Diabetic cardiomyopathy is known to result in increased mortality after ischemic events. Permanently increased oxidative stress with formation of oxygen-free radicals plays a key role in the development of specific heart muscle disease. Associated lesions include structural alterations to cardiomyocytes. Antioxidative treatment in addition to the usual insulin substitution would seem sensible in preventing or delaying long-term diabetic complications and protecting the myocardium against acute ischemic events. We investigated the effects of radical scavenger Ginkgo biloba extract EGb 761 against diabetes-induced damage to cardiomyocytes and additional ischemia/reperfusion injury in spontaneously diabetic BioBreeding/Ottawa Karlsburg (BB/OK) rats, as a model of diabetic myocardium infarction. Morphological and morphometric parameters of heart muscles were analyzed by light and electron-microscopic techniques. We used immunohistochemistry to evaluate parameters of oxidative stress (superoxide dismutase [SOD]) and inducible nitric oxide synthase (iNOS) protein expression. Our results indicated that A) Diabetic myocardium appears more vulnerable to ischemia/reperfusion damage concerning ultrastructure of cardiomyocytes (sarcomeres, vacuoles, mitochondria), expression of antioxidative enzymes (CuZnSOD, MnSOD), and iNOS than normal myocardium; B) Pretreatment of diabetic myocardium with EGb and additional ischemia/reperfusion leads to a relative improvement in myocardial ultrastructure compared to unprotected myocardium. In summary, EGb appears to be promising as an adjuvant therapeutic drug in diabetics with respect to ischemic myocardium injury. It may contribute to the prevention of late diabetic complications in diabetic cardiomyopathy.

Summary. Diabetic cardiomyopathy is known to result in increased mortality after ischemic events. Permanently increased oxidative stress with formation of oxygen-free radicals plays a key role in the development of specific heart muscle disease. Associated lesions include structural alterations to cardiomyocytes. Antioxidative treatment in addition to the usual insulin substitution would seem sensible in preventing or delaying long-term diabetic complications and protecting the myocardium against acute ischemic events. We investigated the effects of radical scavenger Ginkgo biloba extract EGb 761 against diabetes-induced damage to cardiomyocytes and additional ischemia/reperfusion injury in spontaneously diabetic BioBreeding/Ottawa Karlsburg (BB/OK) rats, as a model of diabetic myocardium infarction. Morphological and morphometric parameters of heart muscles were analyzed by light and electron-microscopic techniques. We used immunohistochemistry to evaluate parameters of oxidative stress (superoxide dismutase [SOD]) and inducible nitric oxide synthase (iNOS) protein expression. Our results indicated that A) Diabetic myocardium appears more vulnerable to ischemia/reperfusion damage concerning ultrastructure of cardiomyocytes (sarcomeres, vacuoles, mitochondria), expression of antioxidative enzymes (CuZnSOD, MnSOD), and iNOS than normal myocardium; B) Pretreatment of diabetic myocardium with EGb and additional ischemia/reperfusion leads to a relative improvement in myocardial ultrastructure compared to unprotected myocardium. In summary, EGb appears to be promising as an adjuvant therapeutic drug in diabetics with respect to ischemic myocardium injury. It may contribute to the prevention of late diabetic complications in diabetic cardiomyopathy.

Key words: Cardiomyocytes, Diabetes, Ischemia, SOD, EGb 761

Introduction

Insulin-dependent diabetes mellitus is characterized by a series of complications that affects many organs. Chronic diabetes leads to different structural and functional alterations in the myocardium, known as diabetic cardiomyopathy (Rubler et al., 1972; Kannel et al., 1974). These cardiomyopathic disturbances affecting cardiomyocytes, interstitium, autonomic nerves, and capillaries are responsible for increased cardiac risk in diabetic patients (Mahgoub and Abd-Elfattah, 1998; Nemoto et al., 2006).

Oxygen-free radicals (OFRs) have been implicated in the pathogenesis of diabetes mellitus (Baynes, 1991). Superoxide dismutase (SOD) plays a role in cellular antioxidative capacity, and acts in concert with catalase and glutathione peroxidase towards the elimination of OFRs and protection against oxidative injury (Kakkar et al., 1995). Previous studies have demonstrated complex alterations in the activities of antioxidant enzymes in rats with diabetes induced by streptozotocin and alloxan (Godin et al., 1988; Doi et al., 2001). Furthermore, Ca^{2+}-independent inducible NO synthase generates large quantities of NO with cell-damaging effects, without any regulatory mechanism to control NO generation (Varga et al., 1999; Farhangkhoee et al., 2006).

