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Communication

Aqueous garlic extract alleviates ischaemia–reperfusion-induced oxidative hepatic injury in rats

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Abstract

This study was designed to examine the effects of aqueous garlic extract (AGE) on hepatic ischaemia–reperfusion (I/R) injury in rats. For this purpose, Wistar albino rats were subjected to 45 min of hepatic ischaemia, followed by a 60-min reperfusion period. AGE (1 mL kg^{-1}, i.p., corresponding to 500 mg kg^{-1}) or saline was administered twice, 15 min before ischaemia and immediately before the reperfusion period. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined to assess liver functions. Liver tissues were taken for the determination of malondialdehyde (MDA) levels, an end product of lipid peroxidation; glutathione (GSH) levels, a key antioxidant; and myeloperoxidase (MPO) activity, as an indirect index of neutrophil infiltration. Hepatic collagen content, as a fibrosis marker, was also determined. Plasma ALT and AST activities were elevated in the I/R group as compared with the control group, while these increases were significantly decreased by AGE treatment. Hepatic GSH levels, significantly depressed by I/R, were elevated back to control levels in the AGE-treated I/R group. Increases in tissue MDA levels and MPO activity due to I/R injury were reduced back to control levels by AGE treatment. Similarly, increased hepatic collagen content in the I/R group was reduced to the control level with AGE treatment. Since AGE administration alleviated the I/R-induced injury of the liver and improved the hepatic structure and function, it seems likely that AGE, with its antioxidant and oxidant-scavenging properties, may be of potential therapeutic value in protecting the liver against oxidative injury due to ischaemia–reperfusion.

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Introduction

The liver is highly sensitive to ischaemia–reperfusion (I/R) injury, which occurs clinically during circulatory shock (De La Monte et al 1984), disseminated intravascular coagulation (Yoshikawa et al 1993) and surgery involving this organ, including liver transplantation (Arthur 1988). The tissue changes caused by ischaemia are well known. Upon depletion of energy-rich phosphates (adenosine triphosphate, ATP), the tissue concentration of their degradation products rises. Re-oxygenation of the ischaemic tissue may promote the generation of reactive oxygen metabolites (ROM), which are known to have deleterious effects on various cellular functions (Williams et al 1997). The organ dysfunction that accompanies this condition is generally associated with increased microvascular permeability, interstitial oedema, impaired vasoregulation, inflammatory cell infiltration and parenchymal cell dysfunction and necrosis (Granger & Korthuis 1995). I/R elicits an acute inflammatory response characterized by activation of neutrophils, which are known to induce tissue injury through the production and release of reactive oxygen metabolites and cytotoxic proteins (e.g. proteases, myeloperoxidase, lactoferrin) into extracellular fluid (Kettle & Winterbourn 1997).

Garlic has been used as a folk remedy for a variety of ailments since ancient times. In the past few years, it has been found that garlic preparations, including aged garlic extract, prevented tumour promotion (Dorant et al 1993), cardiovascular diseases (Kleijnen et al 1989), liver damage (Nakagawa et al 1989) and aging (Moriguchi et al 1994) in certain experimental models, which are considered to be associated with

oxygen radical and lipid peroxidation. The intrinsic antioxidant activity of garlic (Rietz et al 1993), garlic extracts (Prasad et al 1996) and some garlic constituents (Rabinkov et al 1998) has been widely documented in-vivo (Augusti & Sheela 1996) and in-vitro (Prasad et al 1996; Rabinkov et al 1998). Garlic extracts increase superoxide dismutase (SOD) (Geng & Lau 1997), glutathione peroxidase (GPx) (Wei & Lau 1998) and catalase (CAT) activity (Wei & Lau 1998) in vascular cell cultures, while a garlic compound, S-allyl-cysteine sulfoxide (alliin), prevents the decreases in hepatic SOD and CAT activity observed in diabetic rats (Augusti & Sheela 1996).

Based on these reports, this study was designed to determine the possible protective effect of aqueous garlic extract (AGE) against oxidative stress during I/R injury of the liver, by determining biochemical parameters and by histological examination.

