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Delayed remodeling in the early period of fracture healing in spontaneously diabetic BB/OK rats depending on the diabetic metabolic state

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Summary. Several clinical series, analyzing fracture healing in patients with insulin-dependent type 1 diabetes (IDDM) demonstrated significant incidence of delayed union, non-union, and pseudarthrosis. The purpose of this study was to examine the detailed histomorphometry and histology of bone formation and remodeling during fracture 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 man.

A standardized fracture 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), 100 spontaneously diabetic BB/OK rats were divided into groups with well-compensated (n=50, 167±77 mg%; 244±68 mg%; 1.8±1.9 IU/kg) or poorly compensated (n=50, 380±89 mg%; 415±80 mg%; 6.0±1.0 IU/kg) metabolic state. Fifty LEW.1A rats served as the normoglycemic controls (97±15 mg%). Ten animals from each group were killed 1, 2, 3, 4 and 6 weeks after fracture and specimens were processed undecalcified for quantitative histomorphometry and for qualitative light microscopy.

In terms of bone histomorphometry, within the first four weeks after fracture, severe mineralization disorders occurred exclusively in the rats with poorly compensated diabetic metabolic states with a significantly decrease of all fluorochrome-based parameters of mineralization, apposition, formation and timing of mineralization in comparison to the spontaneously diabetic rats with well-compensated metabolic states and to the control rats. This was confirmed histologically.

Early fracture healing in the spontaneously diabetic BB/OK rats is delayed exclusively in poorly compensated diabetic metabolic states, and 6 weeks after fracture, histomorphometrically significant deficits in the measured and dynamically calculated parameters remain. This study suggests that strictly controlled insulin treatment resulting in well-compensated diabetic metabolic states will ameliorate the impaired early mineralization and cell differentiation disorders of IDDM fracture healing.

Key words: Spontaneously diabetic rats, Fracture healing, Histomorphometry, Diabetes, Remodeling

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Introduction

Numerous human and experimental studies on the complications of diabetes mellitus have demonstrated alterations in bone and mineral metabolism (for review, see Ref. Seino and Ishida, 1995). The reductions in bone mineral content reported in juvenile (Santiago et al., 1977) and adult-onset diabetics (Krakauer et al., 1995). Clinically, it is often associated with reduced bone mass and delayed fracture healing in humans (Loder, 1988). Experimentally, streptozotocin-induced diabetes impairs fracture healing (Macey et al., 1989) and causes delayed recovery of structural and material strength in femurs with healed fractures in rats (Funk et al., 2000).

Fracture healing is a complex cascade of cellular events, the regulation of which remains poorly understood (Namkung-Matthai et al., 2001). The relationship between fracture healing and insulin-dependent type 1 diabetes (IDDM) is complex. The underlying etiology and the varying effects of a strict insulin regime on the resulting diabetic metabolic state may both potentially affect fracture healing. Fracture healing may also be complicated by accelerated local bone loss and regional osteoporosis (Raisz, 1988). Due to these complexities, animal models for insulin-dependent type 1 diabetes, may be more appropriate to study effects of spontaneous diabetes and to test drugs

on fracture repair.

Experimental models for insulin-dependent type 1 diabetes 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 human patients. 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 (Funk et al., 2000). Nevertheless, the rat model with streptozotocin-induced diabetes is the most widely used experimental model in the study of diabetes (Hough et al., 1981; Shires et al., 1981; Goodman and Hori, 1984; Glajchen et al., 1988; Itaya, 1988; Binz et al., 1990).

In the streptozotocin-induced diabetic rat, quantitative bone histomorphometry has demonstrated a decrease in trabecular bone volume (Binz et al., 1990; Glajchen et al., 1988; Itaya, 1988) as well as reductions in bone formation in acute (Goodman et al., 1984) and chronic (Hough et al., 1981; Shires et al., 1981) forms of the disease. These histologic changes appear to be consistent with decreased collagen synthesis (Schneir et al., 1979, 1990; Spanheimer et al., 1988) and increased collagenolysis (Mohanam and Bose, 1981; Ramamurthy and Golub, 1983).

Extensive histomorphometric research on spontaneously diabetic animals is only to be found in Verhaeghe et al. (1994, 1996, 2000), who deduced lower bone resorption without proof of trabecular or cortical bone loss. Epstein et al. (1994), however, found a significant increase in the eroded perimeter at trabecular bone surfaces in streptozotocin-induced diabetic rat. Histomorphometric indices of bone formation and remodeling during fracture healing have not been reported in spontaneously diabetic rats to date.

Furthermore, most studies arrived at their deficient results in experimental diabetes using untreated streptozotocin-induced diabetic rats (Dixit and Ekstrom, 1978; Macey et al., 1989; Topping et al., 1995; el-Hakim, 1999; Funk et al., 2000). In the usual clinical situation however, the diabetic patient with insulin-dependent type 1 diabetes (IDDM) is treated with insulin, but may still suffer from an overall poor diabetic metabolic state with an uncontrollable or hardly controllable blood glucose level and a high and sometimes changing insulin requirement.

This histomorphometric and histological study examined possible disturbances in fracture healing in spontaneously diabetic BB/O(ttawa)K(arlsburg) rats depending on the diabetic metabolic state instead of in animals with experimentally (Streptozotocin) induced diabetes mellitus. The spontaneously diabetic BB/OK rat develops an autoimmune insulin-dependent type 1 diabetes (IDDM) which bears several close resemblances 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 characterised by glucosuria, hyperglycemia, hypoinsulinemia, and ketoacidosis in both sexes. BB diabetes is characterised morphologically by a ß cell-specific mononuclear cell infiltrate (insulitis) within the pancreatic islets of Langerhans. The autoimmune attack selectively destroys the insulin-producing ß cells. As in human beings, disease development in the BB/OK rat is complex and polygenic which makes BB rats to valuable model of human type 1 diabetes and its complications (Klöting and Voigt, 1991; Klöting et al., 1995).

Materials and methods

Animals

Spontaneously diabetic BB/O(ttawa)K(arlsburg) (F60/61) (Klöting and Voigt, 1991) and non-diabetic LEW.1A rats (F72) used as normoglycemic controls (Klöting, 1987) were bred and kept in our own 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 kept with a rhythm of 12 h light (5 a.m. to 5 p.m.): 12 h dark and housed 3 per cage (Macrolon type III, Ehret GmbH, Emmendingen, Germany) preoperatively but alone 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 >300 mg/dl on two consecutive days as described (Klöting and Voigt, 1991).

Up to surgery diabetic animals were treated with subcutaneous applications of a sustained release insulin implant using a trocar/stylet (LINPLANT™ , LINSHIN Canada, INC., Scarborough, Ontario, Canada). The sustained release insulin implant contains bovine insulin in an erodible palmitic acid matrix and is characterised by an insulin release rate of about 2 IU/day for more than 4 weeks. Following the application of the insulin implant the diabetics 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 diabetics with blood glucose values <200 mg% did not obtain a new implant to avoid hypoglycemia during surgery. They were daily treated with insulin (Lente™ , Novo Nordisk, Denmark). The insulin dose per animal changed between 1 IU/kg and 6 IU/kg depending on stable body weight gain and blood glucose concentrations of animals as described (Klöting and Voigt, 1991).

