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Improving Metabolic Control Reverses the Histomorphometric and Biomechanical Abnormalities of an Experimentally Induced Bone Defect in Spontaneously Diabetic Rats

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Abstract. Insulin-dependent type 1 diabetes mellitus (IDDM) has been shown to alter the properties of bone and to impair fracture-healing in both humans and animals. The objective of this study was to examine changes in the histomorphometric and mechanical parameters of bone and remodeling during bone-defect healing, depending on the diabetic metabolic state in spontaneously diabetic BB/O(ttawa)K(arlsburg) rats, a rat strain that represents a close homology to IDDM in humans.

A standardized bone-defect model was chosen and based on blood-glucose values at the time of surgery (mg%), postoperative blood-glucose course (mg%), and postoperative insulin requirements (IU/kg). A total of 120 spontaneously diabetic BB/OK rats were divided into groups with a well-compensated ($n = 60$; 169 ± 102 mg%; 230 ± 126 mg%; and 2.2 ± 1.1 IU/kg) or poorly compensated ($n = 60$; 380 ± 159 mg%; 359 ± 89 mg%; and 5.4 ± 1.1 IU/kg) metabolic state. Sixty LEW.1A rats served as the normoglycemic controls (93 ± 19 mg%). Fifteen animals from each group were killed on postoperative days 7, 14, 24, and 42, and specimens were processed undecalcified for quantitative bone histomorphometry and for biomechanical testing.

Our study showed in terms of bone histomorphometry, within the first 14 days, that severe mineralization disorders occurred exclusively in the rats with a poorly compensated diabetic metabolic state with a highly significant ($P < 0.001$) or significant ($P < 0.01$) decrease of all fluorochrome-based parameters of mineralization, apposition, formation and timing of mineralization, as well as significantly decreased values of biomechanical properties ($P < 0.05$) in comparison to the spontaneously diabetic rats with a well-compensated metabolic state and to the control rats.

Bone-defect healing in spontaneously diabetic BB/OK rats is retarded exclusively in a poorly compensated diabetic metabolic state. This study suggests that strictly controlled insulin treatment resulting in a well-compensated diabetic metabolic state will ameliorate the impaired early and late parameters of IDDM bone-defect healing.

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Key words: Spontaneously diabetic rats — Bone histomorphometry — Remodeling — Bone defect healing — Bone biomechanics

Introduction

Numerous clinical and experimental studies on the complications of diabetes mellitus have demonstrated alterations of bone and mineral metabolism [1]. Reductions in bone mineral content have been reported in juvenile [2] and adult-onset diabetics [3]. Clinically, diabetes is often associated with reduced bone mass and delayed fracture-healing in humans [4]. Streptozotocin-induced diabetes impairs fracture healing [5] and causes delayed recovery of structural and material strength in femurs with healed fractures in rats [6].

Bone repair is a complex cascade of cellular events, the regulation of which remains poorly understood [7]. The relationship between bone repair and insulin-dependent type 1 diabetes (IDDM) is complex. The underlying etiology and the varying effects of a strict insulin regimen on the resulting diabetic metabolic state may both potentially affect bone-defect healing. Bone-defect healing may also be complicated by accelerated local bone loss and regional osteoporosis [8]. Because of these complexities, animal models for IDDM may be more appropriate for studying the effects of spontaneous diabetes and for testing the effects of drugs on bone repair.

Experimental models for IDDM include the streptozotocin-induced diabetic rat and the spontaneously diabetic BB rat. Streptozotocin-induced diabetes in animals is not the same as clinical diabetes observed in humans. Streptozotocin poisons the pancreatic islets directly, thereby eliminating the production of insulin and causing severe hyperglycemia in the animal. This situation is somewhat analogous to type 1 diabetes in

humans [6]. Nevertheless, the rat model with streptozotocin-induced diabetes is the most widely used experimental model in the study of diabetes [9–14].

In the streptozotocin-induced diabetic rat, quantitative bone histomorphometry has demonstrated a decrease in trabecular bone volume [9, 10, 13] as well as reductions in bone formation in acute [11] and chronic [12, 14] forms of the disease. These histologic changes appear to be consistent with decreased collagen synthesis [15–17] and increased collagenolysis [18, 19].

Extensive histomorphometric data on spontaneously diabetic animals is only to be found in published studies of Verhaeghe et al. [20–22], who deduced lower bone resorption without proof of trabecular or cortical bone loss. Histomorphometric indices of bone formation and remodeling during bone-defect healing have not been reported to data in spontaneously diabetic rats.

Furthermore, most studies arrived at their deficient results in experimental diabetes by using untreated streptozotocin-induced diabetic rats [5, 6, 23–25]. In the usual clinical situation, however, the diabetic patient with IDDM is treated with insulin, but may still have an overall poor diabetic metabolic state with an uncontrollable or hardly controllable blood glucose level and a high and sometimes changing insulin requirement.

The spontaneously diabetic BB/O(ttawa)K(arlsburg) rat develops an autoimmune IDDM that bears close resemblance to the syndrome seen in human type 1 diabetes. Like the human form, the disease has an early age of onset, occurs in lean animals, and is characterized by glucosuria, hyperglycemia, hypoinsulinemia, and ketoacidosis in both sexes. BB diabetes is characterized morphologically by a β-cell-specific mononuclear cell infiltrate (insulitis) within the pancreatic islets of Langerhans. The autoimmune attack selectively destroys the insulin-producing β cells. As in human beings, disease development in the BB/OK rat is complex and polygenic, which makes BB rats a valuable model of human type 1 diabetes and its complications [26, 27].

