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ORGAN TOXICITY AND MECHANISMS

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Inhibitory effect of berberine on *tert*-butyl hydroperoxide-induced oxidative damage in rat liver

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Abstract Berberine, a main protoberberine component of *Coptidis Rhizoma*, was studied for the mechanism of its inhibitory effects on the *tert*-butyl hydroperoxide (t-BHP)-induced cytotoxicity and lipid peroxidation in rat liver. In the preliminary study, berberine expressed an antioxidant property by its capacity for quenching the free radicals of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Further investigations were conducted using t-BHP-induced cytotoxicity in rat primary hepatocytes and hepatotoxicity in rats to evaluate the antioxidative bioactivity of berberine. The results in rat primary hepatocytes demonstrated that berberine, at the concentrations of 0.01–1.0 mM, significantly decreased the leakage of lactate dehydrogenase (LDH) and alanine aminotransferase (ALT), and the formation of malondialdehyde (MDA) induced by 30 min treatment of t-BHP (1.5 mM). Berberine also attenuated the t-BHP-induced depletion of glutathione (GSH) and induced a high level of DNA repair synthesis. The in vivo study showed that the intraperitoneal pretreatment with berberine (0.5 and 5 mg/kg) for 5 days before a single dose of t-BHP (0.1 mmol/kg) significantly lowered the serum levels of hepatic enzyme markers (ALT and aspartate aminotransferase) and reduced oxidative stress in the liver. The histopathological evaluation of the livers revealed that berberine reduced the incidence of liver lesions, including hepatocyte swelling, leukocyte infiltrations, and necrosis induced by t-BHP. These results lead us to speculate that berberine may play a chemopreventive role via reducing oxidative stress in living systems.

Keywords Berberine · *tert*-Butyl hydroperoxide · Cytotoxicity · Genotoxicity · Hepatocyte

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Introduction

Berberine (Fig. 1), an alkaloid purified from *Berberis* species, has been extensively studied and is known to exhibit multiple pharmacological activities such as antiprotozoal, antihypertensive (Bova et al. 1992), antibacterial (Amin et al. 1969), anti-inflammatory (Akhter et al. 1977), anticholinergic (Tsai and Ochillo 1991) and antiarrhythmic activity(Wang and Zheng 1997). Moreover, an anti-HIV activity has recently been reported (Vlietinck et al. 1998).

Berberine has been used in different parts of the world for a long time. Even in modern therapeutics it is used for the treatment of diarrhea (Mekawi et al. 1966; Bhide et al. 1969). This anti-diarrhea effect of berberine may be worked through its anticholinergic (Tsai and Ochillo 1991), α_2 adrenoceptor agonist (Hui et al. 1991), antisecretory (Zhu and Ahrens 1983) and antimicrobial (Amin et al. 1969; Akhter et al. 1979) activities. In the earlier studies using the crude extract of *Berberis aristata* leaves and fruits, this folk medicine was scientifically examined and shown to be hepato-protective, possibly because of its inhibitory action on hepatic drug metabolizing enzymes (Gilani and Janbaz 1992). Recently, it was demonstrated that berberine is the main active component of the crude extract of *Berberis aristata* (Janbaz and Gilani 2000).

t-Butyl hydroperoxide (t-BHP) can be metabolized to free radical intermediates by cytochrome P450 (hepatocytes) or hemoglobin (erythrocytes), which can subsequently initiate lipid peroxidation (Hogberg et al. 1975), affect cell integrity and form covalent bonds with cellular molecules resulting in cell injury (Rush et al. 1985). Alternatively, t-BHP can be rapidly converted by glutathione disulphide (GSSG); GSSG is reduced to

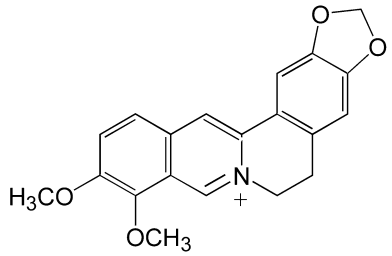


Fig. 1. Structure of berberine

glutathione (GSH) by GSH reductase resulting in pyridine nucleotide (NADPH) oxidation. Loss of GSH and oxidation of pyridine nucleotide are associated with altered Ca^{2+} homeostasis, which is considered to be a critical event in bleb formation on plasma membranes, an early sign of t-BHP-induced toxicity (Thornalley et al. 1983; Davies 1989).

