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Ginkgo biloba Extract reduces Naphthalene-induced Oxidative Damage in Mice

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This investigation elucidated the role of free radicals in naphthalene-induced toxicity and protection by *Ginkgo biloba* extract (EGb). BALB-c mice of either sex were administered with naphthalene (100 mg/kg; i.p.) for 30 days, along with either saline or EGb (150 mg/kg, orally). At the end of the experiment, following decapitation, lung, liver and kidney tissue samples were taken for histological examination or determination of malondialdehyde (MDA), glutathione (GSH), myeloperoxidase (MPO) activity and collagen contents. In addition, proinflammatory cytokines (TNF-α and IL-β) and total antioxidant capacity (AOC) were assayed in the plasma, while lactate dehydrogenase (LDH) activity was assayed in serum samples. The results revealed that naphthalene caused a significant decrease in GSH level, and significant increases in MDA level, MPO activity and collagen content of tissues. Similarly, plasma cytokines, as well as serum LDH activity, were elevated while AOC was decreased in the naphthalene group compared with the control group. On the other hand, EGb treatment reversed all these biochemical indices. The results demonstrate that EGb extract, by balancing the oxidant–antioxidant status and inhibiting the generation of proinflammatory cytokines and neutrophil infiltration, protects against naphthalene-induced oxidative organ injury. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: naphthalene toxicity; *Ginkgo biloba*; cytokine; lipid peroxidation; glutathione; myeloperoxidase.

INTRODUCTION

Naphthalene is a bicyclic aromatic compound that has wide industrial and commercial applications. It is used as the starting material for the synthesis of other compounds, as a moth repellent, soil fumigant and lavatory deodorant. Most exposure occurs through low dose chronic inhalation, dermal contact or ingestion through the food chain (Stucker *et al.*, 1993; Vuchetich *et al.*, 1996).

Toxic manifestations of naphthalene are associated with its oxidative metabolism to various products including quinones, which are known to induce oxidative damage (Bagchi *et al.*, 2001). Previous studies have demonstrated that naphthalene exposure is associated with the development of hemolytic anemia in humans and rats (McMillan *et al.*, 2004; Molloy *et al.*, 2004). Naphthalene has been shown to induce oxidative stress as evidenced by hepatic and brain lipid peroxidation, GSH depletion, DNA single-strand breaks and membrane microviscosity, and excretion of urinary lipid metabolites in rats (Vuchetich *et al.*, 1996). In a study by Yamauchi *et al.*, naphthalene exposure resulted in elevated levels of serum and liver lipid peroxides, while decreased hepatic selenium dependent glutathione peroxidase activity was shown by Germansky and Jamall (1998). Thus, the toxicity of naphthalene is at least in part related to free radicals and free radical-mediated oxidative stress.

An extract of the leaves of *Ginkgo biloba* L., a mixture mainly composed of flavonoid glycosides and terpenoids (ginkgolides and bilobalide), has been shown to exhibit a variety of pharmacological actions (Van Beek *et al.*, 1998). *Ginkgo biloba* extract (Egb) has been reported to be a potent free radical scavenger and an antioxidant. The leaf extracts suppressed platelet aggregation induced by *tert*-butyl hydroperoxide and hydrogen peroxide through its antioxidant action (Akiba *et al.*, 1998). Furthermore, the extract and its ingredients exhibit an antagonistic effect on platelet activating factor (PAF) (Lamant *et al.*, 1987) and an inhibitory effect on the expression of inducible nitric oxide synthase and nitric oxide production (Kobuchi *et al.*, 1997). They also provide protection in myocardial and brain ischemia/reperfusion injury (Shen *et al.*, 1998; Janssens *et al.*, 2000). The antioxidant effects of EGb in mercury- or acetaminophen-induced toxicity, biliary obstruction induced hepatic fibrosis and renal ischemia reperfusion injury were shown previously (Sehirli *et al.*, 2006; Sener *et al.*, 2005 a, b, 2006).

Accordingly, the present study aimed to investigate the possible protective effect of EGb against naphthalene-induced oxidative damage of the lung, liver and kidney tissues by using biochemical approaches, such as the measurement of malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity and collagen content, as well as by the histological analysis of tissue injury.

