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Protective Effects of *Ginkgo biloba* Extract against Mercury(II)-induced Cardiovascular Oxidative Damage in Rats

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This study was designed to determine the possible protective effect of *Ginkgo biloba* extract (EGb) against Hg II-induced oxidative damage and also thromboplastic activity in the aorta and heart tissues. Wistar albino rats of either sex (200–250 g) were divided into four groups. Rats were injected intraperitoneally with (1) control (C) group: 0.9% NaCl; (2) EGb group: *Ginkgo biloba* extract (Abdi Ibrahim Pharmaceutical Company, Istanbul, Turkey) at a dose of 50 mg/kg/day; (3) Hg group: a single dose of 5 mg/kg mercuric chloride ($HgCl_2$); and (4) Hg + EGb group: First day EGb at a dose of 50 mg/kg/day, i.p., 1 hour after $HgCl_2$ (5 mg/kg) injection; following four days EGb at a dose 50 mg/kg/day, i.p. After decapitation of the rats, trunk blood was obtained and serum tumor necrosis factor-α (TNF-α), lactate dehydrogenase (LDH) activity, and malondialdehyde (MDA) and glutathione (GSH) levels were analysed. In the aorta and heart tissues total protein, MDA, GSH levels and thromboplastic activity were determined. The results revealed that $HgCl_2$ induced oxidative tissue damage, as evidenced by increases in MDA levels and decreased GSH levels both in serum and tissue samples. Thromboplastic activity was increased significantly following Hg administration, which verifies the cardiotoxic effects of $HgCl_2$. Serum LDH and TNF-α were elevated in the Hg group compared with the control group. Since EGb treatment reversed these responses, it seems likely that *Ginkgo biloba* extract can protect the cardiovascular tissues against $HgCl_2$-induced oxidative damage. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: mercury; *Ginkgo biloba*; lipid peroxidation; glutathione; thromboplastic activity.

INTRODUCTION

Mercury (Hg) is a heavy metal that affects the activity of the normal function of several enzymes, ion channels or receptors (Moreira *et al.*, 2003). Exposure to toxic mercury is a growing health hazard throughout the world today. It is ubiquitous in the global environment and derives from both natural sources and human enterprise (Counter and Buchanan, 2004). The toxicity of high doses of inorganic mercury on the central nervous system and urinary system has been known for decades (Patrick, 2002). However, few data are available on the effect of mercury on heart and aorta tissues (Boffetta *et al*., 2001; Moreira *et al*., 2003). Inorganic mercury is conjugated with glutathione in the liver and secreted in the bile as a cysteine mercury or glutathione mercury complex (Boffetta *et al*., 2001). Numerous studies have demonstrated that Hg causes lipid peroxidation (Lund *et al.*, 1991; Yonaha *et al.*, 1980), DNA damage (Lund *et al.*, 1991; Yonaha *et al.*, 1980), porphyrinogen oxidation (Woods *et al.*, 1990; Woods and Ellis, 1995) and depletion of reduced glutathione (Gstraunthaler *et al.*, 1983; Sener *et al.*, 2003), which support an oxidative stress-like mechanism for Hg(II) toxicity.

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A growing body of evidence suggests the use of natural antioxidants as therapeutic alternatives against oxidant-induced toxicity. *Gingko biloba* 'EGb 761' extract has been shown to have free radical scavenging and antioxidant properties (Dennis *et al.*, 2001). The extract obtained from the green leaves of the *Gingko biloba* tree, EGb 761, consists of two groups of major substances flavonoid compounds and terpenoids. The main indications for EGb 761 are on cerebral disturbances, cardiovascular diseases, neurosensory and respiratory system disturbances, intellectual deficiency, equilibrium disturbances and inflammation (Dennis *et al.*, 2001).

Based on the studies cited above, this study was designed to determine the possible protective effect of EGb 761 against mercury chloride ($HgCl_2$)-induced thromboplastic activity and oxidative stress in the heart, aorta and serum. This was done by determining glutathione (GSH), lipid peroxidation (LPO), thromboplastic activity and total protein levels.