t-BHP is known to cause lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) leakage, malondialdehyde (MDA) formation and GSH depletion in hepatocyte cells (Joyeux et al. 1990; Tseng et al. 1996). It also mediates DNA-base damage in mammalian cells (Altman et al. 1994). These phenomena are similar to the oxidative stress occurring in the cell and/or tissue. Oxidative stress is considered to play a prominent role in the cause of many diseases, e.g. inflammation, aging and cancer (Cerutti 1985; Breimer 1990). Therefore, this study used t-BHP to induce oxidative damage in primary culture of hepatocytes in order to investigate the protective potential of berberine. Results in rat primary hepatocytes showed that the lethal injury induced by t-BHP was effectively inhibited by the pretreatment of berberine via the actions of inhibiting GSH loss and quenching free radicals.

Material and methods

The experiments performed complied with the current laws of Taiwan.

Chemicals

t-BHP (*tert*-butyl hydroperoxide), berberine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), collagenase, sodium dodecyl sulfate, proteinase K, thiobarbituric acid, [methyl-^{3}H]-thymidine, ethylenediaminetetraacetic acid, GSH, *o*-phthalaldehyde, and kits for LDH and ALT measurements were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Solvents were obtained from E. Merck (Darmstadt, Germany); aquasol-2 (New England Nuclear, Boston, Mass., USA) and protein assay kits were from Bio-Rad Laboratories (Watford, UK), the medium for cell culture was from Gibco (Grand Island, N.Y., USA), and other reagents or dishes (Nunc, Roskilde, Denmark) were obtained from the indicated suppliers.

Determination of free radical quenching capacity

The free radical quenching capacity of berberine was determined by a method involving the bleaching of stable DPPH (Ursini et al. 1994). A reaction mixture containing methanol, DPPH (10 mM, 30 µl) and 100 µl of various concentrations of berberine (0.01, 0.1, or 1.0 mM in dimethyl sulfoxide, DMSO) was allowed to stand at room temperature for 30 min before mixing with redistilled water (1 ml) and toluene (3 ml). The solution was then centrifuged, and the absorbance of the upper phase was read at 517 nm against a blank without berberine, prepared and processed as described above.

Preparation of hepatocyte cultures

Male Sprague-Dawley rats (250–300 g, obtained from Taichung Veterans General Hospital Animal Center, ROC) were used in the experiments. Hepatocytes were prepared by the method of two-stage collagenase perfusion (Bonney et al. 1974), and cultured in Williams E medium supplemented with (1%) PSN antibiotic mixture (penicillin, streptomycin, neomycin), and fetal calf serum (10%), and gassed with O_2/CO_2 (95%/5%). Cells were plated out at a density of 1×10^6 per 60-mm dish, and treated with chemicals, as indicated in the following tests, 3 h after attachment. Test agents were dissolved in redistilled water with the exception of berberine, which was dissolved in DMSO; the final concentration of DMSO was no more than 0.2%.

Microculture tetrazolium (MTT) assay

The range of nontoxic dosage levels of berberine was established using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay with some modification (Alley et al. 1988). Briefly, to the primary cultured rat hepatocytes at a density of 5×10^4 cells/dish, various doses of t-BHP (0.5–1.5 mM) or berberine (0.01, 0.1 and 1.0 mM) were added. After 4 h incubation, the medium was replaced by one containing 20 ml MTT (5 mg/ml) after washing in phosphate-buffered saline. The cells were incubated for another 4 h, and then the blue crystals that are the metabolized product of MTT were extracted by isopropanol. Absorbance at 563 nm was measured and used for estimating the proportion of surviving cells.

