



Peri-implant defect regeneration in the diabetic pig: A preclinical study



Cornelius von Wilmsky^a, Karl Andreas Schlegel^a, Christoph Baran^a, Emeka Nkenke^b,
Friedrich Wilhelm Neukam^a, Tobias Moest^{a,*}

^a Department of Oral and Maxillofacial Surgery, University of Erlangen-Nuremberg, Glückstrasse 11, 91054 Erlangen, Germany

^b Department of Cranio-, Maxillofacial and Oral Surgery, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria

ARTICLE INFO

Article history:

Paper received 6 January 2016

Accepted 5 April 2016

Available online 13 April 2016

Keywords:

Diabetes

Bone regeneration

Osseointegration

Implant

ABSTRACT

Objectives: The study aims to establish a peri-implant dehiscence-type bone defect in a diabetic animal model of human bone repair and to quantify the influence of diabetes on peri-implant bone regeneration. **Material and methods:** Experimental diabetes was induced in three domestic pigs by streptozotocin. Three animals served as healthy controls. After 12 months four standardized peri-implant dehiscence bone defects were surgically created in the ramus mandibulae. The animals were sacrificed after 90 days. Samples were histologically analyzed to quantify new bone height (NBH), bone-to-implant-contact (BIC), area of newly formed bone (NFB), bone-density (BD), and bone mineralization (BM) in the prepared defect (-D) and in a local control region (-L).

Results: After 90 days, diabetic animals revealed a significantly lower BIC ($p = 0.037$) and BD ($p = 0.041$) in the defect area (-D). NBH and BM-D differences within the groups were not significant ($p > 0.05$). Significant more NFB was measured in the healthy control group ($p = 0.046$). In the region of local bone BIC-L was significant less in the diabetic group ($p = 0.028$). In the local control region BD-L and BM-L was lower in the diabetic group compared to the healthy control animals ($p > 0.05$).

Conclusion: Histological evidence indicates impaired peri-implant defect regeneration in a diabetic animal model.

© 2016 European Association for Cranio-Maxillo-Facial Surgery. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Diabetes mellitus (DM) is one of the most prevalent endocrine diseases and the third most common cause of disability and morbidity in the Western world. It has been predicted that the number of diabetics will increase up to 366 million worldwide by the year 2030 (Wild et al., 2004). DM is a chronic metabolic disorder and negatively influences bone metabolism, e.g. bone mineral density, bone matrix protein expression, and osteoblast activity (Von Wilmsky et al., 2011; Retzepi and Donos, 2010). Poor osseous healing characteristics, with an increased risk of osteopenia and osteoporosis as well as an impaired bone regeneration potential, can result from DM. Thus, significantly lower survival rates were observed for dental implants in a hyperglycemic, compromised wound-healing situation where DM represents a

relative contraindication for implant dentistry (Buser et al., 2000; Neukam and Esser, 2000; Marchand et al., 2012). Dental implant osseointegration and implant survival rates comparable to those of patients without diabetes can only be accomplished in diabetic patients with good metabolic control and a serum glycemic level as well as HbA1c in the normal range (Javed and Romanos, 2009). It has been shown that DM is directly associated with an increased prevalence and extent of periodontitis as well as increased bone loss compared with non-diabetic patients (Kaur et al., 2009). Thus, peri-implant bone loss due to infections, especially in combination with a compromised wound-healing condition, is not a rare phenomenon anymore and affects an increasing number of patients.

Peri-implant infections are often associated with the development of bone dehiscence defects. The loss of the bone contours dramatically reduces the regeneration potential for these defects.

Understanding the biology and physiology of peri-implant bone dehiscence defects requires a systematic investigation with an animal model that has anatomical, physiological, and metabolic similarities to the human organism. This allows the experimental

* Corresponding author. Tel.: +49 9131 8534201; fax: +49 9131 8534219.

E-mail address: tobias_moest@uk-erlangen.de (T. Moest).

results to be subsequently extrapolated to the clinical situation. Furthermore, a wound-healing situation that is unaffected by external factors is a prerequisite for evaluating potential regenerative or augmentative treatment applications. In intraoral animal models, the surgical site preparation goes hand in hand with several factors that could negatively influence peri-implant bone regeneration or the treatment application. Potential factors are disturbances of wound healing caused by mastication, colonization of supra-crestal implant parts with the oral flora, plaque accumulation, and disproportionate loading of intraoral implant parts.

To avoid this problem, we established an extraoral dehiscence-type defect model to describe the peri-implant bone regeneration in a diabetically compromised wound-healing situation reliably. Further, we present an innovative evaluation method to quantify newly formed bone in a standardized dehiscence defect model precisely.

The aim of the present study was furthermore to examine the effects of DM on bone regeneration and bone quality. Therefore, we compared *de novo* bone formation (DNBF), bone height (BH), bone density (BD), percent of bone-to-implant contact (BIC), and the proportion of non-mineralized bone area (NMBA) in a standardized peri-implant dehiscence-type bone defect model in the mandible of healthy and diabetic domestic pigs 90 days after the surgical procedures.

Two hypotheses were tested:

- First, we hypothesized that DM has a negative effect on bone formation and implant osseointegration in comparison to the healthy control group.
- Secondly, we assumed that diminished bone regeneration is accompanied by lower bone density and a lower proportion of non-mineralized bone in the diabetic group.

2. Material and methods

2.1. Animals

The domestic pig (*Sus scrofa domestica*) is a valuable model in biomedical research due to anatomical, physiological, and metabolic similarities to humans. It is an established model for bone regeneration (Moest et al., 2014; Schmitt et al., 2014; Von Wilmowsky et al., 2010a; Schlegel et al., 2003, 2004, 2006). Regarding the induction of metabolic disorders, the changes in soft and hard tissue formation showed that pigs are suitable experimental animals to investigate bone formation and osseointegration of enossal implants in a diabetic metabolism (Von Wilmowsky et al., 2011, 2010b). The Committee for Animal Research, Franconia, Germany, approved the study design (Approval No. 54-2531-25/07). The study included 3 healthy control and 3 diabetic experimental female pigs (18 ± 4 months old, mean body weight 95 kg). All animals were kept under circadian day and night rhythm in an open enclosure of 6 m² at an ambient room temperature of 18 ± 1 °C. The pigs received standardized pig mast fodder and water *ad libitum*. All animal handling was in compliance with the ARRIVE guidelines.

