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Ginkgo biloba extract protects rat kidney from diabetic and hypoxic damage

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Abstract

Ginkgo biloba extract EGb 761 was studied for its nephroprotective effects in experimentally diabetic and hypoxic rats. Duration of streptozotocin-induced diabetes was 4 months, that of respiratoric hypoxia of the diabetic group 20 min. The daily dose of 100 mg EGb/kg bodyweight started 1 month after induction of the diabetes. EGb reduced diabetes-induced morphological alterations of the kidney such as increase in volume of glomeruli, capillary tufts, urinary space, and thickening of Bowman's capsule basement membrane. Diabetically increased immunostaining of interstitial collagenes of types I, III, and VI was diminished by the EGb extract. EGb reduced the relative total SOD activity from 163% in diabetic kidney to 46%. Additional hypoxia-induced ultrastructural damage was also diminished.

Keywords: *Ginkgo biloba* extract; Rat; Kidney; Experimental diabetes; Hypoxia

Introduction

Diabetic nephropathy as a late complication in chronic diabetes occurs in 40% of human diabetes cases. Oxidative stress is regarded as an important factor in the pathogenesis of diabetic complications (Rösen et al., 1991, 1995; Reichel and Neundörfer, 1996; Yavuz et al., 2003). Some antioxidative substances has been tested for nephroprotection: beta-carotene (Maritim et al. 2002); vitamin E, probucol and taurine (Koya et al. 2003); captopril and losartan (Yavuz et al. 2003), but not *Ginkgo biloba* extract (EGb 761), a potent radical scavanger (Pincemail and Deby, 1988). The aim of our study was to investigate effects of EGb 761 on structural, histochemical and biochemical alterations caused by diabetes and diabetes + hypoxia. Protective effects of EGb were already demonstrated on diabetic heart and liver (Fitzl et al., 1999, Welt et al., 1999, 2004).

Materials and methods

The experiments were approved by the Leipzig Regierungspräsidium.

The Ginkgo extract EGb 761 (IPSEN, Paris) contained 24% flavonoids, 2.9% bilobalide, 1.3% gingkolide A, 0.6% gingkolide B, 1.2% gingkolide C and >0.1% biflanoide.

Animals

Twenty-five 2-months-old Wistar rats (Crl:(Wi) Br) from Charles River GmbH (Salzfeld, Germany) kept

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separately under standard conditions were used for the experiments.

Experimental groups and procedures

Group I (diabetes; $n = 5$): Diabetes was induced by intraperitoneal injection of streptozotocin (Boehringer, Mannheim, Germany) at 60 mg/kg bodyweight. The blood glucose levels were about 30 mM/l during and at the end of the diabetic interval.

Group II (diabetes with EGb 761-protection; $n = 5$): One month after induction of diabetes the rats received a daily dose of 100 mg EGb 761/kg bodyweight dissolved in 30 ml water administered at night over 3 months. By day, they had free access to drinking water.

Group III (diabetes with additional hypoxia; $n = 5$): Four months after induction of diabetes, the rats were exposed to acute respiratory hypoxia for 20 min at O_2 partial pressure of 5%v/v in N_2O mixture by means of a hypoxia chamber + narcosis apparatus.

Group IV (diabetes with EGb protection and acute hypoxia; $n = 5$): The rats were treated according to group II and exposed to hypoxia as group III.

Group V (control; $n = 5$): Rats from the same litters were kept under identical conditions to the other groups without any treatment.

The data of the rats at the end of the experiments are shown in Table 1.

The animals were killed by cervical vertebral dislocation after brief ether narcosis. The kidneys were quickly removed and processed for various techniques.

Histological techniques

One kidney block per animal from groups I, II and V was fixed in buffered 4% formaldehyde and processed for paraffin embedding; 5 µm slices were used for HE, AZAN, Crossmon, and silver staining (Gomori).

Immunostaining

Three deparaffinized kidney sections per animal of the groups I, II and V were immuno-stained using the DAKO EnVision™ + Conjugate technique. As primary antibodies we used

Rabbit anti-rat collagen type I (polyclonal antibody, Chemicon) (1:1000)
Rabbit anti-rat collagen type III (polyclonal antibody, Chemicon) (1:900)
Rabbit anti-human collagen type VI (polyclonal antibody, Chemicon) (1:600).