Hepatotoxicity assay

After pretreatment with berberine for 1 h at the final concentration of 0.01, 0.1 and 1.0 mM, hepatocytes were incubated with t-BHP (1.5 mM) for 30 min (Tseng et al. 1996). One milliliter of the culture medium was removed, and the activities of LDH and ALT were analyzed by a colorimetric method (340 nm) following procedures supplied by the manufacturer. Cytotoxicity of hepatocytes was expressed in terms of the activity of LDH and ALT released from the treated cells.

Lipid peroxidation assay

Hepatocytes were pretreated with berberine, and then with t-BHP as described above. The lipid peroxidation product was assayed according to an improved thiobarbituric acid (TBA) fluorometric method at 553 nm with excitation at 515 nm, using 1,1,3,3-tetramethoxypropane as a standard (Yagi 1987). The results were expressed as MDA formation per 10^6 cells.

Glutathione assay

GSH assay of the hepatocyte samples, prepared as mentioned before, was performed using a modified version of the method of Hissin and Hilf (1976). In brief, the cells were homogenized, and the proteins were precipitated with 25% *m*-phosphoric acid. After centrifugation (10,000 *g*, 30 min), *o*-phthalaldehyde (1/20 of sample volume, 0.1% in methanol) was added to the supernatant for 15 min at room temperature. Fluorescence at 420 nm was determined with activation at 350 nm. Protein concentration was

determined using a commercial kit (Bio-Rad) with bovine serum albumin as the standard.

Measurement of DNA repair synthesis

The extent of unscheduled DNA repair synthesis induced by t-BHP was used as an indicator of DNA damage (Hsia et al. 1983). Hepatocytes at a density of 1×10^6 cells per 60-mm dish were pretreated with hydroxyurea (1.5 mM) and berberine (0.01, 0.1, 1.0 mM) for 1 h. Cells were then exposed to t-BHP (1.5 mM) for 30 min, washed with phosphate-buffered saline, and incubated in fresh medium containing [methyl-^{3}H]-thymidine (1 μCi/ml) for another 18 h. The cells were subsequently harvested, lysed, and subjected to the measurement of radioactivity with a liquid scintillation counter. DNA content in the lysate was quantitated according to the 3,5-diaminobenzoic acid (DABA) method (Vytasek 1982).

Animal treatment

Male Sprague-Dawley rats (220 ± 10 g) were provided with food and water ad libitum and assigned to five groups (six rats per group). To study the protective effect against t-BHP-induced hepatotoxicity, berberine (0.5 and 5 mg/kg) was given daily by intraperitoneal (i.p.) injection to the animals for 5 consecutive days. On the 5th day, t-BHP (0.1 mmol/kg) was injected (i.p.) into each animal, and 18 h later the rats were killed by decapitation and blood samples were collected for assays of aspartate aminotransferase (AST) and ALT. The livers were excised from the animals and assayed for MDA formation (as described above) and GSH levels, and examined for pathological histology.

Assessment of hepatotoxicity in rats

The hepatic enzymes AST and ALT were used as biochemical markers for early acute hepatic damage. The serum activities of AST and ALT were determined by the colorimetric method of Reitman and Frankel (1957).

Result

Cytotoxicity of berberine

The MTT assay showed that t-BHP expressed little toxicity to the primary culture of hepatocytes up to a concentration of 1.5 mM (Table 1), and the three tested concentrations of berberine exhibited no effect on the cells (Table 1). Therefore, 1.5 mM t-BHP and 0.01, 0.1 and 1.0 mM berberine were used for the subsequent experiments.

Table 1. Cytotoxicity of *tert*-butyl hydroperoxide (t-BHP) and berberine assessed by the microculture tetrazolium (MTT) assay in primary cultured rat hepatocytes. Hepatocyte cultures were treated with various doses of t-BHP or berberine for 30 min. Data represent means ± SD ($n=3$) for absorbance at 563 nm ($OD_{563\ nm}$)