Several studies (Fein et al., 1981; Addicks et al., 1993) have demonstrated that some late complications in chronic diabetes cannot be completely avoided by insulin therapy. Consequently, attempts have been made to find additive therapeutic possibilities. Assuming that diabetic damage is partly caused by increased oxidative stress on the occurrence of oxygen-free radicals, the application of radical scavengers seems promising; indeed, there is evidence that some diabetic alterations
Isochemical lesions of diabetic cardiomyocytes

This study was designed to characterize (ultra)structural alterations in cardiomyocytes in spontaneously diabetic BioBreeding/Ottawa Karlsburg rats, a rat model of human diabetes type 1, additionally exposed to ischemia/reperfusion to simulate myocardial infarction in diabetics and to demonstrate the protective effects of Ginkgo biloba extract EGb, a known radical scavenger that stabilizes cell-membranes. To gain further insight on the antioxidative state of the myocardium, we investigated SOD expression, as well as the expression of inducible nitric oxide synthase (iNOS) as an indicator of cell damage. The diagnostic value of this expression is restricted, but can be improved by biochemical destination of enzyme activity. Increased expression of iNOS occurs after severe stress on the cells, indicating cell damage. Ischemic tolerance of diabetic myocardium was to be compared with that of normoglycemic myocardium.

Alterations in other components of cardiac tissue – connective tissue matrix and microvasculature – will be published in a second part of this study.

Materials and methods

Animals and experimental procedure

The experiments were approved by Leipzig’s regional governing committee (Regierungspraesidium No. 10/00) and have been performed in accordance with local animal welfare legislation. Twenty-two male diabetic BioBreeding/Ottawa Karlsburg (BB/OK) rats aged eight to nine months and sixteen male non-diabetic BB/OK rats (http://www.medizin.uni-greifswald.de/labanim/available_rat.html) kept separately under semisterile conditions were divided into five experimental groups.

Group I rats – 10 non-diabetic BB/OK animals – were not subjected to any treatment. Group II rats – 6 non-diabetic BB/OK animals – were exposed to ischemia and reperfusion using a Langendorff apparatus. Diabetic animals – 22 diabetic BB/OK rats – manifested insulin-dependent diabetes after 102 ±31.6 days as identified by weekly measurement of blood glucose levels. Once plasma glucose levels exceeded 22.1 mmol/l, the rats were treated by subcutaneous sustained-release insulin implant (LINPLANT, LINSHIN Canada, INC., Scarborough, Ontario, Canada). Group III rats – 5 diabetic BB/OK animals – were sacrificed after six months of diabetes. Group IV rats – 12 diabetic BB/OK animals – were exposed to ischemia and reperfusion by preparing isolated hearts using a Langendorff apparatus. After three months of diabetes, the Group V rats – 5 diabetic BB/OK animals – were treated daily with 100 mg/kg body weight of Ginkgo biloba extract (EGb 761, IPSEN Paris, France) dissolved in a limited amount of drinking water and administered overnight. After six months of diabetes and three month of protection by EGb, hearts were exposed to ischemia and reperfusion using a Langendorff apparatus.

Isolated heart perfusion (Langendorff heart)

BB/OK rats from Group II, IV and V were intraperitoneally anesthetized with pentobarbital (180 mg/kg bw). The hearts were excised after thoracotomy, the aorta cannulated, and retrograde perfusion was initiated at a pressure of 81 mm Hg using Tyrode solution saturated with 95% O₂ and 5% CO₂ gas at 37°C. In these experiments, the hearts were perfused for 30 min to allow functional stabilization, and then subjected to 35 min of 37°C global “no flow” ischemia followed by 90 min reperfusion.

Tissue processing for light microscopy

The animals were anesthetized using pentobarbital. The heart was rapidly excised after thoracotomy, and tissue samples from Groups I and III were taken from the left ventricle near the apex by scalpel and processed for histology, electron microscopy, and immunohistochemistry as described below. The biopsies of groups II, IV and V were taken immediately after reperfusion in the same manner.

Tissue processing for electron microscopy

Tissue samples were minced into small blocks of about 1 mm³, fixed in cold Karnovsky’s solution (buffered 2% glutaraldehyde, 2% paraformaldehyde, pH 7.4) for two hours, and contrasted with OsO₄ and phosphotungstic acid, as well as after acetone dehydration embedded in Durcupan (FLUKA).