Materials and Methods

Animals

Male Wistar albino rats, 200–250 g, were housed in an air-conditioned room with a 12-h light–dark cycle, where the temperature ($22 \pm 2^{\circ}C$) and relative humidity (65–70%) were kept constant. All experimental protocols were approved by the Marmara University School of Medicine Animal Care and Use Committee. Rats were anaesthetized (100 mg kg^{-1} ketamine and 0.75 mg kg^{-1} chlorpromazine; i.p.) during all surgical procedures.

Aqueous garlic extract preparation

Peeled garlic (30 g) was crushed with distilled water in a mortar. The crushed material was carefully decanted by pressing and 60 mL of aqueous extract was extracted. One millilitre of aqueous garlic extract (AGE) contained material from 500 mg of garlic (Sener et al 2003). The compounds present in AGE, S-allyl-cysteine, S-allyl-mercaptocystein, S-allyl-cysteinesulfoxide and allicin, are known to have the ability to scavenge ROM (Imai et al 1994).

Experimental protocol

In the rats, under anaesthesia, a midline laparotomy was made using minimal dissection. Total hepatic ischaemia was induced for 45 min by clamping the hepatic artery, the portal vein and the bile duct using a vascular clamp and the rats were then allowed to reperfuse for 1 h. AGE (1 mL kg^{-1}) or saline (0.1 mL kg^{-1}) was administered intraperitoneally on two occasions, 15 min before ischaemia and immediately before the reperfusion period (I/R + AGE or I/R groups, respectively). None of the rats died during these procedures. At the end of the reperfusion period, rats were decapitated and trunk blood samples were collected to determine serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels as indicators of liver function. The hepatic tissue samples were stored at $-70^{\circ}C$. Afterwards, tissue malondialdehyde (MDA) levels, an end product of lipid peroxidation, glutathione (GSH), a key antioxidant, and tissue-associated myeloperoxidase (MPO) activity, as indirect evidence of neutrophil infiltration, were measured in these samples. The hepatic tissue samples were also placed in formaldehyde (10%) for histological evaluation and collagen content.

Malondialdehyde and glutathione assays

The liver tissue samples were homogenized with ice-cold 150 mM KCl for determination of malondialdehyde (MDA) and glutathione levels. The MDA levels were assayed for products of lipid peroxidation (Beuge & Aust 1978). Results were expressed as μmol MDA/g tissue. Glutathione was determined by the spectrophotometric method based on the use of Ellman's reagent (Beutler 1975). Results were expressed μmol GSH/g tissue.

Measurement of myeloperoxidase (MPO) activity

MPO activity was measured in liver tissue in a procedure similar to that documented by Hillegas et al (1990). Liver samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and centrifuged at 41 400 *g* (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41 400 *g* for 10 min. Samples (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Tissue collagen measurement

Tissue samples were cut with a razor blade and immediately fixed in 10% formaldehyde in 0.1 M and in phosphate buffer (pH 7.2) paraffin sections of approximately 15 μm thick were obtained. The measurement of collagen method, originally published by Lopez de Leon & Rojkind (1985), is based on the selective binding of Sirius red and Fast Green FCF to collagenous and non-collagenous components, respectively, when the sections are stained with both dyes dissolved in aqueous saturated picric acid. Both dyes were eluted readily and simultaneously with NaOH–methanol and the absorbances obtained at 540 and 605 nm were used to determine the amount of the collagen and protein.

Histological analysis

For the light microscopic investigations, liver samples, which were placed in 10% formaldehyde, were processed routinely by embedding in paraffin. Tissue sections (5 μm) were stained with haematoxylin and eosin (H&E) and examined under an Olympus BH 2 photomicroscope.

Statistics

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software; San Diego, CA). All data were expressed as means ± s.e.m. 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.

Results

AST and ALT levels were significantly higher in the I/R group when compared with those of the control group. Although AGE treatment decreased both of these values significantly ($P < 0.001$), the AST level was found to be still significantly higher than the control value (Table 1).

