JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 638–643

Received October 23, 2009
Accepted March 4, 2010
DOI 10.1211/jpp/62.05.0012
ISSN 0022-3573

Research Paper

Hepatoprotective and antioxidant effects of gallic acid in paracetamol-induced liver damage in mice

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Abstract

Objectives The aim of this research paper was to investigate the hepatoprotective and antioxidant effects of gallic acid in paracetamol-induced liver damage in mice.

Methods In the present study, the hepatoprotective and antioxidant effects of gallic acid were evaluated against paracetamol-induced hepatotoxicity in mice and compared with the silymarin, a standard hepatoprotective drug. The mice received a single dose of paracetamol (900 mg/kg body weight i.p.). Gallic acid (100 mg/kg body weight i.p.) and silymarin (25 mg/kg body weight i.p.) were administered 30 min after the injection of paracetamol. After 4 h, liver marker enzymes (aspartate transaminase, alanine transaminase and alkaline phosphatase) and inflammatory mediator tumour necrosis factor-alpha (TNF-α) were estimated in serum, while the lipid peroxidation and antioxidant status (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase and glutathione) were determined in liver homogenate of the control and experimental mice.

Key findings Increased activities of liver marker enzymes and elevated TNF-α and lipid peroxidation levels were observed in mice exposed to paracetamol ($P < 0.05$), whereas the antioxidant status was found to be depleted ($P < 0.05$) when compared with the control group. However gallic acid treatment (100 mg/kg body weight i.p.) significantly reverses ($P < 0.05$) the above changes by its antioxidant action compared to the control group as observed in the paracetamol-challenged mice.

Conclusions The results clearly demonstrate that gallic acid possesses promising hepatoprotective effects.

Keywords antioxidant; gallic acid; lipid peroxidation; paracetamol; tumour necrosis factor-alpha

Introduction

Paracetamol (acetaminonphen) is a common antipyretic agent, which is safe in therapeutic doses but with toxic doses can produce hepatic necrosis in man, rats and mice.[1] With therapeutic doses, paracetamol is predominantly eliminated in humans by conjugation (sulphation and glucuronidation). A small amount is partially metabolised by the cytochrome P450 system, which leads to the formation of *N*-acetyl-*p*-benzoquinoneimine (NAPQI). NAPQI is then reduced by glutathione and excreted as the cysteine or mercaptopurine acid conjugate. However, an overdose of paracetamol causes a depletion of the cellular glutathione level in the liver because NAPQI reacts rapidly with glutathione, which leads to oxidative stress and hepatic cell damage.[2] Intentional or unintentional overdose of paracetamol has become the most frequent cause for acute liver failure in the USA, UK, and northern Europe.[3] Protection against paracetamol-induced toxicity has been used as a test for potential hepatoprotective activity in several investigations.[4,5] The efficacy of any hepatoprotective drug to manage paracetamol-induced hepatotoxicity is acting as an inhibitor of cytochrome P-450 (CYP)2E1, which inhibits NAPQI formation, restoring the normal physiology of liver. Some hepatoprotective therapies like *N*-acetylcysteine (the most commonly used antidote for paracetamol overdose) have also been reported to protect the liver against paracetamol-induced injury without the inhibition of CYP2E1.[5]

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Gallic acid (3,4,5-trihydroxybenzoic acid) and its alkyl esters are a group of plant phenols present in berberry, pomegranates and gall nuts.[6] Gallic acid is used extensively as an antioxidant in fats and oils to prevent rancidity and spoilage.[7] It is found in a significant quantity in green tea leaves (4.5 g/kg weight)[8] and is used in cosmetics and in food additives in shortening, baked goods, candy and chewing gum.[9] Gallic acid, a strong natural antioxidant, has been reported to exhibit a free radical scavenging action.[10,11] Gallic acid and its derivatives promote an antiproliferative effect on cancer cells,[12] squalene epoxidase inhibition[13] and a hepatoprotective effect against CCl_4-induced toxicity.[14] However, scientific literature data supporting the use of gallic acid in paracetamol-induced hepatotoxicity is not available, therefore the present study aimed to evaluate the potential of gallic acid against paracetamol-induced hepatotoxicity in mice.