Semithin sections from each block were stained with toluidine blue to select interesting areas for electron microscopy. Ultrathin sections were prepared using the Ultracut E (Reichert-Jung) and contrasted with uranyl acetate and lead citrate solution. Representative electron micrographs were captured using an EM 900 (Zeiss).

Histological techniques

Deparaffinized sections were stained with hematoxilin-eosin, van Gieson and toluidine blue according to Denk et al. (1989).

Morphometric analysis

Measurements at histological level were carried out using classic point-counting, intersection point-counting and SIS image analysis to ascertain the mean cardiomyocyte diameter (Dmyo), the number of cardiomyocyte cross-sections per mm² (NAmyo), and volume fractions of cardiomyocytes (VVmyo) and interstitium (VV ECM). The VV ECM/VV myo ratio was calculated.

For ultrastructural morphometry, we analyzed 25 electron micrographs per animal at 12,000-fold primary
magnification obtained from five tissue blocks. Point counting technique was used to calculate the volume density of the following subcellular structures of cardiomyocytes: myofibrils ($V_{V_{myo}}$), mitochondria ($V_{V_m}$), sarcoplasm ($V_{V_s}$), vacuoles ($V_{V_v}$) and lipid drops ($V_{V_l}$). We also calculated the numeric density ($N_{myo}$) and mean volume ($V_{m}$) of mitochondria.

**Immunohistochemical techniques**

5 serial sections per animal were deparaffinized, rehydrated in a descending alcohol cascade, treated with 3% H$_2$O$_2$ solution, rinsed in distilled water, treated in TBS (Tris buffer saline) and stored in serum protein block, serum-free (DAKO). The sections were stored overnight with the primary antibody at different dilutions ranging from 100 to 1,800:1 at 4°C in a moist chamber, rinsed in TBS, stored for one hour with the diluted secondary antibody at room temperature, rinsed in TBS, stored with the PAP complex (Rabbit or Mouse EnVision DAKO) diluted in Tris buffer or Avidin Biotinylated enzyme Complex (Vectastain ABC Kit, VECTOR LABORATORIES), and rinsed three times in TBS. After reaction with the DAB set, the sections were developed for 1-5 min, rinsed in distilled water, dehydrated in an ascending alcohol cascade, and embedded in Canada balsam.

**Primary antibodies**

Rabbit polyclonal MnSOD antibody and CuZnSOD antibody (Yamanashi Medical University, Japan); Rabbit polyclonal iNOS antibody (Transduction)

**Secondary antibodies**

Goat anti-rabbit IgG/goat anti-mouse IgG EnVision (DAKO).

Controls for immunostaining CuZnSOD, MnSOD and iNOS: Negative controls: Incubation a: without primary antibody; b: without secondary antibody. Positive controls: known positive tissue (rat liver and kidney) incubation.

**Evaluation of immunohistochemical staining**

The extent of SOD protein reaction was subjectively evaluated using semiquantitative 4-level grading. Grade 0 signified no apparent reaction product. Focal and minimal staining intensity was graded (1), and the most prominent staining reaction, covering nearly the whole area of the specimen, was classified as (4). Grades (2) and (3) were intermediate, between (1) and (4). The mean for each group was calculated.

**Statistics**

Data for myocardial morphometric analyses were expressed as means ± SD. Statistical differences between mean values were calculated using Student’s t-test for unpaired values, and were considered significant at values of p<0.05; Wilcoxon's test was used for non-parametric variables. The SPSS+ software package was used for all statistical evaluation.

**Results**

**Light microscopic findings**

Left myocardium exposed to ischemia/reperfusion (Group IV) showed severe structural alterations compared to controls with normal appearance (Group II); a considerable fraction of cardiomyocytes appeared to be increased in diameter with signs of hydropic degeneration, such as swollen nuclei and inhomogeneous cytoplasm with clear areas and vacuoles. Other areas contained groups of cardiomyocytes at varying degrees of cellular disintegration to myocytolysis – dissolution of parts of the sarcolemma and sarcomeres, partly condensed or hydropic cytoplasm, gaps in the cellular compound containing collagen fibril deposits manifesting as replacement fibrosis or fibrotic scars. These collagen patches of varying size were irregularly distributed, partly accompanied by hyperplastic cardiomyocytes. Morphometric analysis of several structural parameters of diabetic myocardium exposed to ischemia/reperfusion confirmed diabetes-induced morphologic alterations (Table 1). The mean diameter of cardiomyocytes ($D_{myo}$ [µm]) was significantly increased, while their cross-section number ($N_{myo}$ [mm$^{-2}$]) and volume fraction ($V_{V_{myo}}$ [%]) were significantly reduced. The interstitial and perivascular connective tissue was generally extended, reflecting a significant increase of ratio interstitium/cardiomyocytes ($V_{V_{ecm}}$/$V_{V_{myo}}$) in unprotected diabetic myocardium exposed to ischemia and reperfusion.