Treatment	$OD_{563\ nm}$	Absorbance (% of control)
Control	0.83 ± 0.04	100
t-BHP (0.5 mM)	0.81 ± 0.02	98
t-BHP (1.0 mM)	0.77 ± 0.01	93
t-BHP (1.5 mM)	0.75 ± 0.02	90
Berberine (0.01 mM)	0.82 ± 0.05	99
Berberine (0.1 mM)	0.81 ± 0.03	98
Berberine (1.0 mM)	0.80 ± 0.02	96

Table 2. Free radical-quenching ability of berberine determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) test. Various concentrations of berberine were mixed with DPPH (10 mM, 30 μl) in methanol. The reaction mixtures were then colored by the addition of toluene, and absorbance read at 517 nm ($OD_{517\ nm}$) against a blank without berberine. The fraction of DPPH bleaching was calculated by comparison with the absorbance of the DPPH solution containing only the vehicle dimethyl sufoxide (100 μl) as 0%. Data represent means ± SD, $n=3$

Berberine concentration	$OD_{517\ nm}$	Absorbance (% of control)
0 (Control)	1.77 ± 0.09	100
0.01 mM	1.24 ± 0.13**	70
0.1 mM	1.05 ± 0.12**	59
1.0 mM	0.43 ± 0.05***	24

**$P<0.01$, ***$P<0.001$, compared with the control

Free radical-quenching capacity of berberine

The results of the DPPH test (Table 2) showed that berberine had a dose-dependent ability to quench free radicals. At the tested concentrations of 0.01, 0.10 and 1.0 mM, berberine scavenged about 30% ($P<0.01$), 41% ($P<0.01$) and 76% ($P<0.001$) of DPPH radicals in the solution, respectively.

Effects of berberine on t-BHP-induced hepatotoxicity

The effects of berberine on the cytotoxicity induced by t-BHP in the primary cultured hepatocytes were expressed by the leakage of LDH and ALT. As shown in Fig. 2, the cellular leakage of LDH and ALT caused by t-BHP (1.5 mM, for 30 min) was significantly suppressed by berberine pretreatment; the maximal inhibition (51% for LDH, $P<0.001$, and 46% for ALT, $P<0.01$) was observed at a concentration of 1.0 mM berberine.

Effects of berberine on t-BHP-induced lipid peroxidation

The concentration of MDA, an index of lipid peroxidation, was increased in the hepatocytes treated for 30 min with 1.5 mM t-BHP alone. Pretreatment with berberine (0.01, 0.10 and 1.00 mM) reduced the formation of MDA to 53% ($P<0.05$), 37% ($P<0.01$) and 20% ($P<0.01$) respectively, compared with that in the absence of pretreatment (Fig. 3).

Effects of berberine on t-BHP-induced loss of GSH

GSH is widely distributed among living cells and is involved in many biological functions. GSH is known to have a protective role in t-BHP-induced toxicity (Joyeux

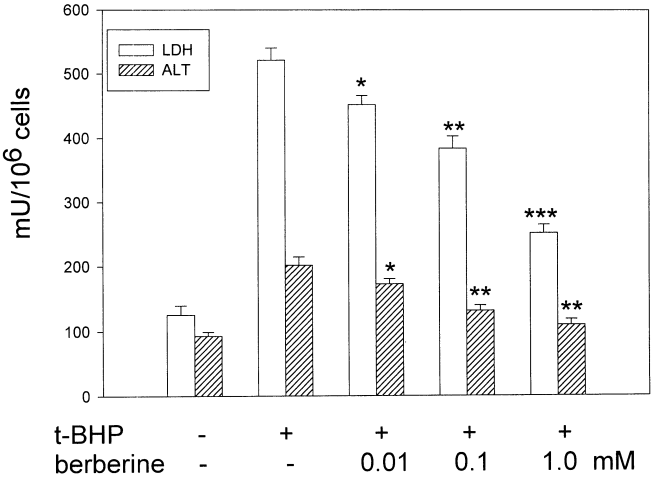


Fig. 2. Effect of berberine on the leakage of lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) induced by *tert*-butyl hydroperoxide (t-BHP; 1.5 mM for 30 min, +) in primary cultured rat hepatocytes. Each column represents the mean and the SD of three independent experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$, compared with the control treatment of t-BHP alone