LDH and TNF-α were also assayed in serum samples for the evaluation of generalized tissue damage.

MATERIAL AND METHODS

Animals. Wistar Albino rats were housed in a room at a constant temperature of 22 ± 2 °C with 12 h light/dark

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cycles, and fed standard pellet chow and water *ad libitum*. The study was approved by the Marmara University (Istanbul, Turkey), School of Medicine, Animal Care and Use Committee.

Experimental design. Wistar Albino rats of either sex (200–250 g) were divided into four groups as follows: control (C) group (n = 6): 0.9% NaCl injection, i.p.; *Gingko biloba* extract (EGb) group (n = 6): EGb 761 (Abdi Ibrahim Pharmaceutical Company, Istanbul, Turkey) at a single daily dose of 50 mg/kg/day, i.p., 1 h after 0.9% NaCl injection, for 5 days; Hg group (n = 7): a single dose of 5 mg/kg mercury chloride ($HgCl_2$), i.p.; Hg + EGb group (n = 7): First day EGb at a dose of 50 mg/kg/day, i.p., 1 hour after $HgCl_2$ (5 mg/kg) injection; following four days EGb at a dose 50 mg/kg/day, i.p.

The animals were killed by decapitation 5 days after the $HgCl_2$ injection.

Biochemical analysis. Trunk blood samples were collected and serum samples were stored at –20 °C until determination of lactate dehydrogenase (LDH), tumour necrosis factor-α (TNF-α), GSH and LPO. Heart and aorta samples also were stored at –20 °C for the measurement of malondialdehyde, glutathione, total protein levels and thromboplastic activity.

Electrophoretic examination of the aorta and heart proteins was carried out by Laemmli SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Malondialdehyde (MDA), glutathione (GSH) and total protein assays. Heart and aorta tissue samples were homogenized with physiological saline solution for the determination of MDA and GSH levels. The MDA levels, an end product of lipid peroxidation, were assayed by monitoring thiobarbituric acid reactive substance formation by the method of Yagi (1984). Glutathione, a key intracellular antioxidant, was measured by the method of Beutler (1975). Total protein levels were measured by the method of Lowry *et al.* (1951).

TNF-α, LDH and thromboplastic activity tests. LDH and TNF-α were assayed in serum samples for the evaluation of generalized tissue damage and cytokine generation respectively. Serum LDH levels were determined spectrophotometrically using an automated analyser (Martinek, 1972). Serum levels of tumor necrosis factor alpha (TNF-α), was quantified according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits specific for the previously mentioned rat cytokines (Biosource International, Nivelles, Belgium). These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intraassay precision, and samll amount of plasma sample required to conduct the assay. TNF-α in the serum samples was expressed as pg/ml.

The thromboplastic activity of heart and aorta samples was evaluated according to Quick's one-stage method using normal plasma (Ingram and Hills, 1976). This was performed by mixing 0.1 mL tissue homogenate with 0.1 mL of 0.02 M $CaCl_2$; the clotting reaction was started by the addition of 0.1 mL of plasma. All reagents were brought to the reaction temperature (37 °C) before mixing. The thromboplastic activity was expressed as seconds. Shortened clot formation time showed an increased thromboplastic activity.

Statistical analysis. A Unistat 5.0™ statistical computer program was used to evaluate the results. Student's t-test, one-way analysis of variance (ANOVA) test and Pearson correlation analysis were used. A value of $p \leq 0.05$ was considered significant.

RESULTS

As shown in Tables 1–3 aorta, heart and serum MDA levels were significantly higher and GSH level was significantly lower in Hg treated rats compared with controls ($p = 0.001$). The groups administered both EGb and Hg had significantly decreased MDA and significantly increased GSH levels ($p = 0.001$) in aorta and serum samples compared with the group given Hg only. In the heart, MDA levels were significantly decreased but the increase in GSH level was not significant, both compared with the group given Hg only. EGb administration alone did not significantly change the aorta, heart and serum MDA and GSH levels when compared with those of the control group.