The liver MDA, which is an index of tissue lipid peroxidation, was found to be significantly higher in the I/R group ($75.5 \pm 2.8\,\text{nmol}\,\text{g}^{-1}$) than that measured in the control group ($50.3 \pm 2.1\,\text{nmol}\,\text{g}^{-1}$) (Fig 1A). Treatment with AGE decreased the elevated MDA level significantly back to control level ($54.3 \pm 2.8\,\text{nmol}\,\text{g}^{-1}$).

The level of the endogenous antioxidant, GSH, in the hepatic tissue was decreased significantly after I/R ($0.94 \pm 0.1\,\mu\text{mol}\,\text{g}^{-1}$) as compared with the levels measured in the control group ($1.96 \pm 0.1\,\mu\text{mol}\,\text{g}^{-1}$), while AGE treatment significantly reversed this I/R-induced GSH reduction ($1.63 \pm 0.1\,\mu\text{mol}\,\text{g}^{-1}$) (Figure 1B).

When compared with the control group ($11.0 \pm 0.7\,\text{U}\,\text{g}^{-1}$), hepatic MPO activity was increased significantly in the I/R group ($16.5 \pm 0.7\,\text{U}\,\text{g}^{-1}$), indicating increased neutrophil infiltration to the tissue. This elevation in the MPO activity induced by I/R was reversed back to the control level with AGE treatment ($11.1 \pm 0.5\,\text{U}\,\text{g}^{-1}$) (Figure 2).

Hepatic collagen content, an index of fibrosis, was found to be significantly higher in the I/R group ($43.1 \pm 2.3\,\mu\text{g}$ (mg protein)$^{-1}$) than in the control group ($29.0 \pm 1.2\,\mu\text{g}$ (mg protein)$^{-1}$). AGE treatment significantly reduced this increase ($31.6 \pm 2.3\,\mu\text{g}$ (mg protein)$^{-1}$) to a level close to that of the control value (Figure 3).

Histological examination

Light microscopic evaluation of the control and AGE groups revealed a regular morphology of liver parenchyma with intact hepatocytes and sinusoids (Figures 4A and 4B). In the I/R group, severe sinusoidal congestion and haemorrhage, central vein dilation, subendothelial oedema and degenerated hepatocytes with perinuclear vacuolisation were observed (Figure 4C). In the AGE-treated I/R group, histological analysis demonstrated a

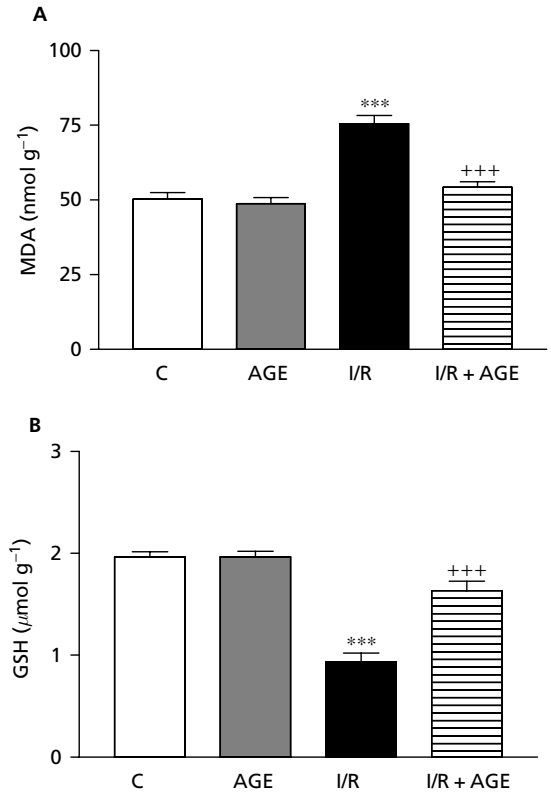

Figure 1 Malondialdehyde (MDA) (A) and glutathione (GSH) (B) levels in the hepatic tissues of C (control), AGE (aqueous garlic extract), I/R (ischemia–reperfusion) and I/R + AGE groups of rats. Data are means ± s.e.m., each group consists of 8 rats. $^{***}P < 0.001$, compared with control group; $^{+++}P < 0.001$, compared with saline-treated I/R group.