Fracture model

The animals were anesthetized with an intraperitoneal injection of a Rompun® (Xylazin, Bayer, Leverkusen, Germany) (0.2 ml/kg)/ Ketanest® (Ketamin, Sanofi, Berlin, Germany) (0.4 ml/kg) mixture. The left femur was shaved and then disinfected with 70% alcohol. A closed femoral fracture was created by three-

point bending (Ekeland et al., 1981), followed by an open stabilization with plate osteosynthesis (Titan-Mini-Plates, MEDICON eG, Tuttlingen, Germany) on the ventral side of the femur (Fig. 1). In brief, the left thigh of the anesthetized rat was placed between three points in the jaw of modified forceps, and the jaws were rapidly closed to deliver a force that resulted in a reproducible fracture of the left femur. With an aseptic surgical technique, a 3-cm incision was made on lateral aspect of the left thigh. Fascia, muscle, and periosteum were sharply incised, and the subperiosteal muscles were dissected. Osteosynthesis was accomplished with a four-hole mini plate (Titan-Mini-Plates, MEDICON eG, Tuttlingen, Germany). The mini-plate was applied to the anterior aspect of the femur with screws of 1.6 mm diameter that perforated the opposing corticalis. The fractures were exactly reduced, but compression was not applied. The fascia and the skin were sutured with DeknatelTM Bondek® (Genzyme, Neu-Isenburg, Germany). Only animals that exhibited non-comminuted, transverse fractures, confirmed by radiographs, were used in this study. In 3 cases, animals were excluded from the study because the radiographs did not show exactly transverse fractures. However, the fracture created by this technique has on the whole been shown to be reproducible, and no infection was observed. At week 6 postfracture, we observed 3 diabetic animals (independent of diabetic metabolic state) and 2 control animals with non-union, which were then also excluded from the study.

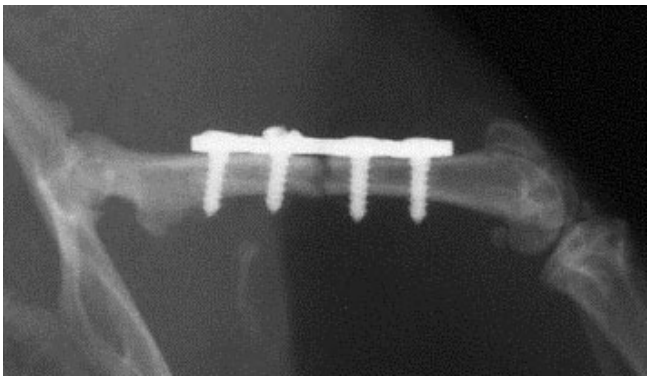

Fig. 1. Radiograph of fractured femur.

Experimental protocol

One hundred spontaneously diabetic BB/OK rats with an average weight of 300±12.5 g (mean±SD), a blood-glucose value of 385±109 mg% at the time of manifestation and at an age of 97±18 days were used in the study after a subsequent diabetes duration of 114±15 days. Therefore, the spontaneously diabetic animals underwent surgery at an age of 203±14 days. Based on 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=50, 167±77 mg%; 244±68 mg%; 1.8±1.9 IU/kg) or poorly compensated metabolic state (n=50, 380±89 mg%; 415±80 mg%; 6.0±1.0 IU/kg). Like in human type 1 diabetes, the autoimmune process destroying ß cells is sometimes stopped before all cells are destroyed. Such animals are diabetic and also insulin-dependent but, they have some few ß cells releasing endogenous insulin which favours glucose metabolism. Animals with residual ß cells are mostly well-compensated whereas animals without endogeneous insulin are more poorly compensated (Klöting and Voigt, 1991).

Fifty LEW.1A rats aged 206±20 d (average weight 305±23.5 g) served as the normoglycemic controls (97±15 mg%). Ten animals from each group were killed 1, 2, 3, 4 and 6 weeks after fracture for callus histomorphometry (n=7 from each group) and for callus morphological analysis (n=3 from each group). The statistical differences in terms of the characteristics

Table 1. Grouping of spontaneously diabetic BB/OK rats.

TIME AFTER FRACTURE	METABOLIC COMPENSATION	BLOOD GLUCOSE AT TIME OF OP IN mg%	POSTOPERATIVE BLOOD GLUCOSE COURSE IN mg%	POSTOPERATIVE INSULIN REQUIEREMENT IN IU/kg BODY MASS
1 week	Well	193±101	219±99	1.8±1.1
	Poor	328±120*	356±82**	5.9±1.9***
2 weeks	Well	179±105	201±98	1.4±1.1
	Poor	378±100**	341±77**	4.6±1.2***
3 weeks	Well	158±99	269±106	2.4±1.9
	Poor	370±169**	388±78**	5.8±1.6***
4 weeks	Well	167±77	244±68	1.8±1.8
	Poor	380±89**	415±80***	6.0±1.0***
6 weeks	Well	199±121	212±69	2.3±1.9
	Poor	388±85**	391±42***	5.9±1.8***

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

chosen for grouping are shown in Table 1 by sampling day.

To assess bone formation rates, the following fluorochrome labels were injected into the rats intraperitoneally (taking into consideration the temporal grouping according to the end of the experiment as mentioned above): beginning on the 2nd postoperative day and then from the 5th day postfracture at 7-day intervals (Welch et al., 1998) during the consolidation phase in the following sequence: tetracycline (15 mg/kg), calcein (20 mg/kg body weight), alizarin (50 mg/kg) and xylenol orange (90 mg/kg) (Fig. 2).

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.

Time of preparation	Multiple Fluorescent Labeling						
	Day 2 Tetracycline	*Day 5* Calcein	*Day 12* Alizarin	*Day 19* Xylenol	*Day 26* Tetracycline	*Day 33* Calcein	*Day 40* Alizarin
1 Week	■	■					
2 Weeks	■	■	■				
3 Weeks	■	■	■	■			
4 Weeks	■	■	■	■	■		
6 Weeks	■	■	■	■	■	■	■

Fig. 2. Schedule of labeling agents administration.

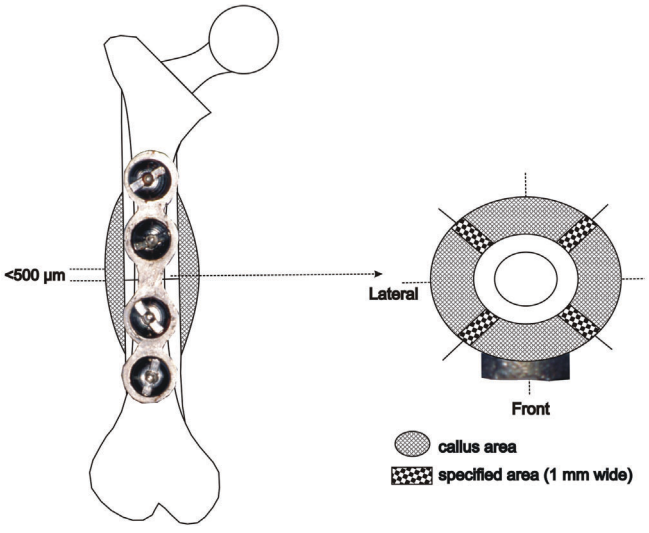

Fig. 3. A diagrammatic representation shows that four 100-μm-thick cross-sections were cut in an area within 500 μm from the original fracture line and then ground to 50 μm thickness for histomorphometry. Histomorphometric measurement was performed on the whole area and further on four specified areas.