This current study examined for the first time, the changes in the histomorphometric and biomechanical parameters of bone with healed bone defect in spontaneously diabetic BB/OK rats, as a function of the diabetic metabolic state, rather than in animals with experimentally (streptozotocin) induced diabetes mellitus compared with normoglycemic controls.

Materials and Methods

Animals

Spontaneously diabetic BB/OK (F60/61) [26] and nondiabetic LEW.1A rats (F72) used as normoglycemic controls [28] were bred and kept in our animal facility under strict hygienic conditions and were free of major pathogens, as described previously. They were given a laboratory diet (Ssniff, Soest, Germany) and acidulated water *ad libitum*. The animals were maintained with 12 hours of light (5 a.m. to 5 p.m.) and 12 hours of dark, and they were housed three per cage (Macrolon type III; Ehret GmbH, Emmendingen, Germany) preoperatively and one per cage postoperatively.

Diabetes in BB/OK rats was diagnosed on the basis of glucosuria (Diabur-Test 5000; Boehringer, Mannheim, Germany) followed by measurement of blood glucose concentrations of >300 mg/dL on two consecutive days as described [26]. Up to the time of surgery, diabetic animals were treated with subcutaneous applications of a sustained-release insulin implant using a trocar/stylet (LINPLANT™; ©LINSHIN Canada, Scarborough, Ontario, Canada). The sustained-release insulin implant contains bovine insulin in an erodible palmitic acid matrix, and is characterized by an insulin release rate of about 2 IU/day for more than 4 weeks. Following the application of the insulin implant, the diabetic rats were monitored weekly for body weight and blood-glucose concentration. When the blood-glucose values exceeded 200 mg%, a new insulin implant was applied. Before surgery, diabetic animals with blood-glucose values <200 mg% did not obtain a new implant to avoid hypoglycemia during surgery. They were treated daily with insulin (Lente™; Novo Nordisk, Denmark). The insulin dose per animal varied between 1 and 6 IU/kg depending on stable body weight gain and blood-glucose concentrations as described [26].

Surgery

The animals were anesthetized with an intraperitoneal injection of a Rompun® (xylazin; Bayer, Leverkusen, Germany) (0.2 mL/kg)/Ketanest® (ketamine, Sanofi, Berlin, Germany) (0.4 mL/kg) mixture. The right distal femur was shaved and then disinfected with 70% alcohol. Subsequently, an incision was made parallel to the long axis of the femur. After dividing the fascia and biceps femoris muscle, the bone was grasped with two, small Hohmann retractors and exposed. By using a 1.1-mm-diameter Kirschner wire, a hole ca. 10 mm proximal to the knee joint space was centrally drilled, also penetrating the cortical bone of the opposite side. The wound was closed with resorbable sutures.

Experimental Protocol

One hundred and twenty spontaneously diabetic BB/OK rats with a blood-glucose value of 385 ± 109 mg% (mean ± SD) at the time of manifestation and at an age of 95 ± 18 days were used in the study after a subsequent diabetes duration of 118 ± 17 days. Therefore, the spontaneously diabetic animals underwent surgery at an age of 206 ± 19 days. On the basis of blood-glucose values at the time of surgery (mg%), postoperative blood-glucose course (mg%), and postoperative insulin requirements (IU/kg), the animals were divided into groups with well-compensated ($n = 60$; 169 ± 102 mg%; 230 ± 126 mg%; 2.2 ± 1.1 IU/kg, respectively) or poorly compensated metabolic state ($n = 60$; 380 ± 159 mg%; 359 ± 89 mg%; 5.4 ± 1.1 IU/kg, respectively). Similar to that in human type 1 diabetes, the autoimmune process destroying β cells in diabetic rats is sometimes stopped before all cells are destroyed. Such animals are diabetic and also insulin-dependent, but they have a few β cells releasing endogenous insulin, which favors glucose metabolism. Animals with residual β cells are mostly well-compensated, whereas animals without endogenous insulin are mostly poorly-compensated [26]. Sixty LEW.1A rats aged 208 ± 23 days, served as the normoglycemic controls (93 ± 19 mg%). Fifteen animals (eight female and seven male) from each group were killed on postoperative days 7, 14, 24, and 42 for callus histomorphmetry ($n = 10$ from each group) and biomechanical testing ($n = 5$ per group). The statistical differences in terms of the characteristics chosen for grouping are shown in Table 1 according to sampling day. We used polychrome sequential labeling [29, 30] to study the dynamics of bone-defect healing. The applied technique, using four, different colored vital

Table 1. Grouping of spontaneously diabetic BB/OK rats

Time of preparation	Metabolic compensation	Blood glucose at time of OP in mg%	Postoperative blood glucose course in mg%	Postoperative insulin requirement in IU/kg body mass
7 Days	Well	175 ± 108	221 ± 91	1.6 ± 1.9
	Poor	359 ± 101**	333 ± 98**	4.8 ± 1.4***
14 Days	Well	169 ± 102	230 ± 126	2.2 ± 1.1
	Poor	380 ± 159**	359 ± 89**	5.4 ± 1.1***
24 Days	Welt	187 ± 110	264 ± 63	2.1 ± 1.2
	Poor	363 ± 59**	406 ± 39***	5.7 ± 0.8***
42 Days	Well	233 ± 112	266 ± 89	2.5 ± 1.7
	Poor	348 ± 55*	381 ± 32**	6.0 ± 1.7***