2.2. Study design

The study was performed in two phases. In the first phase, diabetes was induced in the domestic pigs. Internal parameters were measured, and bone as well as soft tissue biopsies were taken after 0, 6 and 12 months. These were evaluated qualitatively and quantitatively as described and shown previously (Von Wilmowsky et al., 2011, 2010b). After the internal clinical parameters of the

American Diabetes Association for the definition of diabetes mellitus were fulfilled and statistically significant pathological changes in the hard and soft tissue were measurable, which could be confirmed after 12 months, the implants were placed. Buccal standardized peri-implant dehiscence-type bone defects were surgically created following implant site preparation on one side of the lower jaw (4 implants per jaw). The animals were sacrificed after 90 days.

2.3. Induction of the experimental diabetes

A streptozotocin (STZ) solution (Zanosar[®], Pharmacia & Upjohn Company, Kalamazoo, MI, USA) at a concentration of 100 mg/ml was administered at 90 mg/kg body weight into an ear vein 4–5 h after the morning feeding, following sedation of the animals with Azaperone[®] (1 mg/kg body weight) and Midazolam[®] (1 mg/kg body weight). The dosage is based on the experiences of Marshall (1979). To avoid nausea after STZ application, each animal was injected with MCP-ratiopharm[®] (Ratiopharm GmbH, Ulm, Germany) intravenously. To avoid hypoglycemia, food was provided *ad libitum*. Blood glucose levels were checked twice a week over the study period and an intra-venous glucose tolerance test (IvGTT) was carried out. The results are shown in Table 1 and have been displayed previously (Von Wilmowsky et al., 2011) (Table 1). All animals fulfilled the criteria of the World Health Organization and the American Diabetes Association for diabetes. The precise description of the experimental diabetes induction and the verification of histopathological changes in hard and soft tissue were described in the protocol of the establishment of diabetic pigs (Von Wilmowsky et al., 2010b).

2.4. Anesthesia protocol

Before the surgical procedures, the pigs fasted overnight and were handled according to the following anesthesia protocol. After an intramuscular injection of medetomidine (Domitor[®], Pfizer, Karlsruhe, Germany), anesthesia was initiated with an intravenous administration of Ketamine HCl (Ketavet[®]; Ratiopharm, Ulm, Germany). To maintain hydration, animals received a constant infusion of lactated Ringer's solution while anesthetized. Perioperative antibiotics was administered 1 h preoperatively and for 2 d postoperatively to reduce the risk of infection (Streptomycin[®], 0.5 g/day, Grunenthal, Stolberg, Germany). A veterinarian performed the anesthesia and the permanent perioperative control of vital signs. Postoperative pain control was achieved by administering analgesics (0.05 mg/kg every 12 h) (Temgesic[®], Böhringer Mannheim GmbH, Mannheim, Germany) for 3 days following surgery.

2.5. Surgical procedure

12 months after the administration of STZ, 4 implants were placed in the ramus mandibulae in the caudal part of the lower jaw (Fig. 1a). After application of a local anesthetic (Ultracain DS forte[®], Hoechst GmbH, Frankfurt a. M., Germany), an extraoral incision was performed and the soft tissue was mobilized. After mobilization of the periosteum, surgical implant sites were prepared at a distance of 10 mm apart, using a low-trauma surgical technique under copious irrigation with sterile 0.9% Ringer's solution. Following implant site preparation, standardized peri-implant dehiscence-type bone defects, 4 mm in height from the crestal bone, 3 mm in depth from the surface of the buccal bone, and 3 mm in width mesiodistally, were created with the help of a straight fissure carbide bur. The defect sizes were standardized using a periodontal probe (PCP12[®], Hu-Friedy Co., Chicago, IL, USA). After the osteotomy procedures, the 24 Straumann Bone Level[®] implants

Table 1

Blood glucose level of the diabetic and healthy control animals.

Time (mo)	Blood glucose level (mg/dl)		Time (IvGTT) (min)	IvGTT – blood glucose level (mg/dl)	
	Control	Diabetic		Control	Diabetic
0	70.6 ± 11.9	68.9 ± 7.8	Pre-infusion	99.4 ± 57.5	283.6 ± 43.5*
6	64.8 ± 6.2	202.6 ± 50.5*	10	259.2 ± 71.68	582.6 ± 38.9*
12	59.3 ± 8.5	174.9 ± 52.1*	30	175.4 ± 34.4	527.8 ± 45.5*
			60	125.1 ± 27.2	435.1 ± 26.3*

A statistically significant (* $p < 0.05$) difference is visible after 6 and 12 months. Results of the intra-venous glucose tolerance test (IvGTT), performed 4 weeks after induction of the diabetes, are shown on the right side of the table (Von Wilmsowsky et al., 2011).

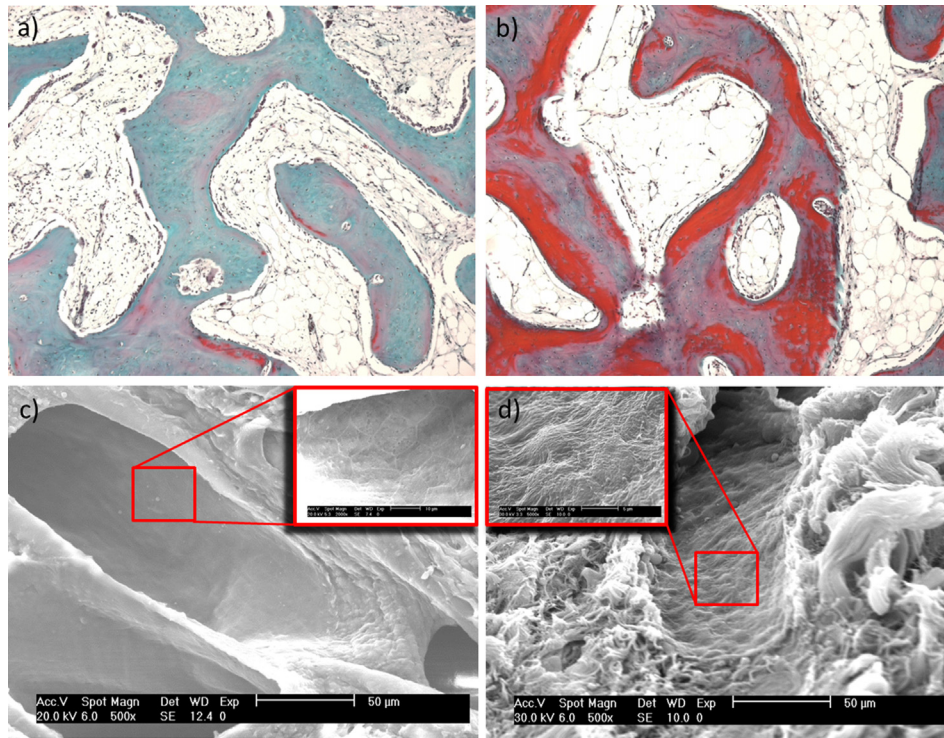


Fig. 1. Masson's trichrome stain staining shows the qualitative differences between the bone biopsies of the healthy control (a) and diabetic animals (b) 12 months after induction of the diabetes. The bone samples of the diabetic group show irregularly shaped, thicker bone trabeculae and expanded mineralization zones, visualized as reddish-stained bone areas, compared to the control group. Representative SEM pictures of the skin vasculatures of the healthy control animals showed a regularly shaped smooth endothelium (c). In contrast, the skin vasculatures of the diabetic animals showed a distinctly altered endothelium with an irregular surface and detached endothelial cells (d).

