Hepatic mitochondrial damage aggravated by azathioprine: Protective effect of quercetin

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A b s t r a c t

Mitochondrian play an important role in the production of energy and cell cycle regulation. Administration of azathioprine (AZA), an immunosuppressant drug, adversely affects the hepatic mitochondria which may culminate hepatotoxicity. The present study was undertaken to evaluate the effect of quercetin (QE), against AZA induced hepatic injury in Wistar rats. AZA (50 mg/kg body weight, i.p.) was administered once on the 7th day of experiment. A significant depletion in the levels of manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPx) and reduced glutathione (GSH) were observed in AZA alone treated rats. Simultaneous decrease in the levels of tricarboxylic acid (TCA) enzymes such as isocitrate dehydrogenase (ICDH), -ketoglutarate dehydrogenase (-KGDH), succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) were observed. Decrease in the levels of these enzymes suggests a loss in mitochondrial function and integrity. Lipids existing in the mitochondrial membrane were peroxidised, and measured by the production of   malondialdehyde (MDA). The supplementation of QE (50mg/kg body weight) restored the depleted levels of enzymes and above hepatic mitochondrial abnormality to near normalcy. The present study highlights the antioxidant property of QE in improving the mitochondrial functions in AZA induced hepatic degradation..

Keywords: Oxidative stress, Liver, Antioxidant, Azathioprine, Mitochondrial enzymes, Quercetin.

Introduction

Liver is the organ of body which performs the detoxification of all substances which are ingested by humans; therefore hepatic cells are most susceptible to damage by metabolites of various allopathic drugs. These drugs cause significant hepatic damage due to formation of highly toxic metabolites. Azathioprine, one of the clinical agents employed in the organ transplantations [1].and autoimmune diseases [2].
is a potent hepatotoxicant.   Mitochondrian is responsible for various functions ATP production, cell cycle regulation, growth and death. Ironically mitochondria become the ultimate targets of free radicals which are generated during the transfer of electrons within enzyme complexes via its own electron transport chain [3]. Therefore in concordance with the previous established theory [4] quercetin was chosen as a novel remedy for ameliorating hepatic mitochondrial damage caused by generation of free radicals due to azathioprine intoxication.

Materials and Methods

Drugs and chemicals

AZA was purchased from Sigma Aldrich Chemical Company, Bangalore, India and QE was obtained from Hi-Media Lab, Nasik, India. All the other chemicals used were of analytical grade.

Experimental Protocol

Animals

The study was performed on male albino rats of Wistar strain (average weight 150-180 g), which were obtained from Experimental Animal Care Centre, Vel"s College of Pharmacy, Chennai, India. The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Ministry of Welfare, Chennai. The animals were housed under 25 ± 20C and acclimatized to 12 ± 1 hr day and night rhythm during the experimental period. They were provided with food supplied by Hindustan Lever Ltd., Mumbai, India under the trade name Gold Mohur rat feed and water ad libitum. Prior to experimentation the animals were deprived of food for 24 hr but allowed free access to water throughout the experiment.

Experiments was conducted under the strict guidelines laid by the committee.

The experimental animals were randomized into four groups of six rats each as follows:

Group I: Control rats received normal saline (2ml/kg body weight) for 7 days.

Group II: A single intraperitoneal injection of AZA (50 mg/kg body weight, suspended in saline) was administered to rats on the 7th day.

Group III: Intraperitoneal injection of QE (50 mg/kg body weight, suspended in saline) was given to rats for 7 days.

Group IV: QE (50 mg/kg body weight, suspended in saline) was administered to rats as in Group III, on the 7th day of experimental period 1hr after administration of QE, single dose of AZA (50 mg/kg body weight, suspended in saline) was given intraperitoneal as in Group II.[5]. After the 7 days of experimental period (i.e., on the 8th day), the animals were anesthetized and decapitated. The liver tissues were immediately excised and rinsed in ice- cold physiological saline. The tissues were homogenized in 0.01 M Tris-HCL buffer (pH 7.4) and aliquots of this homogenate were used for the assays. Blood was collected immediately and the serum was separated by centrifugation. The liver homogenate was centrifuged and mitochondria were isolated. Homogenate and serum were used for several biochemical estimations.

Isolation of liver mitochondria

The mitochondria of liver were isolated by the method of[6]. 10% (w/v) homogenate was prepared in 0.05 M Tris-HCl buffer containing 0.25 M sucrose and centrifuged at 600 x g for 10 minutes. The supernatant fraction was decanted and centrifuged at 15,000 x g for 5 minutes. The resultant mitochondrial pellet was then washed and resuspended in the same buffer.