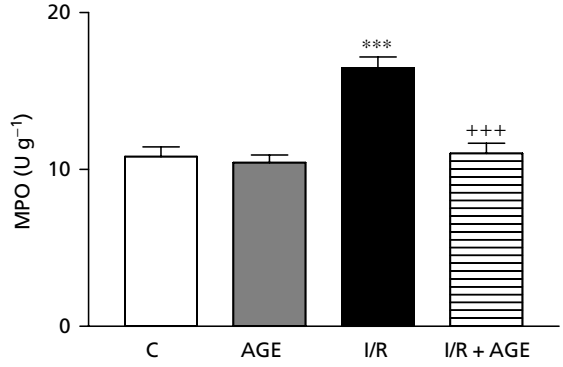

Figure 2 The effect of I/R and AGE treatment on hepatic myeloperoxidase (MPO) activity in rats. Data are means ± s.e.m., each group consists of 8 rats. C, control; AGE, aqueous garlic extract; I/R, ischaemia–reperfusion. $^{***}P < 0.001$, compared with control group; $^{+++}P < 0.001$, compared with saline-treated I/R group.

Table 1 The effect of AGE on plasma alanine aminotransferase (ALT) and (AST) activity in rats

Group	AST ($\text{U}\,\text{L}^{-1}$)	ALT ($\text{U}\,\text{L}^{-1}$)
Control (n = 8)	242 ± 7.5	108 ± 7.5
AGE (n = 8)	228 ± 9.2	106 ± 7.4
I/R (n = 8)	2116 ± 80.3***	1995 ± 152.7***
I/R + AGE (n = 8)	578 ± 30.6***,+++	347 ± 28.5+++

AGE, aqueous garlic extract; I/R, ischaemia–reperfusion. $^{***}P < 0.001$, compared with control group; $^{+++}P < 0.001$, compared with I/R group.

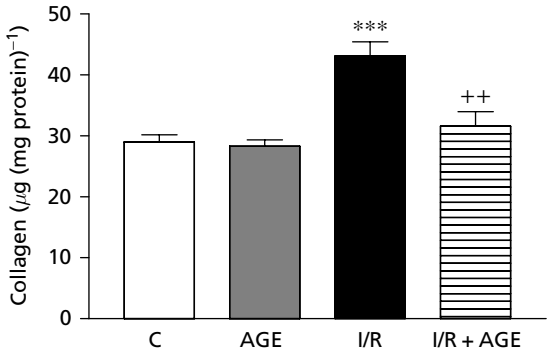

Figure 3 The effect of I/R and AGE treatment on hepatic collagen content in rats. Data are means ± s.e.m., each group consists of 8 rats. C, control; AGE, aqueous garlic extract; I/R, ischaemia–reperfusion. $^{***}P < 0.001$, compared with control group; $^{++}P < 0.01$, compared with saline-treated I/R group.

well-preserved liver parenchyma. Despite the moderate sinusoidal dilatation and haemorrhage, which were in localized areas, usual appearance of the central vein and hepatocytes was observed in most areas (Figure 4D).

Discussion

This study demonstrates that, in rats, AGE treatment improved I/R-induced impairment in liver function, significantly decreased I/R-induced elevation in hepatic lipid peroxidation, myeloperoxidase activity and collagen content, and replenished decreased GSH levels. Histological findings also support the protective effect of AGE in I/R-induced hepatic damage.

The mechanisms underlying I/R damage to the liver are most likely multifactorial and interdependent, involving hypoxia, inflammatory responses and free radical damage (Williams et al 1997). Free-radical-mediated cellular damage can be expected to occur when oxygen is supplied to the tissue by reperfusion and the oxygen radical formation exceeds the high cellular detoxification capacity of the organ. A number of processes have been implicated in the pathogenesis of oxygen-deprivation-induced cell injury (Granger & Korthuis 1995). These include disturbances of cellular calcium metabolism, disruption of the generation of free radicals, activation of phospholipases with production of toxic lipid metabolites, loss of cell volume and impairment in cellular monovalent cation homoeostasis. The result of oxygen radical formation is damage to an array of