The structure of the myocardium was better preserved in the hearts of diabetic rats treated with EGB

<table>
<thead>
<tr>
<th>Group</th>
<th>$D_{myo}$ [µm]</th>
<th>$N_{myo}$ [mm$^{-2}$]</th>
<th>$V_{V_{myo}}$ [%]</th>
<th>$V_{V_{ecm}}$/$V_{V_{myo}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I and II</td>
<td>10.4±0.6</td>
<td>4825.6±129.4</td>
<td>79.4±1.4</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>III and IV</td>
<td>12.6±1.0</td>
<td>3787.2±161.4</td>
<td>70.6±2.3</td>
<td>0.42±0.05</td>
</tr>
<tr>
<td>V</td>
<td>10.6±0.7</td>
<td>4102.8±183.7</td>
<td>74.6±3.0</td>
<td>0.34±0.05</td>
</tr>
</tbody>
</table>
(Group V). This showed reduced numbers and spread of focal loss of cardiomyocytes, which mostly showed normal nuclei and homogeneous cytoplasm. The frequency of fibrotic scars and accumulation of interstitial and perivascular connective tissue was clearly diminished in the EGb-protected group (Fig. 1). Treatment with EGb significantly improved most morphometric parameters. Cardiomyocytes protected by EGb only showed a slightly increased average diameter, whereas cross-section numbers and volume fractions of protected cardiomyocytes were clearly less diminished compared to unprotected diabetic animals.

Ischemia/reperfusion caused no significant morphometric differences at light-microscopic level in control (Groups I and II) or diabetes groups (Groups III and IV).

**Qualitative electron microscopic findings**

The ultrastructural pattern of diabetic cardiomyocytes without experimental ischemia/reperfusion was characterized by the coexistence of components that were either apparently normal or damaged to a greater or lesser degree; scattered areas of degeneration and misalignment were evident. Some cardiomyocytes showed focal loss of myofibrils and disarrangement of the remaining bundles, contraction ribbons and disruption of sarcromeres. Some elements of sarcoplasmic reticulum and transverse tubules were partly swollen. There was evidence of moderate to severe swelling in mitochondria, which showed varying degrees of matrix clearing and partial disintegration of internal mitochondrial membranes. We found a marked increase in the frequency of glycogen granules partly sequestered by membranes, as well as lipofuscin granules and lipid droplets, often closely related to mitochondria. Dehiscence at intercalated disks was frequent in the unprotected diabetic group.

Ischemia and subsequent reperfusion of the diabetic myocardium led to progressed ultrastructural alterations compared to the unprotected diabetic group, characterized by intracellular edema, increased mitochondrial swelling and disintegration or disarrangement of cristae, as well as vacuolization of SR and t-tubules, especially in scattered areas of myofibrilloysis. Sarcomeres of some cells were progressively altered, showing condensed z-disk formation, partial loss of myofibrils and accumulation of intracellular debris. In contrast, ischemia/reperfusion injury of normal myocardium led to less prominent ultrastructural alterations, such as condensation or moderate swelling of a fraction of mitochondria, slight dilatation and vacuolization of SR and t-system, and regionally slight intracytoplasmatic edema of cardiomyocytes.

In the EGb-protected diabetic ischemic group, the ultrastructure of cardiomyocytes appeared less damaged compared to unprotected diabetic ischemic rats. Irregularities of sarcomeric texture, partial loss of
Ischemic lesions of diabetic cardiomyocytes

Fig. 2. Electron microscopic images of rat cardiomyocytes.

a. Non-diabetic control animals (Group I) with normal aspect of cardiomyocytes and regular intracellular structure (sarcomeres [asterisk], mitochondria [arrowhead], SR [arrow]).

b. Non-diabetic control animal exposed to additional ischemia/reperfusion (Group II) with regular myofibrillar structure (asterisk), normal intercalated disk (arrow), slightly condensed mitochondria (right arrowhead), partial degeneration of mitochondria (left arrowhead), sequester with mitochondrial remnants (left chevron), lipofuscin (right chevron) and slight intracellular edema (empty arrow).