Visualization was performed with the DAB set (Sigma).

Table 1. Data of the rats at the end of the experiments (average of the experimental groups)

	Control	Diabetes	Diabetes + EGb
Body weight (g)	573 ± 34	279 ± 73*	247 ± 48*
Blood glucose level (mM)	10.5	> 30	> 33

*$p \leqslant 0.5$ compared with the control group.

Controls for immunostaining

Negative controls: Incubation a: without primary antibody, and b: without secondary antibody. *Positive controls:* For type I collagen: rat tail, for type III and VI collagen: rat gut.

Tissue processing for electron microscopy

Fife small kidney blocks per animal were fixed in 2% paraformaldehyde, 2% glutar dialdehyde in 0.1 M cacodylate buffer (pH 7.4) for 2–4 h, processed in usual manner for electronmicroscopy. Electron micrographs (25 per animal) were randomly taken using the EM 900 electron microscope (ZEISS).

Semiquantitative analysis

The grade of immunostaining for all collagen types was evaluated independently by three persons using a 4-level grading system at selected histotopological localizations. The average for each group was calculated from several single estimations.

Morphometric analysis

The cross-sectional areas of the glomeruli and glomeruli capillary tufts were measured using the SIS automatic image analysing system (Software Imaging System GmbH, Münster). Capsular space was calculated as the difference between these two values. The thickness of the basement membranes of the Bowman's capsule and of glomerular capillary endothelium was measured at several randomly chosen points.

Statistical analysis

The arithmetical means of the parameters from the different experimental groups were compared using the unpaired *t*-test; Wilcoxon's test was used for non-parametric variables. The SPSS + software package was used for all statistical evaluation.

Biochemical investigations of parameters of oxidative stress

Rapidly frozen tissue samples of five animals per experimental group were homogenized, centrifuged for 10 min at 2000 × *g*, and the following parameters were determined in the supernatant:

Superoxide dismutase (SOD): The activity of total SOD was determined by the SOD Assay Kit of CALBIOCHEM (Cat. #574600) with an absorption maximum at 525 nm.

Malondialdehyde (MDA): MDA reacts with thiobarbituric reagent yielding a red coloring, which we extracted using butanol and measured at 535 nm.

The results of each of the methods were calculated using calibration curves.

Results

Light microscopic results

The diabetes-induced enlargement of many glomeruli, capillary tufts and of the subcapsular urinary space is obviously less expressed in the EGb-treated diabetic group (Fig. 1). The normally sparse intertubular connective tissue appears regionally increased in diabetic kidneys, but somewhat less in the protected group. EGb reduces also the apposition of granular or fibrillar matrix at Bowman's capsules.

Immunohistochemical demonstration of collagen

Positive reactions for collagen types I, III and VI in control kidneys are localized mainly around larger blood vessels, and weaker in the tubular interstitium and at some Bowman's capsules. Diabetes causes an increase in collagen I fibers at these locations.

Type III collagen in diabetic kidneys is increased around blood vessels and some capillaries, between collecting tubules and ducts and at isolated Bowman's capsules, type VI-collagen around blood vessels and capillaries (Fig. 2 and 3).

EGb reduces the diabetically induced increase of collagenes except types I and VI between collecting tubules and at Bowman's capsules and type III around vessels (Fig. 3).

Results of negative controls: No immunostaining was obtained in parallel sections, when incubation was carried out without a primary or secondary antibody.

Results of positive controls: Type I collagen antibody stained rat tail collagen; type III and VI collagen antibodies stained rat gut mucosa collagen.