Determination of mitochondrial antioxidant enzymes

The mitochondrial superoxide dismutase activity was assayed by the method of [7]. The mitochondrial glutathione peroxidase activity was assayed by the method of Ro[8]. The reduced glutathione in liver mitochondria was determined according to the method of[9].

Determination of mitochondrial lipid peroxides

The liver mitochondrial lipid peroxide content was determined by the thiobarbituric acid (TBA) reaction described by[10].

Determination of TCA cycle enzymes

The activity of isocitrate dehydrogenase was assayed by the method of[11]. The activity of -ketoglutarate dehydrogenase was assayed by the method of [12]. The activity of succinate dehydrogenase was assayed according to the method of[13]. in which the rate of reduction of potassium ferricyanide was measured by decreased in optical density at 400nm, in the presence of adequate amount of potassium cyanide to inhibit cytochrome C oxidase. The activity of malate dehydrogenase was assayed by the method of [14]. The substrate used was oxaloacetate and

determination of enzyme activity was carried out by measuring the rate of oxidation of NADH.

Statistical analysis

All the grouped data were statistically evaluated with Statistical Package for Social Sciences (SPSS), Version 7.5. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. A „ P' value of less than 0.05 was considered to indicate statistical significance. All the results were expressed as mean + S.D. for six animals in each group.

Results

The activities of MnSOD and GPx were significantly ($p < 0.05$) lower in AZA induced rats (Group II) as compared to that of control rats (group I). On the 7th day QE administration (group IV) significantly reversed all the AZA induced mitochondrial alterations. The rats receiving QE alone (group III), did not show any significant change when compared with control rats indicating that it does not produce oxidative stress up to the concentration tested (Table 1).

Table 1: Effect of Cyclophosphamide and Hesperidin on the activities of cardiac marker enzymes in serum.

Groups	CPK (IU L^{-1})	ALT (IU L^{-1})	LDH (IU L^{-1})	AST (IU L^{-1})
Group I (Control)	125.46 ±2.54	50.95 ±0.74	274.73 ±1.44	83.04 ±2.16
Group II (CP)	290.89 ±7.57*[a]	185.74 ±11.44*[a]	453.26 ±48.31*[a]	275.35 ±18.75*[a]
Group III (HDN)	125.71 ±2.66[NS]	53.59 ±1.72[NS]	280.40 ±7.50[NS]	85.05 ±2.98[NS]
Group IV (HDN+CP)	136.76 ±10.84*[b]	59.15 ±2.66*[b]	299.75 ±14.14*[b]	101.78 ±6.83*[b]

Results are expressed as mean ±S.D. for 6 rats. Comparisons are made between: [a]-group I and group II; [b]-group II and group IV. *Statistically significant ($p < 0.05$); NS – non significant.

The level of hepatic mitochondrial enzymes were significantly depleted in AZA induced rats (group II), when compared to control (group I). These enzyme levels were reversed in QE pretreatment (group IV), which demonstrate amelioration in levels when compared to the AZA induced rats (group II). The rats receiving QE alone (group III), did not exibhit any significant change comparatively control rats (group I) (Table 2). The MDA level was significantly ($p < 0.05$) ameliorated in liver mitochondria of AZA induced rats (group II). The MDA level was found to be lowered by the QE pretreatment (Graph 1) on 7th day (group IV). The QE

alone (group III) did not show any significant change when (Graph 1). The level of mitochondrial GSH was significantly reduced in AZA treated rats (group II).

Table 2: Effect of Cyclophosphamide and Hesperidin on the activities of cardiac marker enzymes in tissue.

Groups	CPK (IU mg^{-1} protein)	ALT (IU mg^{-1} protein)	LDH (IU mg^{-1} protein)	AST (IU mg^{-1} protein)
Group I (Control)	22.59 ±1.48	6.16 ±0.22	33.88 ±1.50	7.00 ±0.44
Group II (CP)	7.43 ±0.45*a	2.68 ±0.10*a	14.52 ±1.08*a	3.32 ±0.15*a
Group III (HDN)	23.30 ±1.53 NS	6.16 ±0.35 NS	34.71 ±0.95 NS	7.19 ±0.87 NS
Group IV (HDN+CP)	17.92 ±0.276*b	4.61 ±0.10*b	28.94 ±1.83*b	7.13 ±0.56*b