c. Untreated diabetic BB rats (Group III) with dehiscent intercalated disk (arrow), focal loss of myofibrils (double arrow) and irregular course of the myofilament bundles (asterisk), degeneration of mitochondria and sequester with mitochondrial remnants (left arrowhead) or myelin figure (question mark), accumulation of glycogen (g) and lipid drops (number sign).

d. Untreated diabetic BB rats (Group IV) exposed to additional ischemia/reperfusion with irregularities of sarcomeres (asterisk), progressive structural disintegration and degeneration of mitochondria with debris (left arrowhead) and dilatation of SR and t-tubule system (arrow).

e. EGb 761-protected diabetic BB rats (Group V) exposed to additional ischemia/reperfusion with better preserved ultrastructure: regular sarcomere structure (asterisk), slight condensation (right arrowhead) or partial disintegration of mitochondrial cristae and clearing of matrix (left arrowhead), slight dilatation of SR (arrow), lipofuscin granule (right chevron). Scale bar: 2.0 μm.
Ischemic lesions of diabetic cardiomyocytes

Fig. 3. Immunohistochemical demonstration of mitochondrial (Mn-) and cytoplasmatic (CuZn-) SOD protein expression in myocardium in experimental groups exposed to ischemia/reperfusion. The majority of cardiomyocytes were unstained. There were some regional patches of cells with a spotted reaction product corresponding mitochondria. 

a. Control rats (Group II), strong immunostaining of MnSOD (arrow). 
b. Diabetic rats (Group IV), weaker immunostaining of MnSOD (arrow). 
c. EGb 761-protected diabetic rats (Group V), strong immunostaining for MnSOD (arrow). In contrast, the majority of cardiomyocytes showed a reaction for CuZnSOD, often near the margin, as an artificial result with dislocation of the reaction product to one side of some cells, or the whole profile of the cells. 

a. Control rats (Group II), strong immunostaining of CuZnSOD (arrow). 
b. Diabetic rats (Group IV), weaker immunostaining of CuZnSOD (arrow). 
c. EGb 761-protected diabetic rats (Group V), stronger immunostaining of CuZnSOD (arrow).
myofilaments, mitochondrial swelling, and matrix alterations, as well as accumulation of glycogen showed decreased expression, while lipid droplets were less frequent (Fig. 2).

Quantitative electron microscopic findings

The quantitative analysis of cardiomyocyte compartments revealed that ischemia and subsequent reperfusion led to a stronger deterioration of morphologic conditions of the diabetic myocardium than in control animals. EGb-treatment could improve some of these ultrastructural compartments after ischemia/reperfusion damage (Table 2).

The volume fraction of myofibrils (VVmyof) was significantly reduced after diabetes induction, and slightly more diminished after ischemia and reperfusion in the unprotected diabetic group. EGb treatment led to a gradual improvement in this parameter. Ischemia/reperfusion damaged the diabetic somewhat more than non-diabetic myocardium. In contrast, the volume fraction of sarcoplasm (VVsp) was significantly increased both after diabetes and ischemia with reperfusion in unprotected diabetic animals, but was significantly less increased in the protected group.

The parameters behaved differently in mitochondria. While the volume density (VVm) was slightly increased after ischemia/reperfusion in diabetes and better preserved in treated myocardium, the numeric density of mitochondria (NVm) was hardly decreased by unprotected diabetes or additional ischemia/reperfusion, which was less expressed in hearts treated with EGb. Moreover, mean mitochondrial volume (Vm) was significantly increased in the diabetic myocardium after additional ischemia/reperfusion, whereas this increase was absent in animals treated with EGb. The ratio of volume density mitochondria to myofibrils (VVm/VVmyof) was remarkably elevated in diabetic animals after additional ischemia/reperfusion. By contrast, this parameter was only slightly increased in

### Table 2. Morphometric parameters of components of cardiomyocytes (left ventricle) in the experimental groups (mean ± SD) (* p ≤ 0.05).