Sample preparation

The fractured femora were removed by disarticulation at the hip and knee joints. All femurs were dissected clean from the surrounding soft tissues. The femora were placed in 1.4% paraformaldehyde-5%-sucrose-0.02M-phosphate buffer 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) (Wolf et al., 1992). Four 100-μm-thick cross-sections (n=7/group) were cut with a band saw (Exakt, Otto Herrmann Co., Norderstedt, Germany) in an area within 500 μm of the original fracture line (Fig. 3) and then ground to 50 μm thickness for histomorphometry (Li et al., 1999). Two slides were stained with the Toluidin (Certistain®, Merck, Darmstadt, Germany) and Giemsa method (Giemsa solution, Merck, Darmstadt, Germany) (Schenk et al., 1984) and used for determining structural and static endpoints. Two slides were coverslipped without further staining and used for determining dynamic endpoints of bone formation. Three 100-μm-thick transverse sections (n = 3/group) were cut with a band saw (Exakt, Otto Herrmann Co., Norderstedt, Germany) in an area within 500 μm of the geometrical middle of the femoral shaft in the region of the original fracture line and then ground to 50 μm thickness (Fig. 4). The middle of the femur in the region of the original fracture line was calculated by measuring the diameter at the thickest part of the callus using Venier calipers (Mitsuyo, Japan). These slides were also stained with the Toluidin (Certistain®, Merck, Darmstadt, Germany) and Giemsa method (Giemsa solution, Merck, Darmstadt, Germany) (Schenk et al.,1984) and examined under a binocular light/epifluorescent microscope (Model BH2-RFC, Olympus®, Tokyo, Japan).

Callus histomorphometry

Histomorphometrical analyses were performed with a semiautomated digitizing image analyzer. The system consisted of a light/epifluorescent microscope (Model BH2-RFC, Olympus®, Tokyo, Japan) and a digitizing

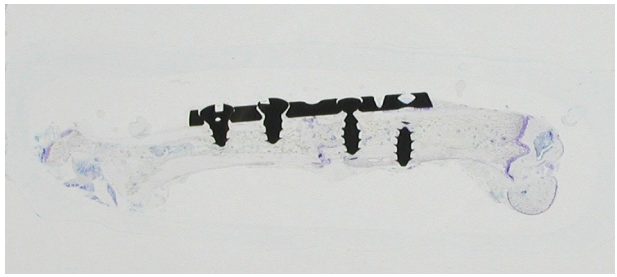

Fig. 4. Histological view of the fractured femur at 3 weeks postfracture. Stain is Toluidin and Giemsa.

pad coupled to a computer with a histomorphometric software (analySIS, Soft Imaging System, Münster, Germany). Nomenclature and symbols used in conventional bone histomorphometry are the same as those described Parfitt et al. (1987).

Total cross-sectional area (C.T.Ar, mm^2), original cortical area (O.Ar, mm^2), and medullary area (M.Ar, mm^2) were measured at x125 magnification and callus area (Ca.Ar = C.T.Ar – O.Ar – M.Ar, mm^2) was calculated. Using the method described by Li et al. (1999), further measurements were made at x200 magnification in four standardized quarters: anteromedial, posteromedial, posterolateral, anterolateral given an exactly ventrally positioned mini-plate (Fig. 2). The following histomorphological parameters were measured: total trabecular area (Tb.Ar, mm^2), trabecular perimeter (Tb.Pm, mm^2), osteoid perimeter (O.Pm, mm), osteoclast perimeter (Oc.Pm, μm), eroded perimeter (E.Pm, μm) (Measurement from week 2 postfracture because Hownship's lacunae were not demonstrable any earlier.), osteoid width (O.Wi, μm), single- and double-labeled perimeter (sL.Pm and dL.Pm, μm), the interlabel width (Ir.L.Wi, μm) of double labels, wall width (W.Wi, μm) (measurement 6 weeks postfracture). The following structural calculations were made: bone surface (BS = Tb.Pm, μm), osteoid surface [OS = (O.Pm/Tb.Pm) x 100, %], osteoclast surface [Oc.S = (Oc.Pm/Tb.Pm) x100, %], eroded surface [ES = (E.Pm/Tb.Pm) x 100,%] (calculations from week 2 postfracture), quiescent surface [QS=BS - (OS+ES),%]. The following dynamic calculations were made: single-labeled surface [sLS = (sL.Pm/Tb.Pm) x 100,%], double-labeled surface [dLS = (dL.Pm/Tb.Pm) x 100,%], mineralizing surface (MS = dLS + 0.5sLS,%), mineral apposition rate [MAR = Ir.L.Wi/Ir.L.t (interlabel time period), μm/d], surface referent bone formation rate (BFR/BS = MS x MAR, $\mu m^3/\mu m^2$/d), adjusted apposition rate [Aj.Ar = MAR x (MS/OS), μm/d], mineralization lag time (Mlt = O.Wi/Aj.Ar, d). To estimate the remodeling period and its subdivisions, the following calculations were made 6 weeks postfracture: formation period (FP = W.Wi/Aj.Ar, d), resorption period [Rs.P = FP x (Oc.S/OS), d], reversal period [Rv.P = FP x (ES x Oc.S)/OS, d], remodeling period (Rm.P = Rs.P + Rv.P + FP, d), quiescent period [QP = FP x (QS/BS), d], activation frequency (Ac.f = FP + Rs.P + QP^{-1}, $year^{-1}$).

Data analysis

The significance of differences between groups was calculated with the unpaired Student's t test corrected by Bonferroni-Holm procedure (Aickin and Gensler, 1996). The level of significance was pre-set at $p<0.05$. All statistical analyses were carried out according to Steel and Torrie (1988) using a computer program (SPSS/PC+TM 4.0, Base Manual for the IBM PC/XT/AT and PS/2V, Release 4.0, SPSS Inc., Chicago, USA, 1990).

Results

Callus histomorphometry

One week after fracture

In the two diabetic groups, the blood glucose values at the time of the surgery ($p<0.05$), the postoperative blood glucose course ($p<0.01$) and the postoperative insulin requirement ($p<0.001$) were statistically significantly increased in the poorly compensated diabetic rats (Table 1).

Table 2 summarizes the calculated results of bone histomorphometry 1 week after fracture (measured data not shown). Comparing the diabetic groups, single-labeled surface (sLS, 228%, $p<0.01$), double-labeled surface (dLS, 223%, $p<0.05$), mineralizing surface (MS, 221%, $p<0.01$), mineral apposition rate (MAR, 450%, $p<0.001$), adjusted apposition rate (Aj.Ar, 685%, $p<0.001$) and bone formation rate (BFR/BS, 1200%, $p<0.001$) (Fig. 5) were significantly increased in the well-compensated diabetic rats. Comparing the poorly compensated diabetic rats to the control group, the mineralizing surface-related parameters single-labeled surface (sLS, 245%, $p<0.01$), double-labeled surface (dLS, 259%, $p<0.01$), mineralizing surface (MS, 234%, $p<0.01$), mineral apposition rate (MAR, 440%,

Table 2. Callus histomorphometry, calculated values 1 week after fracture.