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MATERIALS AND METHODS

Animals. BALB/c mice of either sex (25–30 g) were housed in a room at a mean constant temperature (22 ± 2 °C) with a 12 h light-dark cycle, 50%–60% relative humidity and free access to standard pellet chow and water. Mice were maintained in these facilities for at least 1 week before the experiment. The study was approved by the Marmara University School of Medicine Animal Care and Use Committee.

Experimental groups. Since differences in toxicity between rats and mice were reflected in the ability of naphthalene much more severely to deplete nonprotein sulphydryls in mouse tissues compared with rats (O'Brien *et al.*, 1985), this study used a mouse model.

BALB-c mice of either sex 25–30 g were divided into four groups each consisting of 12 animals. In the naphthalene groups, naphthalene was administered at a dose of 100 mg/kg (dissolved in corn oil) either with saline or Egb (150 mg/kg, orally) for 30 days. In other control mice corn oil either with saline or with Egb were administered for 30 days. *Ginkgo biloba* used in this study comes as an extract from the leaves of the plant and contains 24% flavone glycosides and 6% ginkgolides (terpenoids). Extraction was carried out by Abdi Ibrahim Pharmaceuticals (Istanbul, Turkey) who kindly donated the extract. Briefly, following primary extraction with acetone/water, hydroethanol liquid purification and methylene chloride washing, precipitation and filtration were done. Then the solvent was removed and the extract dried.

After decapitation of the animals, trunk blood was collected and liver, lung and kidney were carefully dissected and stored at –70 °C for the determination of tissue MDA and GSH levels, MPO activity and collagen content. Tissue samples were also examined histologically.

Biochemical analysis. Plasma levels of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β were 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 intra-assay precision and the small amount of plasma sample required to conduct the assay. The total antioxidant capacity in plasma was measured by using a colorimetric test system (ImAnOx, cataloge no.KC5200, Immunodiagnostic AG, D-64625 Bensheim), according to the instructions provided by the manufacturer. In serum samples, LDH activity (Martinek, 1972) was determined spectrophotometrically using an automated analyser.

Malondialdehyde (MDA) and glutathione (GSH) assays. Tissue samples were homogenized with ice-cold trichloracetic acid (1 g tissue plus 10 mL 10% TCA) in an Ultra Turrax tissue homogenizer. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (Beuge and Aust, 1978). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and the results are expressed as nmol MDA/g tissue. Glutathione measurements were performed using a modification of the Ellman procedure (Beutler, 1975). Briefly, after centrifugation at 2,000 × g for 10 min, 0.5 mL of supernatant was added to 2 mL of 0.3 mol/L $Na_2HPO_4.2H_2O$ solution. A 0.2 mL solution of dithiobisnitrobenzoate (0.4 mg/mL 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. Glutathione levels were calculated using an extinction coefficient of 1.36×10^5 M^{-1} cm^{-1}. The results are expressed in μmol GSH/g tissue.

Measurement of myeloperoxidase activity. Tissue MPO activity was assessed by measuring the H_2O_2-dependent oxidation of o-dianizidine. 2 HCl. Briefly, tissue samples (0.2–0.3 g) were homogenized in 10 vol of ice-cold potassium phosphate buffer (50 mmol/L K_2HPO_4, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5%, w/v). The homogenate was centrifuged at 30,000 × g for 10 min at 4 °C and the supernatant was discarded. The pellet was then rehomogenized with an equivalent volume of 50 mmol/L K_2HPO_4 containing 0.5% (w/v) hexadecyltrimethylammonium bromide and 10 mmol/L ethylenediaminetetraacetic acid (EDTA, Sigma). MPO activity was assessed by measuring the H_2O_2-dependent oxidation of o-dianizidine. 2 HCl. One unit of enzyme activity was defined as the amount of the MPO present per gram of tissue that caused a change in absorbance of 1.0/min at 460 nm and 37 °C (Hillegas *et al.*, 1990).

Tissue collagen measurement. Tissue collagen was measured as a free radical-induced fibrosis marker. Tissue samples were cut with a razor blade, immediately fixed in 10% formalin then samples were embedded in paraffin, and sections, approximately 15 μm thick were obtained. The evaluation of collagen content was based on the method published by Lopez de Leon and Rojkind (1985), which is based on selective binding of the dyes Sirius Red and Fast Green FCF to collagen and noncollagenous components, respectively. Both dyes were eluted readily and simultaneously by using 0.1 N NaOH–methanol (1:1, v/v). Finally, the absorbances at 540 and 605 nm were used to determine the amount of collagen and protein, respectively.