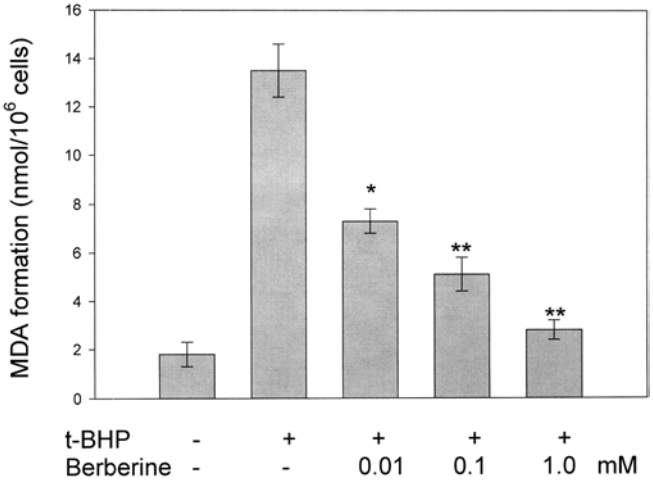


Fig. 3. Effect of berberine on the lipid peroxidation induced by *tert*-butyl hydroperoxide (t-BHP; 1.5 mM, for 30 min, +) in primary cultured rat hepatocytes. Lipid peroxidation was evaluated by malondialdehyde (MDA) formation. Each column represents the mean and the SD of three independent experiments. $*P<0.05$, $**P<0.01$, compared with the control treatment of t-BHP alone

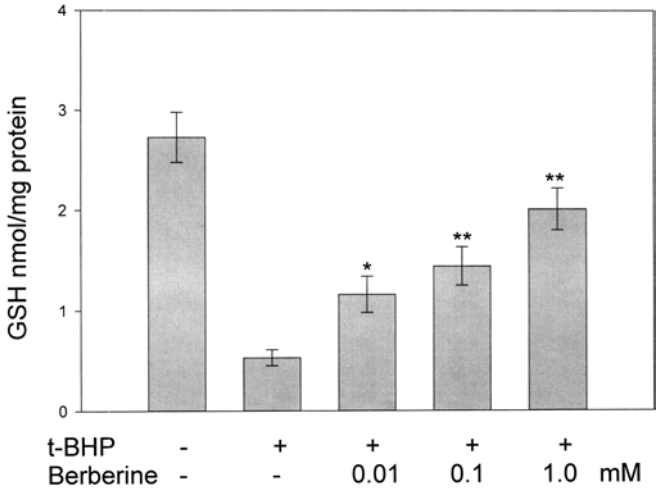


Fig. 4. Effect of berberine on the depletion of glutathione (GSH) induced by *tert*-butyl hydroperoxide (t-BHP; 1.5 mM for 30 min) in primary cultured rat hepatocytes. Each column represents the mean and the SD of three independent experiments. $*P<0.05$, $**P<0.01$, compared with the control treatment of t-BHP alone

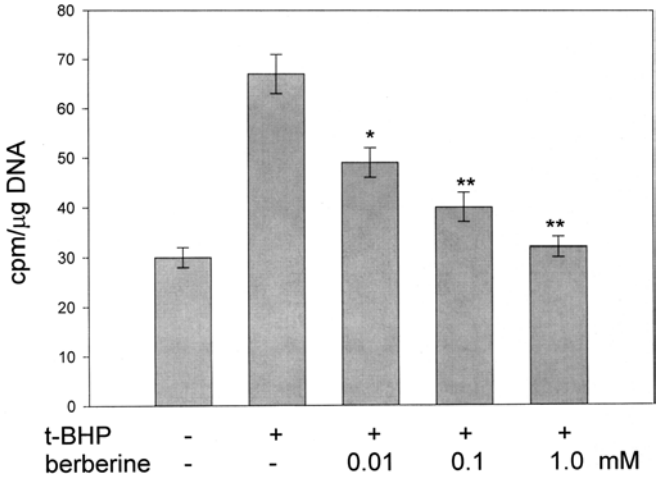


Fig. 5. Effect of berberine on the DNA damage induced by *tert*-butyl hydroperoxide (t-BHP; 1.5 mM for 30 min, +) in primary cultured rat hepatocytes. DNA damage was evaluated as unscheduled DNA repair synthesis (UDS) and expressed as incorporation of [^{3}H]-thymidine in DNA. Each column represents the mean and the SD of three independent experiments. $*P<0.05$, $**P<0.01$, compared with the control treatment of t-BHP alone