Serum lactate dehydrogenase activity, a marker of generalized tissue damage, showed a significant increase in Hg treated animals ($p < 0.01$) compared with the controls, while animals treated with both Hg and EGb showed significantly decreased enzyme activity ($p < 0.01$) compared with the Hg treated group (Fig. 1).

In the Hg treated group, TNF-α levels were significantly increased ($p < 0.001$) when compared with the

Table 1. Mean levels of aorta total protein, malondialdehyde and glutathione levels for all groups

	(1)	(2)	(3)	(4)	
Aorta	C Mean ± SD (n = 6)	EGb Mean ± SD (n = 6)	Hg Mean ± SD (n = 7)	Hg + EGb Mean ± SD (n = 7)	p
Total protein (% mg)	282.67 ± 52.68	244.00 ± 30.78	256.57 ± 39.36	205.71 ± 34.78	0.017
MDA (nmol MDA/g tissue)	18.44 ± 5.03	15.03 ± 2.98	54.55 ± 17.20	22.01 ± 2.51	0.0001
GSH (% mg GSH/g tissue)	33.17 ± 8.09	29.38 ± 2.58	23.83 ± 3.28	29.88 ± 2.90	0.001

MDA, malondialdehyde; GSH, glutathione; C, control; EGb, *Gingko biloba*; Hg, mercury.
Values are given as mean ± SD. Important significant differences, where *** $p < 0.01$, ** $p < 0.05$. For example, MDA (1–3)*** means that the difference in MDA between groups 1 and 3 is significant for $p < 0.01$. Total protein (1–4)***, MDA (1–3, 2–3, 2–4, 3–4)***, GSH (2–3, 3–4)***, (1–3)**.

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Table 2. Mean levels of heart total protein, malondialdehyde and glutathione levels for all groups

Heart	(1) C Mean ± SD (n = 6)	(2) EGb Mean ± SD (n = 6)	(3) Hg Mean ± SD (n = 7)	(4) Hg + EGb Mean ± SD (n = 7)	p
Total protein	1015.33 ± 179.66	994.66 ± 72.73	1096.00 ± 196.50	1049.71 ± 80.03	0.543
MDA (nmol MDA/g tissue)	4.45 ± 0.52	4.27 ± 0.30	9.64 ± 1.46	6.28 ± 0.45	0.0001
GSH (% mg GSH/g tissue)	3.54 ± 0.57	2.97 ± 0.35	2.16 ± 0.19	2.49 ± 0.34	0.0001

MDA, malondialdehyde; GSH, glutathione; C, control; EGb, *Gingko biloba*; Hg, mercury.
Values are given as mean ± SD. Important significant differences, where *** $p < 0.01$, ** $p < 0.05$. For example, MDA (1–3)*** means that the difference in MDA between groups 1 and 3 is significant for $p < 0.01$.
MDA (1–3, 1–4, 2–3, 2–4, 3–4)***, GSH (1–3, 1–4, 2–3)*** (2–4)**.

Table 3. Mean levels of serum malondialdehyde and glutathione levels for all groups

Serum	(1) C Mean ± SD (n = 6)	(2) EGb Mean ± SD (n = 6)	(3) Hg Mean ± SD (n = 7)	(4) Hg + EGb Mean ± SD (n = 7)	p
MDA (nmol MDA/mL plasma)	3.39 ± 0.28	3.35 ± 0.15	6.62 ± 0.42	4.35 ± 0.33	0.0001
GSH (% mg GSH/mL plasma)	1.28 ± 0.13	1.45 ± 0.15	0.89 ± 0.07	1.27 ± 0.56	0.001

MDA, malondialdehyde; GSH, glutathione; C, control; EGb, *Gingko biloba*; Hg, mercury.
Values are given as mean ± SD. Important significant differences, where *** $p < 0.01$, ** $p < 0.05$. For example, MDA (1–3)*** means that the difference in MDA between groups 1 and 3 is significant for $p < 0.01$.
MDA (1–3, 1–4, 2–3, 2–4, 3–4)***, GSH (1–3, 2–3, 3–4)*** (2–4)**.