All values are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Time of preparation	Multiple Fluorescent Labeling				
	Day 2 Calcein	Day 5 Tetracycline	Day 12 Alizarin	Day 22 Xylenol	Day 40 Calcein
7 Days	■	■			
14 Days	■	■	■		
24 Days	■	■	■	■	
42 Days	■	■	■	■	■

Fig. 1. Schedule of labeling agents administration.

markers, gives detailed information about the site and time of new bone formation in the bone defect. The following fluorochrome labels were injected into the rats intraperitoneally postoperatively (taking into consideration the temporal grouping according to the end of the experiment as mentioned previously): calcein (20 mg/kg body weight) on days 2 and 40, tetracycline (15 mg/kg) on day 5, alizarin (50 mg/kg) on day 12, and xylenol orange (90 mg/kg) on day 22 (Fig. 1).

The experimental protocol adhered to the guidelines of the National Health and Medical Research Council of Germany for the use of animals and was approved by the animal ethics and care committee of the University of Greifswald.

Sample Preparation

Femurs were cut distally, 5 mm proximal to the medial condyle articular surface and proximally, 5 mm from the drilling hole ($n = 10$ per group). The cut specimens were placed in 1.4% paraformaldehyde-5%-sucrose-0.02 M-phosphate buffered solution (pH = 7.4) at 4°C for 48 hours. They were then dehydrated in a graded series of ethanol and acetone, and embedded in modified methylmethacrylate (Technovit® 9100; Heraeus Kulzer, Wehrheim, Germany) [31]. 5-µm Frontal thick, sections, were cut from each femur with a microtome (Model Leica RM 2165; Leica, Bensheim, Germany) and affixed to slides. Two slides were stained with the Toluidine (Certistain®; Merck, Darmstadt, Germany) and Giemsa method (Giemsa solution; Merck, Darmstadt, Germany) [32] and used for determining structural and static endpoints. Two slides were coverslipped without further staining and used for determining dynamic endpoints of bone formation.

At the end of the experiment, both hindlimbs ($n = 5$ per group) were disarticulated at the hip and stored at −80°C until biomechanical analyses were performed.

Histomorphometric Analysis

Bone histomorphometry in the cancellous area of femur was performed with a light/epifluorescent microscope (Model BH2-RFC; Olympus®, Tokyo, Japan) interfaced with analySIS software (Soft Imaging System, Münster, Germany). Nomenclature and symbols used in conventional bone histomorphometry are the same as those described by Parfitt et al. [33]. For data collection, the entire drill hole was measured. The following histomorphologic parameters were measured: total tissue area (Tt.Ar, μm^2), total trabecular area (Tb.Ar, μm^2), trabecular perimeter (Tb.Pm, µm), single- and double-labeled perimeter (sL.Pm and dL.Pm, µm), the interfabel width (Ir.L.Wi, µm) of double labels, osteoid perimeter (O.Pm, µm). osteoblast perimeter (Ob.Pm, µm), osteoclast perimeter (Oc.Pm, µm), osteoid width (O.Wi, µm, eroded perimeter (E.Pm, µm), measurement from the 14th postoperative day, because Hownship lacunae were not demonstrable any earlier), osteoclast number (N.Oc), and wall width (W.Wi, µm, measurement on postoperative day 42). The following structural calculations were made: cancellous bone surface (BS = Tb.Pm, µm), cancellous bone volume (BV/TV = Tb.Ar/Tt.Ar, %), trabecular thickness [Tb.Th = (BV/TV)/0.5 BS, µm], trabecular number [Tb.N = (BV/TV)/Tb.Th, mm^{-1}], trabecular spacing [Tb.Sp = (1/Tb.N) − Tb.Th, µm], osteoid surface [OS = (O.Pm/Tb.Pm) × 100, %], osteoblast surface [Ob.S = (Ob.Pm/Tb.Pm) × 100, %], osteoclast surface [Oc.S = (Oc.Pm/Tb.Pm) × 100, %], and eroded surface [ES = (E.Pm/Tb.Pm) × 100, %] (calculations from the 14th postoperative day). The following dynamic calculations were made: single-labeled surface [sLS = (sL.Pm/Tb.Pm) × 100, %], double-labeled surface [dLS = (dL.Pm/Tb.Pm) × 100, %], mineralizing surface (MS = dLS + 0.5sLS, %), mineral apposition rate [MAR = Ir.L.Wi/lr.Lt (intertabel time period), µm/day, surface referent bone formation rate (BFR/BS = MS × MAR, $\mu m^3/\mu m^2$/day), adjusted apposition rate [Aj.Ar = MAR × (MS/OS), µm/day], osteoid maturation time (Omt = O.Wi/MAR, day), and mineralization lag time (Mlt = O.Wi/Aj.Ar, day).