et al. 1990). Therefore, the influence of berberine on the t-BHP-induced loss of GSH in the cultured rat hepatocytes was investigated. According to the results, berberine at the tested concentrations effectively attenuated the loss of GSH that was induced by t-BHP treatment ($P<0.05$ and $P<0.01$; Fig. 4).

Effects of berberine on t-BHP-induced DNA damage

To examine the effect of berberine on the DNA repair synthesis induced by t-BHP in rat hepatocytes, unscheduled DNA repair synthesis was performed by quantitating the incorporation of [methyl-^{3}H]-thymidine into DNA. Berberine was shown to inhibit t-BHP-induced DNA repair synthesis by 52%, 40% and 29% at concentration of 1.00 mM, 0.1 mM and 0.01 mM ($P<0.01$, $P<0.01$ and $P<0.05$, respectively; Fig. 5), respectively, demonstrating an inhibitory effect of berberine on the t-BHP-induced genotoxicity in hepatocytes.

Effect of berberine on t-BHP-induced rat hepatotoxicity in vivo

The hepatic enzymes, AST and ALT, were used as biochemical markers for early acute hepatic damage.

Table 3. Effect of berberine on serum enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and hepatic malondialdehyde (MDA) and glutathione (GSH) levels in rats treated with *tert*-butyl hydroperoxide (t-BHP). Data represent means ± SD ($n = 6$)

Treatment	AST (U/l)	ALT (U/l)	MDA (nmol/g liver)	GSH (μg/g liver)
Control	124 ± 14	55 ± 10	104 ± 15	1636 ± 160
Berberine (5 mg/kg)	130 ± 18	62 ± 14	110 ± 17	1668 ± 236
t-BHP (0.1 mmol/kg)	595 ± 79	214 ± 36	201 ± 21	1219 ± 219
Berberine (0.5 mg/kg) + t-BHP	265 ± 28*	131 ± 28*	138 ± 19*	1515 ± 227*
Berberine (5 mg/kg) + t-BHP	185 ± 22**	98 ± 25**	115 ± 18**	1653 ± 245**

*$P < 0.05$, **$P < 0.01$, compared with the group treated with t-BHP alone

When a single dose of t-BHP was given to rats by i.p. injection, it caused elevations of serum AST and ALT and an increased formation of MDA in the liver (Table 3). It also led to a significant depletion of GSH in the liver as compared with the control group. Pretreatment with berberine suppressed all these acute hepatotoxicity reactions induced by t-BHP (Table 3).