PARAMETER	BB/OK, METABOLIC COMPENSATION		CONTROL ANIMALS
	Well	Poor	
Callus area (Ca.Ar, mm^2, $x10^{-4}$)	12.35±4.33	8.03±2.33[a]*	13.64±3.17b*
Osteoid surface (OS, %)	17.34±6.58	12.85±4.04	16.83±8.08
Osteoclast surface (Oc.S, %)	7.56±4.82	6.46±2.90	8.33±4.67
Single-labeled surface (sLS, %)	14.23±6.52	6.22±2.57[a]**	15.09±7.04[b]**
Double-labeled surface (dLS, %)	9.11±4.11	4.08±1.26[a]*	10.56±4.74[b]**
Mineralizing surface (MS, %)	16.55±6.07	7.50±4.45[a]**	17.57±6.45[b]**
Mineralization lag time (Mlt, d)	2.54±1.36	19.87±4.45[a]***	2.35±1.41[b]***

All values are expressed as mean±SD.[a]: well-compensated vs. poorly compensated. [b]: poorly compensated vs. control animals. *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$

p<0.001), adjusted apposition rate (Aj.Ar, 725%, p<0.001) and the bone formation rate (BFR/BS, 1250%, p<0.001) (Fig. 5) were significantly higher in the control animals. The mineralization lag time (Mlt) was significantly prolonged in the poorly compensated diabetic rats in comparison to both the well-compensated diabetic rats (782%, p<0.001) and to the control animals (845%, p<0.001). There were no significant differences between the well-compensated diabetic rats and the control rats.

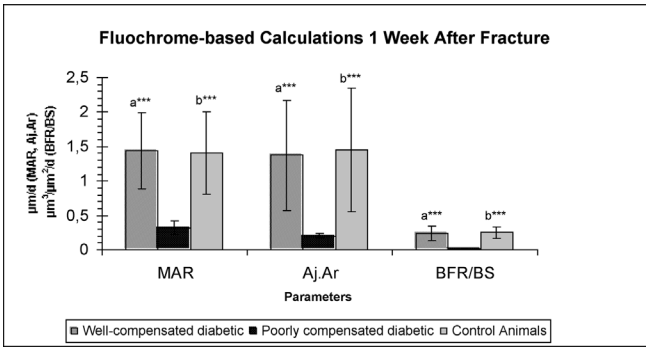

Fig. 5. Fluochrome-based calculations 1 week after fracture.

Two weeks after fracture

The differences in the diabetic animals were the significantly increased blood glucose values at the time of surgery and postoperative blood glucose curves (p<0.01), as well as the significantly increased postoperative insulin requirement (p<0.001) (Table 1) of the poorly compensated diabetic rats.

Table 3 gives an overview of the calculated results of bone histomorphometry two weeks after fracture. The differences in the measured fluorochome-based indices (Fig. 6) (measured data not shown) resulted in significantly higher dynamic calculations [mineral apposition rate (MAR, 364%, p<0.001), bone formation rate (BFR/BS, 833%, p<0.001), adjusted apposition rate (Aj.Ar, 767%, p<0.001), mineralizing surface (MS, 235%, p<0.01), single-labeled surface (sLS, 293%, p<0.01), double-labeled surface (dLS, 225%, p<0.05)]

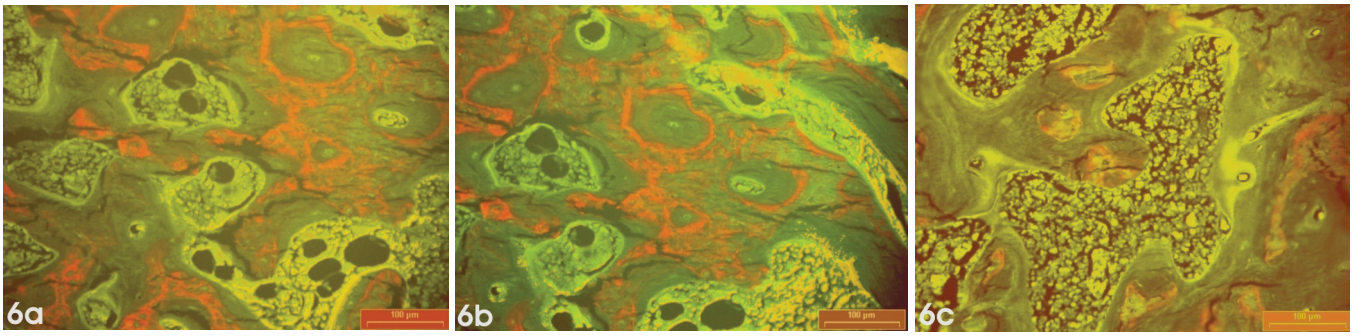

Fig. 6. Fluorescent photomicrographs (yellow: tetracycline; green: calcein; red: alizarin) of the fracture callus at 2 weeks postfracture. Gap callus in control **(a)**, well-compensated diabetic **(b)**, and poorly compensated diabetic **(c)** animals. Note the substantial decreases in alizarin uptake in the poorly compensated diabetic animals. Original magnification x 200. Bar represents 100 µm. Unstained.

Table 3. Callus histomorphometry, calculated values 2 weeks after fracture.

PARAMETER	BB/OK, METABOLIC COMPENSATION		CONTROL ANIMALS
	Well	Poor	
Callus area (Ca.Ar, mm^2, x 10^{-4})	15.36±7.73	10.03±5.73a*	16.00±6.77b*
Osteoid surface (OS, %)	28.52±11.23	22.78±10.35	27.82±9.62
Osteoclast surface (Oc.S, %)	9.87±5.36	7.92±4.12	8.26±5.56
Eroded surface (ES, %)	13.87±5.72	11.28±7.53	12.18±6.83
Quiescent surface (QS, %)	55.44±14.75	65.95±22.86	60.11±24.65
Single-labeled surface (sLS, %)	15.45±7.44	5.27±1.56a**	16.28±9.22b**
Double-labeled surface (dLS, %)	8.98±2.66	3.98±0.88a*	10.22±3.75b*
Mineralizing surface (MS, %)	15.76±6.58	6.68±2.06a**	18.39±8.25b**
Mineralization lag time (Mlt, d)	3.87±1.66	20.76±6.65a***	3.21±1.22b***

All values are expressed as mean±SD. a well-compensated vs. poorly compensated. b poorly compensated vs. control animals. *p < 0.05, **p < 0.01, ***p < 0.001

for the well-compensated diabetic animals in both diabetic groups (Fig. 7). It also resulted in significantly higher dynamic calculations [mineral apposition rate (MAR, 391%, p<0.001), bone formation rate (BFR/BS, 1066%, p<0.001), adjusted apposition rate (Aj.Ar, 958%, p<0.001), mineralizing surface (MS, 275%, p<0.01), single-labeled surface (sLS, 308%, p<0.01), double-labeled surface (dLS, 255%, p<0.05)] for the control animals in comparison to the poorly compensated diabetic animals (Fig. 7). The mineralization lag time (Mlt) was significantly prolonged in poorly compensated diabetic rats in comparison to both the well-compensated diabetic rats (536%, p<0.001) and to the control animals (646%, p<0.001). There were no significant differences between the well-compensated diabetic and the control rats.