Histological preparation and analysis. Samples of liver, lung and kidney tissues were fixed in 10% formaldehyde and processed routinely for embedding in paraffin. Paraffin sections were stained with hematoxylin and eosin and examined under a light microscope (Olympus-BH-2, Tokyo, Japan) by an experienced histologist, who was unaware of the treatment conditions.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± SEM. Groups of data were compared with an analysis of variance followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were regarded as significant.

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RESULTS AND DISCUSSION

Naphthalene exposure is associated with several toxic manifestations in humans and laboratory animals, with the lens of the eye and the lungs being the most sensitive (Stohs *et al.*, 2002). Most human toxicities involve low dose, chronic exposure to naphthalene (O'Brien *et al.*, 1985). In mice, 200 mg/kg intraperitoneal naphthalene administration caused pulmonary damage (O'Brien *et al.*, 1985). Koch *et al.* (1976) reported the development of cataract following exposure to naphthalene. In tests with Chinese hamster ovary cells, naphthalene induced sister chromatid exchanges with and without exogenous metabolic activation. Naphthalene caused an increase in the incidence and severity of olfactory epithelial metaplasia of respiratory epithelium in the nose and chronic inflammation in the lungs (Abdo *et al.*, 2001). Previously, studying the toxic effects of naphthalene in mice, it was demonstrated that when administered chronically, naphthalene caused multiorgan damage by increasing lipid peroxidation of the tissues. Furthermore when aqueous garlic extract was administered concomitantly with naphthalene, this damage was prevented, suggesting that naphthalene caused oxidative injury and that antioxidative agents could be beneficial against naphthalene toxicity (Omurtag *et al.*, 2005). As a free radical generating system, lipid peroxidation has been suggested to be closely related to oxidant-induced tissue damage, and MDA is a good indicator of the degree of lipid peroxidation (Vuchetich *et al.*, 1996; Stark, 2005). In this study, it was observed that chronic naphthalene administration resulted in a significant increase in MDA levels in all the tissues (lung, liver and kidney) compared with control animals. However, these elevations were significantly reversed with EGb administration ($p < 0.05$–0.001, Table 1).

Naphthalene undergoes extensive microsomal metabolism. The first step in the naphthalene metabolism is oxidative in nature and catalysed by cytochrome P-450 oxygenases in the microsome that produces an electrophilic arene epoxide intermediate, 1,2-naphthalene oxide (Jerina *et al.*, 1970). The epoxides can rearrange spontaneously to form naphthols (predominantly 1-naphthol), which undergo further metabolism to naphthaquinons. Naphthoquinones are directly toxic to mononuclear leukocytes and deplete GSH to 1% of the control levels (Wilson *et al.*, 1996). Thus, as a result of naphthalene metabolism, the formation of reactive oxygen radicals causes tissue injury, because GSH is depleted. Yamauchi *et al.* (1986) demonstrated that naphthalene elevated the serum lipid peroxides with a concomitant decrease in GSH levels in lenses, suggesting enhanced lipid peroxidation. Similarly Honda *et al.* (1990) have also demonstrated a significant depletion of pulmonary GSH levels following naphthalene exposure. On the other hand, the GSH precursor, *N*-acetylcysteine, and the free radical spin trapping agent, alpha-phenyl-*N*-t-butylnitrone, have been shown to decrease naphthalene-induced cataracts effectively (Wells *et al.*, 1989). Since the roles of oxidative stress-reactive oxygen species and the critical role of GSH in preventing naphthalene toxicity are well documented, agents, which have an antioxidant property, would be beneficial. As shown in this study the antioxidant EGb treatment significantly inhibited MDA production with a concomitant replenishment of tissue GSH content, implying a reduction in lipid peroxidation and cellular injury, which protects the liver, lung and kidney tissues against naphthalene-induced oxidative damage (Table 1). Furthermore, naphthalene administration significantly reduced the total antioxidant capacity of plasma while EGb treatment also replenished the plasma antioxidant status (Fig. 2a) and protected against this toxicity.