<table>
<thead>
<tr>
<th></th>
<th>group I</th>
<th>group II</th>
<th>group III</th>
<th>group IV</th>
<th>group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>myofibrils (VVmyof)</td>
<td>0.464±0.03</td>
<td>0.447±0.04</td>
<td>0.387±0.05</td>
<td>0.345±0.04</td>
<td>0.399±0.03</td>
</tr>
<tr>
<td>mitochondria:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>volume density (VVm)</td>
<td>0.381±0.02</td>
<td>0.391±0.03</td>
<td>0.399±0.02</td>
<td>0.409±0.03</td>
<td>0.396±0.03</td>
</tr>
<tr>
<td>numeric density (NVm)</td>
<td>0.343±0.05</td>
<td>*</td>
<td>0.279±0.05</td>
<td>0.291±0.03</td>
<td>*</td>
</tr>
<tr>
<td>volume (Vm) [µm³]</td>
<td>1.11±0.1</td>
<td>*</td>
<td>1.32±0.2</td>
<td>1.28±0.1</td>
<td>*</td>
</tr>
<tr>
<td>sarcoplasm (VVsp)</td>
<td>0.120±0.04</td>
<td>0.131±0.02</td>
<td>0.163±0.03</td>
<td>*</td>
<td>0.199±0.03</td>
</tr>
<tr>
<td>vacuoles (VVvac)</td>
<td>0.006±0.002</td>
<td>*</td>
<td>0.010±0.002</td>
<td></td>
<td>0.016±0.006 *</td>
</tr>
<tr>
<td>lipid drops (VVl)</td>
<td>0.009±0.002</td>
<td>0.012±0.003</td>
<td>0.0021±0.006</td>
<td>0.0018±0.004</td>
<td>0.025±0.006</td>
</tr>
<tr>
<td>ratio VVsp/VVmyof</td>
<td>0.821</td>
<td>0.875</td>
<td>1.031</td>
<td>1.19</td>
<td>* 0.992</td>
</tr>
</tbody>
</table>

Fig. 4. Semi-quantitatively determined grade and volume of the immunoreaction (4-level grading system) (isch/rep.: ischemia/reperfusion). a. of MnSOD. b. of CuZnSOD.
the EGb-protected group.

The volume fraction of cytoplasmic vacuoles ($V_{Vac}$) was strongly increased in diabetes and significantly more increased after additional ischemia/reperfusion, but significantly less increased after protection by EGb. Diabetes led to significantly increased volume density of lipid drops ($V_{Lip}$), which showed somewhat lower expression after ischemia/reperfusion. In contrast, this parameter increased under EGb treatment.

**Immunohistochemical demonstration of SOD expression**

Immunostaining of mitochondrial (Mn-) and cytoplasmatic (CuZn-) SOD protein in cardiomyocytes from all experimental groups was observed in an inhomogeneous spotted pattern with areas of stronger staining reaction, surrounded by areas with weak or no immunostaining.

Regarding MnSOD, staining intensity was weak; the majority of cardiomyocytes were unstained. In some regional patches of cells, the whole profile contained a spotted reaction product corresponding to mitochondria.

In contrast, the majority of cardiomyocytes showed a moderate reaction for CuZnSOD, often near the margin, as an artificial result, with dislocation of the reaction product to one side of some cells, or the whole profile of the cells.

MnSOD immunostaining (Fig. 4) was weak in cardiomyocytes from control rats, but strong after ischemia/reperfusion (Fig. 3a$_1$), moderate in diabetic rats, somewhat stronger after additional ischemia/reperfusion (Fig. 3b$_1$), and somewhat more intense, but below the level of ischemic control rats, after EGb pre-treatment (Fig. 3c$_1$). Figure 4a shows the staining intensity of all groups according to their semiquantitative evaluation.

CuZnSOD immunostaining (Fig. 4) showed a decreasing intensity from control, control + ischemia/reperfusion (Fig. 3a$_2$), diabetes and diabetes + ischemia/reperfusion (Fig. 3b$_2$). The strongest reaction was seen in the diabetic ischemic group with EGb pre-treatment (Fig. 3c$_2$). Figure 4b shows an overview of all groups after semiquantitative evaluation.

**Immunohistochemical demonstration of iNOS expression**

In general, an inhomogeneous striated pattern of iNOS immunostaining was apparent in cardiomyocytes from all experimental groups with only slight differences in semiquantitative evaluation (not shown). The staining intensity was only weak or moderate. The majority of cardiomyocytes were unstained; clusters of cells in some regions contained reaction product either filling small regions, often near the margin, or the whole profile of the cells. A cytoplasmic distribution to a greater or lesser extent of unevenness was seen with obvious artificial dislocation of the reaction product to one side of some cells.

![Fig. 5. Immunohistochemical demonstration of iNOS protein expression in myocardium in experimental groups after ischemia/reperfusion, with an inhomogeneous striated pattern of iNOS immunostaining in cardiomyocytes in all experimental groups. Most cardiomyocytes were unstained. a. Control rats (Group II), moderate immunostaining of iNOS (arrow). b. Diabetic rats (Group IV), weaker immunostaining of iNOS (arrow). c. EGb 761-protected diabetic rats (Group V), weak immunostaining of iNOS (arrow).](image)
Staining intensity was relatively strong in diabetic cardiomyocytes and control cardiomyocytes exposed to ischemia/reperfusion (Fig. 5a), and slightly reduced in diabetic myocytes after ischemia/reperfusion (Fig. 5b). Treatment with EGB reduced the intensity of iNOS protein expression to close to healthy control levels in the ischemic-diabetic group (Fig. 5c).

