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Original Article

Ameliorative Potential of Quercetin Against Paracetamol-induced Oxidative Stress in Mice Blood

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ABSTRACT

The aim of the present study was to evaluate the ameliorative potential of quercetin (QC) against paracetamol (PCM)-induced oxidative stress and biochemical alterations in mice blood. A total of 36 mice were randomly allocated into six groups, six mice in each. Group I served as healthy controls, while groups II and III were administered with *N*-acetylcysteine (NAC) and QC alone respectively. Group IV was administered with PCM alone. Groups V and VI were administered with PCM on day 0 followed by NAC and QC, respectively, for 6 consecutive days. On day 7th blood samples were obtained and subjected for the assays of oxidative stress and serum biochemical panels. Erythrocytic lipid peroxides contents of alone PCM-intoxicated mice were significantly higher, while reduced glutathione contents were found to be significantly lower in comparison with the healthy controls. The activities of antioxidant enzymes were also found to be singnificantly lower in these mice. Additionally, significantly increased activities of serum aspartate transaminase, alanine transaminase and alkaline phosphatase, as well as levels of bilirubin, urea and creatinine were revealed by these mice. Postadministration with QC remarkably alleviated the over production of MDA and improved GSH levels in PCM-intoxicated mice blood. In addition, antioxidant enzymes; glutathione peroxidase, glutathione-S-transferase, superoxide dismutase and catalase activities were also improved significantly in these mice. QC had also considerably ameliorated the altered biochemical parameters toward normalcy. Thus, it can be concluded that QC may constitute a remedy against PCM-induced oxidative stress and reno-hepatic injuries.

Key words: Antioxidant, lipid peroxidation, oxidative stress, paracetamol, quercetin

INTRODUCTION

In recent years there has been growing interest in understanding the role of antioxidants in management of many diseases such as cancer, arteriosclerosis, aging and many compounds, including clinically useful drug-induced toxicities. Many clinically useful drugs can cause cellular damage after metabolic activation to highly reactive compounds. One of the commonly used over-the-counter analgesics is paracetamol (PCM). PCM is a drug of para-aminophenol group, which is considered quite safe at recommended doses, and is commonly used in humans to relieve mild to moderate pain, as well as to reduce fever.[1] The main problem with this medication is misuse through intentional or unintentional ingestion of supratherapeutic dosages, which usually lead to hepatic necrosis. When administered at normal doses, PCM is primarily metabolized by conjugation with sulfate and glucuronic acid. A minor

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pathway through CYP450 has been also reported to yield a highly reactive metabolite, *N*-acetyl-p-benzoquinonimine (NAPQI). This metabolite is generally stabilized through conjugation with glutathione (GSH) and eliminated *via* the kidney. However, when overdose of PCM is administered, the production of NAPQI overleads the capacity of GSH to detoxify it. The excess NAPQI then causes liver damage associated with oxidative stress.[2] PCM overdose is also known to be associated with inflammation, marked by an increase in the inflammatory cytokines; tumor necrosis-α (TNF-α), interleukin-1α and interleukin-1β, as well as the upregulation of nitrogen oxide (NO) from serum, macrophages and hepatocytes.[3,4] Oxidative stress is reported to constitute a major mechanism in the pathogenesis of PCM-induced liver and renal damage in experimental animals.[4, 5]