Biomechanical Analysis

Biomechanical testing was carried out on the well-compensated and poorly compensated diabetic and control rats

Table 2. Histomorphometry on postoperative day 7

Parameter	BB/OK, Metabolic compensation		
	Well	Poor	Control animals
Cancellous bone volume (BV/TV, %)	17.73 ± 3.92	14.38 ± 4.94	19.53 ± 2.22[c]*
Trabecular thickness (Tb.Th, μm)	27.38 ± 6.89	20.80 ± 7.06[a]*	21.79 ± 7.52
Trabecular number (Tb.N, mm^{-1})	0.66 ± 0.14	0.70 ± 0.19	0.96 ± 0.25[b]*[c]*
Trabecular spacing (Tb.Sp, μm)	1542.31 ± 392.46	1475.25 ± 360.29	1106.48 ± 384.70
Osteoblast surface (Ob.S, %)	5.43 ± 4.54	6.05 ± 3.98	4.65 ± 3.45
Osteoid surface (OS, %)	17.14 ± 20.56	14.27 ± 12.37	16.01 ± 20.21
Osteoclast surface (Oc.S, %)	0.90 ± 1.18	1.14 ± 1.52	0.34 ± 0.38
Single-labeled surface (sLS, %)	34.61 ± 16.87	7.62 ± 5.31[a]***	32.90 ± 20.73[c]**
Double-labeled surface (dLS, %)	17.82 ± 10.04	5.52 ± 4.69[a]**	15.20 ± 9.38[c]*
Mineralizing surface (MS, %)	35.12 ± 15.21	9.04 ± 7.28[a]***	31.65 ± 18.65[c]**
Osteoid maturation time (Omt, d)	1.78 ± 0.85	1.94 ± 1.65	1.15 ± 0.55
Mineralization lag time (Mlt, d)	1.27 ± 1.87	14.90 ± 25.06	0.95 ± 1.46

All values are expressed as mean ± SD
[a] Well-compensated vs. poorly compensated
[b] Well-compensated vs. control animals
[c] Poorly compensated vs. control animals
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

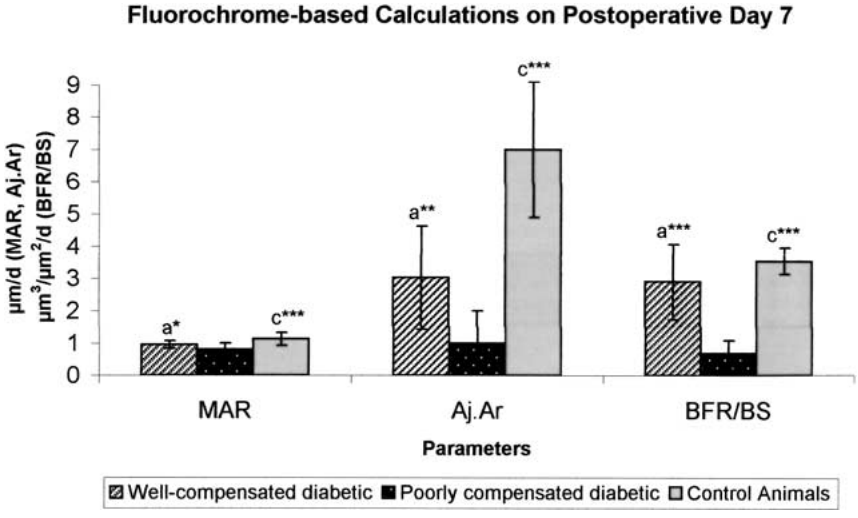

Fig. 2. Fluorochrome-based calculations on postoperative day 7. Mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. [a]Well-compensated vs. poorly compensated. [c]Poorly compensated vs. control animals.

using the experimental protocol described by Diwan et al. [34]. In brief, femurs ($n = 5$ per group) were subjected to three-point bending to failure on an AG-50KNE material testing machine (Shimadzu, Kyoto, Japan) with a jig span of 18 mm at a displacement rate of 5 mm/minute. Load displacement data were acquired with a 50-N load cell for the rate of 200 per second. Load-displacement curves were plotted, and failure load, stiffness, and energy were calculated for each sample [35]. The unoperated left side was used as internal control.

Data Analysis

The significance of differences between groups was calculated with the unpaired Students t test corrected by Bonferroni-Holm procedure [36]. The level of significance was pre-set at $P < 0.05$. All statistical analyses were carried out according to Steel and Torrie [37] using a computer program (SPSS/PC+TM 4.0, Base Manual for the IBM PC/XT/AT and PS/2 V, Release 4.0, SPSS Inc., Chicago).

Results

Bone Histomorphometry

Postoperative day 7. There were statistically significant differences between animals with well-compensated and those with poorly compensated metabolic states in the blood-glucose values at the time of surgery and the postoperative blood-glucose course ($P < 0.01$), and the postoperative insulin requirement also differed significantly ($P < 0.001$) (Table 1).

Table 2 summarizes the results of bone histomorphometry on the 7th postoperative day. Compared with the diabetic groups; Tb.Th (135%, $P < 0.05$) of the structural indices; sLS (486%, $P < 0.001$), MS (389%, $P < 0.001$), and dLS (356%, $P < 0.01$) of the mineralizing surface; Aj.Ar (290%, $P < 0.01$) and MAR