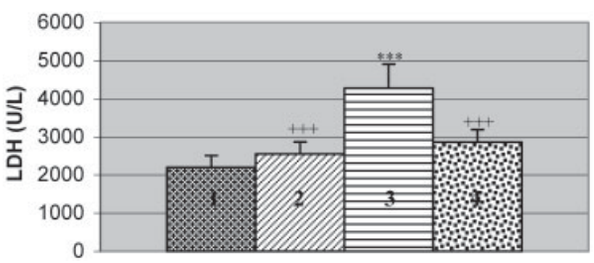

Figure 1. Mean levels of lactate dehydrogenase (LDH) in serum. (**1**) control; (**2**) *Ginkgo biloba*; (**3**) mercury; (**4**) mercury + *Ginkgo biloba.* *** $p < 0.001$ compared with control groups. +++ $p < 0.001$ compared with mercury group.

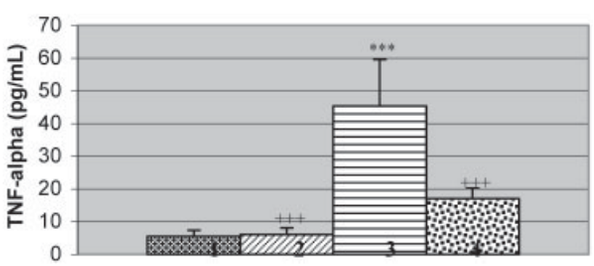

Figure 2. Mean levels of TNF-alpha (TNF-α) in serum. (**1**) control; (**2**) *Ginkgo biloba*; (**3**) mercury; (**4**) mercury + *Ginkgo biloba.* *** $p < 0.001$ compared with control groups. +++ $p < 0.001$ compared with mercury group.

control group. In the group treated with both Hg and Egb, the TNF-α level decreased significantly ($p < 0.05$, $p < 0.001$) (Fig. 2).

In the Hg-treated group, the aorta and heart thromboplastic activity showed a significant increase compared with the control group. The group treated with EGb and Hg showed a slight, but insignificant, decrease in thromboplastic activity when compared with

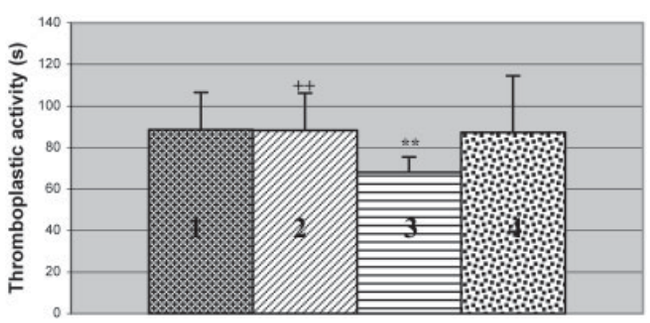

Figure 3. Mean levels of thromboplastic activity in aorta. (**1**) control; (**2**) *Ginkgo biloba*; (**3**) mercury; (**4**) mercury + *Ginkgo biloba.* ** $p < 0.05$ compared with control groups. ++ $p < 0.05$ compared with mercury group.

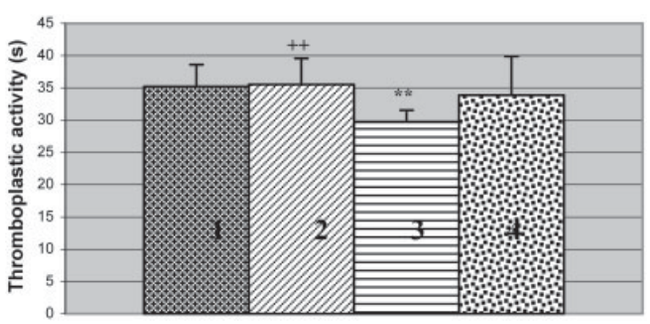

Figure 4. Mean levels of thromboplastic activity in heart. (**1**) control; (**2**) *Ginkgo biloba*; (**3**) mercury; (**4**) mercury + *Ginkgo biloba.* ** $p < 0.05$ compared with control groups. ++ $p < 0.05$ compared with mercury group.

the Hg group. The differences between the control, EGb and Hg + EGb groups were not significant. The EGb treated group did not exhibit thromboplastic activity that was different from the controls (Figs 3 and 4).