On the other hand, there are numerous reports indicating that PCM-mediated oxidative stress or hepatotoxicity is attenuated by use of naturally occurring antioxidants and/or free radical scavengers such as vitamins, medicinal plants and flavonoids.[6-8] The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. The antioxidant capacity of these molecules seems to be responsible for many of their beneficial effects and confers a therapeutic potential in diseases such as cardiovascular diseases, gastric or duodenal ulcers, cancer and hepatic pathologies.[9] Quercetin (3,3′,4′,5,7-pentahydroxyflavone) (QC) is a naturally occurring polyphenolic compound, commonly found in vegetables, fruits, nuts and beverages such as coffee, tea and red wine.[10] Its average human consumption is in the range of 25–50 mg per day.[11] QC has been reported to have biological, pharmacological and medicinal activities[12] that are believed to arise from its antioxidant potentials. It prevents oxidative stress by scavenging $O_2^{-\cdot}$, ^{-}OH and singlet oxygen. It also prevents lipid peroxidation and membrane damage by inhibiting cyclooxygenase (COX) and lipooxygenase (LOX) enzymes. Measurement of lipid peroxidation in terms of malondialdehyde (MDA), reduced glutathione (GSH) level and activities of antioxidant enzymes for instance glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) in biological samples are widely used to determine the state of oxidative stress. The compromised antioxidant defense and increased peroxidation products in blood are indicative for the oxidative stress. The studies on PCM-induced oxidative stress in blood of the experimental animals and its amelioration by using QC are meager. Considering the ubiquitous prevalence of PCM toxicity, present study was undertaken with a very common dietary component, QC, to evaluate the possible ameliorative effects on PCM-induced oxidant/antioxidant imbalance and altered biochemical parameters.

MATERIALS AND METHODS

Chemicals

The tested compounds were purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used in this experiment were of analytical grade. The dose selection for each compound was based on previously published studies.

Animals and experimental design

A total of 36 male Swiss albino mice, weighing 26-28 g were obtained from the Laboratory Animal Resource Section, SRMS Institute of Medical Sciences (Bareilly). All mice were maintained under standard laboratory conditions (27 ± 1oC temperature; 12:12 h light/dark and 50-60 % humidity) and quarantined for 7 days prior to the start of study. Standard rodent chow and tap water were provided *ad libitum* to the experimental animals. The experiment performed was in full compliance with Institutional Animal Ethics Committee.

All mice were randomly allocated into six groups (group I-VI), 6 mice in each. Group I served as healthy control and was administered with normal saline orally for 7 days. Groups II and III were administered with *N*-acetylcysteine (NAC) (150 mg/kg b.w, *p.o.* in distilled water) and QC (20 mg/kg b.w, *p.o.* in normal saline), respectively, for 6 days. Group IV was administered with PCM (640 mg/kg b.w, *p.o.* in distilled water) on day 0 followed by normal saline for 6 consecutive days. Groups V and VI were administered with PCM (640 mg/kg b.w, *p.o.* in distilled water) on day 0 followed by NAC (150 mg/kg b.w, *p.o.* in distilled water) and QC (20 mg/kg b.w, *p.o.* in normal saline), respectively, for 6 consecutive days.

Blood samples

On day 7 approximately 1.5 ml of blood samples were obtained from each mice from retro-orbital plexus using mice bleeding tubes. Of this, 0.75 ml was transferred in EDTA-containing tubes and used for the estimation of oxidative stress markers. While, remaining 0.75 ml was used for harvesting the serum to determine the biochemical parameters.

Assays of oxidative stress

Preparation of hemolysate and RBC suspension

Blood samples obtained for oxidative stress assays were centrifuged at 200× g for 10 min to harvest the erythrocytes. Erythrocytes were washed thrice with normal saline solution and finally, 10% hemolysate was prepared by adding chilled distilled water. For estimation of GSH, RBC suspension was prepared by adding equal volume of erythrocytes and normal saline solution. Hemolysate

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and RBC suspension were kept at -70°C and used for antioxidant assay within 6 h. Hemoglobin concentration was estimated by cyanomethemoglobin method.[13] All assessments executed were performed in triplicate.

Lipid peroxides assay

The concentration of MDA, a reliable marker of lipid peroxidation, was estimated in hemolysate following the method suggested by Placer *et al*.[14] Lipid peroxidation was calculated on the basis of molar extinction coefficient of MDA (1.56×10^5) and expressed in terms of μmol of MDA/mg of Hb.

Reduced glutathione assay

The concentration of glutathione (GSH) in RBC suspension was estimated by 5, 5-dithiobis-(2-nitro- benzoic acid) (DTNB) method as per the procedure of Prins and Loos.[15] GSH concentration in the test samples was calculated by employing the molar extinction coefficient of DTNB-GSH conjugate (ηmol/mg Hb), 13600/M/cm.