Table 3. Histomorphometry on postoperative day 14

Parameter	BB/OK, Metabolic compensation		
	Well	Poor	Control animals
Cancellous bone volume (BV/TV, %)	44.49 ± 12.52	29.73 ± 12.59	40.44 ± 12.29
Trabecular thickness (Tb.Th, µm)	49.28 ± 28.43	31.02 ± 16.09	29.29 ± 8.82
Trabecular number (Tb.N, mm^{-1})	1.02 ± 0.45	1.03 ± 0.45	1.39 ± 0.18
Trabecular spacing (Tb.Sp, µm)	1097.70 ± 417.10	1063.15 ± 343.89	699.29 ± 94.07[b]*[c]*
Osteoid surface (OS, %)	74.05 ± 19.63	73.11 ± 25.38	57.08 ± 21 45
Osteoblast surface (Ob.S, %)	13.43 ± 11.45	14.45 ± 9.65	16.86 ± 12.05
Osteoclast surface (Oc.S, %)	8.92 ± 7.55	9.10 ± 6.38	8.31 ± 6.86
Eroded surface (ES, %)	13.30 ± 10.39	10.82 ± 7.41	12.37 ± 7.05
Single-labeled surface (sLS, %)	22.96 ± 12.43	6.41 ± 3.39[a]**	20.15 ± 10.10[c]**
Double-labeled surface (dLS, %)	21.18 ± 13.25	5.27 ± 2.66[a]**	16.36 ± 7.25[c]***
Mineralizing surface (MS, %)	32.67 ± 15.43	8.68 ± 4.16[a]***	26.44 ± 12.09[c]***
Osteoid maturation time (Omt, d)	2.06 ± 0.72	6.96 ± 2.58[a]***	2.01 ± 0.77[c]***
Mineralization lag time (Mlt, d)	5.34 ± 2.54	72.08 ± 45.50[a]***	5.83 ± 5.24[c]***

All values are expressed as mean ± SD
[a] Well-compensated vs. poorly compensated
[b] Well-compensated vs. control animals
[c] Poorly compensated vs. control animals
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

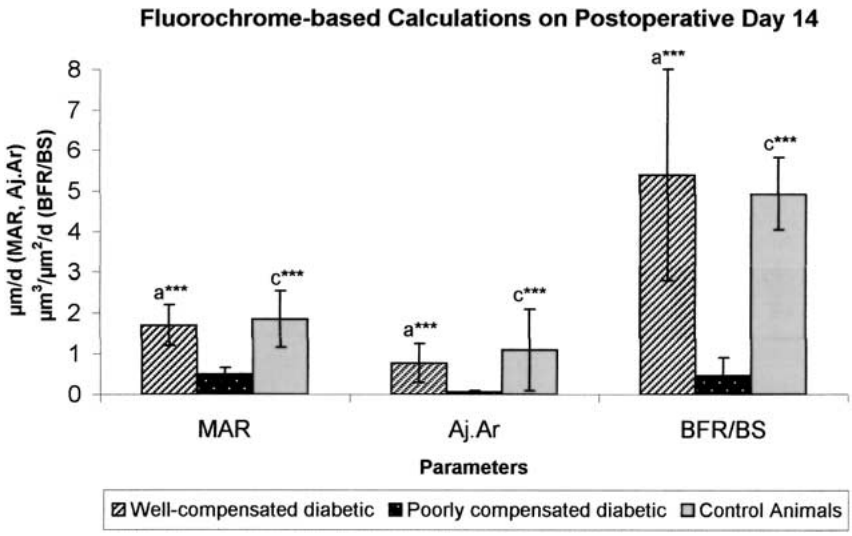

Fig. 3. Fluorochrome-based calculations on postoperative day14. Mean ± SD. ***$P < 0.01$. [a]Well-compensated vs. poorly compensated. [c]Poorly compensated vs. control animals.

(119%, $P < 0.05$) of the apposition rates; and BFR/BS (432%, $P < 0.001$) were significantly increased in the well-compensated rats. When comparing the poorly compensated diabetic rats to the control group, the cancellous bone volume (BV/TV) (136%, $P < 0.01$), Tb.N (137%, $P < 0.05$), sLS (434%, $P < 0.01$), dLS (276%, $P < 0.05$), MS (351%, $P < 0.01$), MAR (141%, $P < 0.01$), Aj.Ar (704%, $P < 0.001$), and the BFR/BS (522%, $P < 0.01$) were highly significantly or significantly higher in the control animals (Fig. 2).

Postoperative day 14. There were a statistically significant differences between animals with well-compensated and those with poorly compensated metabolic states in the blood-glucose values at the time of surgery and the postoperative blood-glucose course ($P < 0.01$), and the postoperative insulin requirement also differed significantly ($P < 0.001$) (Table 1).

Table 3 gives an overview of the results of bone histomorphometry on the 14th postoperative day. The highly significant differences in the measured fluorochome-based indices (data not shown) resulted in highly significant ($P < 0.001$) [MS (375%), MAR (341%), Aj.Ar (1283%), BFR/BS (1176%)], and significantly ($P < 0.01$) [sLS (354%), dLS (401%)] higher dynamic calculations for the well-compensated diabetic animals in both diabetic groups. It also resulted in highly significant ($P < 0.001$) [dLS (310%), MS (304%), MAR (362%), Aj.Ar (1816%), BFR/BS (1072%)] (Fig. 3) and significantly ($P < 0.01$) [sLS (314%)] higher dynamic calculations for the control animals in comparison to the poorly compensated