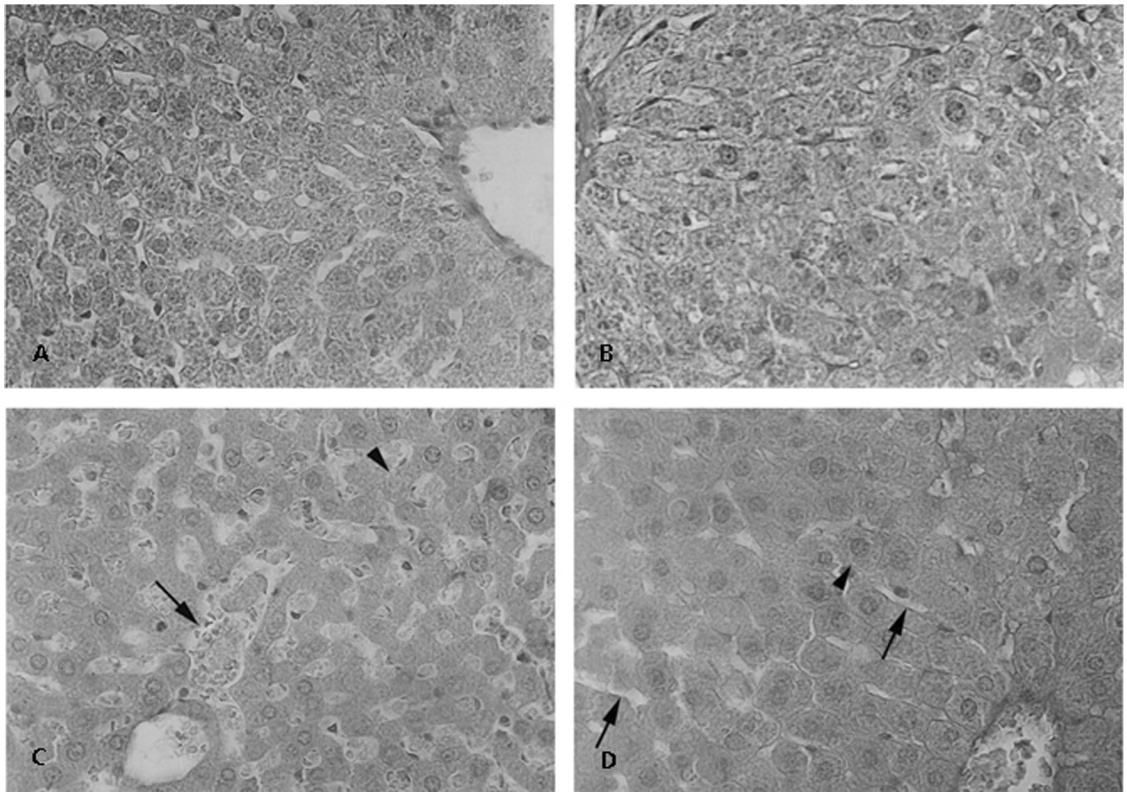

Figure 4 Control group of rats (A) and AGE-treated group of rats (B) showing regular liver parenchyma with normal appearance of hepatocytes and sinusoids. I/R group of rats (C) show severe sinusoidal congestion (arrow) and haemorrhage, dilated central vein and degenerated hepatocytes (arrow head) showing perinuclear vacuolization. I/R + AGE group of rats (D) show moderate sinusoidal dilatation (arrow) and haemorrhage in localized areas, with the usual appearance of central vein and hepatocytes in most areas. H&E staining, scale bar: 10 μm.