Glutathione peroxidase assay

Glutathione peroxidase (GSH-Px) activity was determined by the method of Paglie and Valentie.[16] GSH-Px activity was standardised against protein concentrations and expressed as NADPH ηmol oxidized per minute per mg of hemoglobin (mU/mg Hb) by using the molar extinction coefficient (ε) of 6200 at 340 nm.

Glutathione -S-transferase

The Glutathione-S-transferase (GST) activity in erythrocytes was determined according to the standard procedure of Habig *et al*.[17] The specific activity of GST is expressed as mmol of GSH–CDNB conjugate formed /min/mgHb using an extinction coefficient of 9.6/mM/cm.

Superoxide dismutase assay

Superoxide dismutase (SOD) activity in hemolysate was measured by using nitro blue tetrazolium as a substrate after suitable dilution as per the method suggested by Menami and Yoshikawa.[18] One unit of SOD activity was defined as the amount of enzyme that inhibited auto-oxidation by 50% under the given experimental condition and the values were expressed as U/mg of hemoglobin.

Catalase assay

Catalase (CAT) activity in hemolysate was estimated by using H_2O_2 as a substrate as per the method of Bergmayer.[19] One unit of activity is equal to mmol of H_2O_2 degraded per minute and is expressed as units/mg of hemoglobin.

Biochemical parameters

Serum aspartate transaminase (AST; EC 2.6.1.1), alanine transaminase (ALT; EC 2.6.1.2) activities, alkaline phosphatase (ALP; EC 3.1.3.1), urea and creatinine were determined with kits from ERBA (India). Stored serum samples were analyzed for total protein, albumin and bilirubin, concentrations with kits from ACCUREX (India).

Statistical analysis

The values were expressed as mean ± S.E. and comparisons between the groups were performed using the MANOVA and post hoc Tukey's test. The level of statistical significance for all the comparisons made was established at $P \leq 0.05$. All data were analyzed by means of the statistical package SPSS 15 (SPSS Sciences, Chicago, USA).

RESULTS

The means ± S.D values of the markers of oxidative stress for all groups are depicted in Table 1. The marker of lipid peroxides (LPO), MDA contents of alone PCM-intoxicated mice (Group IV) were significantly higher ($P \leq 0.01$) in comparison with the healthy controls (Group I). Whereas, the levels of MDA in PCM intoxicated followed by QC-treated mice (Group VI) was significantly lower ($P \leq 0.01$) in comparison with the PCM alone intoxicated mice (Group IV). The levels of MDA in this group were

Table 1: The markers of oxidative stress (lipid peroxides, reduced glutathione, glutathione peroxidase, glutathione-S-transferase, superoxide dismutase and catalase) in erythrocytes of control and trial groups (Mean ± S.D)

Groups	LPO (μmol MDA/mg Hb)	GSH (ηmol/mg Hb)	GSH-Px (mU/mg Hb)	GST (mU/mg Hb)	SOD (U/mg Hb)	CAT (K/mg Hb)
Group I	0.43±0.02	25.8±2.63	1141±16	1.11±0.06	1.13±0.04	157±7
Group II	0.42±0.02	27.3±1.75	1148±12	1.13±0.06	1.12±0.03	154±8
Group III	0.42±0.04	28.3±3.77	1153±19	1.13±0.04	1.12±0.02	158±7
Group IV	1.65±0.11[a]	7.0±1.58a	804±30[a]	0.75±0.05[a]	0.61±0.05[a]	82±7[a]
Group V	0.48±0.05[b]	22.6±2.06[b]	1118±14[b]	1.06±0.03[b]	1.07±0.01[b]	147±10[b]
Group VI	0.44±0.04[b]	22.1±2.31[b]	1117±9[b]	1.07±0.01[b]	1.03±0.01[b]	148±11[b]

Group I- healthy control; Groups II- N-acetylcysteine (NAC) @ 150 mg/kg; Groups III- Quercetin (QC) @ 20 mg/kg; Group IV- Paracetamol (PCM) @ 640 mg/kg; Groups V- PCM (640 mg/kg) + NAC (150 mg/kg); VI- PCM (640 mg/kg) + QC (20 mg/kg). [a] - Statistically significant (P<0.01), When compared with Group I. [b] - Statistically significant (P<0.01), When compared with Group IV.