Ginkgo biloba extract contains many different flavone glycosides and terpenoids. Flavonoids seem to be responsible for the antioxidant activity that is considered to be one of the main mechanisms involved in

Table 1. The effects of naphthalene and *Ginkgo biloba* extract (EGb) treatment on the malondialdehyde (MDA), glutathione (GSH), myeloperoxidase activity (MPO) and collagen contents of the lung, liver and kidney tissues of groups. Each group consists of 12 animals

	Control		Naphthalene	
	Saline-treated	EGb-treated	Saline-treated	EGb-treated
MDA (nmol/g)				
Lung	41.15 ± 2.6	41.02 ± 2.5	71.5 ± 3.7^{c}	54.05 ± 3.72^{ay}
Liver	52.08 ± 1.9	53.73 ± 3.3	87.0 ± 3.6^{c}	62.35 ± 4.34^{z}
Kidney	32.3 ± 2.7	29.75 ± 3.0	53.8 ± 3.1^{c}	41.35 ± 2.48^{x}
GSH (μmol/g)				
Lung	2.16 ± 0.14	2.11 ± 0.13	0.72 ± 0.11^{c}	1.65 ± 0.15^{z}
Liver	1.42 ± 0.16	1.46 ± 0.13	0.56 ± 0.07^{c}	1.22 ± 0.10^{y}
Kidney	1.43 ± 0.13	1.53 ± 0.14	0.64 ± 0.08^{c}	1.21 ± 0.06^{y}
MPO (U/g)				
Lung	11.43 ± 0.9	10.05 ± 1.1	38.48 ± 3.1^{c}	18.65 ± 1.87^{z}
Liver	10.37 ± 1.3	10.10 ± 1.3	27.55 ± 3.0^{c}	13.73 ± 1.41^{z}
Kidney	4.7 ± 0.5	4.98 ± 1.6	14.73 ± 1.3^{c}	7.02 ± 0.61^{z}
Collagen (μg/mg prot)				
Lung	25.8 ± 1.5	25.37 ± 1.3	40.18 ± 3.1^{c}	28.12 ± 1.48^{y}
Liver	20.82 ± 2.3	20.58 ± 2.3	34.13 ± 2.2^{b}	23.72 ± 1.62^{x}
Kidney	15.65 ± 1.3	14.1 ± 1.5	27.70 ± 1.7^{c}	16.93 ± 1.24^{z}

Data are mean ± SD. ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$ compared with the saline-treated control group. ^{x} $p < 0.05$, ^{y} $p < 0.01$, ^{z} $p < 0.001$ compared with the saline-treated naphthalene group.

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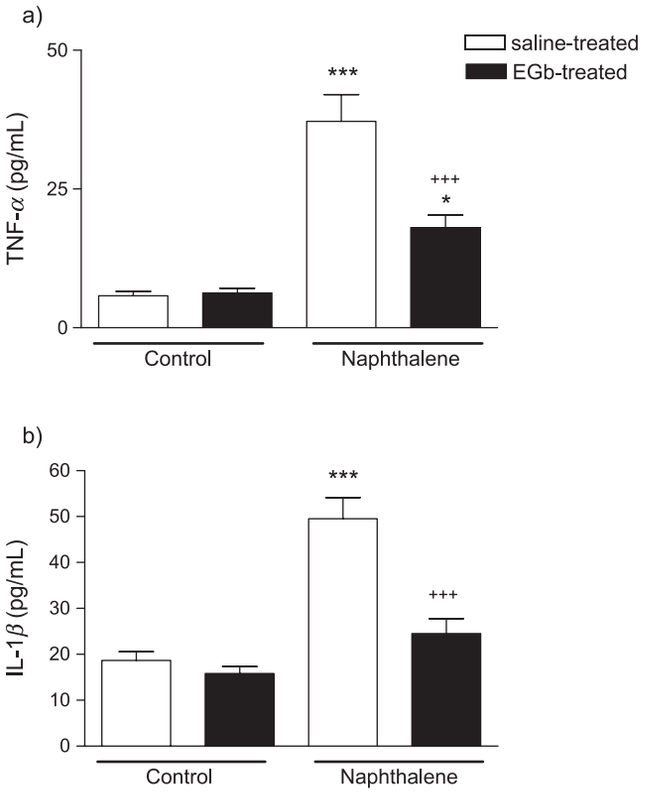

Figure 1. Plasma (a) tumor necrosis factor-alpha (TNF-α) and (b) interleukin 1-beta (IL-1β), levels in saline or EGb treated naphthalene and control groups. Each group consists of 12 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *** $p < 0.001$, * $p < 0.05$ versus saline-treated control group; $^{+++}$ $p < 0.001$ versus saline treated-naphthalene group.