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Table 4. Correlation analysis of aorta, heart and serum parameters

	p	*r*
Aorta MDA–Aorta GSH	0.021	−0.450
Aorta GSH–Heart MDA	0.014	−0.487
Heart MDA–Heart thromboplastic activity	0.006	0.534
Heart MDA–Heart GSH	0.001	−0.642
Heart GSH–Aorta MDA	0.002	−0.600
Serum MDA–Aorta thromboplastic activity	0.034	0.464
Serum MDA–Aorta GSH	0.016	−0.476
Serum MDA–Heart thromboplastic activity	0.0001	0.441
Serum GSH–Aorta MDA	0.0001	−0.862
Serum GSH–Heart MDA	0.0001	−0.793
Aorta MDA–Heart MDA	0.0001	0.794
Aorta GSH–Heart GSH	0.029	0.436
Heart GSH–Heart thromboplastic activity	0.016	−0.476
Aorta MDA–Heart MDA	0.0001	0.794
Serum MDA–Aorta MDA	0.0001	0.877
Serum MDA–Heart MDA	0.0001	0.901
Serum GSH–Aorta GSH	0.015	0.482
Serum GSH–Heart thromboplastic activity	0.007	−0.524
Serum GSH–Heart GSH	0.002	0.585

The Hg administration did not significantly change the total protein levels in either the aorta or the heart samples. In the aorta samples from the group treated with both EGb and Hg there was a significant decrease in total protein levels (Table 1). The aorta and heart protein band developed through SDS polyacrylamide gel electrophoresis were not significantly influenced in any treatment group (Figure not shown).

The results of correlation analysis are shown in Table 4.

DISCUSSION

In the present study, acute administration of $HgCl_2$ to rats was observed to cause toxic effects in the aorta and heart, and this damage was observed to be associated with increases in lipid peroxides, indicating oxidative tissue damage, and a significant reduction in glutathione levels. Elevated TNF-α and LDH levels demonstrated the severity of Hg-induced systemic inflammatory response. Thus, it seems likely that the alleviation of Hg-induced oxidative tissue damage by EGb involves the suppression of a variety of pro-inflammatory mediators produced by leucocytes and macrophages. On the other hand, EGb prevented the $HgCl_2$-induced toxicity, since the biochemical parameters were restored to control levels. EGb is a known free radical scavenger (Mahady, 2002); therefore, the involvement of free radicals in the pathogenesis of Hg-induced toxicity is supported.

Mercury compounds have strong toxicity, and because of the high affinity of mercury to thiol groups, they are known to affect living organisms by damaging proteins and thiol enzymes.

Lund *et al.* suggested that Hg(II) administration selectively depletes mitochondrial GSH, and produces local toxicity by altering membrane permeability and decreasing the efficiency of oxidative phosphorylation (Lund *et al.*, 1991). This renders the mitochondria more susceptible to oxidative damage, especially during increased free radical production.

The concentration of intracellular GSH is essential for the protection of thiol and other nucleophilic groups in proteins from the toxic metabolites. Therefore, it is the key determinant of the extent of Hg induced tissue injury. A number of sulfur-containing compounds, both naturally occurring and synthetic, are known to have a protective effect against free radical-induced oxidative organ damage (Bulat *et al.*, 1998). Previous studies demonstrated that the antioxidant agents melatonin and *N*-acetylcysteine prevented toxicity induced by Hg (Sener *et al.*, 2003). Similarly in the present study, acute Hg treatment significantly depleted the aorta and heart GSH stores. This indicates that GSH is used as an antioxidant for the detoxification of toxic metabolites of this metal while the susceptibility of the tissue to oxidative injury was enhanced. Due to its antioxidant activity, EGb treatment reduced the Hg-induced oxidative injury and restored the GSH levels significantly. As reported by Ross, cell injury and enhanced cell susceptibility to toxic chemicals are related to the efflux of GSH precursors and hence to diminished GSH biosynthesis (Ross, 1988). In this sense, GSH and other antioxidants play a critical role in the propagation of free-radical reactions leading to lipid peroxidation.