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Table 2: Serum biochemical parameters (ALT, AST, ALP, bilirubin, urea nitrogen, creatinine, total protein and albumin) of control and trial groups

Groups	AST (IU)	ALT (IU)	ALP (IU)	Bilirubin (mg/dl)	Urea nitrogen (mg/dl)	Creatinine (mg/dl)	Total protein (mg/dl)	Albumin (mg/dl)
Group I	78±4.8	59±10.80	92±7.79	0.23±0.05	49±2.52	0.33±0.08	6.5±0.33	2.96±0.16
Group II	79±6.6	57±6.04	92±7.14	0.23±0.05	52±2.31	0.31±0.01	6.3±0.14	2.98±014
Group III	82±4.7	55±7.80	93±7.38	0.23±0.05	50±4.25	0.18±0.07	6.3±0.23	3.05±0.16
Group IV	156±9.8[a]	242±21.44[a]	302±6.65[a]	0.86±0.11[a]	97±9.73[a]	1.14±0.28[a]	5.3±0.22[a]	2.06±0.24[a]
Group V	79±4.2[b]	69±8.39[b]	91±11.44[b]	0.23±0.05[b]	47±4.79[b]	0.30±0.08[b]	6.6±.32[b]	2.86±0.19[b]
Group VI	81±4.2[b]	68±7.06[b]	95±9.57[b]	0.22±0.06[b]	50±3.93[b]	0.31±0.09[b]	6.8±0.31[b]	3.01±0.14[b]

Group I- healthy control; Groups II- *N*-acetylcysteine (NAC) @ 150 mg/kg; Groups III- Quercetin (QC) @ 20 mg/kg; Group IV- Paracetamol (PCM) @ 640 mg/kg; Groups V- PCM (640 mg/kg) + NAC (150 mg/kg); VI- PCM (640 mg/kg) + QC (20 mg/kg). [a] - Statistically significant (P<0.01), When compared with Group I. [b] - Statistically significant (P<0.01), When compared with Group IV.

also comparable to the healthy controls as well as standard therapeutic controls (Group V). No significant differences existed, when the values for MDA were compared among these groups (Groups I, V and VI).

Erythrocytic GSH contents were found to be significantly lowered ($P \leq 0.01$) in alone PCM-intoxicated mice (Group IV) in comparison with the healthy controls. The activities of antioxidant enzymes, GSH-Px, GST, SOD and CAT were also found to be singnificantly lower in these mice, when compared to the healthy controls ($P \leq 0.01$). Whereas, the level of GSH in PCM intoxicated followed by QC treated mice (Group VI) was significantly higher ($P \leq 0.01$) in comparison with the only PCM-intoxicated mice (Group IV). The activities of antioxidant enzymes, GSH-Px, GST, SOD and CAT were also found to be singnificantly higher in these mice, when compared with the PCM-intoxicated mice ($P \leq 0.01$). The levels of GSH and activities of GSH-Px, GST, SOD and CAT in this group were comparable to the healthy controls as well as standard therapeutic controls (Group V). No significant differences existed, when the values for GSH, GSH-Px, GST, SOD and CAT were compared among these groups (Groups I, V and VI). The values for MDA, GSH, GSH-Px, GST, SOD and CAT in alone QC (Group II) and NAC (Group III) administered mice were comparable to the healthy controls and no significant differences were existed among these groups.