Light microscopic-morphometric results

The mean cross-section area of diabetic glomeruli is significantly increased by 20%, that of glomerular capillary tufts by 13%, and that of the subcapsular urinary space by more than 100%. Additional hypoxia of diabetic rats leads to slight increase of these

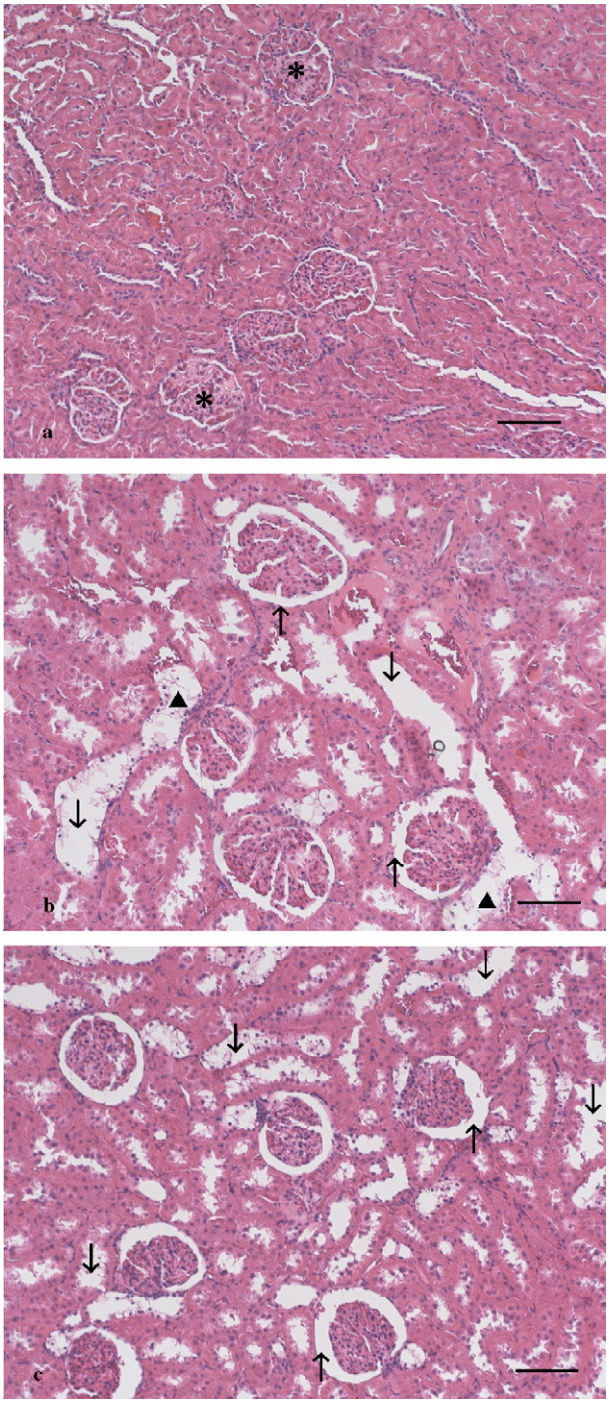

Fig. 1. HE-stained sections of rat kidneys. (bar = 100 μm). (a) control; normal size of glomeruli with small subcapsular space (*). (b) diabetic rat; glomeruli are larger, the subcapsular space appears dilated (↑). Some tubules (↓) and singular Maculae densae (▲) are composed of Armanni-Ebstein cells. (c) EGb-protected diabetic rat; glomeruli are smaller than in b, the subcapsular space remains dilated (↑), many Armanni-Ebstein cells (↓) are present.

