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Probiotic *Enterococcus lactis* IITRHR1 protects against acetaminophen-induced hepatotoxicity

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ABSTRACT

Objective: Acetaminophen (APAP), an antipyretic/analgesic drug, is reported to cause toxicity on overdose. Dietary supplements are currently being explored to decrease toxicity. In the present study, the protective effect of probiotic *Enterococcus lactis* IITRHR1 was evaluated at different doses (10^7, 10^8, and 10^9 colony-forming units) against APAP-induced liver damage.
Methods: Male Wistar rats were administered APAP (1 g/kg of body weight orally) for 14 d, and hepatotoxicity was assessed by marker enzymes in serum and observation of histopathologic changes. Rats were pretreated with probiotic *E. lactis* IITRHR1 for 7 d and modulation of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase), redox ratio, and ferric reducing antioxidant power was assessed. Oxidative damage by APAP to membrane lipids, proteins, and DNA was also observed. Involvement of Bax, Bcl2, cytochrome *c* (pro-/anti-apoptotic proteins), caspases, and their modulation was assessed by immunoblot analysis and reverse transcriptase polymerase chain reaction.
Results: The *E. lactis* IITRHR1 pretreatment lowered the level of biomarkers of hepatotoxicity in serum. A significant increase was observed in the level of antioxidant enzymes and redox ratio and decreased oxidative damage to membrane lipids and proteins. Probiotic *E. lactis* IITRHR1 also modulated key apoptotic/anti-apoptotic proteins such as cytochrome-*c*, Bcl2, Bax, expression of caspases, and resultant DNA damage.
Conclusion: Probiotic strain *E. lactis* IITRHR1 was found to have antioxidant capacity and afforded protection against APAP-induced hepatotoxicity by modulating antioxidant status, pro-/anti-apoptotic proteins, caspases, and DNA damage.

Introduction

Acetaminophen (APAP) is a commonly used over-the-counter analgesic/antipyretic drug. It is safe at therapeutic doses but an overdose is reported to cause severe liver injury [1,2]. Glucuronyl transferases/sulfotransferases directly conjugate a large portion of the therapeutic dose of APAP. The remaining part is converted to a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), by cytochrome P450 2E1 (CYP2E1) [3]. NAPQI forms a glutathione (GSH) adduct that is excreted in bile [4], leading to depletion of hepatocellular GSH. After exhaustion of GSH, the remaining NAPQI reacts with other cellular proteins. Binding of NAPQI to mitochondrial proteins is the key initiator of APAP-induced cell death, leading to liver toxicity [5]. An exploration of dietary antioxidants that would offer protection against APAP-induced hepatic injury is being performed by many research groups [6]. Reported protective agents are *N*-acetylcysteine, activated charcoal, and some medicinal plants, but contraindications in immunocompromised individuals, allergic reactions, and gastrointestinal disturbances have been reported [7].

Probiotics, the live microbial food supplements, have been used for the prevention of bacterial infections, alcohol-induced oxidative stress [8], hepatic encephalopathy [9], cancer therapy [10], and non-steroidal anti-inflammatory drug enteropathy [11].

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The combination of different probiotics has been shown to have a significant effect in modulating the makeup of intestinal flora, resulting in lower levels of ammonia and endotoxins in liver [12,13]. Its protective effect against carbon tetrachloride (CCl_4)-induced liver injury has been reported [14]. Probiotics mainly consist of lactic acid bacteria, including many strains of *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus*. Among these the genus *Enterococcus* is of particular interest for environmental, food, and clinical research [15,16]. *Enterococci* are present or deliberately added to fermented foods, where they contribute to the organoleptic properties [17].

Enterococcus lactis IITRHR1 (GenBank accession no. FJ447353) is a novel strain that was isolated from cottage cheese and demonstrated probiotic potential, including an efficient adhesion to intestinal epithelial cell lines (intestinal epithelial cell line 6, CRL 1592), tolerance of an acid/bile environment, and cytoprotection [18]. Dose standardization is essential for probiotics because an inadequate dose may cause complications in immunocompromised individuals. In the present study, we evaluated the effect of three different doses of *E. lactis* IITRHR1 against APAP induced hepatotoxicity in male Wistar rats. Antioxidant enzymes (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx], glutathione-S-transferase [GST]), redox ratio, and ferric reducing antioxidant power (FRAP) were assessed in all experimental groups. Oxidative damage by APAP to membrane lipids, proteins, DNA and involvement of critical control points of apoptosis such as Bax, Bcl2 (pro-/anti-apoptotic proteins), release of cytochrome-*c*, activation of caspases, and DNA damage were also assessed.

Materials and methods

Chemicals

Primary antibodies against cytochrome-*c*, Bax, Bcl2, β-actin, cytochrome oxidase-IV (COX-IV), and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). de Man-Rogosa-Sharpe medium, de Man-Rogosa-Sharpe medium Man-Rogosa-Sharpe broth, and vitamin C were obtained from Himedia Laboratories (Mumbai, India). RNA was isolated using an RNAspin mini-isolation kit (GE Healthcare, Buckinghamshrine, UK) and a cDNA synthesis kit was purchased from Roche Diagnostics (Mannheim, Germany). All other chemicals used throughout the study were commercial products of the highest purity grade and purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Microorganisms

Three different doses of *E. lactis* IITRHR1 (2×10^{10} colony-forming units [CFU] per gram of IITRHR1 lyophilized powder; 10^7, 10^8, and 10^9 CFU) were prepared (0.05, 5, and 50 mg of lactic acid bacteria powder, respectively) and administered per 200 g of rat body weight. The bacterial suspension was prepared in 0.5% carboxy methyl cellulose (CMC) and administered orally by gavage to each rat in respective groups.

