Microsystems GmbH, Germany).* The parafin blocks were cut into 5µm sections using Microtome SLEE CUT 6062 *(SLEE Medical GmbH, Germany).* The sliced sections were deparaffinized and colored with Masson tricrom techniques.

Histological examination and histomorphometric analysis

The qualitative histologic analysis was realized on colored sections, using a Leica DM 750 microscope *(Leica Microsystems GmbH, Germany)* connected to a digital camera Leica ICC50 HD *(Leica Microsystems GmbH, Germany).* The histomorphometric study was performed using an image analysis system Leica Application Suit (LAS) version 4.2 (oct/2012). This study performed the following measurements: percentage of newly formed bone, residual graft material and ne-mineralized connective tissue.

Statistics

Variation analysis was performed using SPSS 19.0 for Windows (SPSS Inc, Chicago, IL, United States of America) and identified the significant differences for average and SD. Then the average and SD of these values were calculated for each variable. A significant difference of the compared data was assumed if the probability was less than .05. Individual differences and graft positioning were not considered significant.

Results and discussions

The evaluation results of serum glucose level

Before surgery, serum glucose concentrations were evaluated at 72 h and 1 week after diabetes induction. After the diagnose of diabetes was confirmed, the surgery was performed.

Postoperative, serum glucose concentrations were evaluated weekly, with a glucometer. Insulin treatment significantly reduced the high values of serum glucose concentrations (groups DI and DIS). Serum glucose concentration have a tendency of getting higher in subjects without insulin treatment. These information indicate that the experimental conditions were safe (fig. 1).

Results of histological and histomorphometric analysis group H

In the implant area, the histological examination reveals resorption of the entire graft and young bone formation. There can be observed newly formed haversian channels, limited by concentric bony lamellae (fig. 2). The histomorphometric analysis reveals that newly formed bone represents 82.3 ± 1.5%, residual graft represents 6.8 ± 2.3 % and connective tissue represents 10.9 ± 1.4 %.

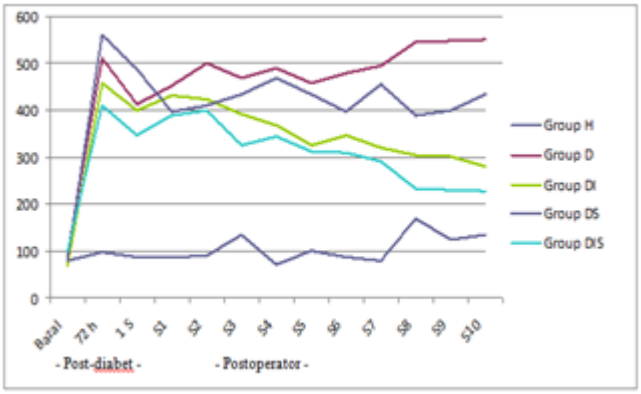

Fig. 1. Dinamic evaluation of serum glucose levels before diabetes induction, at 72h and 1 week after streptozotocine injection and weekly postoperative

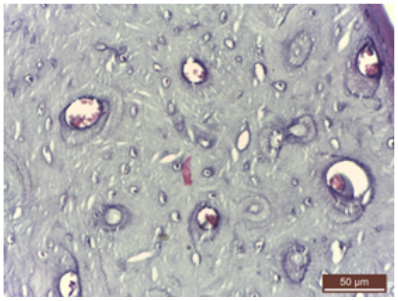

Fig. 2. Histological section of the augmented site – group H. (*HE staining*, 400X)

Results of histological and histomorphometric analysis group D

The graft is almost completely non - resorbed. The adjacent area has inflammatory character containing macrophages with vacuolated cytoplasm, differentiated osteoblasts, neo-formation vessels and rare connective fibers (fig. 3). The histomorphometric analysis reveals that newly formed bone represents 12.9 ± 1.7%, residual graft represents 67.9 ± 2.8 % and connective tissue represents 19.2 ± 1.2 %.

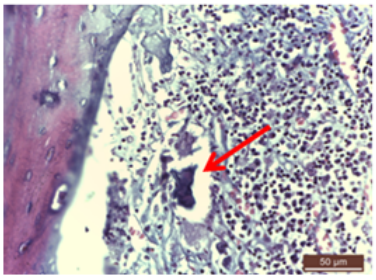

Fig. 3. Histological section of the augmented site – group D, marked the presence of residual graft material. (*HE staining*, 400X)

Results of histological and histomorphometric analysis group DI

The primitive bone callus is formed of cancellous bone that contains in its spaces conjunctive-vascular buds derived from bone marrow, periosteum and vessels of haversian systems. The newly formed bone is poorly mineralized and has a reduced osteogenic activity (fig. 4). The histomorphometric analysis reveals that newly formed bone represents 67.3 ± 2.4%, residual graft represents 12.3 ± 3.2 %, and connective tissue represents 20.4 ± 2.1 %.