(Straumann GmbH, Freiburg, Germany), with a diameter of 4.1 mm and a length of 12 mm, were inserted with good clinical primary stability (Fig. 1b). The correct depth was reached when the implant shoulder had exactly the same height as the adjacent bone. A bio-resorbable membrane was applied to eliminate the invasion of osteogenic precursor cells from the periosteum, which affects osseous regeneration (Bio-Gide®, Geistlich Biomaterials, Baden-Baden, Germany) (Fig. 1c). The periosteum and skin were sutured in two layers (Vicryl 1-0 and Vicryl 3-0, Ethicon GmbH & Co. KG, Norderstedt, Germany). The same experienced operator performed all surgical procedures.

2.6. Animal sacrifice

After the designated healing period of 90 days, the animals were sacrificed. The pigs were sedated by an intramuscular injection of Azaperone® (1 mg/kg) and Midazolam® (1 mg/kg). Euthanasia was performed by an intravascular injection of 20% pentobarbital solution into an ear vein until cardiac arrest. During the experimental period, all pigs remained in good health. At the time of sacrifice, no

macroscopic signs of inflammation or adverse tissue reactions were apparent. The lower jaws were immediately removed and stored at -80°C .

2.7. Retrieval of specimen

To identify the precise implant localization, CT analyses (Department of Radiology, University of Erlangen-Nuremberg; Director: Prof. Dr. M. Uder) were performed on all jaws in the frontal and transversal plane. Afterwards, the specimens were dissected at the lower jaws and subsequently fixed by immersion in 1.4% paraformaldehyde at room temperature to render the organic matrix insoluble. The specimens were dehydrated in an ascending alcohol series at room temperature (Shandon Citadel 1000, Shandon GmbH, Frankfurt, Germany). Xylol was used as an intermediate fixative. Technovit 9100 (Heraeus Kulzer, Kulzer Division, Wertheim Germany) was used for embedding. To avoid any negative influence of polymerization heat, the polymerization was performed in a cold atmosphere (4°C). After 20 h, the specimens were completely polymerized.

2.8. Histological preparation

For histological preparation, the embedded bone samples were cut in the middle through the implants and the prepared defect. The specimens were ground into thin sections (30 μm) using a precision saw and a special grinding machine (both Exakt Apparatebau GmbH, Norderstedt, Germany), high-gloss polished, and transferred to 10% H_2O_2 solution for 5 min. After being rinsed under cold running water, the specimens were stained for 10 min with Toluidine blue O (Sigma–Aldrich Chemie GmbH, Munich, Germany). Excess stain was removed by rinsing the specimens under running water again. The stained specimens were examined by digitalizing the specimens with a slide scanner (Axio Scan.Z1, Carl Zeiss Microscopy GmbH, Goettingen, Germany). With the

Toluidine-Blue O stain, mineralized, laminated tissue appears uncolored to pale blue, whereas cells, cell cores, osteoid fringes, and soft tissue are colored in different shades of blue. Early wound-healing areas were metachromatic red/violet and the un-calcified matrix was dark blue as suggested by Donath and Breuner (1982) (Fig. 2a and b). The pictures were stored in .tif format.

2.9. Histomorphometrical analyses

One experienced investigator, masked to the experimental conditions, performed the histomorphometrical analyses with Bioquant Osteo[®] software 2013 v13.2.6 (Bioquant Image Analysis Corporation, Nashville, TN, USA) as well as the microscopic observations. The software distinguishes between different tissue fractions by their individual color spectra, marks them in a specific color, and assigns a metric variable that allows calculation of different bone indices.

The landmarks and parameters we chose for analyses are related to the method of Schwarz et al. (2007) (Fig. 3). In order to quantify parameters of newly formed bone, the landmarks of bone crest (BC) and defect bottom (DB) were defined first.

BC was equivalent to the implant shoulder since bone level implants were used, and DB represents the preparation margin of the peri-implant defect. The section from BC and DB characterized the defect length (DL). Within the DL, the distance from the most coronal level of newly formed bone that was in contact with the implant to the DB described the new bone height (NBH), which was stated in percent (%). Within the section of NBH, the percentage of direct contact between mineralized bone and the implant surface, the bone-to-implant contact defect (BIC-D), was evaluated. In order to evaluate newly formed bone (NFB), the bone volume within the defect was studied (Fig. 4a and c). An additional slide 2 mm adjacent to the implant was prepared (Fig. 4b). The additional slide, whose volume was set as 100%, was superimposed on the slide of the defect (Fig. 4d). The ratio of defect regeneration was then determined in percent. Within the volume of regenerated bone, the bone density of defect (BD-D) (osteoid plus mineralized bone proportion) and bone mineralization of defect (BM-D) (mineralized bone portion) were evaluated and also stated in percent. The bone density of local bone (BD-L), bone mineralization of local bone (BM-L) as well as bone-to-implant contact in local bone (BIC-L) were evaluated on the opposite side of the implant (Fig. 3).

2.10. Statistical analysis

Statistical analyses were performed using SPSS version 22.0 for Windows (SPSS Inc., Chicago, USA). Mean and median values as well as standard deviations among animals were calculated for each group. To determine distribution, the data rows were examined using the Kolmogorov–Smirnov test. The Mann–Whitney U test was used for between-group comparison. A *p*-value [*] of less than 0.05 was considered to be significant.

3. Results

3.1. General results

Regarding the clinical situation of the animals during the study period, we found the animals in a stable condition without signs of any physical impairment. The diabetes appeared to be in a stable condition, even without insulin therapy. The wound healing seemed to be prolonged in the diabetic animals but was unremarkable. An apparent diabetes according to the American Diabetes Association (ADA) criteria was verifiable in all animals of the experimental group (American Diabetes, 2015).