Results are expressed as mean ±S.D. for 6 rats. Comparisons are made between: a-group I and group II; b-group II and group IV. *Statistically significant (p < 0.05); NS – non significant.
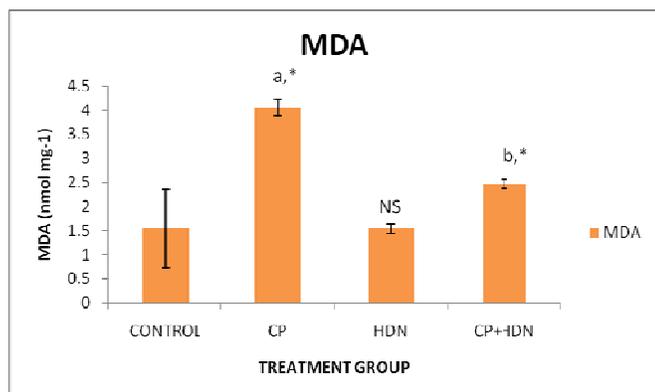
Figure 1 :   Levels of MDA in the heart of experimental animals.

Results are given as mean ±S.D. For 6 rats. Comparisons are made between: [a]-group I and group II; [b]-group II and group IV. *Statistically significant (p < 0.05); NS – non significant.

The mitochondrial GSH level was normalized by QE on the (group IV). The rats receiving QE alone did not show any significant change (group III) when compared to control rats (group I),indicating that it does not produce any harmful effects (Graph 2). Therefore through the above enzyme estimations, it was proved that quercetin is efficient in protecting hepatic mitochondria degradation induced by AZA administration.

Discussion

There is a substantial evidence to prove that quercetin protects azathioprine induced hepatic cellular damage [5]. Therefore, we examined the protective effect of quercetin against azathioprine induced deterioration at the subcellular level. The mitochondrial electron transport chain comprising of several enzyme complexes is responsible for production of ATP which is utilized by the cell [15]. Evidence of data revealed that free radicals generated during the electron transfer in the respiratory complexes may lead to deposition of these radicals in mitochondria that might impinge on its physiology [16]. The homeostasis of reactive oxygen species in the mitochondria is regulated by GSH [17]. GSH is a tripeptide [18].which counteracts against oxidative stress in the mitochondria by quenching free radicals [19]. The attenuation in the antioxidant system in mitochondrial matrix as a consequence of hampered GSH levels has been shown to enhance oxidative damage to the electron transport chain (ETC). This severe effect on ETC indirectly inhibits GSH synthesis there by exacerbating the effect of mitochondrial oxidative free radicals ultimately leading to hepatic damage [20,21]. Culminating evidences proved that during azathioprine disposition, GSH is consumed as a co substrate, so that it leads to depleted levels in mitochondria [22]. It was observed in our study that on treatment with azathioprine, mitochondrial GSH was reduced, also our results depicted an increment in the GSH levels after quercetin administration that may be due to fact that it affects GSH/GSSG ratio in hepatic mitochondria. These free radical species initiate the oxidation of mitochondrial polyunsaturated fatty acids (PUFA), protein, and sterol as revealed by mounting literatures [23]. The formation of conjugated dienes by the PUFA through oxidative radicals may propagate synthesis of lipid peroxides that cause impairment in the integrity and function of hepatic mitochondrial membrane [24]. We observed an alteration in mitochondrial MDA levels in azathioprine intoxicated rats, which is in sync with the previous literatures indicating increased lipid peroxidation that could be attributed to deterioration of antioxidant defense mechanism [25]. The quercetin treatment in our study exhibited near normalcy in MDA levels by quenching peroxide radicals thereby proving its efficacy in maintaining membrane integrity. Mitochondrial endogenous radical load is diminished by enzymatic scavengers like Mn- SOD and GPx [5]. Superoxide radical is formed directly by accepting electron from the molecular oxygen, its accumulation leads to inhibition of ETC [15]. This superoxide is dismutated into H_2O_2 by the MnSOD [26]. GPx converts H_2O_2 in to H_2O [25]. in mitochondria. Activities of Mn-SOD depict hampered levels after induction with azathioprine as documented by previous reports. The hampered levels of GPx in AZA intoxicated rats may be due to the unavailability of the thiol substrate [27]. Pre-treatment with the quercetin significantly restored the mitochondrial levels of Mn-SOD and GPx to near normal levels [28,5].

Due to the degradation of mitochondrial membrane via free radicals, aldehydic products are formed which inhibit the activity of -KGDH enzyme as corroborated earlier [29]. Hydrogen peroxide

attacks the enzyme; leading to its diminished activity thereby stopping the formation of NADH which consequently does not participates in ETC, resulting in depleted ATP levels [30]. ICDH controls he redox balance in mitochondria. It restores NADPH which is adept in regenerating GSH. Due to the free radical attack, electron transfer gets hampered leading to its loss in activity [31,32]. Activity of SDH and MDH get lost due to thiolation of cysteine residues [33,34]. We observed a significant decrease in the levels of mitochondrial enzymes such as ICDH, SDH, MDH and -KGDH in AZA intoxicated rats which were in concordance with previous studies [5]. In our experiment quercetin protected rats depicted an increase in the activities of ICDH, SDH, MDH and -KGDH.