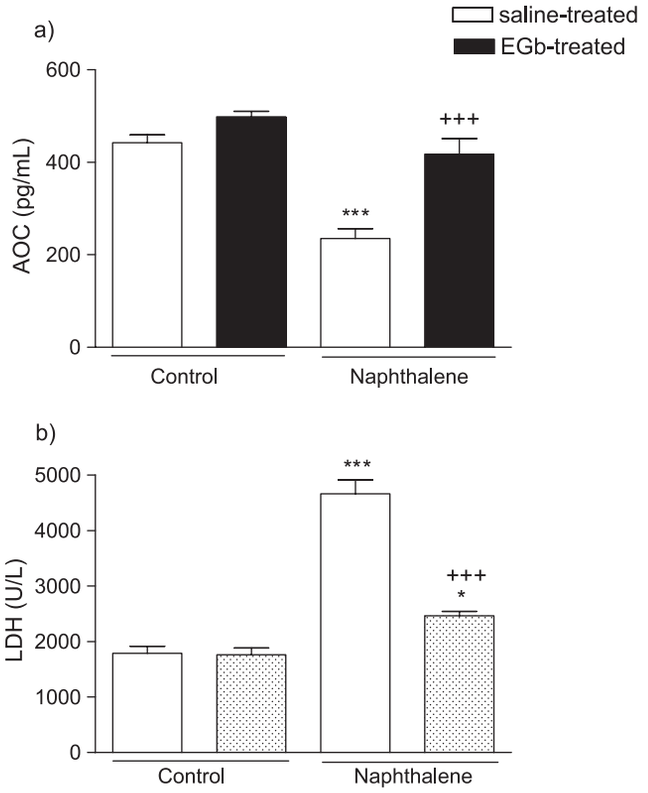

Figure 2. Plasma (a) total antioxidant capacity (AOC) and (b) serum lactate dehydrogenase activity (LDH) of saline or EGb treated naphthalene and control groups. Each group consists of 12 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *** $p < 0.001$, * $p < 0.05$ versus saline-treated control group; $^{+++}$ $p < 0.001$ versus saline treated-naphthalene group.

the pharmacological effects of the extract. The protective activity of EGb against myocardial ischemia/reperfusion injury is usually associated with the free radical scavenging activity of flavonoid components (Shen *et al.*, 1998). On the other hand, more recently Pietri *et al.* demonstrated that terpenic components of the extract could be involved in the cardioprotective activity, probably by inhibiting free radical formation (Pietri *et al.*, 1997). In the study by Zhang *et al.* (2004) the protective effect of EGb on hepatic endothelial cells in rats with chronic liver injury induced by carbon tetrachloride was attributed to the inhibition of PAF and lipid peroxidation. Furthermore EGb was also shown to enhance the activity of antioxidant enzymes, superoxide dismutase and glutathione peroxidase, and protected brain tissues against ischemia/reperfusion injury (Janssens *et al.*, 2000).