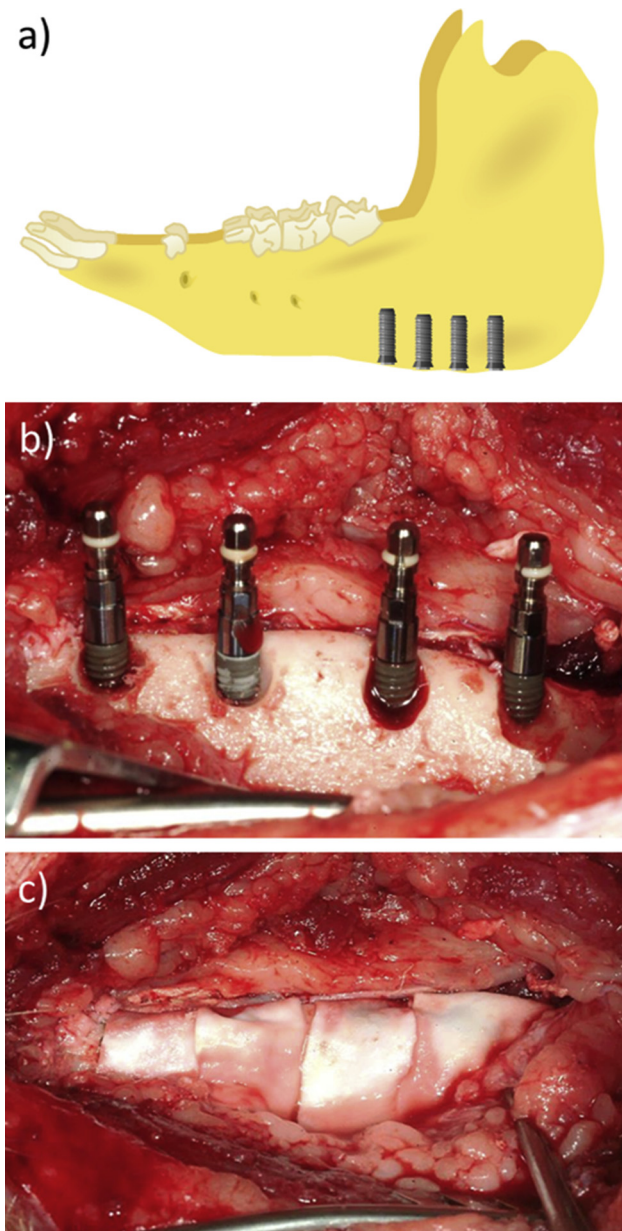


Fig. 2. The experimental setup is shown. Four implants were placed on the caudal side of the lower jaw by an extraoral approach to the ramus mandibulae (a). A standardized periimplant dehiscence bone defect (height: 3 mm, width: 3 mm) was surgically created at every implant at the buccal side (b). The defects were then covered with a collagenous membrane to shield them from the periosteum (c).

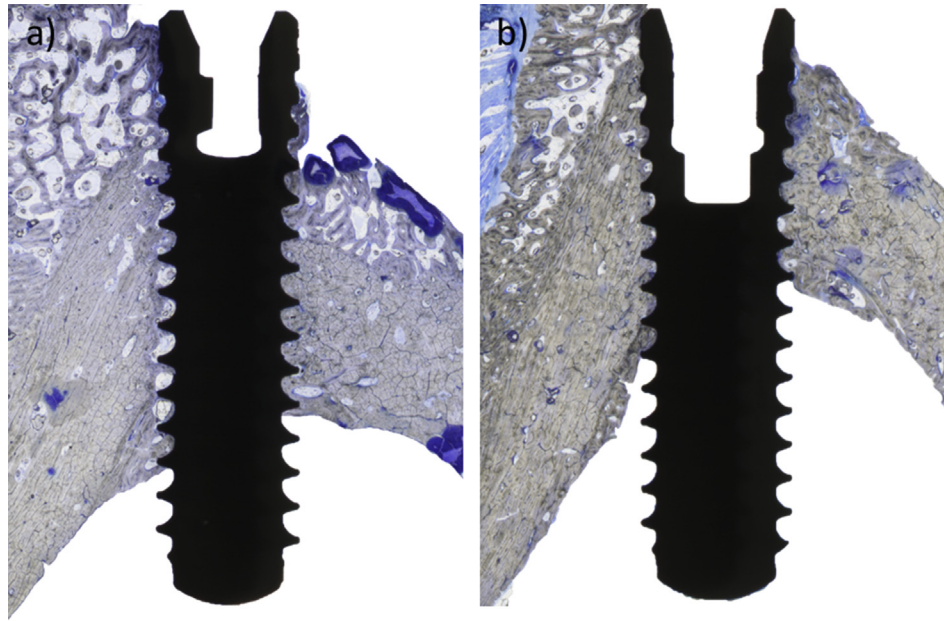


Fig. 3. Representative histological sections show the bone regeneration in a diabetic animal (a) and in an animal of the healthy control group (b) after 90 days.

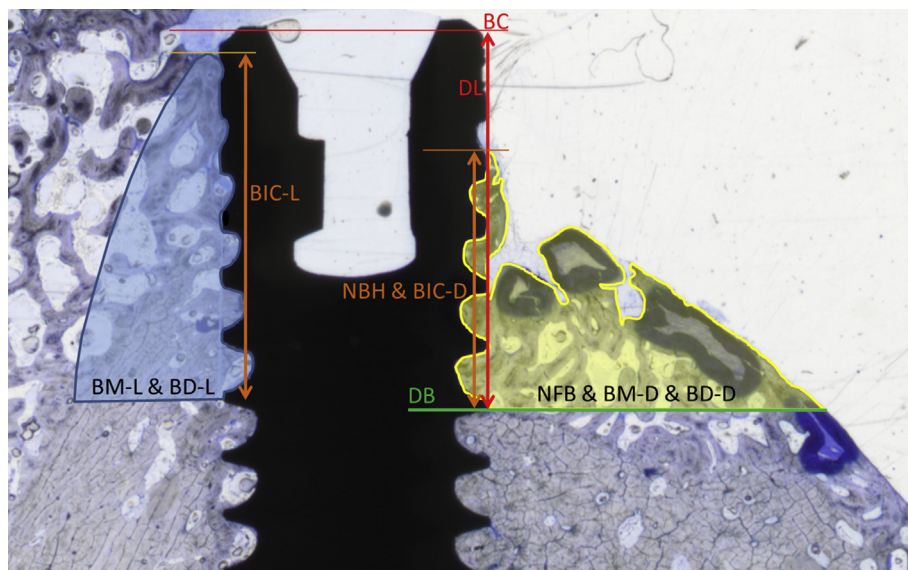


Fig. 4. Landmarks for histomorphometrical analysis: the defect length (DL) represents the distance from the bottom of the defect (DB) to the implant shoulder (BC). The new bone height (NBH) is defined by the distance from the bottom of the defect (DB) to the most coronal level of bone in contact with the implant. Bone-to-implant contact was evaluated in the area between the DB and the most coronal level of bone adjacent to the implant on the defect side (BIC-D) as well as on the local bone side (BIC-L). Newly formed bone (NFB) represents the bone volume in the defect area. Bone mineralization and bone density were evaluated in the defect area (BM-D & BD-D) as well as in the local bone (BML and BD-L).