Three weeks after fracture

In the diabetic animals, the blood-glucose values at the time of the surgery, the postoperative blood-glucose course (each p<0.01) and the postoperative insulin requirement (p<0.001) were statistically significantly increased in the poorly compensated diabetic rats (Table 1).

Table 4 summarizes the calculated results of bone histomorphometry 3 weeks postfracture (measured data not shown). Comparing the diabetic groups, single-labeled surface (sLS, 144%, p<0.05), double-labeled surface (dLS, 193%, p<0.05) and mineralizing surface (MS, 170%, p<0.01), mineral apposition rate (MAR, 361%, p<0.001) and adjusted apposition rate (Aj.Ar, 346%, p<0.001) of the apposition rates and the bone formation rate (BFR/BS, 636%, p<0.001) (Fig. 8) were significantly increased in the well-compensated diabetic rats. Comparing the poorly compensated diabetic rats to the control group, the mineralizing surface-related parameters single-labeled surface (sLS, 153%, p<0.05), double-labeled surface (dLS, 207%, p<0.01), mineralizing surface (MS, 187%, p<0.01), mineral apposition rate (MAR, 397%, p<0.001), adjusted apposition rate (Aj.Ar, 398%, p<0.001) and the bone formation rate (BFR/BS, 763%, p<0.001) (Fig. 8) were significantly higher in the control animals. The mineralization lag time (Mlt) was significantly prolonged in poorly compensated diabetic rats in

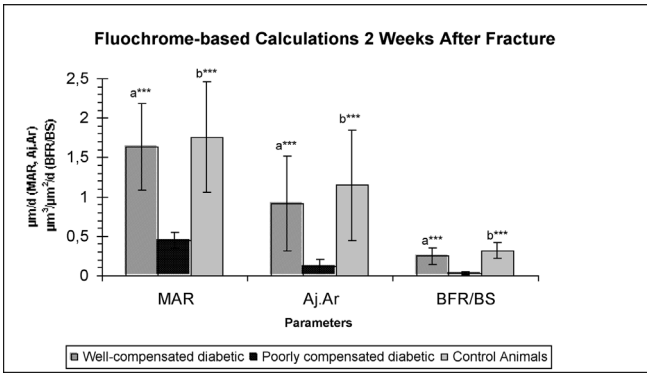

Fig. 7. Fluochrome-based calculations 2 weeks after fracture.

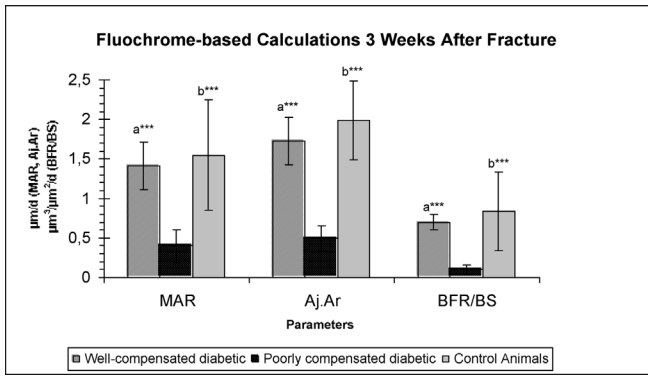

Fig. 8. Fluochrome-based calculations 3 weeks after fracture.

Table 4. Callus histomorphometry, calculated values 3 weeks after fracture.

PARAMETER	BB/OK, METABOLIC COMPENSATION		CONTROL ANIMALS
	Well	Poor	
Callus area (Ca.Ar, mm^2, x 10^{-4})	17.36±4.93	11.63±3.73^{a*}	18.51±4.22^{b**}
Osteoid surface (OS, %)	40.64±21.45	20.99±12.08^{a**}	41.83±23.04^{b**}
Osteoclast surface (Oc.S, %)	11.72±9.78	10.85±6.55	12.28±9.99
Eroded surface (ES, %)	15.38±6.56	14.11±7.54	15.16±4.94
Quiescent surface (QS, %)	54.11±26.58	60.98±32.26	54.19±22.24
Single-labeled surface (sLS, %)	43.34±21.56	29.93±14.97^{a*}	45.99±12.55^{b*}
Double-labeled surface (dLS, %)	28.36±11.28	14.67±10.22^{a*}	30.38±9.54^{b**}
Mineralizing surface (MS, %)	49.27±14.86	29.05±11.65^{a*}	54.34±13.32^{b**}
Mineralization lag time (Mlt, d)	4.69±1.32	18.56±5.76^{a***}	4.94±2.11^{b***}

All values are expressed as mean±SD. [a] well-compensated vs. poorly compensated. [b] poorly compensated vs. control animals. *p< 0.05, **p<0.01, ***p<0.001

comparison to both the well-compensated diabetic rats (395%, p<0.001) and to the control animals (375%, p<0.001). There were no significant differences between the well-compensated diabetic and the control rats.

Four weeks after fracture

In the diabetic animals, the postoperative insulin requirement and blood glucose course (p<0.001), and the blood glucose values at the time of surgery were significantly increased (p<0.01) in the poorly compensated diabetic rats (Table 1).

Table 5 summarizes the calculated results of bone histomorphometry (measured data not shown) 4 weeks postfracture. Comparing the diabetic groups, mineral apposition rate (MAR, 221%, p<0.01), adjusted apposition rate (Aj.Ar, 248%, p<0.01) and the bone formation rate (BFR/BS, 270%, p<0.01) were significantly increased in the well-compensated diabetic rats. The dynamic calculations double-labeled surface (dLS, 153%, p<0.05), mineralizing surface (MS, 138%, p<0.05), mineral apposition rate (MAR, 227%, p<0.01), adjusted apposition rate (Aj.Ar, 279%, p<0.01) and the bone formation rate (BFR/BS, 307%, p<0.01) of the

Table 5. Callus histomorphometry, calculated values 4 weeks after fracture.

PARAMETER	BB/OK, METABOLIC COMPENSATION		CONTROL ANIMALS
	Well	Poor	
Callus area (Ca.Ar, mm^2, $x10^{-4}$)	15.07±4.03	13.19±2.03	15.33±4.60
Osteoid surface (OS, %)	46.54±21.65	42.11±24.63	44.72±12.66
Osteoclast surface (Oc.S, %)	10.07±4.82	10.65±6.33	9.22±4.15
Eroded surface (ES, %)	12.03±8.56	11.72±5.61	13.65±7.54
Quiescent surface (QS, %)	41.27±17.82	46.22±27.34	41.65±17.82
Single-labeled surface (sLS, %)	53.32±23.57	45.33±22.56	55.23±26.65
Double-labeled surface (dLS, %)	31.23±17.55	23.12±13.34	35.45±12.44^{b*}
Mineralizing surface (MS, %)	58.45±21.27	46.03±24.60	63.56±31.80^{b*}
Mineral apposition rate (MAR, μm/d)	1.93±1.22	0.87±0.43^{a*}	1.98±1.11^{b**}
Adjusted apposition rate (Aj.Ar, μm/d)	2.41±1.07	0.97±0.71^{a**}	2.71±1.16^{b**}
Bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/d)	1.11±0.65	0.41±0.09^{a**}	1.26±0.64^{b***}
Mineralization lag time (Mlt, d)	4.94±2.16	6.32±2.41	5.34±2.86

All values are expressed as mean±SD. [a]: well-compensated vs. poorly compensated. [b]: poorly compensated vs. control animals. *p<0.05, **p<0.01, ***p<0.001

Table 6. Callus histomorphometry, calculated values 6 weeks after fracture.