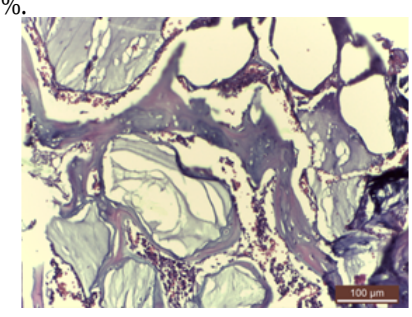

Fig. 4. Histological section of the augmented site – group DI. (*HE staining*, 200X)

Results of histological and histomorphometric analysis group DS

The histological evaluation of the place where the graft was inserted reveals almost completely resorption of the graft. The graft was replaced by a conjunctive area formed of bands that penetrate the bone, highlighting a process of osteogenesis with late onset and with poor osteoblasts differentiation. We can identify capillary neoformation and fibroblasts (fig. 5). The histomorphometric analysis reveals that newly formed bone represents 68.9 ± 1.8%, residual graft represents 9.7 ± 1.3 %, and connective tissue represents 21.4 ± 3.1 %.

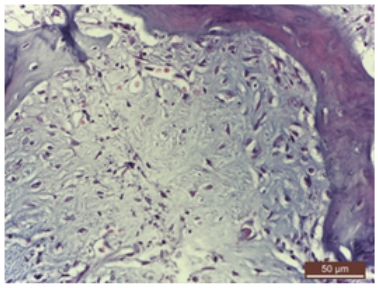

Fig. 5. Histological section of the augmented site – group DS. (*HE staining*, 400X).

Results of histological and histomorphometric analysis group DIS

Histologically, it can be observed advanced bone regeneration and cancellous bone transformation in compact, lamellar bone (fig. 6). The histomorphometric analysis reveals that newly formed bone represents 79.8 ± 1.7%, residual graft represents 8.7 ± 2.4 % and connective tissue represents 11.5 ± 1.3 %.

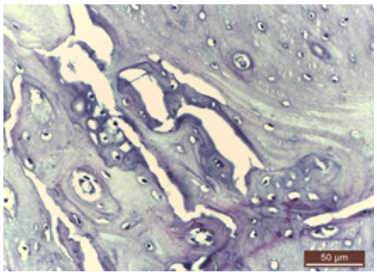

Fig. 6. Histological section of the augmented site – group DIS. (*HE staining*, 400X)

For many years, bone substitution was studied a lot, due to bone augmentation necessities in oral and maxillofacial surgery. The current demand in clinical dentistry is for materials that can accelerate bone regeneration processes.

There are three mechanisms that govern the success in bone regeneration: osteogenesis, osteoinduction and osteoconduction [23]. The ideal substitute that combines the three features is autologous bone, *the gold standard* in regeneration [24]. Studies that use blocks of autologous bone [25], indicate a low rate of morbidity. Patients accuse moderate pain until the third day, postoperative. Despite that, surgeons look for alternatives to harvesting autologous bone in order to eliminate unwanted postoperative phases [26, 27].

The efficiency of porcine xenografts, and their high rate of osteoconductivity was demonstrated in different studies [28]. Our study confirms the biocompatibility of xenograft *(Osteobiol® mp3, Tecnoss Dental s.r.l., Torino, Italy)*, [29], which is a mixture of cortical – lamellar bone (90%), of porcine origin (600-1000µ granulometry) combined with a collagen gel (10%) (*OsteoBiol® Gel 0, Tecnoss Dental s.r.l., Torino, Italy)*, in healthy and diabetic subjects with controlled status of serum glucose concentration.

Among the diabetic subjects , the best osseointegration was seen in the group that was under insulin treatment and benefited of strontium ranelate (*Osseor®, Les*

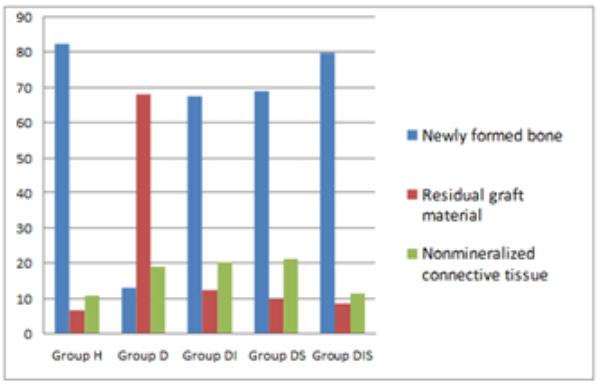

Fig. 7. Quantitative distribution of newly formed bone, residual graft material and nonmineralized connective tissue for each group

Laboratoires Servier Industrie, France) administration for improving bone quality. It has been previously reported that strontium ranelate positively influences biomaterial properties of bone in a rat model [20], and it is therefore plausible that this treatment is able to improve this tissue quality in the region of dental implants.