Materials and Methods

Animals

Male crossbreed Swiss albino mice weighing about 20–25 g were purchased from the Karigiri Hospital and Research Centre, Vellore, India. The animals were housed in large spacious cages. They were acclimatised for a week in a light- and temperature-controlled room with a 12 h dark–light cycle and fed with commercial pelleted feed acquired from Hindustan Lever Ltd (Mumbai, India) and water *ad libitum*. The animals used in this study were treated and well cared for in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Culture, Government of India, Chennai, India. The experimental protocol was also approved by our departmental ethics committee.

Drugs and chemicals

The commercially available gallic acid (yellowish-white crystalline powder, >98% purity by HPLC) was obtained from the Natural Remedies Pvt. Ltd (Bangalore, India). Silymarin, a standard hepatoprotective drug, and paracetamol were obtained from the Micro Labs Ltd (Goa, India). All other reagents and chemicals used were of analytical grade.

Experimental protocol

In this experiment, mice were randomly allocated into five groups, each consisting of six animals. All animals were made to fast 24 h before the experiment. Group I (control), received saline. Group II (paracetamol) was treated with a single dose of paracetamol (900 mg/kg body weight i.p.).[15] Paracetamol was first dissolved in water at 70°C, and then cooled to 37°C before administration. Group III, (gallic acid + paracetamol) was given gallic acid (100 mg/ kg body weight i.p. dissolved in saline) 30 min after the single injection of paracetamol. Group IV (silymarin + paracetamol) was given silymarin (25 mg/kg body weight i.p. dissolved in saline) 30 min after the single injection of paracetamol. Group V (gallic acid alone) received gallic acid (100 mg/kg body weight i.p. dissolved in saline). The dose selection for paracetamol, silymarin and gallic acid was based on our preliminary and previous experiments.[14–17] The mice were decapitated 4 h after paracetamol injection, the trunk blood was collected, and the serum was separated and stored at −70ºC. Tissue samples from the liver were obtained for biochemical analysis.

Biochemical parameters

The activities of alanine transmaniase (AST), aspartate transaminase (ALT) and alkaline phosphatases (ALP) in serum were estimated by using commercial kits (Span Diagnostics, India).

In the hepatic tissue samples, lipid peroxidation was determined by the procedure of Ohkawa *et al.*[18] Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of oxidative stress. Superoxide dismutase was assayed according to the method of Marklund and Marklund.[19] The unit of enzyme activity was defined as the amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation. Catalase was assayed by the method of Sinha.[20] In this method, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced was measured colorimetrically at 610 nm. Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al.*,[21] based on the reaction between glutathione remaining after the action of GPx and 5,5′-dithiobis-(2-nitrobenzoic acid) to form a complex that absorbs maximally at 412 nm. Glutathione reductase, which utilises NADPH to convert oxidised glutathione to the reduced form, was assayed by the method of Bellomo *et al.*,[22] glutathione-*S*-transferase and total reduced glutathione (GSH) were determined by the method of Habig *et al.*[23] and Moron *et al.*,[24] respectively. The protein content was determined by the method of Lowry *et al.*[25] using bovine serum albumin as a standard. Tumour necrosis factor-alpha (TNF-α) level in serum of control and experimental mice was determined by enzyme-linked immunosorbent assay (ELISA, Cayman Chemicals, USA), according to the manufacturer's instructions.

Histopathological studies

Immediately after sacrifice, a portion of the liver was fixed in 10% formalin, then washed, dehydrated in descending grades of isopropanol and finally rinsed with xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 μm thickness, stained with haematoxylin and eosin and observed microscopically for histopathological changes.

Statistical analysis

Results were expressed as mean ± SD and statistical analysis was performed using ANOVA, to determine the significant differences between the groups, followed by Student Newman–Keul's test. $P < 0.05$ implied significance.

Results

The activities of ALT (92%), AST (106%) and ALP (72.5%) in serum were significantly increased ($P < 0.05$) in paracetamol-treated mice when compared to the control group (Table 1). However, the levels of the above enzymes were significantly altered on treatment with gallic acid.