Table 2

Means, standard deviations, and statistical significance of the histomorphometric results after 90 days.

	Diabetic group	Healthy group	<i>p</i> Value
New bone height (NBH) [%]	66.88 (±19.81)	75.80 (±10.94)	0.109
Newly formed bone (NFB) [%]	57.62 (±17.11)	73.76 (±13.64)	0.046
Bone-to-implant contact defect (BIC-D) [%]	40.40 (±20.11)	67.47 (±8.37)	0.037
Bone-to-implant contact local bone (BIC-L) [%]	41.28 (±15.78)	62.88 (±13.35)	0.028
Bone density defect (BD-D) [%]	57.32 (±10.52)	81.28 (±17.22)	0.041
Bone density local bone (BD-L) [%]	67.66 (±13.53)	75.27 (±12.21)	0.522
Bone mineralization defect (BM-D) [%]	42.39 (±16.57)	49.60 (±6.95)	0.078
Bone mineralization local bone (BM-L) [%]	42.23 (±16.19)	45.62 (±7.95)	0.282

3.2. Histomorphometric analysis

The mean values and standard deviations of the measurement parameters are presented in Table 2. The *p*-value shown in Table 2 represents the statistical significance between the diabetic and the healthy group after 90 days.

Evaluation of the new bone height (NBH) showed no significant difference between both groups ($p = 0.109$), whereas the quantification of newly formed bone (NFB) showed significant more bone quantity in the healthy control group ($p = 0.046$). Furthermore, there was less bone in contact with the implant on the defect side (bone-to-implant contact defect (BIC-D)) in the diabetic group compared to the healthy animals. The same effect was measurable on the other side of the implant. In the non-defect area, there was less bone-to-implant contact in the diabetic group compared with the control group (bone-to-implant contact of local bone (BIC-L)). There was 21.6% less bone in contact with the implant, which was statistically significant ($p = 0.028$).

The bone density in the defect (bone density defect (BD-D)) area was significantly lower ($p = 0.041$) in the diabetic animals (57.32 ± 10.52) compared to the control group (81.28 ± 17.22). The same was measurable for the bone density in the local bone (bone density of local bone (BD-L)). The difference between both groups (diabetic: 67.66 ± 13.53 vs healthy: 75.27 ± 12.21) was not statistically significant ($p = 0.522$).

Histological evaluation of the specimen revealed that the mineralization rate of the newly formed bone (bone mineralization defect (BM-D)) in the defect area of the diabetic group was lower in comparison to the healthy group. A statistical significance was not present ($p = 0.078$). In addition, the mineralization rate of the local bone (bone mineralization of local bone (BM-L)) measured on the opposite site of the defect area (lingual) showed a difference, but no significant difference was measurable ($p = 0.282$).

Evaluating the paraffin cross sections for pathological changes in bone structure, the bone of the healthy control animal presented a regular structure with physiological bone remodeling areas after 12 months (Fig. 5a). Examination of the bone samples of the diabetic animals after 12 months revealed wider and irregularly shaped

bone trabecels with expanded mineralization zones (Fig. 5b). Biopsies from the ear lobe presented blood vessels in regular conditions with a smooth, correctly shaped endothelium without any signs of disconnection in the healthy control group (Fig. 5c). In comparison, the vessels of the diabetic animals showed pathological changes characterized by a distinctly altered endothelium after 12 months (Fig. 5d).

4. Discussion

It was aim of this preclinical study to establish a peri-implant dehiscence-type bone defect in a diabetic animal model of human bone repair. Furthermore, we systematically evaluated the influence of a diabetically compromised wound-healing situation on peri-implant bone regeneration. The surgical creation of standardized dehiscence-type bone defects is a commonly used procedure to evaluate peri-implant defect regeneration. The used technique is well cited for dogs (Oh et al., 2003; Casati et al., 2002; Schwarz et al., 2007; Hockers et al., 1999; Becker et al., 1990). In our study, the domestic pig was the animal of choice due to a bone regeneration rate ($1.2\text{--}1.5\text{ }\mu\text{m/day}$) that is comparable to that of humans ($1.0\text{--}1.5\text{ }\mu\text{m/day}$). The results with pigs are thus better applicable to the clinical situation in humans, since the bone remodeling turnover rate in dogs has been reported to be approximately four times higher than that of humans (Draper, 1994; Eitel et al., 1981).

For defect localization and implant placement, we chose the ramus mandibulae region. The ramus mandibulae is easy to access surgically from the outside and offers enough space and bone for the insertion of several implants. Defect preparation and implant insertion were performed in one surgical stage, in order to avoid wound dehiscence and infection due to an additional surgical intervention. Furthermore, standardized defect preparation after implant placement could be problematic, due to drill-implant contact, which destroys modified implant surfaces and distributes titanium particles in the wound area.

The defect localization in the ramus mandibulae region offers ideal conditions for osseous regeneration, since wound healing is