Azathioprine provoked mitochondrial membrane damage and subsequent degradation of respiratory enzymes thus leading to imbalanced transaction of substrates and ions in mitochondria, thereby pushing mitochondrial membrane to expand leading to mitochondrial swelling [34].

In conclusions, the quercetin may reduce oxidative mitochondrial damage which evoked by AZA intoxication.

References

[1]. Raza M, Ahmad M, Gado A, Al-Shabanah OA. A comparison of hepatoprotective activities of aminoguanidine and N-acetylcysteine in rat against the toxic damage induced by azathioprine. Comp Biochem Physiol C Toxicol Pharmacol. 2003; 134(4):451-6.

[2]. Nash CL, Sutherland LR. Medical management of inflammatory bowel disease: old and new perspectives. Curr Opin Gastroenterol 2001;17:336–341.

[3]. Katalin Sas., Hermina Robotka., Jozsef, Toldi. Laszlo vecsei. Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. Journal of the Neurological Sciences. 2007; 221-239.

[4]. guzy J, ku.nír J, mareková M, chavková Z, dubayová K, mojzisová G, mirossay L, mojzis J. Effect of Quercetin on Daunorubicin-Induced Heart Mitochondria Changes in Rats. Physiol. Res. 2003.; 52: 773-780.

[5]. Shanmugarajan TS, Prithwish N, Somasundaram I, Arunsundar M, Niladri M, Lavande JP, Ravichandiran V. Mitigation of Azathioprine-Induced Oxidative Hepatic Injury by the Flavonoid Quercetin in Wistar Rats. Toxicology Mechanisms and Methods, 2008;Volume 18, pages 653 – 660.

[6]. Johnson D and Lardy H, Isolation of liver or kidney mitochondria. Methods Enzymol 1967;10: 94-96.

[7]. Misra HP and Fridovich I, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;247: 3170-3175.

[8]. Rotruck JT, Pope AL, Ganther H, Swanson AB, Hafeman DG, Hoektsra WG. Selenium: biochemical role as a component of glutathione peroxidase. Science 1973;179: 588-590.

[9]. Moran MS, Depierre JW and Mannervik B., Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. Biochim Biophys Acta1979; 582: 67-68.

[10]. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95: 351-358.

[11]. King J. The hydrolases-acid and alkaline phosphatases. In: Practical clinical enzymology (King JC, ed.), Van D Nostrand Company, London 1965;a.pp.199-208.

[12]. Reed, LJ, Mukherjee, BB. a-Ketoglutarate dehydrogenase complex from Escheriachia coli. Method Enzymol. 1969;13, 55–61.

[13]. Slater EC and Bonner WD, The effect of fluoride on succinic oxidase system. Biochem J 1952;52: 185-196.

[14]. Mehler AH, Kornberg A, Grisolia S and Ochoa S, The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate. J Biol Chem1948; 174: 961-977.

[15]. Vali S, Mythri RB, Jagatha B, Padiadpu J, Ramanujan KS, Andersen JK, Gorin F, Bharath MM. Integrating glutathione metabolism and mitochondrial dysfunction with implications for Parkinson's disease: a dynamic model. Neuroscience. 2007;149:917-30.

[16]. Marc W. Fariss, Catherine B. Chan, Manisha Patel, Bennett Van Houten5, and Sten Orrenius., Role of mitochondria in toxic oxidative stress. Molecular interventions: 2005;Volume 5, Issue 2.

[17]. Carmen Garcia-Ruiz. Albert Morales, Anna Colell, Joan Rodes, Jiau-R, Yi, Neil Kaplowitz, and Jose C. Fernandez-Checa, Evidence that the rat hepatic mitochondrial carrier is distinct from the sinusoidal and canalicular transporters for reduced glutathione. The journal of biological chemistry 1995.vol. 270, no. 27, pp. 15946-15949.

[18]. Bjorn Vergauwen, Frederik Pauwels, Mario Vaneechoutte, and Jozef J. Van Beeumen, Exogenous Glutathione Completes the Defense against Oxidative Stress in Haemophilus influenza. Journal of bacteriology, 2003; Vol. 185, p. 1572–1581.