**Discussion**

Our spontaneously diabetic BB/OK-rats, the closest animal counterpart to human insulin-dependent diabetes, developed the typical signs of diabetic cardiomyopathy as described by Rösen et al. (1991). Both compensative and degenerative processes, as described by others (Zhou et al., 1990; Fitzl et al., 1999; Nemoto et al., 2006) were seen in our study at varying levels of expression – qualitative light-microscopic and morphometric analyses showed diabetes-induced swelling or hypertrophy confirmed by increased mean diameter and diminished number of cross sections per unit area in cardiomyocytes. In contrast, we observed an extensive myocytolysis, which has been discussed in the literature (Zhu et al., 1993; Poornima et al., 2006), together with focal necrosis as a result of OFR-induced apoptosis, mitochondrial dysfunction, and activation of endogenous endonucleases. This loss of myocytes is compensated by an increase of interstitial connective tissue, which is morphometrically expressed as a reduced fraction of cardiomyocytes and increased interstitium/cardiomyocyte ratio.

What is referred to as replacement fibrosis is accompanied by increased perivascular fibrosis due to diabetic microangiopathy (Tayebjee et al., 2005; Marwick, 2006). The development of cell loss, as well as compensative hypertrophy of remaining cardiomyocytes accompanied by an increase in interstitial collagen and matrix, leads to alterations in myocardial architecture, which Swynghedauw described as cardiac remodeling (1999).

To avoid or delay late complications of chronic diabetes, it appears logical to apply adjuvant therapies to scavenge OFRs that occur during diabetic metabolism as investigated for Vitamin E (Tocopherol) by Rösen et al. (1995). In our case, the combined activities of the constituents of EGB 761 (24% flavonoids, 6% terpenoids, 8% ginkgolides) are probably responsible for the therapeutic benefit observed. The protective effects of EGB on the myocardial structure of BB rats can be attributed to the direct antioxidative and free radical quenching potential of EGB (Pietri et al., 1997), counteracting free radical-induced apoptosis, as well as activation of endogenous nucleases responsible for the loss of cardiomyocytes in untreated diabetes (Pincemail et al., 1987). A direct membrane-stabilizing effect and inhibition of lipid peroxidation of biological membranes at the early beginning of alterations has been described in the literature (Huang et al., 1981; Janssens et al., 1995). The lipophil property of the mean components explains the high affinity to the biological membranes. Further, the anti-inflammatory potential of EGB (Hammond and Hess, 1985) inhibits hypertrophy-stimulating cytokines.

Pathophysiological aspects of OFRs as a general mechanism of cell injury in ischemia and reperfusion damage have been sufficiently investigated in literature (Hammond and Hess, 1985; Burton et al., 1990; Shattock, 1997). From our ultrastructural results, we conclude that diabetes does not improve ischemia/reperfusion tolerance in the myocardium, in contrast to DaTorre et al. (1991), who postulated that "no-flow" ischemia desensitizes the diabetic heart against ischemia/reperfusion. We found increased vulnerability with considerable ultrastructural and enzymatic alterations of cardiomyocytes, resulting from energy deficit and liberation of radicals, as seen by Punkt et al. (1997) and Fitzl et al. (2000, 2001) in hypoxic STZ-diabetic rats:

A. Ischemia/reperfusion led to a reduction in the volume fraction of myofibrils, accompanied by a complementary increase in the sarcoplasmic fraction in untreated diabetes, which may be explained either by intracellular edema of cardiomyocytes or by substantial loss of myofilaments, or both.

B. The ratio of energy-liberating structures (mitochondria) to energy-consuming structures (myofibrils) was markedly increased in the unprotected diabetes group after additional ischemia/reperfusion, indicating alterations in both compartments, regardless of cellular edema.

C. Diabetes with ischemia/reperfusion led to an enormous increase in cellular vacuolization, indicating disturbances of Ca2+ and water movement, possibly due to sarcoplasm vacuolization and/or disintegration of SR into vacuoles, which is difficult to distinguish.