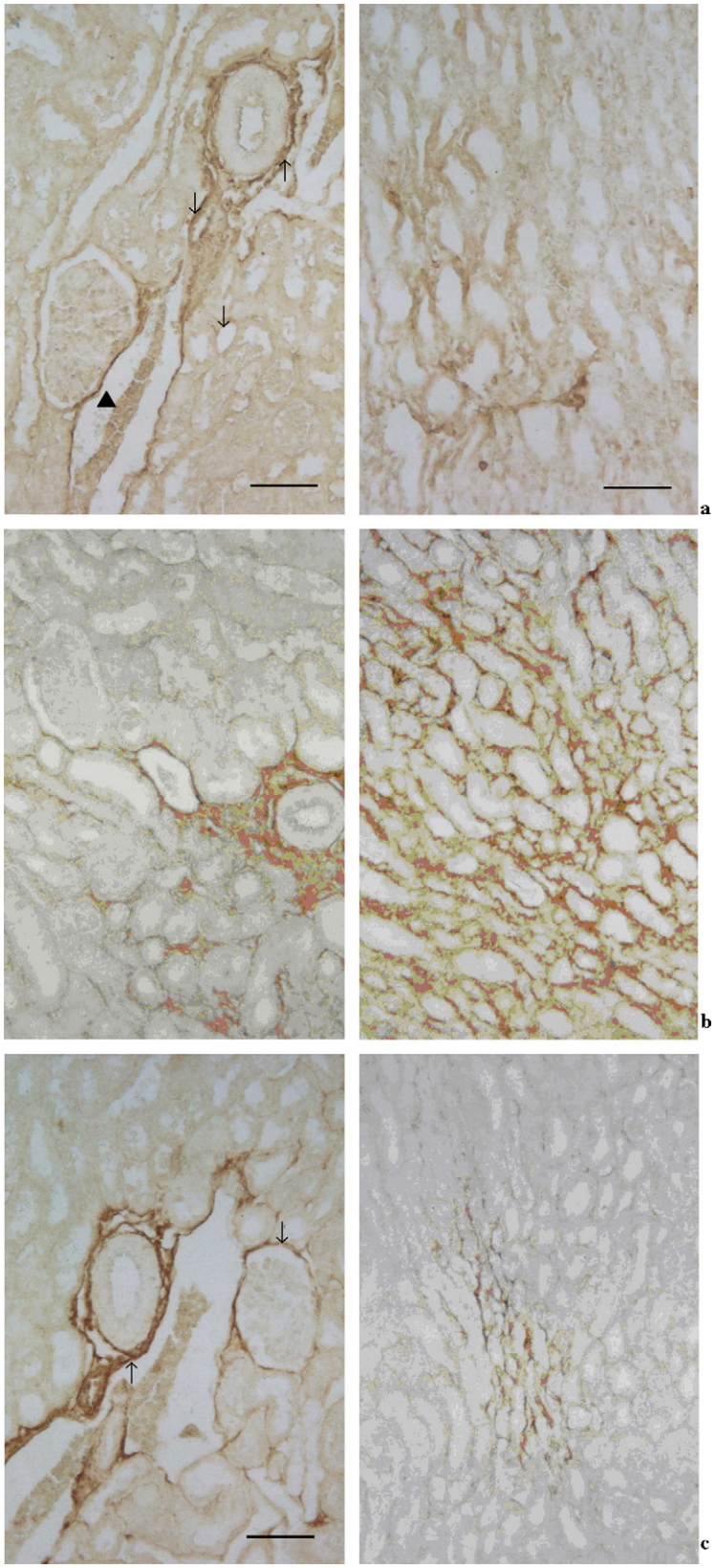

Fig. 2. Rat kidney, immunostaining of type III collagen. (bar = 100 μm). (a) control; positive fibers lay around large vessels (↑) and partly capillaries (↓), at some Bowman's capsules (▲), and regionally around collecting tubules and -ducts (right picture). (b) diabetic rat; the amount of positive fibers appears markedly increased, especially around collecting tubules and -ducts (right picture). (c) EGb-protected diabetic rat; the staining intensity around vessels (↑), and some Bowman's capsules (↓) remains high, between collecting tubules and-ducts only small areas show positive fibers (right picture).

parameters. EGb prevents glomerulus hypertrophy and diminishes capsular space enlargement (Table 2).

Electronmicroscopic results

Fig. 4a shows the ultrastructure of a normal glomerulus. Diabetic glomeruli (Fig. 4b) appear larger, Bowman's capsule BMs are partially thickened and/or covered with granular or fibrillar matrix. The BMs of glomerula capillaries appear normal, only small segments are thickened. The endothelium show local irregularities in profile and pore arrangement, some mitochondria are moderately swollen; some podocyte processes appear broadened or show degenerative features such as vacuolization, blebbing and myelin figures (Fig. 4b). In EGb-protected diabetic kidneys, the alterations mentioned above are less expressed (Fig. 4c). Hypoxia-induced aggravation of these parameters (Fig. 4d) is also diminished by EGb (Fig. 4e).