Table 4. Histomorphometry on postoperative day 24

Parameter	BB/OK, Metabolic compensation		
	Well	Poor	Control animals
Cancellous bone volume (BV/TV, %)	54.07 ± 6.60	44.75 ± 14.86	64.14 ± 9.76[c]**
Trabecular thickness (Tb.Th, μm)	52.26 ± 5.84	42.61 ± 31.94	53.41 ± 10.58[c]*
Trabecular number (Tb.N, mm^{-1})	1.05 ± 0.18	0.96 ± 0.28	1.22 ± 0.22
Trabecular spacing (Tb.Sp, μm)	931.90 ± 172.70	1084.85 ± 367.86	784.22 ± 137.91
Osteoid surface (OS, %)	65.81 ± 15.47	65.75 ± 18.97	56.69 ± 10.02
Osteoblast surface (Ob.S, %)	12.94 ± 10.88	14.12 ± 7.87	15.28 ± 12.87
Osteoclast surface (Oc.S, %)	11. 95 ± 7.31	10.39 ± 5.09	9.81 ± 3.42
Eroded surface (ES, %)	17.70 ± 10.12	14.58 ± 5.49	13.97 ± 3.60
Single-labeled surface (sLS, %)	70.85 ± 17.24	72.45 ± 19.02	69.39 ± 15.96
Double-labeled surface (dLS, %)	60.15 ± 15.96	43.25 ± 19.36[a]*	60.40 ± 16.90[c]*
Mineralizing surface (MS, %)	94.59 ± 24.21	76.48 ± 26.93[a]*	95.10 ± 23.71[c]*
Mineral apposition rate (MAR, μm/d)	1.04 ± 0.11	1.06 ± 0.10	1.06 ± 0.15
Adjusted apposition rate (Aj.Ar, μm/d)	1.31 ± 0.35	1.20 ± 0.28	1.84 ± 0.66[b]*[c]*
Bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/d)	0.77 ± 0.38	0.89 ± 0.22	1.79 ± 0.34
Osteoid maturation time (Omt, d)	3.65 ± 0.97	2.77 ± 0.76	3.57 ± 1.16
Mineralization lag time (Mlt, d)	3.26 ± 1.38	2.32 ± 0.66	2.21 ± 0.86

All values are expressed as mean ± SD
[a] Well-compensated vs. poorly compensated
[b] Well-compensated vs. control animals
[c] Poorly compensated vs. control animals
*$P < 0.05$, **$P < 0.01$

Table 5. Histomorphometry on postoperative day 42

Parameter	BB/OK, Metabolic compensation		
	Well	Poor	Control animals
Cancellous bone volume (BV/TV, %)	83.00 ± 6.70	72.42 ± 12.75	85.74 ± 5.87[c]*
Trabecular thickness (Tb.Th, μm)	140.14 ± 53.56	112.78 ± 44.44	127.47 ± 66.87
Trabecular number (Tb.N, mm^{-1})	0.67 ± 0.24	0.70 ± 0.20	0.79 ± 0.27
Trabecular spacing (Tb.Sp, μm)	1556.00 ± 638.87	1460.81 ± 549.32	1335.15 ± 612.88
Osteoid surface (OS, %)	69.47 ± 22.74	60.96 ± 20.45	61.96 ± 27.48
Osteoblast surface (Ob.S, %)	25.45 ± 14.67	19.56 ± 11.08	20.56 ± 15.67
Osteoclast surface (Oc.S, %)	14.71 ± 11.26	16.22 ± 9.30	11.96 ± 10.36
Eroded surface (ES, %)	22.58 ± 14.70	23.20 ± 12.18	14.93 ± 12.41
Single-labeled surface (sLS, %)	78.81 ± 25.23	58.21 ± 24.91	84.21 ± 20.40
Double-labeled surface (dLS, %)	30.32 ± 11.74	22.09 ± 15.44	31.95 ± 11.29
Mineralizing surface (MS, %)	75.73 ± 22.60	50.19 ± 26.07[a]*	74.06 ± 19.96[c]*
Mineral apposition rate (MAR, μm/d)	1.03 ± 0.20	0.90 ± 0.23	1.01 ± 0.23
Adjusted apposition rate (Aj.Ar, μm/d)	0.95 ± 0.27	0.76 ± 0.31	1.42 ± 0.72[c]*
Bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/d)	5.43 ± 1.98	4.88 ± 3.47	7.53 ± 2.74
Osteoid maturation time (Omt, d)	3.39 ± 0.90	4.34 ± 1.26	3.55 ± 1.17
Mineralization lag time (Mlt, d)	4.60 ± 1.78	5.51 ± 1.85	2.99 ± 1.61[c]*

All values are expressed as mean ± SD
[a] Well-compensated vs. poorly compensated
[c] Poorly compensated vs. control animals
* $P < 0.05$

diabetic animals. The dynamic calculations Omt (337%, $P < 0.001$) and Mlt (1349%, $P < 0.001$) were significantly prolonged in the poorly compensated compared to the well-compensated diabetic animals, and Omt (346%, $P < 0.001$) and Mlt (1236%, $P < 0.001$) were also significantly prolonged in the poorly compensated diabetic animals in comparison to the control animals.

Postoperative day 24. There were statistically significant differences between animals with well-compensated and those with poorly compensated metabolic states in the

Table 6. Biomechanical effect of poorly metabolic diabetic state on bone defect healing