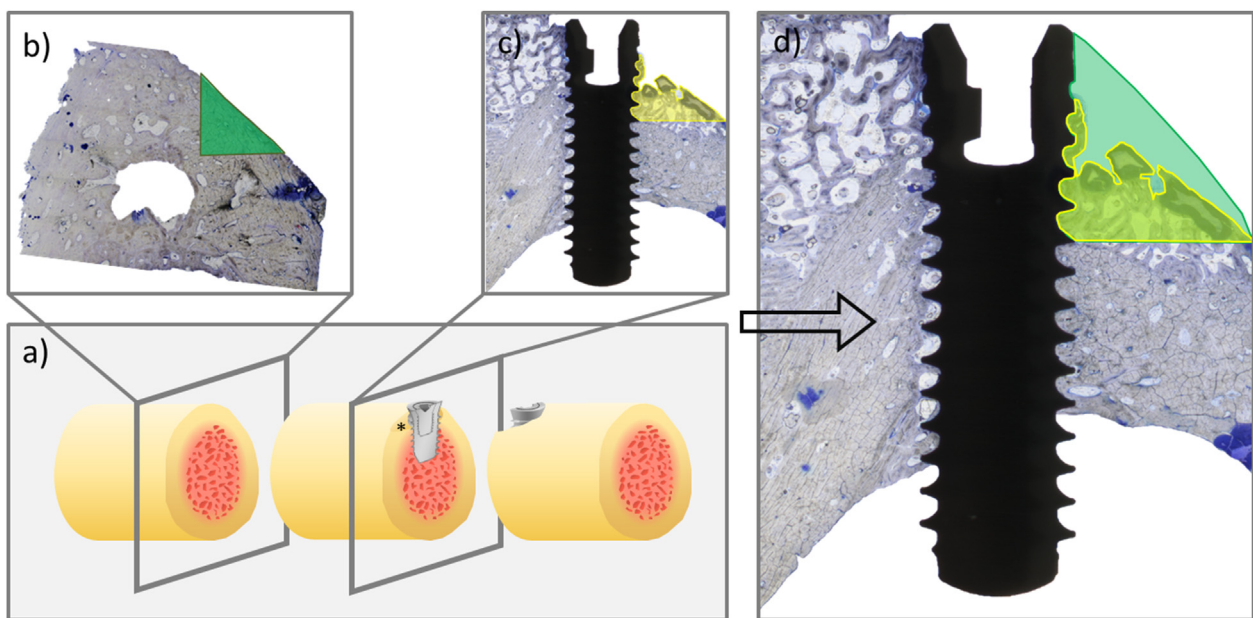


Fig. 5. Experimental setting for the evaluation of the new bone formation in the peri-implant defect (asterisk) (a). Histological sections were prepared from the defect (c) and distal to the defect (b). By overlaying the two histological sections (d), it is possible to see the newly formed bone (yellow) in relation to the original boundaries (green) of the defect.

not affected by mastification or the colonization of oral bacteria. A bioresorbable membrane was applied to eliminate biological effects of the periosteum on the osseous regeneration. Several studies have shown that the periosteum contains a high number of osteogenic precursor cells, causing higher rates of bone volume and accelerated defect regeneration that affects the results (Agata et al., 2007). In the context of guided bone regeneration (GBR), membranes should maintain its barrier function until the provisional matrix and woven bone are present, which means that barrier duration is considered to be necessary for 7–14 days. Membranes with a longer barrier function were established, however, studies have proven that Geistlich Bio-Gide® supports bone regeneration on an equivalent level, even with a reduction of complications during wound healing (Tal et al., 2008; Becker et al., 2009; Schwarz et al., 2008). The bilayered structure of the membrane leads to effective prevention of soft invasion as well as an increased invasion of bone forming cells and angiogenesis (Schwarz et al., 2008).

In order to quantify peri-implant defect regeneration as precisely as possible, we modified the evaluation technique that was published by Schwarz (2007). Thus, the *de novo* formed bone volume can be put into relation to the bone defect shape dimensions. Small bone defect shape dimensions go hand in hand with small volumes of regenerated bone. Thus we established a new evaluation technique that is independent from dehiscence defect configuration. With the aim of quantifying defect regeneration in percent, we defined the original defect volume by preparing an additional slide adjacent to the implant. Compared to the small size of the alveolar ridge of dogs, the straight and homologous anatomical shape of the ramus mandibulae in pigs allows an additional slide preparation 1–2 mm adjacent to the created peri-implant defect. To define the volume, the slices are superimposed and the volume can be determined precisely.

In order to understand the influence of DM on the osseointegration of dental implants, the progress of peri-implant *de novo* bone formation must be viewed as a complex series of discrete and overlapping stages (Schwartz and Boyan, 1994; Davies, 1998, 2003). Bone formation involves a cascade of various cellular and extracellular events, which can each be negatively influenced by DM (Retzepi and Donos, 2010).

Due to this complexity, compromised wound healing may have a major impact on the physiology of bone formation. Thus, the influence of a metabolic disorder, e.g. diabetes mellitus, on peri-implant defect regeneration can be shown by our results.

The histomorphologic evaluations clearly show an impaired hard tissue regeneration situation that is characterized by a lower volume of *de novo* formed bone in the standardized peri-implant defect in comparison to the healthy control group. In addition to impaired bone quantity, the bone quality of newly formed bone is lower compared to the healthy controls. Bone mineralization, bone density, as well as the proportion of non-mineralized bone surface area (osteoid) were lower in the regenerated bone volume of diabetic animals.

Our results of deficient bone formation and bone mineralization in an experimental diabetic animal model can be confirmed by histological and histomorphological studies in *in vivo* osteotomy and fracture models. Studies have shown a suppressed bone formation with less developed marrow spaces, suppressed cellular activity, and vascularization, whereas bone formation was inhibited by 40% in the diabetic group, irrespective of the defect size in a calvarial defect animal model (Santana et al., 2003). Furthermore, it could be shown that especially in larger defect sizes (≥ 1.2 mm), the poorly controlled glycemic status correlates with severe materialization disorders as indicated by the suppressed expression of dynamic histomorphometric parameters, such as the apposition, formation, and timing of mineralization (Follak et al., 2004).

In addition to impaired bone quality and quantity, a diminished bone-to-implant contact was measurable in the area of *de novo* bone formation as well as in the percentage of the implant in contact with the local bone. Reduced bone-to-implant contact is a sign of compromised hard tissue regeneration, which is effected by DM (Von Wilmsowky et al., 2011; Schlegel et al., 2013). A reduction of peri-implant bone density has also been reported in literature and confirms our results (Gerritsen et al., 2000).

Reasons for diminished bone regeneration with diabetes may stem from deficits in the recruitment and differentiation of mesenchymal stem cells in the osteoblastic lineage, since suppressed mRNA expressions of the transcription factors *Dlx5* and *Runx-2*, which are required for the acquisition of an osteoblastic phenotype, has been observed (Lu et al., 2003; McCabe, 2007).