The means ± S.D values of biochemical panels for all groups are depicted in Table 2. PCM-intoxicated mice revealed significantly increased AST, ALT and ALP activities ($P \leq 0.01$), when compared with the healthy controls. Levels of bilirubin, urea and creatinine were also significantly elevated in these mice as compared to the healthy controls. Whereas, intoxication with PCM resulted in significant ($P \leq 0.01$) decrease in serum total protein (TP) and albumin (A) levels. QC administration significantly ($P \leq 0.01$) ameliorated these altered biochemical panels toward normalcy in comparison with the PCM controls and the values were comparable to the healthy and standard therapy controls. No significant alterations for these biochemical parameters were exhibited by the mice of alone QC and NAC administered groups, when compared with the healthy controls. In alone PCM-intoxicated group mortality of one mouse was recorded on day 3rd of study start. Whereas, no mortality was recorded in remaining groups.

DISCUSSION

In the present study, PCM intoxication caused a significant elevation in MDA levels and reduction in GSH levels with simultaneous inhibition in the activities of antioxidant enzymes; GSH-Px, GST, SOD and CAT in mice erythrocytes. Lipid peroxidation is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress in cells and tissues. Lipid hydroperoxides (LOOH) are byproducts of lipid peroxidation and increased levels of lipid peroxidation products are associated with a variety of chemical-induced toxicities including PCM.[20,21] LPO are known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerizaton of polysaccharide, as well as protein cross linking and fragmentation.[22] Kanbur and coworkers have reported LPO contents to increase in the liver tissues of PCM-induced liver damage in mice.[23] A rapid loss of GSH and lipid peroxidation in both liver[3] and kidney[4] of PCM-intoxicated animals is also found. PCM toxicity in the liver is mainly interceded by the covalent binding of NAPQI, the reactive metabolite of PCM, to sulfhydryl groups of GSH and various proteins and their subsequent oxidation. Overproduction of free radical in alone PCM-intoxicated mice might have upshot the erythrocytic lipid peroxidation, and consequently increased MDA contents. This may also be implicated with diminished erythrocytic GSH contents, as to combat the over production of free radicals, erythrocytic GSH stores might have been exhausted.

Oxidative stress plays a critical role in the cellular toxicity and is implicated as a major factor in the pathogenesis of several diseases.[24,25] It is known that when ROS generation overleads the antioxidant defense, the free radicals can then interact with endogenous macromolecules and alter the cellular functions. Administration of PCM alone had also resulted in diminution of antioxidant enzymes activities

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in the mice erythrocytes. Among the various antioxidant molecules, SOD and CAT mutually functions as important enzymes in the elimination of ROS. Decrease in SOD and CAT activities in alone PCM-intoxicated mice may be due to the overproduction of superoxide radical anions. In contrast to the report of no significant alteration in the activities of SOD and CAT in liver tissues of PCM-intoxicated mice,[23] we demonstrated a significant reduction of these enzymes activities in erythrocytes of the PCM intoxicates mice. This may be as a result of a hasty exhaustion of erythrocytic stores for these enzymes. Various scientific reports have demonstrated significant reduction of GSH-Px activity in PCM-intoxicated animal's liver and kidney.[4,23] GSH-Px and GST are enzymes which prevent the generation of hydrogen peroxide and alkyl hydroperoxides in association with GSH and GSH-reductase, as well as the generation of more harmful metabolites such as the hydroxyl radical.[24] In the present study, decreased erythrocytic GST and GSH-Px activities in alone PCM-intoxicated mice can be explained by the consumption of these enzymes during the detoxification of reactive oxygen metabolites generated due to PCM, as well as consumption of erythrocytes GSH store. Decrease in GSH content due to PCM intoxication can simultaneously decrease the activities of GST as well as GSH-Px.[4] The results of the present study positively indicate the possibility of PCM mediated oxidative stress in erythrocytes.