Time after bone defect	Parameters	BB/OK, Metabolic compensation		
		Well	Poor	Control animals
7 days	Failure load (N)	6.66 ± 1.27	3.49 ± 0.76[a]*	6.98 ± 0.99[b]*
	Stiffness (N/mm)	3.03 ± 0.46	2.74 ± 0.87	3.12 ± 0.45
	Stress (MPa)	2.07 ± 0.87	2.04 ± 0.67	2.13 ± 0.78
	Energy (N/mm)	5.90 ± 2.18	2.70 ± 0.73[a]*	5.80 ± 2.73[b]*
14 days	Failure load (N)	8.36 ± 2.17	4.09 ± 0.59[a]*	8.31 ± 3.42[b]*
	Stiffness (N/mm)	3.99 ± 0.66	3.25 ± 0.24	3.41 ± 1.88
	Stress (MPa)	2.64 ± 0.54	2.13 ± 0.56	2.55 ± 1.09
	Energy (N/mm)	7.65 ± 2.11	3.59 ± 0.12[a]*	7.87 ± 2.83[b]*
24 days	Failure load (N)	10.63 ± 3.21	10.42 ± 1.56	10.89 ± 3.32
	Stiffness (N/mm)	5.07 ± 1.34	5.04 ± 0.88	5.33 ± 1.00
	Stress (MPa)	3.25 ± 0.50	3.09 ± 0.73	3.33 ± 1.64
	Energy (N/mm)	10.08 ± 1.97	10.40 ± 0.82	10.79 ± 2.98
42 days	Failure load (N)	11.53 ± 6.61	10.88 ± 4.56	11.49 ± 4.62
	Stiffness (N/mm)	5.56 ± 1.74	5.64 ± 2.09	5.15 ± 1.77
	Stress (MPa)	3.95 ± 0.59	3.89 ± 0.99	3.93 ± 1.34
	Energy (N/mm)	10.58 ± 3.67	10.70 ± 3.82	11.39 ± 4.68

All values are expressed as mean ± SD, *$P < 0.05$
[a] Well-compensated vs. poorly compensated
[b] Poorly compensated vs. control animals

postoperative insulin requirement and postoperative blood-glucose course ($P < 0.001$), and the blood-glucose values at the time of surgery also differed significantly ($P < 0.01$) (Table 1).

Table 4 summarizes the results of bone histomorphometry on the 24th postoperative day. The cancellous bone volume (BV/TV) was significantly increased (142%, $P < 0.01$), the structural parameter Tb.Th (125%, $P < 0.05$), as well as the dynamic calculations dLS (139%, $P < 0.05$), MS (125%, $P < 0.05$), and Aj.Ar (153%, $P < 0.05$) of the control animals were significantly increased compared with the poorly compensated diabetic animals. Apart from significantly increased dLS (139%, $P < 0.05$) and MS (123%, $P < 0.05$) in the well-compensated diabetic animals in comparison to the poorly compensated ones, there were no statistical differences between the diabetic animals.

Postoperative day 42. There were no statistically significant differences between animals with well-compensated and those with poorly compensated metabolic states in blood-glucose values at the time of surgery ($P < 0.05$) and in the postoperative blood-glucose course ($P < 0.01$), and the postoperative insulin requirement also differed significantly ($P < 0.001$) (Table 1).

Table 5 summarizes the results of bone histomorphometry on the 42nd postoperative day. When the control animals were compared with the poorly compensated diabetic animals, the control animals showed statistically significantly higher calculations in BV/TV (119%, $P < 0.05$), MS (147%, $P < 0.05$), and Aj.Ar (186%, $P < 0.05$). Mlt (184%, $P < 0.05$) was significantly prolonged in the poorly compensated compared to the control animals. MS ($P < 0.05$) was significantly increased in the well-compensated diabetic animals in comparison to the poorly compensated diabetic animals.

Biomechanical Analysis

On a three-point bending test, all femora displayed a load-displacement curve typical for a long bone, with an initial nonlinear response followed by an upward-sloping linear component, and then a failure response at the point of break.

Table 6 summarizes the biomechanical properties on the 7th, 14th, 24th and 42nd postoperative days. On the 7th and 14th postoperative days, biomechanical data from the healing femur of the poorly compensated diabetic animals revealed a twofold decrease in the energy required to break the femur ($P < 0.05$) and a twofold decrease in peak failure load ($P < 0.05$) in comparison to both the well-compensated diabetic and to the control animals.

All healing femoral bone defects failed at the fracture site on three-point bending. The unoperated femur failed in all cases, with a transverse break at the midshaft. There was no significant alteration in the mechanical properties of the unfractured right femur in the three groups (data not shown).

Discussion

This study demonstrated the influence of the diabetic metabolic state on bone-defect healing in spontaneously diabetic rats. Until the end of the second week, the extensive histomorphometric data on the different time-

points of bone-defect healing showed for the first time, a considerable mineralization disorder, which is the expression of a reduced and retarded mineralization process in animals with a poorly compensated diabetic metabolic state. These early disorders manifest themselves in significantly decreased fluorochrome-based indices and results in highly significant or significant differences in all parameters of the mineralization, the apposition, the formation, the timing of the mineralization in the dynamic calculations between the well-compensated and the poorly compensated diabetic animals and between the poorly compensated animals and the control animals up to the 14th postoperative day.

From a histomorphometric standpoint, this proves in detail that the poorly compensated genetically determined diabetes mellitus of the test animals impairs mineral metabolism in the form of a decreased and retarded mineralization in the early stages. In correlation with this, decreased values of biomechanical properties until the end of the 14th postoperative day were morphologically proven exclusively in poorly compensated diabetic rats.

Until now, only Beam et al. [38] had shown that the values of cellular proliferation, biomechanical properties and callus bone content of blood-glucose-controlled BB rats during fracture healing do not differ significantly from those of nondiabetic control animals. If the blood glucose values are successfully controlled in animal experiments with genetically determined spontaneous diabetes [38] or if—as we have shown—a well-compensated diabetic metabolic state is achieved by postoperatively controlling the blood glucose value and the insulin consumption, the histomorphometrically detected mineralization disorders and the decrease in the biomechanical values almost disappear in comparison to the normoglycemic control animals.