It has been suggested that an increase in lipid peroxidation may be due partly to the free radicals generated by neutrophils. Because activated neutrophils are known to induce tissue injury through the production and release of reactive oxygen metabolites and cytotoxic proteins (e.g. proteases, myeloperoxidase, lactoferrin) into the extracellular fluid. When neutrophils are stimulated by various stimulants, myeloperoxidase, as well as other tissue-damaging substances, is released from the cells (Kettle and Winterbourn, 1997). Since neutrophil infiltration is an important event for acute inflammation, an increase in MPO activity due to naphthalene may cause inflammation and damage in the organs. MPO activity, which is an indicator of tissue neutrophil infiltration, was increased in all the studied tissues due to naphthalene ($p < 0.001$) and EGb significantly reduced the tissue MPO activity ($p < 0.001$; Table 1). As an indicator of enhanced tissue fibrotic activity, the collagen contents of hepatic, pulmonary and renal tissues were increased ($p < 0.01$–0.001) following naphthalene administration, and EGb treatment on the other hand, reduced the increased collagen levels back to the control levels ($p < 0.05$–0.001; Table 1). On the other hand, the proinflammatory cytokines, TNF-alpha and IL-1β, were found to be significantly increased, also verifying that naphthalene toxicity is closely related with inflammatory mechanisms and oxidative damage (Fig. 1). Since EGb treatment significantly decreased these cytokines and prevented the infiltration of neutrophils into the damaged tissue, the results suggest that the protective effects of EGb is mediated in part by blocking plasma cytokines and tissue neutrophil infiltration. This might also result in reduced lipid peroxidation and less accumulation of MDA since activation of neutrophils might lead to the generation of oxygen reactive metabolites (Kettle and Winterbourn, 1997).

The histological results also verified the naphthalene-induced oxidative injury, as demonstrated by biological parameters. Treatment with naphthalene led to a prominent liver tissue injury with congestion in the central vein, severe deterioration of hepatocytes, high accumulation of inflammatory cells besides Kuppfer cells and

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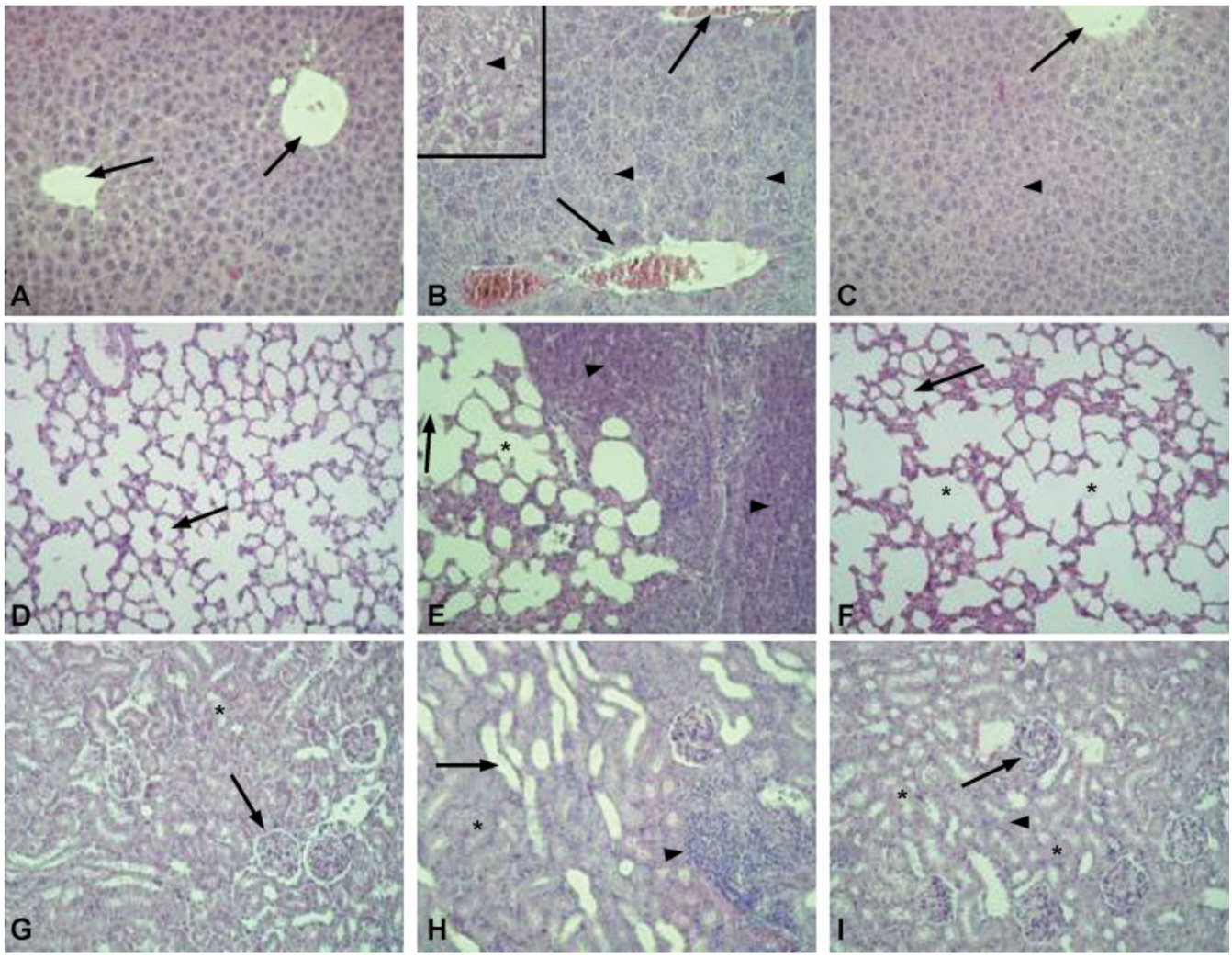