Sharp increase in serum ALT level is considered to be a significant indicator of PCM-induced acute liver damage.[26] ALT is an enzyme specific to liver damage. In the present study, we demonstrated significant increase in the serum ALT levels of alone PCM-intoxicated mice, signifies PCM-induced acute liver damage to have developed. In these mice levels of AST and ALP were also found to be increased. Serum AST and ALP levels increase not only in liver damage but also in case of various other tissues and organs damage. In experimental acute PCM intoxications, in addition to ALT levels, serum AST and ALP levels also increase.[26] Similarly, in this study, serum AST and ALP levels were determined to have increased in PCM-intoxicated mice. In the present study, PCM intoxication also decreased serum total protein and albumin, while increased serum bilirubin. The liver is the major source of most of the serum proteins, in which the parenchymal cells are responsible for synthesis of albumin, fibrinogen and other coagulation factors and most of the α- and β-globulins.[27] The observed decrease in albumin by PCM could be a result of a decline in the number of cells responsible for albumin synthesis in the liver through necrosis. The direct interference with the albumin-synthesizing mechanism in the liver as result inflammation may also be implicated for decrease in albumin. PCM overdose is known to be associated with inflammation, marked by an increase in the inflammatory cytokines; tumor necrosis-α (TNF-α), interleukin-1α and interleukin-1β, as well as the upregulation of nitrogen oxide (NO) from macrophages and hepatocytes.[3,4] Such cytokines produced during inflammation shunt amino acids to increase the synthesis of proteins important to the inflammatory process, thus decreasing albumin synthesis as it is not essential to inflammation. Accumulation of bilirubin is a measure of alterations in binding, conjugation and excretory capacity of hepatocytes. The elevated level of bilirubin is usually an indication of biliary obstruction, hemolysis, and in some cases renal failure.[27] Our observation of increased level of serum bilirubin in alone PCM-intoxicated mice indicates PCM-induced hepatic damage. Results also revealed a significant renal impairment in animals treated with PCM alone, demonstrated by the increase in serum urea and creatinine. Various experimental studies have demonstrated PCM-induced renal injury in experimental animals.[4]

Results obtained in the present study indicated that post-administration of QC in PCM-intoxicated mice had effectively ameliorated the increased MDA contents toward normalcy. It had also maintained the GSH contents in erythrocytes of PCM-intoxicated mice. In addition, it restored the activities of antioxidant enzymes in erythrocytes of PCM-intoxicated mice. In the present study, we also demonstrated the amelioration of altered oxidant/antioxidant balance in PCM-intoxicated mice by QC was comparable to the postadministration of standard antioxidant, NAC. This indicates the antioxidant potential of QC against PCM-induced oxidative stress in mice blood. The antioxidant efficacy of QC may be due to its higher diffusion into the membranes allowing it to scavenge ROS at several sites through the lipid bilayer. In addition, it also inhibits LOX-1 activity, hinders the progression of the free radical chain reaction, and hence decreasing the lipid peroxidation in the membranes.[28] QC also acts as antioxidant by inhibiting oxidative enzymes such as xanthine oxidase, lipoxygenase and NADPH oxidase. Inhibition of these enzymes is also responsible for the attenuation of oxidative stress as they play key roles in the initial process of free radical-induced cellular damage.

In the present study, QC also protected the liver damage of PCM intoxicated as demonstrated by the decrease in serum AST, ALT and ALP activities, with consequent restoration of serum total protein, albumin and bilirubin, when compared with the alone PCM-intoxicated mice. The observed hepato-protective effect might be a consequence of the amelioration of oxidative stress and maintenance of the antioxidant capacity conferred by QC. This suppresses the leakage of these enzymes into blood circulation. Results of the presents are in agreement with the various scientific reposts enlightening protective effect of QC against the different chemicals including PCM-induced increase in the plasma AST and ALT levels.[8,20,21] In the present study, we demonstrated the reno-protective potential of QC, as post-treatment with QC significantly ameliorated

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the increased serum levels of creatinine and urea nitrogen toward normalcy. Reno protective potential QC has been demonstrated by previous scientific reports.[29,30]

Thus it can be concluded that the post-treatment of QC has a potentials antioxidant action to restore the erythrocytic antioxidants stores of PCM-intoxicated mice. It has also protective activity against PCM-induced hepatic and renal injuries. Finally, these results warrant further investigation of QC as a potential therapeutic agent against the ailments where erythrocytic antioxidants store is compromised and/or reno-hepatic damage is manifested.

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