Table 1 Effect of gallic acid on liver marker enzymes in serum of paracetamol-intoxicated mice

Parameter	Group I (control)	Group II (paracetamol)	Group III (paracetamol + gallic acid)	Group IV (paracetamol + silymarin)	Group V (gallic acid)
Alanine transaminase (U/dl)	80.75 ± 5.76	166.50 ± 13.87[a,*]	94.50 ± 7.26[a,*,b,*]	91.1 ± 5.69[a,*,c,*]	85 ± 6.53
Aspartate transaminase (U/dl)	44.42 ± 3.41	85.27 ± 6.56[a,*]	51.67 ± 3.97[a,*,b,*]	60.67 ± 4.33[a,*,c,*]	50.67 ± 3.75
Alkaline phosphatase (K.A.units/I)	110.53 ± 7.36	190.75 ± 14.4[a,*]	131.42 ± 9.38[a,*,b,*]	129.92 ± 8.12[a,*,c,*]	114.75 ± 9.56

Each value represents the mean ± SD of six mice. Comparisons were made as follows: [a]group I vs groups II, III, IV; [b]group II vs group III; [c]group II vs group IV. The symbols represent statistical significance at $^{*}P < 0.05$. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test.

Table 2 Effect of gallic acid on liver lipid peroxidation levels and antioxidant enzyme activities in paracetamol-intoxicated mice

Parameter	Group I (control)	Group II (paracetamol)	Group III (paracetamol + gallic acid)	Group IV (paracetamol + silymarin)	Group V (gallic acid)
Lipid peroxidation	1.38 ± 0.12	3.22 ± 0.23[a,*]	1.72 ± 0.15[a,*,b,*]	1.80 ± 0.09[a,*,c,*]	1.55 ± 0.11
Superoxide dismutase	3.22 ± 0.24	1.35 ± 0.19[a,*]	2.58 ± 0.14[a,*,b,*]	2.75 ± 0.18[a,*,c,*]	2.95 ± 0.15
Catalase	52.09 ± 3.11	42.91 ± 2.86[a,*]	48.50 ± 3.03[a,*,b,*]	45.60 ± 3.80[a,*,c,*]	49.60 ± 2.61
Glutathione peroxidase	26.08 ± 1.97	18.52 ± 1.37[a,*]	23.5 ± 1.67[a,*,b,*]	25.83 ± 1.61[c,*]	24.20 ± 1.23
Glutathione reductase	20.65 ± 1.54	14.53 ± 0.80[a,*]	18.41 ± 1.08[a,*,b,*]	17.55 ± 1.17[a,*,c,*]	19.80 ± 1.65
Glutathione-*S*-transferase	103.25 ± 6.45	86.10 ± 6.15[a,*]	97.50 ± 7.5[a,*,b,*]	99.53 ± 7.11[a,*,c,*]	98.50 ± 6.32
Total reduced glutathione	31.58 ± 2.92	15.85 ± 1.21[a,*]	27.25 ± 1.52[a,*,b,*]	28.8 ± 2.05[a,*,c,*]	29.21 ± 1.71

Units: lipid peroxidation, nmol of MDA formed/mg protein; catalase, μmol of H_2O_2 consumed/min per mg protein; superoxide dismutase, units/mg protein (1 U = amount of enzyme that inhibits the auto-oxidation of pyrogallol by 50%); glutathione peroxidase, μg of GSH utilised/min per mg protein; glutathione reductase, nmol of NADPH oxidised/min per mg protein; glutathione-*S*-transferase, nmol of 1-chloro-2,4-dinitrobenzene–GSH conjugate formed/min per mg protein; total reduced glutathione, nmol/mg protein. Each value represents the mean ± SD of six mice. Comparisons were made as follows: [a]group I vs groups II, III, IV; [b]group II vs group III; [c]group II vs group IV. The symbols represent statistical significance at $^{*}P < 0.05$. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test.

In paracetamol-treated mice, MDA level was increased significantly ($P < 0.05$), whereas superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase and glutathione were found to be decreased when compared to the control group (Table 2). However, gallic acid treatment altered the above changes by regulating the MDA and antioxidant enzymes to levels similar to those in paracetamol-induced mice.

Figure 1 shows the levels of pro-inflammatory cytokine TNF-α in the serum of the control and experimental animals. A significant elevation in the level of TNF-α was noticed in the serum of paracetamol-treated mice. However, the elevated level of TNF-α was reduced in the gallic acid administered mice treated with paracetamol.

Figure 2 shows the results of liver histological observation of control and experimental mice. Under the photomicroscope, normal liver parenchyma and hepatocytes arranged around the central vein were observed in the group I (control) mice (Figure 2a). Group II (paracetamol) mice exhibited extensive microvesicular degenerative change and necrosis in some hepatocytes (Figure 2b). However, the findings were significantly decreased in group III (paracetamol + gallic acid) and group IV (paracetamol + silymarin) mice when compared to the group treated with paracetamol alone. On the other hand, group V (gallic acid alone) animals did not show any significant change in their liver histology (Figure 2e).