Various reports note a preventive or reverse effect of treatment with insulin in experimental [39] and in spontaneous diabetes [21, 40]. Insulin treatment appears to revert active osteoblasts into inactive bone-lining cells, from which the suggestive evidence of decreased proliferation of preosteoblastic cells is concluded [39]. There are also reports of disorders in the early phases of fracture-healing in untreated streptozocin-induced animals, in the form of a 50% to 55% decrease in the collagen content of the callus between the 4th and 11th postoperative days [5], and a decrease of type X collagen expression in the fracture callus of between 54% and 70% on the 14th postoperative day [25].

Histomorphometric results on bone-defect healing have not been reported in spontaneously diabetic rats to date. So far, only Follak et al. [41] have found retarded bone-defect healing in spontaneously diabetic rats, which was significant up to postoperative day 24, both within the diabetic groups dependent on their metabolic state and in comparison to the diabetes-resistant control animals, as seen with a scanning electron microscope.

Shyng et al. [42] found that cancellous bone volume and bone formation in the femur were greatly reduced in the streptozotocin-induced diabetic model. These observations are also consistent with published reports of impaired osteoid formation [14], and decreased synthesis of both collagen [16, 17] and proteoglycan [43] in streptozotocin-induced diabetes. Because osteoclast numbers are also reduced in diabetic rats [12, 14], an overall reduction in bone turnover, rather than excessive bone resorption, is implicated in the pathogenesis of diabetic osteopenia [44].

Skeletal changes in diabetic rats have also been attributed to metabolic abnormalities that accompany the malnutrition of insulin deficiency [14]. However, several studies have shown that undernutrition can account for only 30% of the net deficit in collagen production in the streptozotocin-induced diabetic rats [45]. Spanheimer [17] has shown that decreases in collagen production in streptozotocin-induced diabetic rats are a consequence of the chronic diabetic state and not the result of streptozotocin toxicity in the days immediately after injection of this diabetogenic drug. Furthermore, exposing bone and cartilage in culture to streptozotocin for short periods had no direct effect on connective tissue metabolism [42].

In contrast to Verhaeghe et al. [46], who found a normalization of the histomorphometric parameters under insulin substitution, except for MAR in spontaneously diabetic rats, in comparison to the normoglycemic control animals—in our study, statistically significant differences in the static and dynamic calculations remained in comparison to the control group in the course of bone-defect healing, for which the significantly or highly significantly decreased fluorochrome-based indices were responsible. The histomorphometric bone data of this study also contradict the scanning electron microscopic results of Follak et al. [41], since, at the end of the experiment after 42 days, no differences were found either in the semiautomatic quantitative analysis between the spontaneously diabetic animals independent of their metabolic state, or in comparison to the normoglycemic control animals.

Several biomechanical studies have been conducted in streptozotocin-induced diabetic animals [6, 47–49, 51], but our findings are the first to demonstrate that the diabetic metabolic state may affect the bone repair process in spontaneously diabetic rats. The inferior mechanical properties seen in poorly compensated diabetic animals may reflect poor bone quality, less bone mineral, and alterations in the bonding interactions between the mineral and organic constituents of bone matrix.

In contrast to studies with untreated streptozotocin-induced [5, 6, 23–25] or poorly controlled spontaneous diabetes [38], in the present study, all animals were

treated with insulin according to their current blood-glucose value. Still, one of the experimental groups was in a poor diabetic metabolic state because of the post-operative blood-glucose course and the insulin requirements, due to a lack of endogenous insulin. This is in comparison to the mostly well-compensated diabetic animals with some few β cells releasing endogenous insulin, which favors glucose metabolism. This fact is closer to the real clinical situation of an insulin-substituted diabetic with an uncontrollable or hardly controllable blood-glucose level and a high and sometimes changing insulin consumption than to untreated diabetes mellitus, and is especially important when searching for clinically relevant complications in bone repair.

As Bouillon [52] aptly described, it is altogether difficult to form a comprehensive picture of the pathomoiphologic phenomena in diabetes, since they have not been studied in every detail. It is assumed that the most prominent effect of insulin deficiency on bone structure is probably a decreased osteoblast recruitment, either directly or in concert with abnormal production of other hormones or growth factors [52]. This is the approach of several recent studies concerned with the optimization of the bone-defect healing process or the bone turnover in experimental diabetes. Thaller et al. [53] reported that insulin-like growth factor type 1 (IGF-1) exerts a potentiating effect on the repair of critical-sized calvarial defects in adult, male diabetes-induced rats. Furthermore, el-Hakim [24] demonstrated that fibrin stabilizing factor (factor XIII) may enhance early stages of bone healing in uncontrolled streptozotocin-induced diabetic rats. On the other hand, factor XIII did not significantly affect healing in nondiabetic rats. Finally, Tsuchida et al. [54] showed that parathyroid hormone (PTH) enhanced bone turnover and bone mass but not trabecular connectivity in the late stage of streptocotocin-induced diabetes in rats.

In conclusion, we have shown the influence of the diabetic metabolic state on the bone repair process in spontaneously diabetic BB/OK rats. This study has demonstrated that in poorly compensated diabetes of spontaneously diabetic animals, a mineralization disorder (a reduced and retarded mineralization process) occurs in the first 2 weeks of bone disorder healing. With strictly controlled insulin therapy and a resulting well-compensated diabetic metabolic state, severe mineralization disorders, as well as the decreased values of biomechanical properties in the bone repair process in the poorly compensated diabetic metabolic state of the experimental animal can be avoided.

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