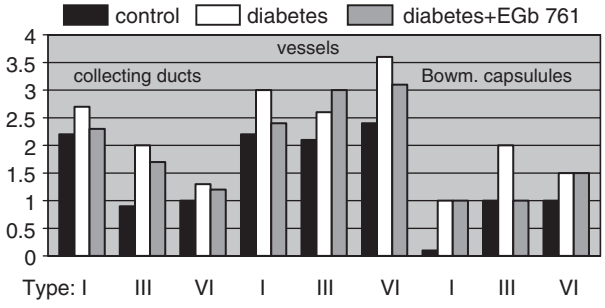

Fig. 3. Semiquantitative evaluation of collagen types I, III, VI in different localizations. (collect. duct: between collecting ducts (medullar interstitium); vessels: around blood vessels; Bowm. caps.: at Bowmann's capsules (cortical interstitium).

Morphometric electron microscopic results

The Bowman's capsule BM shows a significant increase of 8% in average thickness under diabetic conditions but only of 3% in EGb-protected animals without significant difference to the controls (Table 2).

Biochemical parameters of oxidative stress

Biochemically determined malondialdehyde (MDA) content related to moist weight is highest in kidney from the control group and significantly diminished in the other groups regardless of hypoxia or EGb administration (Fig. 5a).

Related to protein content the total superoxide dismutase (SOD) activity is markedly increased in diabetic and more increased in diabetic-hypoxic kidneys. EGb pretreatment causes a strong reduction in activity under these conditions (Fig. 5b). The differences between the groups are statistically not significant.

Discussion

Antioxidative protection as performed in our experiment in diabetic rat kidney is focussed on delay or prevention of late diabetic complications. Our morphologic findings in STZ-diabetic rat kidney agree in general with results in the literature (Osterby and Gundersen, 1980, Hirose et al., 1982; Nyengaard and Rasch, 1993; Shirato et al., 1993). The expansion in capsular space is probably caused by enhanced angiotensin II level leading to increased intraglomerular pressure and filtration rate (Wolf et al., 1996). In our experiment,

Table 2. Morphometrical parameters of glomeruli (mean and SD)

	Control	Diabetes	Diabetes + EGb	Diabetes + hypoxia	Diabetes + hypoxia + EGb
Mean cross-section area of glomeruli (μm^2)	95.8 ± 31	115.1 ± 32*	91.1 ± 30	102.4 ± 35	98.3 ± 35
Mean cross-section area of the glomerular capillaries (μm^2)	90.5 ± 30	102.5 ± 31**	83.8 ± 26	91.5 ± 32	87.5 ± 33
Capsular space:					
Difference between mean cross-section area of glomeruli and glomerular capillaries (μm^2)	5.3	12.6**	7.3	10.9	10.8
Mean thickness of basement membran (μm) of:					
Bowmanns-capsules	1.33 ± 0.05	1.44 ± 0.01**	1.37 ± 0.01		
Glomerular capillaries	0.39 ± 0.08	0.38 ± 0.08	0.39 ± 0.10		

*$p \leqslant 0.5$; **$p \leqslant 0.01$, compared with the control group.