Figure 3. Tissue micrographs. Liver tissues: (A) control group, regular structure with central vein (arrows) and sinusoids, (B) naphthalene group, severe inflammation with high congestion of central vein (arrows), increased number of inflammatory cells besides Kuppfer cells in the sinusoids, severe degenerations in the hepatocytes (arrowheads, inset), (C) EGb-treated naphthalene group: the reversal of severe inflammation was clear but the cytoplasmic degeneration (arrowhead) with paler staining was still present, central vein (arrow), HE ×200, inset ×400. Lung tissues: (D) control group, regular lung morphology with alveoli (arrow), (E) naphthalene group, severe interstitial edema with reduced and distended alveoli (arrow), prominent inflammation (arrowheads) and saccular alveolar morphology (*), (F) EGb-treated naphthalene group, a decrease in the interstitial edema (arrow) and reshaped alveoli (*), HE ×200. Kidney tissues: (G) control group, regular morphology of both glomeruli (arrow) and tubuli (*), (H) naphthalene group, the severe degeneration of kidney tissue with congestion of vessels in the interstitium and dilation of tubuli (arrow), severe inflammation in the interstitium (arrowhead) (I) EGb-treated naphthalene group, the interstitial congestion diminished to limited regions (*) and tubuli appeared regenerated (arrowhead), regular glomerulus (arrow), HE ×200.

dilated sinusoids (Fig. 3B) when compared with regular control group (Fig. 3A). *Ginkgo biloba* treatment reduced the overall degeneration where mild hepatocellular degeneration persisted (Fig. 3C). In the lung tissues, the saline-treated control group demonstrated regular alveolar structure with no distension in the walls (Fig. 3D). However, in the saline-treated naphthalene group there was severe interstitial edema, which led to a decrease in alveolar space in most of the tissues. Moreover, in some regions the alveoli united with each other resulting in large distended alveolar spaces (Fig. 3E). In the EGb-treated naphthalene group the interstitial edema decreased prominently and the alveolar structure appeared to gain its integrity (Fig. 3F). When the kidney tissues were analysed, in the saline-treated control group, regular morphology with glomeruli and tubuli were observed (Fig. 3G). However, in the naphthalene group the kidney showed severe degenerations in the structure of tubuli morphology with debris in the tubuli being the result of epithelial cell desquamation. Furthermore in the interstitium edema was apparent (Fig. 3H). In the EGb-treated naphthalene group the overall kidney morphology changed as congestion in the interstitium became mild and the tubular epithelium maintained its integrity (Fig. 3I).

In the present study, increases in lipid peroxidation, myeloperoxidase activity and collagen content due to the toxic effects of naphthalene were accompanied by significant reductions in glutathione levels of the hepatic, pulmonary and renal tissues, and plasma total AOC suggesting the presence of oxidative tissue damage. Furthermore, the elevated plasma level of the cytokines TNF-α and IL-1β and the histological analyses demonstrated the severity of the naphthalene-induced systemic inflammatory response. EGb, as an antioxidant agent, ameliorated the oxidative injury in

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the hepatic, pulmonary and renal tissues, and elevated the antioxidant capacity in the plasma and inhibited the cytokine release.

In conclusion, the protective effects of EGb can be attributed, at least in part, to its ability to inhibit neutrophil infiltration, to balance oxidant–antioxidant status, and to regulate the generation of inflammatory mediators, suggesting a future role in the treatment of multiorgan damage due to drug or chemical toxicities.

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