D. Ischemia/reperfusion led to somewhat stronger MnSOD expression in diabetic cardiomyocytes, but lower expression compared to ischemic control followed by reperfusion. This may indicate that the synthetic capacity of diabetic cells is disturbed by excess radical formation during reperfusion. Furthermore, increasing oxidative stress in the diabetes – diabetes + ischemia/reperfusion system seems to decrease the expression of CuZnSOD gradually, probably through specific fragmentation and inactivation by toxic free radicals (Taniguchi et al., 1989).

E: In agreement with D, immunostaining iNOS as an indicator for cell damage showed strong expression in untreated diabetes as a result of the continuous oxidative stress and of toxic diabetic metabolites, leading to liberation of different cytokines and catecholamines known to induce the expression of the iNOS in cardiac myocytes (Bierhaus et al., 1997; Arstall et al., 1999). The diminished enzyme expression in the experimental diabetic group may be due to ischemia/reperfusion-induced intracellular acidification and activation of cellular proteases, which may result in protein degradation (Giraldez et al., 1997).
These data represent the progression of the negative energetic balance due to tissue oxygen suppression under diminished oxygen utilization conditions by diabetes. Concerning volume density of myofibrils, sarcoplasm, vacuoles, mitochondria, average volume and numeric density, EGb may slightly to moderately or significantly improve these parameters, which may gradually improve tolerance against ischemia with reperfusion. EGb diminishes cardiomyocyte swelling in BB rats via its anti-edematous potential. In this way, an improvement in glucose and O2 uptake and stabilized mitochondrial function regulate the metabolic, ionic (Ca2+-glucose and O2 uptake and stabilized mitochondrial tolerance against ischemia with reperfusion). EGb improve these parameters, which may gradually improve density, EGb may slightly to moderately or significantly diminish oxygen utilization conditions by diabetes. energetic balance due to tissue oxygen suppression under diabetic state, and are probably responsible for the development of secondary complications (Kakkar et al., 1995). A connection between mitochondrial damage and generation of reactive oxygen species and changes in SOD status has been suggested (Godin et al., 1988). EGb evidently improves antioxidative capacity in cardiomyocytes with respect to SOD availability under high oxidative stress conditions. MnSOD and CuZnSOD expression nearly reaches ischemic control levels. EGb may act via direct influence on enzyme activity and synthesis, the suppression of free radical formation and scavenging (Tosaki et al., 1994; Punkt et al., 1997).

The weaker reaction of iNOS protein in protected diabetic cardiomyocytes after ischemia/reperfusion may be due to the action of EGb as an NO scavenger, which also inhibits iNOS mRNA formation, NO production (Marcocci et al., 1994) and TGF-β expression (Varga et al., 1999). Furthermore, flavonoid components of EGb inhibit protein kinase C, which is directly involved in NOS induction in rats.

The terpenoid constituents and the flavonoid metabolites of the EGb probably act in a complementary manner to protect against myocardial ischemia/reperfusion injury (Liebgott et al., 2000). We conclude that EGb may directly activate enzyme activity/synthesis towards reducing free radical formation and stabilizing membranes rather than scavenging free radicals (Tosaki et al., 1994). Ginkgolide A is thought to play a particularly important role in hemodynamic and antioxidant protection, with protection involving the inhibition of free radical formation rather than direct free radical-scavenging (Pietri et al., 1997). EGb-induced activation of antioxidant enzymes has been observed by Punkt et al. (1997).

In summary, cardiomyocytes from spontaneously diabetic BB/OK rats develop the typical morphological pattern of diabetic alterations under low-dose insulin therapy after six months of diabetes.

After exposure to additional ischemia/reperfusion injury, unprotected diabetic cardiomyocytes reveal reduced tolerance towards O2 deficiency in myocardium. Ultrastructural integrity in diabetic cardiomyocytes is also damaged along with the deterioration of antioxidant enzyme status. EGb pre-treatment may slightly to moderately shift these parameters towards normal values, which could be interpreted as an improvement in ischemia/reperfusion tolerance. With respect to ultrastructure of ischemic diabetic myocardium and parameters of antioxidant stress, the protective effect of EGb substitution seems to consist of stabilizing membranes and enzymes as well as scavenging radicals. This implicates EGb as a promising candidate for preventation and/or adjuvant therapy of acute ischemic injury in diabetes. Moreover, it may contribute towards preventing diabetes-induced structural alterations in cardiomyocytes while reducing late diabetic complications.

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References
Doi K., Sawada F., Toda G., Yamachika S., Seto S. and Urata Y. (2001). Alteration of antioxidants during the progression of heart...
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