biomolecules found in tissues, including nucleic acids, membrane lipids, enzymes and receptors. Membrane-associated polyunsaturated fatty acids are readily attached by •OH in a process that leads to peroxidation of lipids, which can disrupt membrane fluidity and cell compartmentation, resulting in cell lysis. Thus, oxygen radical-initiated lipid peroxidation may contribute to the impaired cellular function and necrosis associated with reperfusion of ischaemic tissues (Reiter et al 2000). In this study, I/R caused significant increases in the hepatic malondialdehyde levels, end products of lipid peroxidation. This observation is in agreement with previous studies, wherein elevated levels of lipid peroxidation products were increased from 40 to 80% above basal values (Cuzzocrea et al 2000). AGE treatment abolished the increase in malondialdehyde, probably partly by scavenging the very reactive hydroxyl and peroxyl radicals. Horie et al (1992) suggested that aged garlic extract and diallyl polysulfides inhibit the formation of thiobarbituric acid-reactive and fluorescent substances induced by iron-ascorbic acid in isolated liver microsomal membranes, indicating the protective effect of garlic against lipid peroxidation. Alliin scavenges the •OH radical and garlic powder scavenges both •OH and 1,1-diphenyl-2-picrylhydrazyl radicals (Kourounakis & Rekka 1991). Yamasaki et al (1994) have shown that aged garlic extract protects vascular endothelial cells from H_2O_2-induced oxidative damage by inhibiting lipid peroxidation. Moreover, it has been reported that chronic garlic intake significantly decreased lipid peroxidation and increased endogenous antioxidants, such as SOD, CAT, GSH and GPxe (Banerjee et al 2002). Oxidative-stress-induced tissue damage can be prevented or ameliorated by favouring the balance towards a lower oxidative status.

Glutathione is an important constituent of intracellular protective mechanisms against noxious stimuli, including oxidative stress. On the other hand, reduced glutathione, the main component of the endogenous non-protein sulfhydryl pool, is known to be a major low-molecular-weight scavenger of free radicals in the cytoplasm (Ross 1988). Because of their exposed sulfhydryl groups, non-protein sulfhydryls bind a variety of electrophilic radicals and metabolites that may be damaging to cells (Szabo et al 1992). The results of this study further support the notion that depletion of tissue GSH, as observed in the I/R-induced hepatic injury, is one of the major factors that permit lipid peroxidation and subsequent tissue damage. Since administration of AGE prevented the hepatic GSH depletion, it appears that the protective effect of garlic extract involves the maintenance of antioxidant capacity in protecting the hepatic tissue against oxidative stress.

Observations suggest that ROM play a role in the recruitment of neutrophils into post-ischaemic tissue, but activated neutrophils are also a potential source of ROM (Zimmerman et al 1990; Granger & Korthuis 1995). Although it is not certain whether neutrophil accumulation and activation are the causes or the result of reperfusion injury, increasing evidence suggests that mesengial cells and neutrophils release chemotactic substances (e.g., interleukin-8), which further promote neutrophil migration to the tissue, activate neutrophils and increase the damage (Cuzzocrea et al 2000). Several methods have been used to define the role of neutrophils in reperfusion tissue injury. One of them is neutrophil-specific enzyme, MPO, activity. MPO, which is an essential enzyme for normal neutrophil function, is released as a response to various stimulatory substances (Kettle & Winterbourn 1997). In this study, the presence of elevated MPO activity in the liver indicates that I/R-induced injury involves the contribution of neutrophil infiltration. Grisham et al (1986) have examined the influence of ischaemia and reperfusion on neutrophil fluxes in the cat intestinal mucosa using tissue-associated myeloperoxidase activity. They observed a five-to-seven-fold increase in mucosal enzyme activity during the ischaemic period, whereas reperfusion produced an 18-fold enhancement of activity. The neutrophil accumulation initiated by reperfusion was shown to be significantly attenuated by pretreatment with either SOD, CAT, allopurinol, hydroxyl radical scavenger (dimethylthiourea) or desferoxamine (Zimmerman et al 1990). In our study, increased MPO activity due to I/R injury was effectively reversed by AGE treatment. Moreover, I/R-induced increase in fibrotic activity, as assessed by the hepatic collagen content, was reduced by AGE treatment. This finding suggests that AGE has an additional protective effect on inflammation-induced production and deposition of extracellular matrix components that result in hepatic fibrosis.

In conclusion, in view of previous observations and our present data, aqueous garlic extract, with its potent free radical scavenging and antioxidant properties, seems to be a highly promising agent in protecting hepatic tissue against oxidative damage and in preventing hepatic dysfunction due to ischaemia–reperfusion. However, these effects may be species dependent and further studies are required to extend these results to man.

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