[19]. Samantha M, BeerEllen R, Taylor, Stephanie E, Brow Christina C, Dahm, Nikola J, Costa, Michael J, Runswick Michael P. Murphy Glutaredoxin 2 Catalyzes the Reversible Oxidation and Glutathionylation of Mitochondrial Membrane Thiol Proteins. The journal of biological chemistry, 2004;279: 47939–47951.

[20]. Julie Ehrhart, Martin Gluck, John Mieyal, Gail D. Zeevalk, Functional glutaredoxin (thioltransferase) activity in rat brain and liver mitochondria. Parkinsonism and related disorders 2002; 8:395-400.

[21]. Cesar Menor, Maria D. Fernandez – Moreno, Jesus A. Fueyo, Oscar Escribano, Tomas lleros, Encarna Arriaza, Carlos Cara, Michele Lorusso, Marco Di Paola, Irene D.Roman and Luis G. Guijarro, Azathioprine acts upon rat hepatocyte mitochondria and stress activated protein kinases leading to necrosis: Protective role of N-acetyl –L-cysteine. The Journal of Pharmacology and Experimental Therapeutics 2004; 311: 668-676.

[22]. F MJ, Jones BE, Wu WM, Farrell GC., Toxicity of low dose azathioprine and 6- mercaptopurine in rat hepatocytes. Roles of xanthine oxidase and mitochondrial injury. J Hepatol. 2004;40 (3):454-63.

[23]. Alves E, Summavielle T, Alves CJ, Gomes-da-Silva J, Barata JC, Fernandes E, Bastos Mde L, Tavares MA, Carvalho F., Monoamine oxidase-B mediates ecstasy-induced neurotoxic effects to adolescent rat brain mitochondria. J Neurosci. 2007;27(38):10203-10.

[24]. Higuchi H, Adachi M, Miura S, Gores GJ, Ishii H. The Mitochondrial Permeability Transition Contributes to Acute Ethanol-Induced Apoptosis Induced Apoptosis in Rat Hepatocytes. Hepatology 2001;Vol. 34. No. 2.

[25]. Thomas J. Preston, Arkan Abadi, Leigh Wilson, G. Singh., Mitochondrial contributions to cancer cell physiology: potential for drug development. Advanced Drug Delivery Reviews 2001;49, 45-61.

[26]. Kebieche M, Lakroun Z, Lahouel M, Bouayed J, Meraihi Z, Soulimani R., Evaluation of epirubicin-induced acute oxidative stress toxicity in rat liver cells and mitochondria, and the prevention of toxicity through quercetin administration. Exp Toxicol Pathol. 2008.

[27]. Francisco Martinez – Cruz, Carmen Osuna, Juan M. Guerrero, Mitochondrial damage induced by fetal hyperphenylalaninemia in the rat brain and liver: Its prevention by melatonin, Vitamin E and Vitamin C. Neuroscience Letters 2006; 392:1-4.

[28]. Laszlo Tretter and Vera Adam-Vizi, Alpha-ketoglutarate dehydrogenase: a target and generator of oxidative stress Phil. Trans. R. Soc. B 2005; 360, 2335-2345.

[29]. Lee SM, Koh HJ, Park DC, Song BJ, Huh TL, Park JW. Cytosolic NADP(+)-dependent isocitrate dehydrogenase status modulates oxidative damage to cells. Free Radic Biol Med. 2002;32(11):1185-96.

[30]. Plaut GW, Cook M, Aogaichi T. The subcellular location of isozymes of NADP- isocitrate dehydrogenase in tissues from pig, ox and rat. Biochim Biophys Acta. 1983;760(2):300-8.

[31]. Khanh Le-Quoc, Danielle Le-Quoc, and Yves Gaudemer, Evidence for the existence of classes of sulphydryl groups essential for membrane-bound succinate dehydrogenase activity. Biochemistry 1981;20, 1705-1710.

[32]. Fernandez-checa Jose C., kaplowitz Neil, Hepatic mitochondrial glutathione: transport and role in disease and toxicity, Toxicology and applied pharmacology. 2005;204: 263-273.

[33]. Shanmugarajan TS, and Devaki T. Hepatic Perturbations Provoked by Azathioprine: A Paradigm to Rationalize The Cytoprotective Potential Of Ficus Hispida Linn. Toxicology Mechanisms and methods. 2009;18.1.6.

[34]. Arti B. Sharma, Jie Sun, Linda L. Howard, Arthur G. Williams, Jr, and Robert T. Mallet, Oxidative stress reversibly inactivates myocardial enzymes during cardiac arrest. Am J Physiol Heart Circ Physiol 2007; 292: H198–H206.