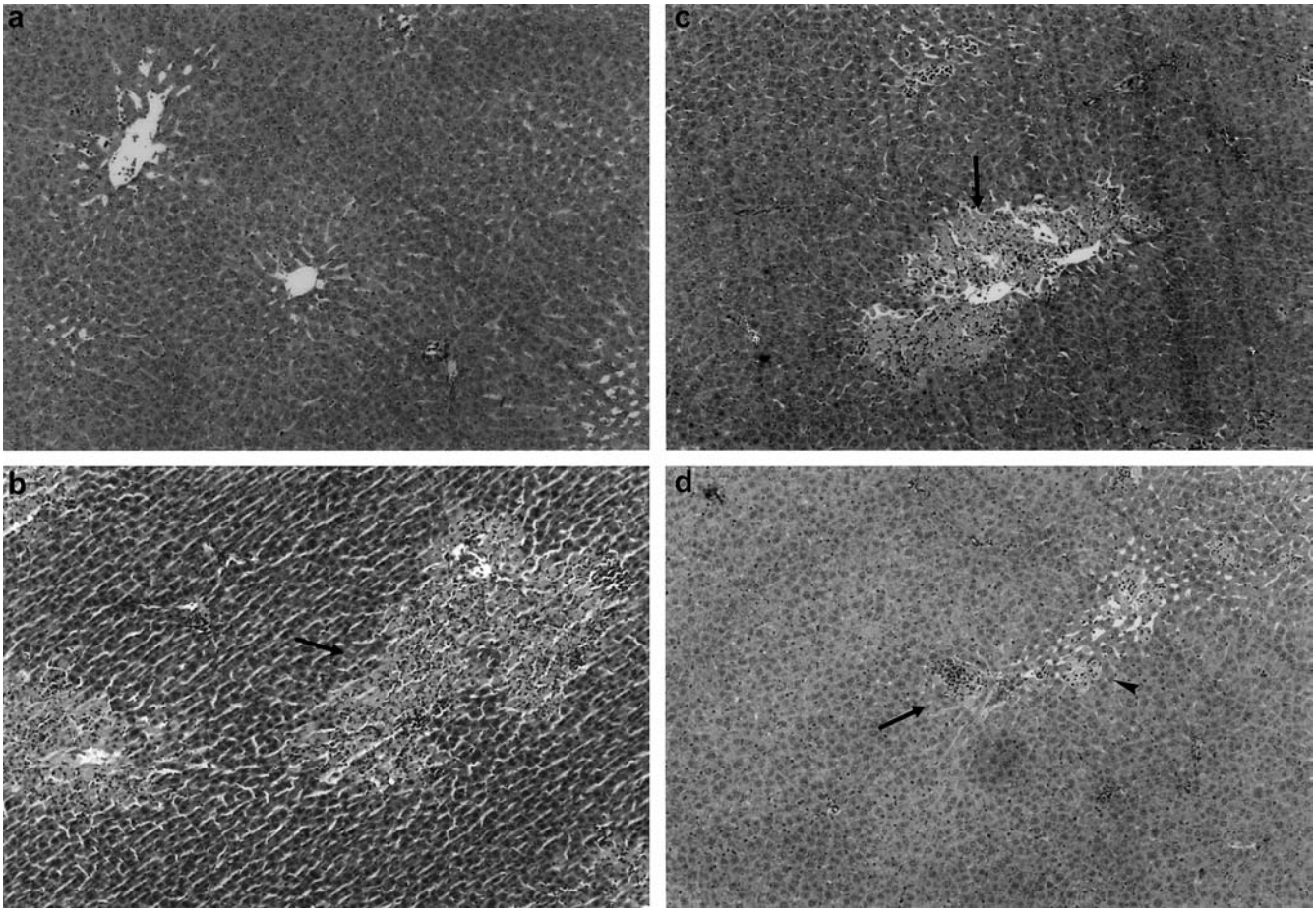
Fig. 6A–D. Effect of berberine on *tert*-butyl hydroperoxide (t-BHP) -induced liver damage in rats. **A** Representative section of liver from control animals treated with solvent. **B** Representative section of liver from animals treated with 0.1 mmol/kg t-BHP, showing severe neutrophil leukocyte infiltration (*small arrow*), degeneration and necrosis (*large arrowhead*). **C** Representative section of liver from animals pretreated with 0.5 mg/kg berberine and then with t-BHP, showing partial hepatocyte degeneration and swelling (*large arrowhead*). **D** Representative section of liver from animals pretreated with 5 mg/kg berberine and then with t-BHP, showing no pathological change (H&E staining, ×200)

Histopathology of the liver

The treatment of t-BHP led to several large areas of necrosis in rat livers with severe inflammatory cell infiltration (Fig. 6B). Histological examination showed that berberine pretreatment (0.5 mg/kg) reduced the extent of the liver lesions (Fig. 6C). Using a higher dose of berberine (5 mg/kg) as pretreatment, the extent of inflammatory cell infiltration and the area of hepatocyte necrosis were effectively reduced further (Fig. 6D).