Animals

Male Wistar rats (*Rattus norvegicus*, $n = 42$) weighing 200 ± 10 g were procured from the animal house of the Indian Institute of Toxicology Research. Animals were kept under standard conditions of humidity (60–70%), temperature (25 ± 2°C), and a controlled 12-h light/dark cycle. Rats were fed a pellet diet and water *ad libitum*. Animals were acclimatized for 7 d to the experimental animal room conditions. The study was conducted according to the protocol approved by the institutional animal ethics committee (ITRC/IAEC/01/2010).

Experimental design

The experimental design for the present in vivo study is summarized in Figure 1. Rats were divided into seven groups of six animals each and administered oral doses of APAP/*E. lactis* IITRHR1/vitamin C by gavage according to the following schedule: group I (control) received the vehicle (0.5% CMC) for 21 d; Group II received APAP (1 g/kg of body weight in 0.5% CMC) for 14 d; groups III, IV, and V received *E. lactis* IITRHR1 (10^7, 10^8, and 10^9 CFU, respectively) for 7 d followed by APAP treatment for 14 d; group VI received *E. lactis* IITRHR1 (10^9 CFU) for 21 d and served as the treatment control to check the effect of treatment without the drug in normal rats; and group VII (vitamin C + APAP) received vitamin C (500 mg/kg of body weight in 0.5% CMC) for 7 d (positive recovery control) followed by APAP administration for 14 d.

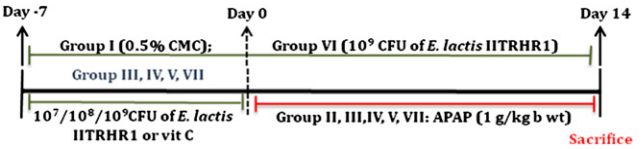

Fig. 1. Treatment schedule. APAP, acetaminophen; b wt, body weight; CFU, colony-forming units; CMC, carboxy methyl cellulose.

Evaluation of serum marker enzymes

All animals were euthanized using chloroform and sacrificed after 21 d of treatment. Blood was collected from each animal and serum was separated according to the standard protocol. The liver marker enzymes serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum alkaline phosphatase (SAP), and bilirubin and cholesterol level were determined by an automated clinical analyzer (Chemwell, 2910, Palm city, FL, USA) using commercially available kits (Spin React, Girona, Spain).

Preparation of homogenate for measurement of antioxidant enzymes

Liver tissues from all groups were collected, washed twice in ice-cold phosphate buffered saline (pH 7.4) and homogenized. After homogenization, samples were centrifuged at 800 × *g* (3K18, Sigma, Osterode am Harz, Germany) for 10 min, the supernatant was collected, and the protein content was measured by a bicinchoninic acid method [19].

Histopathologic studies

Liver tissues from rats of each group were collected, fixed, and processed at the central pathology laboratory of the Indian Institute of Toxicology Research using a paraffin-embedding technique. Liver sections (5 μm) were stained with hematoxylin, and eosin and semiqualitative scaling (125×, Leica, Cambridge, UK) was performed for each section.

Measurement of enzymatic and non-enzymatic antioxidant activities

The SOD activity in liver homogenate was estimated using the method of Kakkar et al. [20] by measuring spectrophotometrically the inhibition of nitroblue tetrazolium/reduced nicotinamide adenosine dinucleotide/phenazine methosulfate-mediated formazan formation at 560 nm. SOD activity was expressed as units per minute per milligram of protein. CAT activity was assayed spectrophotometrically using the method of Aebi [21]. The decrease in absorbance was observed on a spectrophotometer (Spectramax plus 384, Molecular Devices, Sunnyvale, CA, USA) for 60 s at every 15-s interval at 240 nm. CAT activity was expressed as nanomoles of H_2O_2 decomposed per minute per milligram of protein. FRAP assay was performed in serum, which measured the change in absorbance at 593 nm from the formation of a blue FeII-tripyridyltriazine (Fe-TPTZ) compound [22] and was expressed as micromoles per liter of trolox equivalent antioxidant capacity.

Glutathione-S-transferase catalyzes the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. It was measured according to the method of Habig and Jakoby [23], monitored spectrophotometrically at 340 nm for 5 min, and expressed as activity per minute per milligram of protein. GPx activity was measured using the method of Paglia and Valentine [24]. The activity was expressed as nanomoles of reduced nicotinamide adenosine dinucleotide phosphate per minute per milligram of protein using a molar extinction coefficient of 6.22×10^3 nmol L^{-1} cm^{-1}. Total glutathione and oxidized glutathione were measured by the method of Griffith [25] using the Ellman's reagent. The change in optical density was measured at 412 nm after 10 min and expressed in a redox ratio, i.e., ratio of reduced glutathione to oxidized glutathione.

Estimation of lipid peroxidation and protein oxidation

Lipid peroxidation level was measured by an estimation of malondialdehyde, an endproduct of lipid peroxidation, by the method of Wallin et al. [26]. Absorbance was measured at 530 and 600 nm and results are expressed as nanomoles