Another plausible mechanism for inferior bone regeneration is the reduced proliferation of osteoblastic cells. Studies confirm decreased cellularity with an up to 50% reduction in immunohistochemical indices of the cell proliferation rate under diabetic metabolism circumstances (Beam et al., 2002; Gebauer et al., 2002; Gandhi et al., 2005; Tyndall et al., 2003). The evaluation of gene expression thereby demonstrated down-regulation of pathways related to cell division, energy production, and osteogenesis during bone healing (Retzepi and Donos, 2010). In poorly controlled diabetic situations, suppressed mRNA and protein expression levels of critical growth factors, e.g. fibroblast growth factor (FGF) (Kawaguchi et al., 1994), platelet derived growth factor (PDGF), transforming growth factor $\beta 1$ (TGF- $\beta 1$), insulin-like growth factor I (IGF-I), and vascular endothelial growth factor (VEGF), are hypothesized as responsible reasons (Gandhi et al., 2005). In addition to a diminished cell number, osteoblasts are characterized by a reduced activity in the presence of diabetes (Lu et al., 2003). Studies showed reduced mRNA expression of the bone matrix protein osteocalcin and collagen I (Lu et al., 2003), which correlates to reports of a significantly reduced content of total collagen (Macey et al., 1989). A pathogenic mechanism, e.g. including modulation of the redox state, increases the activity of the polyol pathway, the activation of the protein kinase C pathway, and the non-enzymatic glycosylation of key proteins, e.g. collagen type I and IGF-I, and may be responsible for impaired osteoblastic cell activity due to prolonged hyperglycemia (McCabe, 2007). Furthermore, the accumulation of advanced glycosylation end products (AGEs) in the osseous tissue, as a result of non-enzymatic glycosylation, has been implicated in the pathogenesis of diminished bone formation (Santana et al., 2003). AGEs negatively influence growth, differentiation, and activity of osteoblastic cells, due to their interaction with specific osteoblastic receptors (McCarthy et al., 2001). In addition, AGEs lead to modification of collagen I, which impairs the integrin-mediated adhesion of osteoblasts on the extracellular matrix and therefore compromises normal bone forming activity (McCarthy et al., 2001; 2004).

Reduced bone formation volume and bone quality in diabetes is the result of the suppressed differentiation, proliferation, and bone-forming capacity of osteoblastic as well as mesenchymal stem cells due to increased blood glucose levels.

In this context, we confirm our hypotheses of the negative effects of DM on bone formation with diminished bone density and reduced bone-to-implant contact compared to healthy controls.

5. Conclusion

We were able to establish a peri-implant dehiscence-type bone defect model in a compromised wound-healing situation. Histological evidence indicates impaired bone and peri-implant defect regeneration in a diabetic animal model.

Conflict of interest

The authors declare that there are no conflicts of interest related to this study.

Funding

This project was founded by the ELAN-Fond of the University of Erlangen (ELAN-07.02.26.1), the ITI Foundation/Research Committee (ITI-5052007) and the IZKF (Interdisciplinary Centre of Clinical Research of the University of Erlangen-Nuremberg, 20051011).

Acknowledgments

The work of Susanne Schönherr, Andrea Krautheim-Zenk and Heidemarie Heider is highly appreciated. Furthermore, we would like to thank Elfriede Tissera of the Department of Manufacturing Technology for the electron microscopy scanning pictures and Sandra Romeis (Department of Method and Product Development [GfK SE], Nuremberg, Germany) for the statistical advisory.