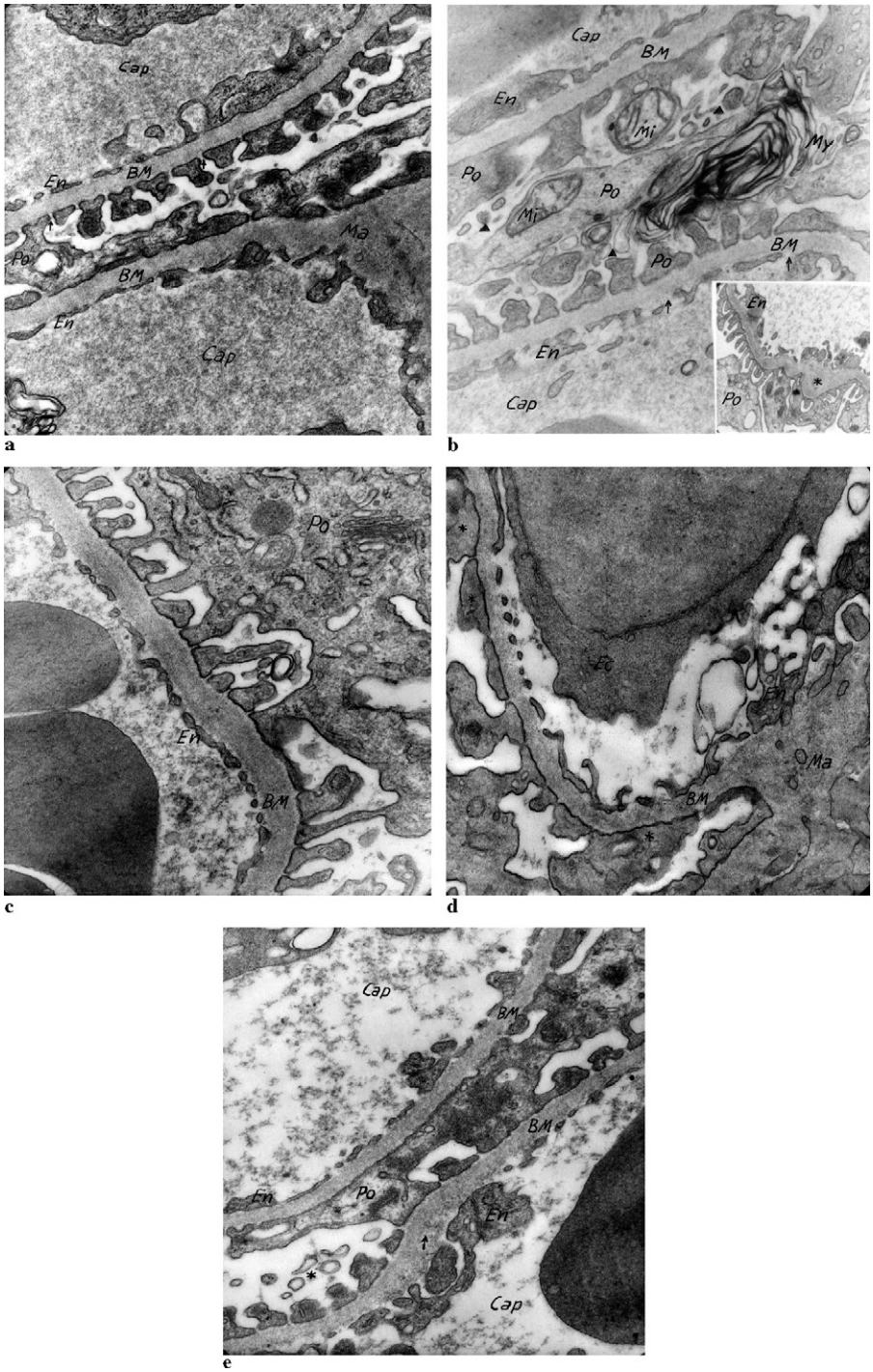

Fig. 4. Electronmicroscopic images of rat kidney. (× 40 000). (a) Kidney of control rat. Glomerular capillaries (cap) show porous endothelium (En). Regularly arranged podocyte processes (Po) with slit pores (↑) are separated from the endothelium by a thick basement membrane (BM). The BM is continuous with the mesangial matrix (Ma). (b) Kidney of diabetic rat. Glomerular endothelium (En) contains gaps (↑) instead of regular pores. A podocyte (Po) exhibits signs of degeneration: myelin figure (My), swollen mitochondria (Mi), and membrane detritus in the interstitium (▲). The glomerular BM appears normal; a local thickening (∗) is shown in the inset (lower magnification). (c) Kidney of an EGb 761-protected rat. The porous endothelium (En) and podocyte (Po) appear better preserved than in b. The basement membrane is thickened compared with the control. (d) Kidney of a diabetic rat additionally exposed to hypoxia. The damage of capillary endothelium (En) is much more prominent than in b. The bulging endothelial cell (Ec) is structureless, some podocyte processes appear broadened (∗). Ma = mesangial matrix. (e) Kidney of an EGb 761-protected diabetic hypoxic rat. The glomerular endothelium shows normal structure. The basement membrane is locally thickend (↑) and detritus (∗) lies between podocyte processes.