PARAMETER	BB/OK, METABOLIC COMPENSATION		CONTROL ANIMALS
	Well	Poor	
Callus area (Ca.Ar, mm^2, x 10^{-4})	8.38±2.23	9.82±3.92	8.17±1.08
Osteoid surface (OS, %)	55.94±22.63	50.54±25.68	57.00±24.11
Osteoclast surface (Oc.S, %)	10.77±6.88	12.93±10.64	10.40±9.14
Eroded surface (ES, %)	12.22±4.46	14.76±7.28	12.73±6.63
Quiescent surface (QS, %)	35.09±14.85	34.75±21.43	33.08±12.63
Single-labeled surface (sLS, %)	57.56±25.86	46.93±16.76	58.68±20.74
Double-labeled surface (dLS, %)	30.90±16.55	26.37±11.34	31.35±12.55
Mineralizing surface (MS, %)	59.44±23.22	49.56±21.76	60.78±25.97
Mineral apposition rate (MAR, μm/d)	1.87±1.09	1.35±1.65	1.89±1.22b*
Adjusted apposition rate (Aj.Ar, μm/d)	2.02±1.00	1.13±0.44^{a*}	2.03±1.15b*
Bone formation rate (BFR/BS, μm3/μm2/d)	1.11±0.56	0.70±0.23	1.13±1.01
Mineralization lag time (Mlt, d)	4.45±3.34	6.99±3.43^{a*}	4.23±2.67^{b*}
Formation period (FP, d)	3.45±1.74	6.12±2.84^{a*}	3.51±1.56^{b*}
Resorption period (Rs.P, d)	0.79±0.12	1.68±1.13^{a**}	0.76±0.26^{b**}
Reversal period (Rv.P, d)	0.10±0.01	0.24±0.09^{a**}	0.14±0.07^{b*}
Remodeling period (Rm.P, d)	4.35±1.45	8.04±3.55^{a*}	4.41±1.73^{b*}
Quiescent period(QP, d)	1.60±0.73	2.89±1.46^{a*}	1.54±0.83^{b*}
Activation frequency (Ac.f, year-1)	0.17±0.02	0.06±0.01^{a**}	0.18±0.03^{b***}

All values are expressed as mean±SD. [a]: well-compensated vs. poorly compensated. [b]: poorly compensated vs. control animals. *p<0.05, **p<0.01, ***p<0.001

control animals were significantly increased compared with the poorly compensated diabetic animals. Between the well-compensated diabetic and the control rats, no significant differences were histomorphometrically demonstrable.

Six weeks after fracture

In the diabetic animals, the postoperative insulin requirement and the postoperative blood glucose course were significantly increased ($p<0.001$) in the poorly compensated diabetic rats, and the blood glucose values at the time of surgery were significantly increased ($p<0.01$) (Table 1).

Apart from a significantly increased adjusted apposition rate (Aj.Ar, 178%, $p<0.05$) in the well-compensated diabetic animals in comparison to the poorly compensated rats and a significantly prolonged mineralization lag time (Mlt, 157%, $p<0.05$) in the poorly compensated diabetic animals in comparison to the well-compensated, there were no statistical differences between the diabetic animals, as shown in an overview in Table 6. The mineral apposition rate (MAR, 140%, $p<0.05$) and adjusted apposition rate (Aj.Ar, 179%, $p<0.05$) were significantly increased in the control animals in comparison to the poorly compensated diabetic animals. The mineralization lag time (Mlt) was significantly prolonged in the poorly compensated diabetic animals (165%, $p<0.01$) in comparison to the control animals.

In the diabetic animals, the indices of remodeling cycle duration and its subdivisions (Table 6) resulted in a significantly prolonged formation period (FP, 177%, $p<0.05$), resorption period (Rs.P, 212%, $p<0.01$), reversal period (Rv.P, 240%, $p<0.01$), remodeling period (Rm.P, 184%, $p<0.05$) and quiescent period (QP, 180%, $p<0.05$) of the poorly compensated diabetic animals. A comparison between the control animals and the poorly compensated diabetic animals showed a significantly prolonged formation period (FP, 174%, $p<0.05$), resorption period (Rs.P, 221%, $p<0.01$), reversal period (Rv.P, 171%, $p<0.05$), remodeling period (Rm.P, 182%, $p<0.05$) and quiescent period (QP, 187%, $p<0.05$) of the poorly compensated diabetic animals. The activation frequency (Ac.f) was statistically decreased in the poorly compensated animals compared to both the well-compensated diabetic animals (282%, $p<0.01$) the control animals (300%, $p<0.001$).

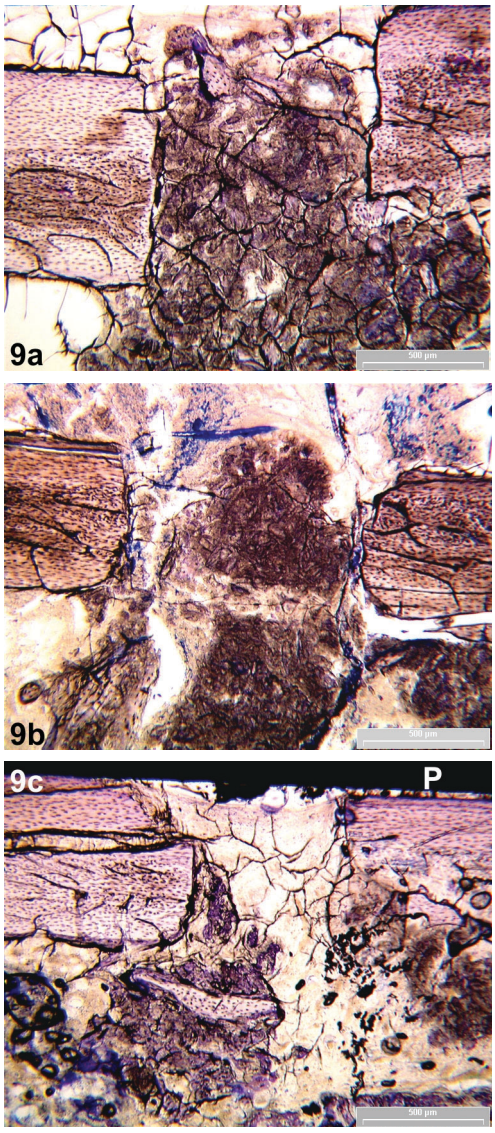

Fig. 9. Histology of the fracture callus at 2 weeks postfracture. Gap callus in control **(a)**, well-compensated diabetic **(b)**, and poorly compensated diabetic **(c)** animal. Stain is Toluidin and Giemsa. Original magnification x 63. Bar represents 500 μm.

Callus morphology

One week after fracture

Table 7 summarizes the temporal sequence of early histological events at the fracture site (Namkung-Matthai et al., 2001). At one week postfracture, the fractured bone was bound by early callus with lamella and woven trabecular bone differentiating subperiostally in the spontaneously diabetic rats independent of their diabetic metabolic state as well as in the control rats. Hyaline cartilage, foci of woven bone, and undifferentiated mesenchymal tissue immediately adjacent to the fracture line were present in all three samples. However, the callus forming in the fractured femurs of the poorly compensated diabetic animals appeared to have more undifferentiated mesenchymal tissue and thinner trabecular bone, which extended beyond that found in the well-compensated diabetic and in the control animals.