It has also been reported that strontium ranelate treatment is able to stimulate preosteoblastic and osteoblastic cell replication and synthesis of collagenous matrix [30, 31], and to promote the differentiation of osteoblast precursors into mature osteoblasts [32]. This effect of strontium ranelate may induce an ingrowth of bone into the etched surface of the implants, an important factor for their integration, as well as an improvement of the microarchitecture around the implant.

In the current study, strontium ranelate increased bone regeneration capacity and newly formed volume when compared with controls. Strontium ranelate improves bone density in the region where the porcine graft was inserted. This is the first time when there are reported benefits of strontium ranelate therapy on bone regeneration capacity, bone density and newly formed bone volume in diabetic patients.

The initial phase of remodeling, after graft implantation, is characterised through an increase in osteoclast activity [26]. Strontium ranelate is known to decrease markers of bone resorption in human studies, reduce osteoclastic bone resorption [19] and decrease osteoclast formation [32], as well as induce osteoclast apoptosis in vitro [33]. It is likely, therefore, that this action of strontium ranelate also contributes to the improved osseointegration seen in the present study.

The results of the study demonstrated that the porcine graft acts like a matrix for the bony cells and has an osteoconductive capacity [34]. The presence of collagen in each particle gives the hygroscopic features that facilitates the sequential mixing with pure collagen. Collagen plays a fundamental role in the osteoconduction process, which acts as a valid substrate for platelet activation and aggregation.

Also, it attracts and stimulates the mesenchymal stem cells present in the bone marrow and can augment the proliferation of osteoblasts [35], two or three times more [36].

Previous studies with these types of grafts [11], showed the ability of osteoblast for proliferation, differentiation and matrix mineralization, a fact which is confirmed by our study.

In addition, this study investigated the regeneration process of porcine grafts in diabetic patients.

This study showed that uncontrolled diabetes has a negative impact on the quality of guided bone regeneration. Insulin and strontium ranelate therapy can increase the volume of newly formed bone in diabetic patients.

A study [28], compares porcine xenograft with and without added collagen and found no significant differences in the process of resorption. According to the authors, the mixture of collagen and porcine bone particles facilitates clinical manipulation of the graft, but did not affect bone responses to the material. Studies on porcine xenografts that were covered by membranes, [37], in order to preserve the bone socket, showed also a small rate of residual bone graft (24.5%) at four months after the insertion of the implant.

However, a recent study [11] using porcine bone as augmentation material showed that after 4 to 6 months, no evidence of graft resorption could be observed, only a few osteoclasts were observed in the samples examined at 6 months.

Conclusions

The results of this study suggest that this xenograft *(Osteobiol® mp3, Tecnoss Dental s.r.l., Torino, Italy)*, which is a mixture of cortico-cancellous bone (90%) of porcine origin, with particle size of 600-1000 microns, appropriately combined with collagen gel (10%) (*OsteoBiol® Gel 0 Tecnoss Dental SRL, Torino, Italy*), may be a biocompatible material, causing only a minor inflammatory response in the early stage.

In addition, the material has osteoconductive properties, acting as a matrix for bone cells, which leads to a gradual increase in bone growth in the xenograft. We also observed the replacement of osteoid by adipose tissue and hematopoietic bone marrow, which indicates the ability of this material to resorb partially and sequentially.

This biomaterial can be considered a satisfactory substitute for bone tissue, a material that does not influence the normal reparative processes of bone. Bone regeneration with this type of material took place in optimum conditions both for both healthy subjects and those with diabetes who have had a controlled glycemic.

In contrast, subjects who did not receive insulin treatment showed poor results of bone regeneration capacity at the end. In addition, subjects with diabetes who received insulin and strontium ranelate (*Osseor®, Les Laboratoires Servier Industrie, France*) have shown significant results comparable to the healthy group.

These results, which need to be confirmed by clinical studies may support the potential benefits of strontium ranelate in oral and maxillofacial surgery.

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