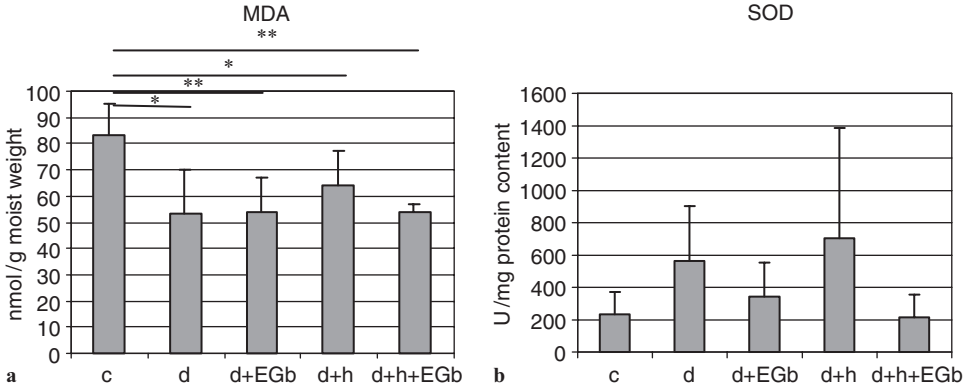

Fig. 5. Effects of experimental conditions on biochemically determined parameters of oxidative stress in rat kidney. (a) MDA content; b: SOD activity. (* = $p \leqslant 0.05$; ** $\leqslant 0.01$; *** $\leqslant 0.001$) (c = control, d = diabetes, h = hypoxia).

EGb treatment diminished the diabetes-induced enlargement of glomerular capillaries and capsular space probably by regulation of the filtering blood pressure.

Diabetes-induced glomerular hypertrophy and ultrastructural damage of glomerular endothelium and podocyte processes, thickening of BMs as seen in our diabetic rats may partly be caused by free radicals, which may be also responsible for gradual ultrastructural deterioration by additional hypoxia. Only this part of damage is accessible for the protective effects of EGb as seen in our experiment.

Chronic diabetes leads to renal parenchyma destruction compensated by tubular cell hypertrophy and interstitial fibrosis. Tubular cells, interstitial myofibroblasts and mesangium cells produce increased amounts of type I and IV collagen (Razzaque et al. 1995; Wolf et al., 1996; Phillips and Steadman, 2002). Immunostaining for collagen types I, III and VI in our rats revealed diabetes-induced increase in renal collagenization. EGb diminished the increase in collagen types I, III and VI at different locations. Abrass et al. (1988) found a type III collagen increase even in kidneys of insulin-treated STZ-diabetic rats. Later stages of diffuse and nodular glomerulosclerosis are irreversible and responsible for functional disturbances (Olgemöller and Schleicher, 1993, Schleicher et al., 1996). These facts elucidate the necessity of adjuvant therapy of diabetes.

MDA as a biochemical marker of lipid peroxidation was decreased in our diabetic and diabetic-hypoxic rats, EGb was without effect. Maritim et al. (2002) observed its increase. We explain the decreased MDA level by the upregulation of SOD activity in our diabetic and hypoxic-diabetic rat kidneys. EGb diminished this increase of SOD activity, indicating its efficacy in radical quenching. Increased SOD activity in diabetic kidney was also reported by Mekinova et al. (1995), Yadav et al. (1997), Jang et al. (2000), and Strother et al. (2001).

Conclusion

EGb 761 showed some beneficial effects in STZ diabetic rat kidneys: glomerular hypertrophy, dilation of the capsular space, thickening of Bowman's capsule BM, ultrastructural lesions on capillaries and podocytes were reduced, increase in collagen fibers of type I, III, and VI in the tubular interstitium was diminished, increased SOD activity was equalized. Ultrastructural damage after additional hypoxia was also reduced. EGb as an adjuvant antidiabetic drug could be able to delay late renal complications.

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