Two weeks after fracture

At 2 weeks, callus in the samples of the well-compensated diabetic and control animals had minimal undifferentiated mesenchymal tissue (Fig. 9). A

significant proportion of hyaline cartilage had undergone endochondral osteogenesis. The differentiation in the callus formed in the poorly compensated diabetic animals appeared to lag behind that seen in the samples of the well-compensated diabetic and control animals at 2 weeks (Table 7).

Three weeks after fracture

After week 3 postfracture, calluses indicated that endochondral ossification in the well-compensated diabetic and control animals had progressed extensively displaying large portions of lamellar bone undergoing remodeling. Prominent hypertrophic chondrocytes undergoing ossification, however, persisted in the poorly compensated diabetic animals, indicating a delay in cellular differentiation (Table 7).

Four weeks after fracture

Four weeks postfracture, the callus in femurs of the well-compensated diabetic and control animals was composed of mature lamellar bone, although hyaline cartilage persisted in the femurs of the poorly compensated diabetic animals, still suggesting a delay in differentiation (Fig. 10).

Six weeks after fracture

6 weeks, the microscopic appearance of the callus was similar to that of the control and the spontaneously diabetic rats independent of their diabetic metabolic state (Fig. 11). The dominant tissue was well formed trabecular bone.

Discussion

The extensive and detailed histomorphometrical data at various times of fracture healing collected in our study do not show any differences between the spontaneously diabetic BB/OK rats with well-compensated diabetic metabolic state and the control animals. Until the end of the 4th week postfracture, the spontaneously diabetic animals with poorly compensated diabetic metabolic states showed a considerable mineralization disorder,

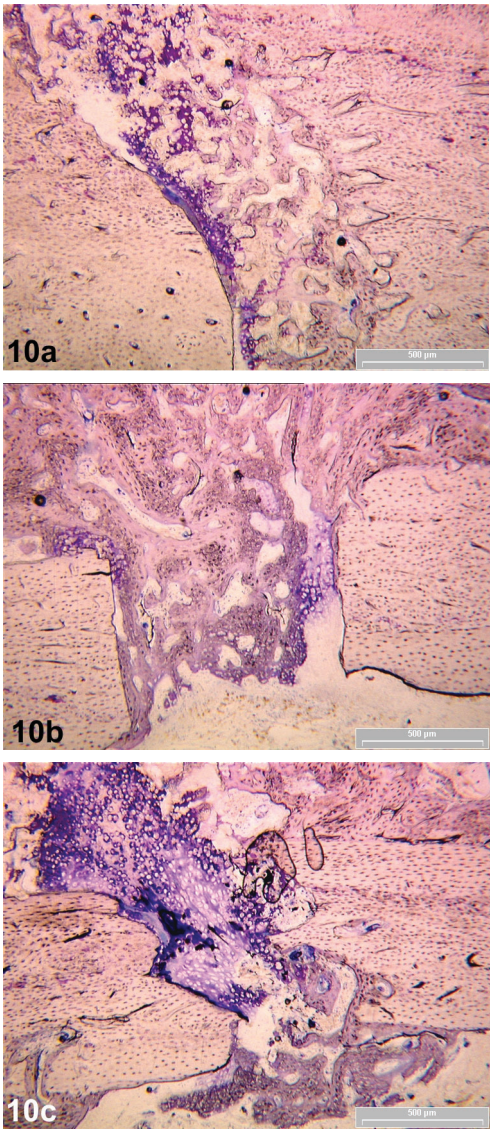

Fig. 10. Histology of the fracture callus at 4 weeks postfracture. Gap callus in control **(a),** well-compensated diabetic **(b)**, and poorly compensated diabetic **(c)** animals. Original magnification x 63. Bar represents 500 μm. Stain is Toluidin and Giemsa.

Table 7. Temporal sequence of early histological events at the fracture site.

HISTOLOGICAL EVENT	POSITIVE FINDINGS/NUMBER OF ANIMALS/GROUP			
	Week 1	Week 2	Week 3	Week 4
Soft callus				
Hypertrophic chondrocyte				
Well-compensated rats	3/3	3/3	3/3	3/3
Poorly compensated rats	3/3	3/3	3/3	3/3
Control rats	3/3	3/3	3/3	3/3
Endochondral ossification				
Well-compensated rats	3/3	3/3	3/3	3/3
Poorly compensated rats	3/3	3/3	3/3	3/3
Control rats	3/3	3/3	3/3	3/3
Hard callus				
Periostal callus formation				
Well-compensated rats	3/3	3/3	3/3	3/3
Poorly compensated rats	2/3	2/3	3/3	3/3
Control rats	3/3	3/3	3/3	3/3
Bone remodeling				
Well-compensated rats	0/3	1/3	2/3	3/3
Poorly compensated rats	0/3	0/3	1/3	2/3
Control rats	0/3	1/3	3/3	3/3

which is the expression of a reduced and delayed mineralization process. These early disorders manifest themselves 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- and poorly compensated diabetic animals and between the poorly compensated animals and the control animals. Histomorphometrically, this proves in detail that the poorly compensated genetically determined diabetes mellitus of the test animals impairs the mineral metabolism in form of a decreased and delayed mineralization in the early stages of fracture healing. In correlation with this, a delay of the cell differentiation until the end of the 4th week postfracture was morphologically proven exclusively in poorly compensated diabetic rats.

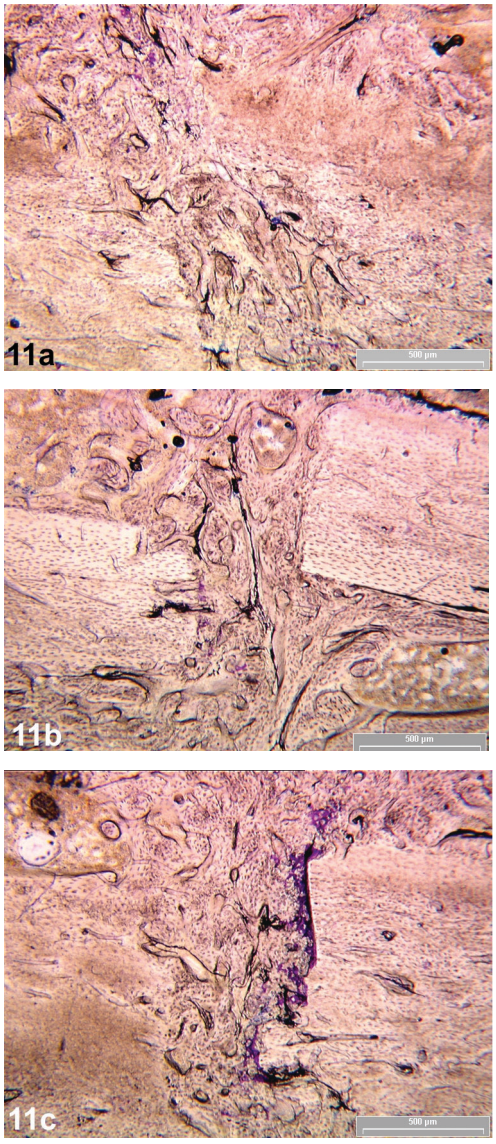

Fig. 11. Histology of the fracture callus at 6 weeks postfracture. Gap callus in control **(a)**, well-compensated diabetic **(b)**, and poorly compensated diabetic **(c)** animals. Original magnification x 63. Bar represents 500 μm. Stain is Toluidin and Giemsa.