References

- Agata H, Asahina I, Yamazaki Y, Uchida M, Shinohara Y, Honda MJ, et al: Effective bone engineering with periosteum-derived cells. *J Dent Res* 86: 79–83, 2007
- American Diabetes A: 2. Classification and diagnosis of diabetes. *Diabetes Care* 38: S8–S16, 2015
- Beam HA, Parsons JR, Lin SS: The effects of blood glucose control upon fracture healing in the BB Wistar rat with diabetes mellitus. *J Orthop Res* 20: 1210–1216, 2002
- Becker J, Al-Nawas B, Klein MO, Schliephake H, Terheyden H, Schwarz F: Use of a new cross-linked collagen membrane for the treatment of dehiscence-type defects at titanium implants: a prospective, randomized-controlled double-blinded clinical multicenter study. *Clin Oral Implants Res* 20: 742–749, 2009
- Becker W, Becker BE, Handlesman M, Celletti R, Ochsenbein C, Hardwick R, et al: Bone formation at dehiscent dental implant sites treated with implant augmentation material: a pilot study in dogs. *Int J Periodontics Restorative Dent* 10: 92–101, 1990
- Buser D, Von Arx T, Ten Bruggenkate C, Weingart D: Basic surgical principles with ITI implants. *Clin Oral Implants Res* 11(Suppl. 1): 59–68, 2000
- Casati MZ, Sallum EA, Nociti Jr FH, Caffesse RG, Sallum AW: Enamel matrix derivative and bone healing after guided bone regeneration in dehiscence-type defects around implants. A histomorphometric study in dogs. *J Periodontol* 73: 789–796, 2002
- Davies JE: Mechanisms of endosseous integration. *Int J Prosthodont* 11: 391–401, 1998
- Davies JE: Understanding peri-implant endosseous healing. *J Dent Educ* 67: 932–949, 2003
- Donath K, Breuner G: A method for the study of undecalcified bones and teeth with attached soft tissues. The Sage-Schliff (sawing and grinding) technique. *J Oral Pathol* 11: 318–326, 1982
- Draper HH: Bone loss in animals. *Adv Nutr Res* 9: 53–71, 1994
- Eitel F, Seiler H, Schweiberer L: [Morphologic examination of animal-experiment results: comparison with regeneration of the human bone-structure. I. Research methods (author's transl)]. *Unfallheilkunde* 84: 250–254, 1981
- Follak N, Klotting I, Wolf E, Merk H: Histomorphometric evaluation of the influence of the diabetic metabolic state on bone defect healing depending on the defect size in spontaneously diabetic BB/OK rats. *Bone* 35: 144–152, 2004
- Gandhi A, Beam HA, O'Connor JP, Parsons JR, Lin SS: The effects of local insulin delivery on diabetic fracture healing. *Bone* 37: 482–490, 2005
- Gebauer GP, Lin SS, Beam HA, Vieira P, Parsons JR: Low-intensity pulsed ultrasound increases the fracture callus strength in diabetic BB Wistar rats but does not affect cellular proliferation. *J Orthop Res* 20: 587–592, 2002
- Gerritsen M, Lutterman JA, Jansen JA: Wound healing around bone-anchored percutaneous devices in experimental diabetes mellitus. *J Biomed Mater Res* 53: 702–709, 2000
- Hockers T, Abensur D, Valentini P, Légrand R, Hammerle CH: The combined use of bioresorbable membranes and xenografts or autografts in the treatment of bone defects around implants. A study in beagle dogs. *Clin Oral Implants Res* 10: 487–498, 1999
- Javed F, Romanos GE: Impact of diabetes mellitus and glycemic control on the osseointegration of dental implants: a systematic literature review. *J Periodontol* 80: 1719–1730, 2009
- Kaur G, Holtfreter B, Rathmann W, Schwahn C, Wallaschofski H, Schipf S, et al: Association between type 1 and type 2 diabetes with periodontal disease and tooth loss. *J Clin Periodontol* 36: 765–774, 2009
- Kawaguchi H, Kurokawa T, Hanada K, Hiyama Y, Tamura M, Ogata E, et al: Stimulation of fracture repair by recombinant human basic fibroblast growth factor in normal and streptozotocin-diabetic rats. *Endocrinology* 135: 774–781, 1994
- Lu H, Kraut D, Gerstenfeld LC, Graves DT: Diabetes interferes with the bone formation by affecting the expression of transcription factors that regulate osteoblast differentiation. *Endocrinology* 144: 346–352, 2003
- Macey LR, Kana SM, Jingushi S, Terek RM, Borretos J, Bolander ME: Defects of early fracture-healing in experimental diabetes. *J Bone Joint Surg Am* 71: 722–733, 1989
- Marchand F, Raskin A, Dionnes-Hornes A, Barry T, Dubois N, Valero R, et al: Dental implants and diabetes: conditions for success. *Diabetes Metab* 38: 14–19, 2012
- Marshall M: Induction of chronic diabetes by streptozotocin in the miniature pig. *Res Exp Med (Berl)* 175: 187–196, 1979
- Mccabe LR: Understanding the pathology and mechanisms of type I diabetic bone loss. *J Cell Biochem* 102: 1343–1357, 2007
- Mccarthy AD, Etcheverry SB, Bruzzone L, Lettieri G, Barrio DA, Cortizo AM: Non-enzymatic glycosylation of a type I collagen matrix: effects on osteoblastic development and oxidative stress. *BMC Cell Biol* 2: 16, 2001
- Mccarthy AD, Uemura T, Etcheverry SB, Cortizo AM: Advanced glycation end-products interfere with integrin-mediated osteoblastic attachment to a type-I collagen matrix. *Int J Biochem Cell Biol* 36: 840–848, 2004
- Moest T, Koehler F, Prechtel C, Schmitt C, Watzek G, Schlegel KA: Bone formation in peri-implant defects grafted with microparticles: a pilot animal experimental study. *J Clin Periodontol* 41: 990–998, 2014
- Neukam FW, Esser E: [Implantology]. *Mund Kiefer Gesichtschir* 4(Suppl. 1): S249–S256, 2000
- Oh TJ, Meraw SJ, Lee EJ, Giannobile WV, Wang HL: Comparative analysis of collagen membranes for the treatment of implant dehiscence defects. *Clin Oral Implants Res* 14: 80–90, 2003
- Retzeppi M, Donos N: The effect of diabetes mellitus on osseous healing. *Clin Oral Implants Res* 21: 673–681, 2010
- Santana RB, Xu L, Chase HB, Amar S, Graves DT, Trackman PC: A role for advanced glycation end products in diminished bone healing in type 1 diabetes. *Diabetes* 52: 1502–1510, 2003
- Schlegel KA, Donath K, Rupprecht S, Falk S, Zimmermann R, Felszeghy E, et al: De novo bone formation using bovine collagen and platelet-rich plasma. *Biomaterials* 25: 5387–5393, 2004
- Schlegel KA, Kloss FR, Schultze-Mosgau S, Neukam FW, Wiltfang J: [Osseous defect regeneration using autogenous bone alone or combined with Biogran or Aligpore with and without added thrombocytes. A microradiologic evaluation]. *Mund Kiefer Gesichtschir* 7: 112–118, 2003
- Schlegel KA, Lang FJ, Donath K, Kulow JT, Wiltfang J: The monocortical critical size bone defect as an alternative experimental model in testing bone substitute materials. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 102: 7–13, 2006
- Schlegel KA, Prechtel C, Most T, Seidl C, Lutz R, Von Wilmowsky C: Osseointegration of SLActive implants in diabetic pigs. *Clin Oral Implants Res* 24: 128–134, 2013
- Schmitt CM, Moest T, Lutz R, Neukam FW, Schlegel KA: Anorganic bovine bone (ABB) vs. autologous bone (AB) plus ABB in maxillary sinus grafting. A prospective non-randomized clinical and histomorphometrical trial. *Clin Oral Implants Res* 26: 1043–1050, 2014
- Schwartz Z, Boyan BD: Underlying mechanisms at the bone-biomaterial interface. *J Cell Biochem* 56: 340–347, 1994
- Schwarz F, Herten M, Sager M, Wieland M, Dard M, Becker J: Bone regeneration in dehiscence-type defects at chemically modified (SLActive) and conventional SLA titanium implants: a pilot study in dogs. *J Clin Periodontol* 34: 78–86, 2007
- Schwarz F, Rothamel D, Herten M, Wustefeld M, Sager M, Ferrari D, et al: Immunohistochemical characterization of guided bone regeneration at a dehiscence-type defect using different barrier membranes: an experimental study in dogs. *Clin Oral Implants Res* 19: 402–415, 2008
- Tal H, Kozlovsky A, Artzi Z, Nemcovsky CE, Moses O: Long-term bio-degradation of cross-linked and non-cross-linked collagen barriers in human guided bone regeneration. *Clin Oral Implants Res* 19: 295–302, 2008
- Tyndall WA, Beam HA, Zarro C, O'Connor JP, Lin SS: Decreased platelet derived growth factor expression during fracture healing in diabetic animals. *Clin Orthop Relat Res* 319–330, 2003
- Von Wilmowsky C, Schwarz S, Kerl JM, Srour S, Lell M, Felszeghy E, et al: Reconstruction of a mandibular defect with autogenous, autoclaved bone grafts and tissue engineering: an in vivo pilot study. *J Biomed Mater Res A* 93: 1510–1518, 2010a
- Von Wilmowsky C, Stockmann P, Harsch I, Amann K, Metzler P, Lutz R, et al: Diabetes mellitus negatively affects peri-implant bone formation in the diabetic domestic pig. *J Clin Periodontol* 38: 771–779, 2011
- Von Wilmowsky C, Stockmann P, Metzler P, Harsch I, Amann K, Schlegel KA: Establishment of a streptozotocin-induced diabetic domestic pig model and a systematic evaluation of pathological changes in the hard and soft tissue over a 12-month period. *Clin Oral Implants Res* 21: 709–717, 2010b
- Wild S, Roglic G, Green A, Sicree R, King H: Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* 27: 1047–1053, 2004