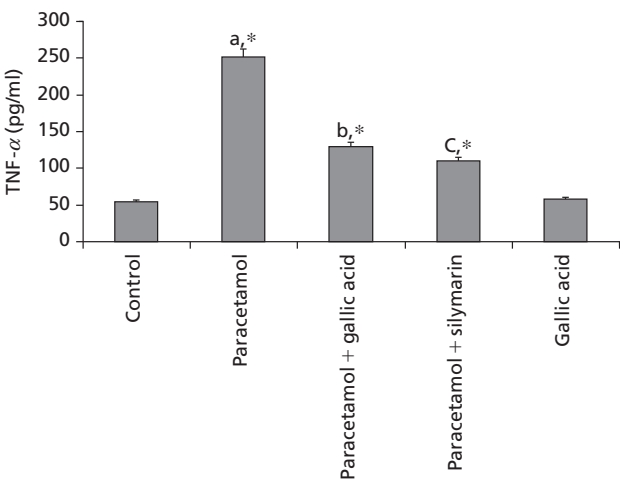

Figure 1 Hepatoprotective effect of gallic acid on serum tumour necrosis factor-alpha (TNF-α) levels in paracetamol-intoxicated mice. Figure 1 presents the TNF-α level in serum samples of group I (control), group II (paracetamol), group III (paracetamol + gallic acid), group IV (silymarin + gallic acid) and group V (gallic acid). Each value is expressed as mean±SD of six animals. Comparisons were made as follows: [a]group I vs groups II, III, IV; [b]group II vs group III; [c]group II vs group IV. The symbols represent statistical significance at $^{*}P<0.05$. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test