Until now, only Beam et al. (2002) has shown that the values of cellular proliferation and callus bone content of blood-glucose-controlled BB rats during fracture healing do not significantly differ from non-diabetic control animals. If the blood glucose values are successfully controlled in animal experiments with genetically determined spontaneous diabetes (Beam et al., 2002) 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 morphologically shown cellular differentiation disorders disappear completely in comparison to the normoglycemic control animals.

Regarding bone repair, Shyng et al. (2001) did not show any histological differences in the bone remodeling of regenerating bone of calvarial defects between insulin-treated streptozotocin-induced and control rats 4 weeks postoperatively, whereas he observed considerable deficits in untreated streptozotocin-induced rats. However, Follak et al. (2003) had 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.

Various reports state a preventive or reverse effect of treatment with insulin in experimental (Weiss and Reddi, 1980) and in spontaneous diabetes (Verhaeghe and Bouillon, 1996; Verhaeghe et al., 1997). It appears to revert active osteoblasts into inactive bone-lining cells, from which the suggestive evidence of decreased proliferation of preosteoblastic cells is concluded (Weiss and Reddi, 1980).

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 day (Macey et al.,1989) and a decrease of type X collagen expression in the fracture callus of between 54% and 70% on the 14th postoperative day (Topping et al., 1995).

Histomorphometric results on fracture healing have not been reported in spontaneously diabetic rats to date. Shyng et al. (2001) found that cancellous bone volume and bone formation in the femur were greatly reduced in the streptozotocin-induced diabetic model, indicating either a defect of mineralization or osteoid formation. These observations are also consistent with published reports of impaired osteoid formation (Shires et al., 1981), and decreased synthesis of both collagen (Schneir et al., 1979, 1990; Spanheimer, 1988) and proteoglycan (Weiss et al., 1981), in streptozotocin-induced diabetes. Since osteoclast numbers are also reduced in diabetic rats (Hough et al., 1981; Shires et al., 1981) an overall reduction in bone turnover, rather than excessive bone resorption, is implicated in the pathogenesis of diabetic

osteopenia (Bain et al., 1997).

Skeletal changes in diabetic rats have also been attributed to metabolic abnormalities which accompany the malnutrition of insulin deficiency (Shires et al., 1981). However, recent studies have shown that undernutrition can account for only 30% of the net deficit in collagen production in the streptozotocin-induced diabetic rats (Umpierrez et al., 1989). Spanheimer et al. (1988) has shown that decreases in collagen production in the streptozotocin-induced diabetic rats are a consequence of the chronic diabetic state and not due to Streptozotocin toxicity in the days immediately after injection of this diabetogenic drug. Furthermore, exposing bone and cartilage in culture to Streptozotocin for a short time had no direct effect on connective tissue metabolism (Spanheimer, 1989).

In contrast to Verhaeghe et al. (1992), 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, there were no histomorphometrically different parameters during fracture healing in well-compensated diabetic animals. In spite of the daily treatment with insulin and resulting overall poor diabetic metabolic state, statistically significant differences in dynamic calculations remained 6 weeks postfracture in the poorly compensated animals, which were only detectable with very sensitive quantitative histomorphometrical measurements, but not with qualitative morphology.

In contrast to studies with untreated streptozotocin-induced (Dixit and Ekstrom, 1978; Macey et al., 1989; Topping et al., 1995; el-Hakim, 1999; Funk et al., 2000) or poorly controlled spontaneous diabetes (Beam et al., 2002), all animals were treated with insulin according to the current blood glucose value. Still, one of the experimental groups was in a poor diabetic metabolic state because of the postoperative blood glucose course and the insulin requirements due to a lack of endogenous insulin, in comparison to the mostly well-compensated diabetic animals with some few ß cells releasing endogenous insulin, which favours glucose metabolism (Klöting and Voigt, 1991). 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 untreated diabetes mellitus, and is especially important when searching for clinically relevant complications (Loder, 1988) in fracture repair.

In contrast to streptozotocin-induced diabetes, BB/OK rats spontaneously develop Type 1 diabetes with a number of analogies to human diabetes (Yoon and Yun, 2001). As is true of Type 1 diabetes in human, Type 1 diabetes in BB rats is familial, but the mode of inheritance is non-Mendelian. In both species, it is more clearly associated with certain class II genes of the major histocompatibility complex (MHC). BB diabetes requires class II genes of RT1u haplotype (Iddm1) (Klöting et al., 1995, 1998). That these genes are essential for diabetes development is supported by two additional rat models of type 1 diabetes, the Komeda Diabetes-Prone and the LEW.1AR1/Ztm-iddm rat. Both rat strains are also characterised by the MHC class II genes of the RT1u haplotype (Lenzen et al., 2001; Yokoi et al., 1997). This fact and recent findings in class II specimen mice indicating the importance of these genes in bone properties prompted us to use normoglycemic LEW.1A rats with diabetes-resistant MHC class II genes of the RT1a haplotype as controls (Simske et al., 2002).

As Bouillon (1991) aptly described, it is altogether difficult to form a comprehensive picture of the pathomorphological 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 (Bouillon, 1991). This is the approach of several recent studies concerned with the optimization of the fracture healing or the bone turnover in experimental diabetes. Kawaguchi et al. (1994) showed that local application of recombinant human basic fibroblast growth factor (bFGF) may facilitate bone union in streptozotocin-induced rats with impaired fracture repair. Hoshino et al. (2000) observed an enhancement of fracture healing in rats with streptozotocin-induced diabetes with a single injection of biodegradable microcapsules containing a bone formation stimulant, TAK-778. Finally, Tsuchida et al. (2000) showed that PTH enhanced bone turnover and bone mass but not trabecular connectivity in the late stage of streptocotocin-induced diabetes in rats. Bain et al. (1997) reported that minocycline treatment of the streptocotocin diabetic rat maintains normal bone formation, normalizes growth plate thickness, and prevents cancellous bone loss.

Taking the duration of the whole remodeling process and the appertaining partial parameters during fracture repair into account, there were no statistical differences between the well-compensated diabetic and the control animals. As an expression of the delayed remodeling, the remodeling period in the poorly compensated diabetic animals was prolonged by a factor of almost 2 compared to the well-compensated diabetic animals (184%) and the control rats (182%) 6 weeks after fracture .

In conclusion, we have shown that a delay in mineralization and cell differentiation occurs in the early stage of fracture repair in poorly compensated diabetes of spontaneously diabetic animals. According to the histomorphometrical and histological evaluation, there are no differences in the fracture healing in the well-compensated metabolic state, whereas histomorphometrically quantitative significant deficits in measured and dynamically calculated parameters remain in the poorly compensated diabetic metabolic state.

With a strictly controlled insulin therapy and a resulting well-compensated diabetic metabolic state, severe mineralization and cell differentiation disorders in the early phase of fracture healing in the poorly

compensated diabetic metabolic state of the experimental animal can be avoided.

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