It is known that mercury promotes the formation of free radicals (Ganther, 1980) and the peroxidative degradation of lipids (Benow *et al.*, 1990; Sunderman, 1986). As a free radical generating system, lipid peroxidation has been suggested to be closely related with Hg-induced tissue damage, and MDA is a good indicator of the degree of lipid peroxidation. In the present study a significant increase in MDA content was observed in both serum and tissues during Hg-induced toxicity This agrees with previous studies where lipid peroxidation products were increased from 40% to 120% above baseline values (Lund *et al.*, 1991; Mahady, 2002; Sunderman, 1986; Seppanen *et al.*, 2004; Yonaha *et al.*, 1980). Our results show that EGb treatment significantly inhibits MDA production, implying a reduction in lipid peroxidation and cellular injury that protect the tissues against Hg-induced oxidative damage.

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Ginkgo biloba extract (EGb 761) is one of the pharmacological agents that have been proposed as a free radical scavenger in the past decade. Standardized *Ginkgo biloba* extract contains ginkgo flavone glycosides and terpenoids (ginkgolides A, B, C and bilobalide) (Dennis *et al.*, 2001; Kose *et al.*, 1997; Robak and Gryglewski, 1988). These active constituents of EGb 761 contribute in varying degrees to the therapeutic actions of the extract (Robak and Gryglewski, 1988). EGb 761 has also been shown to have antioxidant activity. It is assumed that the EGb 761 antioxidant action is caused by the flavanoid glycosides that remove $O_2^{\cdot}$ and the terpenoids that scavenge $OH^{\cdot}$ radicals. It is a potent vasodilator for cerebral dysfunction (Robak and Gryglewski, 1988). EGb 761 also has an antiatherosclerotic effect (Jiao *et al.*, 2005; Satoh and Nishida, 2004).

Both clinical and experimental studies have shown that any noxious event is preceded by tissue macrophages and monocytes, which in turn secrete cytokines such as interleukin-1 (IL-1) and TNF-α (Ziemba *et al.*, 2005). As evidenced in the present study, Hg treatment resulted in increased serum TNF-α, indicating the role of this cytokine in this toxicity, while EGb depressed the TNF-α response.

An increase in thromboplastic activity both in aorta and heart samples following mercury administration is reversed by EGb treatment. Thromboplastin, also known as tissue factor or Factor III, is an important coagulation factor that initiates extrinsic blood coagulation with FVII. It is not actively found in the blood but as a component of the cell membranes (Bachlie, 2000; Lwaleed, 2002). It has been shown that some body tissues and fluids have thromboplastic activities (Ashkinazi, 1977; Lwaleed *et al.*, 2000; Tutuarima *et al.*, 1985; Utter *et al.*, 2002; Yarat *et al.*, 2004; Zacharski and Rosenstein, 1979). Decreased thromboplastic activity in tissue samples contributes to high thromboplastin levels and cellular damage. In the present study thromboplastic activity was decreased in rats treated with both EGb and Hg. This result shows that the cell damage induced by mercury administration in aorta and heart samples was improved by EGb treatment as evidenced by the MDA and GSH results.

Correlation analysis suggests that an increase in heart MDA in the mercury treated group is related to a decrease in thromboplastic activity. It also suggests that an increase in serum MDA in the mercury treated group caused a decrease in both aorta and heart thromboplastic activity. The increase in heart and aorta GSH by EGb treatment is associated with an increase in thromboplastic activity. It can be concluded that thromboplastic activity is related to cell damage.

CONCLUSION

The findings of the current study illustrate for the first time that exogenously administered *Ginkgo biloba* extract reduced aorta, heart and serum oxidant damage and normalized activated thromboplastic activity induced by mercury toxicity. As *Ginkgo biloba* reduces the aorta and heart oxidant damage after mercury administration, tissue factors will not appear in the blood stream and clot formation will be prevented.

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