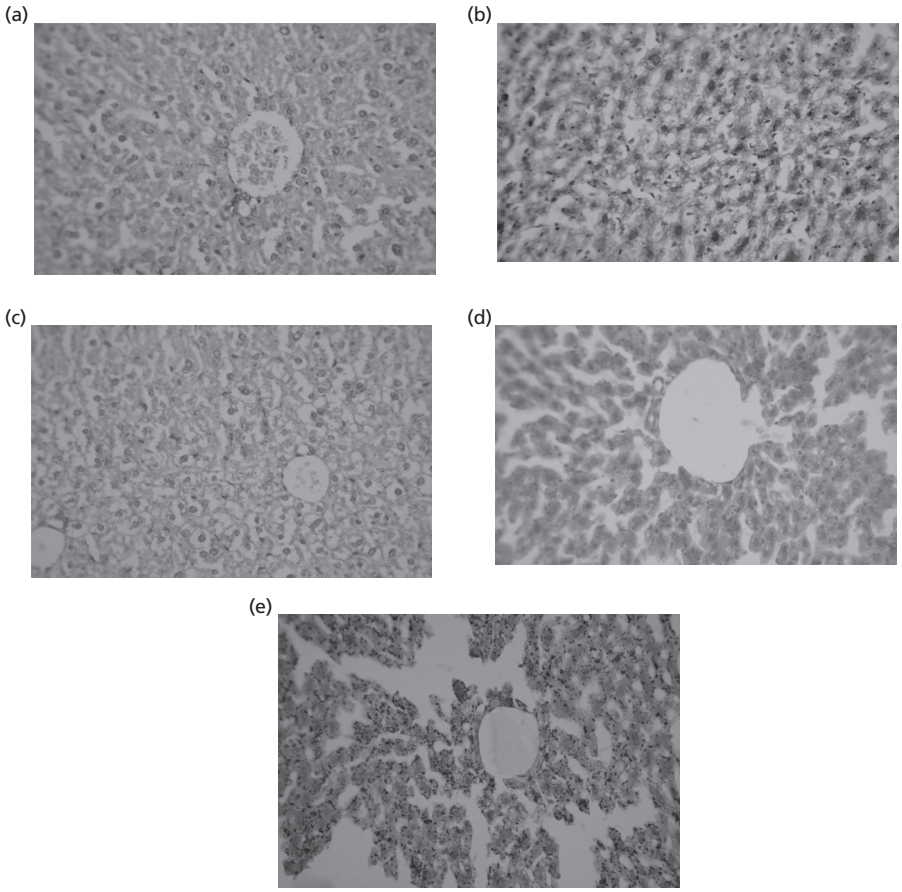

Figure 2 Hepatoprotective effect of gallic acid in paracetamol-intoxicated mice. (a) Group I (control) shows normal morphology and hepatocytes arranged around the central vein. (b) Group II (paracetamol) shows extensive microvesicular degenerative change and necrosis in some hepatocytes. (c) Group III (paracetamol + gallic acid)) shows marked improvement with no necrosis. (d) Group IV (paracetamol + silymarin) shows almost normal architecture of liver. (e) Group V (gallic acid) shows normal morphology and hepatocytes arranged around the central vein. Haematoxylin and eosin staining, original magnification 400×

Discussion

Paracetamol-induced hepatic failure is the second leading cause of liver transplantation and accounts for considerable levels of morbidity and mortality.[3] The estimation of enzymes in the serum is a useful quantitative marker of the extent and type of hepatocellular damage. The rise in serum AST and ALT levels has been attributed to the damaged structural integrity of the liver because these are cytoplasmic in location and are released into circulation after cellular damage.[26] In this study, a single dose of paracetamol (900 mg/kg body weight) treatment caused a significant elevation of enzyme levels such as AST, ALT and ALP when compared to the control group. Our result is in agreement with a previous report.[2] The reversal of increased serum enzymes in paracetamol-induced liver damage by gallic acid may be due to the prevention of the leakage of intracellular enzymes by its membrane-stabilising and antioxidant activity, which was supported by the limited extent of histological changes. The results are in agreement with previous reports that gallic acid decreased plasma AST and ALT activities that had been elevated by acute hepatic damage.[14] This is in accordance with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes.[27]

Free radicals play an important role in the physiopathological situations involving lipid peroxidation reactions. It is well established that at normal doses paracetamol is converted via the cytochrome P-450 pathway to a highly toxic metabolite, NAPQI, which is normally conjugated with glutathione and excreted in the urine as conjugates. Overdoses of paracetamol deplete the glutathione stores, leading to the accumulation of NAPQI, mitochondrial dysfunction and the development of acute hepatic necrosis.[28] Wendel and his co-workers reported that the paracetamol metabolism triggers lipid peroxidation, which is responsible for liver injury.[29]

The peroxidases are enzymic antioxidants widely distributed in all animal tissues, which decompose hydrogen peroxide and protect the tissue from highly reactive hydroxyl radicals. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide.[30] GSH plays an essential role in the detoxification of paracetamol and protects hepatocytes by uniting with reactive metabolites of paracetamol. Thus, it prevents them from binding covalently with liver proteins. Intracellular

decrease of the reduced GSH exposes the cell to the destructive effects of oxidative stress.[31]

Similar to our previous report,[16] paracetamol induction (900 mg/kg body weight) in mice significantly increases the levels of lipid peroxidation and causes the depletion of antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase and glutathione). However, gallic acid treatment significantly reversed these changes in mice. The inhibitory activity of gallic acid on free radical production could be due to its action on cytochrome P450-dependent mono-oxygenase activities and the enhancement of epoxide hydrolase activity.[32,33] Moreover, the free radical scavenging action of gallic acid is already well established from earlier investigations.[10,11]

TNF-α is a major endogenous inflammatory mediator of hepatotoxicity in several experimental liver injuries through its direct cytotoxicity, nitric oxide production and the triggering of an inflammatory cascade.[34] The production of TNF-α is known to be one of the earliest events in the hepatic inflammatory response, which induces cytotoxicity, hepatocyte apoptosis and necrosis;[35] therefore, TNF-α is considered to be an important target in research to discover hepatoprotective agents. In accordance with this report, our results demonstrate that paracetamol increases serum TNF-α, indicating the role of this cytokine in paracetamol-induced hepatotoxicity. However, our findings reveal that gallic acid treatment alleviates the progression of the inflammatory mediator TNF-α in paracetamol-exposed mice.

Conclusions

According to the results of the present study, gallic acid possesses a potent hepatoprotective effect in paracetamol-intoxicated mice due to its antioxidant activity. However, further pharmacological evidence at the molecular level is required to establish the actual mechanism of the action of the drug. Research into this area is underway.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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