Discussion

Reactive oxygen species (ROS; $^{\bullet}OH$, O_2^-, RO, ROO, NO) can originate from a number of internal and external sources, such as metabolic reactions, infections,



dietary intake and cigarette smoking. The body, however, possesses defense mechanisms to reduce the oxidative damage, and such mechanisms use both enzymes and antioxidant nutrients or medicine to arrest the damaging properties of excited oxygen species. Only when the normal protective mechanism breaks down, or when the effectiveness of antioxidant sources is reduced, which leads to the amount of free radicals in the body increasing beyond control, may irreversible oxidative damage occur. A great number of studies have suggested that antioxidant nutrients and/or medicines play a protective role in human health. In the present study, berberine was observed to inhibit the oxidative damage in intact cells. This provides biological evidence supporting the use of berberine for liver disorders in Chinese folk medicine.

ROS are well known to lead to lipid peroxidation and subsequent cell injury and toxicity (Hogberg et al. 1975; Rush et al. 1985). An increase in the production of ROS might be a primary factor for hepatotoxicity. t-BHP is metabolized in hepatocytes via two distinct pathways. One pathway involves cytochrome P450 (CYP), which leads to the formation of toxic peroxyl and alkoxyl radicals, and initiates lipid peroxidation. The second pathway is a detoxification reaction involving GSH peroxidase, which gives rise to *t*-butanol and oxidized GSH (Davies 1989), that in turn alters Ca^{2+} homeostasis and increases physiological formation of ROS. In the cytotoxicity assays of this study, pretreatment with berberine (0.01–1.00 mM) significantly protected the cultured rat hepatocytes against oxidative damage caused by t-BHP, as indicated by the reduced leakage of LDH and ALT, the decreased formation of MDA, and the suppression of loss of GSH (Figs. 2 and 3). LDH and ALT are known to be general indices of hepatic cytotoxicity. MDA, on the other hand, is the major oxidative degradation product of membrane unsaturated fatty acid, having toxic and genotoxic properties (Husain et al. 1987). The physiological role of GSH is as an essential intracellular reducing agent for maintenance of thiol groups on intracellular proteins and for antioxidant molecules. GSH protects cells against oxidative stress and other types of damage, which may arise from compounds of endogenous and exogenous sources.

Increasing evidence suggests that free radical damage is involved in both the initiation and promotion stages of carcinogenesis. The effect of antioxidants in decreasing oxidative damage is believed to contribute, to some extent, to lower the cancer incidence (Ho 1992; Newmark 1992). From our observations, berberine displayed moderate quenching capacity on DPPH radicals. Furthermore, berberine inhibited the genotoxicity caused by t-BHP, which mediates the formation of hydroxyl radicals in close proximity to DNA (Altman et al. 1994).

Collectively, we speculate that berberine might block the formation of hydroxyl radicals via a detoxification reaction involving a GSH system that metabolizes t-BHP to *t*-butanol. Therefore, the anti-hepatotoxicity effect of berberine against t-BHP could result from its abilities to quench free radicals and attenuate the loss of GSH. However, we cannot rule out the possibility that berberine may interact directly with t-BHP in the medium, which subsequently alleviates the cell damage caused by t-BHP. Furthermore, the study of Janbaz and Gilani (2000) pointed out that the preventive effects of berberine (oral dose of 4 mg/kg) against acetaminophen- and carbon tetrachloride-induced hepatotoxicity were executed via its inhibitory effect on microsomal drug metabolizing enzymes, CYPs. They also observed a selective curative effect of berberine against acetaminophen but not carbon tetrachloride, suggesting an antioxidant role of berberine, which was justified by reports that the acetaminophen toxicity following metabolism by CYPs is chiefly due to oxidative stress and can be effectively improved by antioxidants (Harman 1985). The present results concur with their speculation, proving berberine is an effective antioxidant.

Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on the mechanisms of their action. Many natural antioxidant products are capable of preventing or inhibiting the process of carcinogenesis (Rice-Evans 1999; Surh 1999). The results of this study demonstrated that berberine inhibits the hepatotoxicity induced by t-BHP via its antioxidant potential. We therefore suggest that berberine could function as a chemopreventive agent in living system.

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