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Pioglitazone, quercetin and hydroxy citric acid effect on cytochrome P450 2E1 (CYP2E1) enzyme levels in experimentally induced non alcoholic steatohepatitis (NASH)

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Abstract. – OBJECTIVE: Non-Alcoholic Steatohepatitis (NASH) is a severe form of Non Alcoholic Fatty Liver Disease (NAFLD) spectrum, which progresses to the end stage liver disease. A common denominator in the pathogenesis of insulin resistance and nonalcoholic steatohepatitis is increased oxidative stress. Hepatic induction of the pro-oxidant enzyme Cytochrome P450 2E1 (CYP2E1) occurs in both NAFLD and type-2 diabetes. In this study, the comparative effect of pioglitazone, quercetin and hydroxy citric acid on liver CYP2E1 enzyme levels in experimentally induced NASH has been studied.

MATERIALS AND METHODS: The experimental protocol consists of 5 groups viz. Control (n = 6); NASH Induced (n=6); NASH + Pioglitazone (n=6); NASH + Quercetin (n=6); NASH + Hydroxy Citric Acid (n=6). CYP2E1 enzyme levels were detected in liver by immunoblot analysis in all the groups.

RESULTS: CYP2E1 catalytic activity was increased in experimentally induced NASH group compared to control group as evidenced by the Immunoblot analysis. It revealed low CYP2E1 in the experimentally induced NASH, treated with pioglitazone, quercetin and hydroxy citric acid. Mild decrease in the CYP2E1 level was observed in experimental NASH treated with pioglitazone compared to NASH group. Treatment with hydroxy citric acid also showed mild decrease in the levels of CYP2E1. On contrary to the action of pioglitazone and hydroxy citric acid, quercetin showed an approximate 2-fold decrease in the level of CYP2E1 in experimental NASH treated with quercetin compared to NASH group.

CONCLUSIONS: Being a powerful antioxidant, quercetin offers absolute protection to liver against NASH by reducing the levels of CYP2E1 and, thereby, reducing CYP2E1 mediated oxidative stress, which is believed to be the one of the key factor in the pathogenesis of NASH. On the other hand, pioglitazone and hydroxy citric acid exerted limited effect on the levels of CYP2E1. This study showed the therapeutic value of quercetin, pioglitazone and hydroxy citric acid in treating NASH.

Key Words:
Pioglitazone, Quercetin, Hydroxy citric acid, Cytochrome, CYP2E1, Non-Alcoholic Fatty Liver Disease (NAFLD), Non-Alcoholic Steatohepatitis (NASH).

Introduction

Non alcoholic steatohepatitis (NASH) a severe, asymptomatic disease in the spectrum of non-alcoholic fatty liver disease (NAFLD) which finally leads to cirrhosis of the liver, an end-stage liver disease if not diagnosed and treated properly[1-4]. A common denominator in the pathogenesis of insulin resistance and nonalcoholic steatohepatitis is increased oxidative stress[5]. Oxidative stress and diminished antioxidants within the liver initiate the progression from steatosis alone to NASH and ultimately to cirrhosis, a fatal end stage liver disease[6]. The mechanism of progression from the steatosis of NAFLD to the necro-inflammatory state of NASH is poorly understood. However, it is hypothesized that increased production of pro-inflammatory mediators plays an important role in the pathogenesis of NASH[6]. Reactive Oxygen Species (ROS) formation also increased proportionately for a given level of fatty acid oxidation and worsens the oxidative stress[7].

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Hepatic induction of the pro-oxidant enzyme CYP2E1 occurs in both NAFLD and type 2 diabetes[8]. Both insulin resistance and increased cytochrome P450 2E1 (CYP2E1) expression are associated with and mechanistically implicated in the development of nonalcoholic fatty liver disease[8]. Insulin resistance and CYP2E1 expression may be interrelated through the ability of CYP2E1-induced oxidant stress to impair hepatic insulin signaling[8]. Up-regulation of microsomal proteins viz. CYP2E1 and CYP4A have been observed in patients with NASH and constitute the most important factors in the oxidation of FFAs. The CYP2E1 activity is directly related to the hepatic steatosis[9]. It has been well documented that alcohol-mediated up regulation of cytochrome P450 2E1 (CYP2E1) may initiate lipid peroxidation by the production of ROS[10-12]. In our previous studies we have reported the effect of pioglitazone, quercetin, and hydroxy citric acid on the hepatic bio markers, lipid profile and lipoproteins in experimentally induced non-alcoholic steatohepatitis (NASH)[13,14].

We have also studied the comparative effect of pioglitazone, quercetin and hydroxy citric acid on the status of lipid peroxidation and antioxidants in experimental non-alcoholic steatohepatitis[15]. But very little information is known about the role of CYP2E1 in NASH. This study explores the comparative effects of pioglitazone, quercetin, and hydroxy citric acid on CYP2E1 in experimentally induced NASH.

Materials and Methods

The experimental model of NASH in rats was established by feeding the animals a high-fat diet for eight weeks[15,16], and this model was used to conduct a comparative study of the roles of pioglitazone, quercetin, and hydroxy citric acid on various parameters in non-alcoholic steatohepatitis. Male Wistar rats weighing approximately 250 g were housed in solid-bottomed polypropylene cages under strict veterinary supervision and maintained in control rooms with a 12-h light/12-h dark cycle. The animals received water and a commercial rat diet, standard diet, or high-fat diet *ad libitum* according to the experimental protocol. This study conformed to the guiding principles of the Institutional Animal Ethical Committee (IAEC), the Committee for the Purpose of the Control and Supervision of Experiments on Animals (CPCSEA), and the Guide for the Care and Use of Laboratory Animals (IAEC Approval Numbers: 001/006/2010 and 01/007/2011).

The male Wistar rats selected for the study were divided into eight groups as follows (15-17):

- **Group 1, Controls (n = 6):** The control rats received the regular standard diet for eight weeks.
- **Group 2, NASH (n = 6):** The rats were fed a high-fat diet for eight weeks to induce NASH.
- **Group 3, pioglitazone control (n = 6):** These rats were fed the standard diet for four weeks and were then fed the standard diet and intragastrically administered pioglitazone (4 mg/kg. b.wt.; 0.5% methyl cellulose w/v) for the next four weeks.
- **Group 4, quercetin control (n = 6):** These rats were fed the standard diet for four weeks and were then fed the standard diet intragastrically administered quercetin (20 mg/kg. b.wt.) dissolved in 1% dimethyl sulfoxide (DMSO) v/v for the next four weeks.
- **Group 5, hydroxy citric acid control (n = 6):** These rats were fed the standard diet for four weeks and were then fed the standard diet and intragastrically administered hydroxy citric acid (150 mg/kg. b.wt.) for the next four weeks.
- **Group 6, NASH + pioglitazone (n = 6):** These rats were fed a high-fat diet for four weeks and were then fed the high-fat diet and intragastrically administered pioglitazone (4 mg/kg. b.wt.; 0.5% methyl cellulose w/v) for the next four weeks.
- **Group 7, NASH + quercetin (n = 6):** These rats were fed a high-fat diet for four weeks and were then fed the high-fat diet and intragastrically administered quercetin (20 mg/kg. b.wt.) dissolved in 1% dimethyl sulfoxide (DMSO) v/v for the next four weeks.
- **Group 8, NASH + hydroxy citric acid (n = 6):** These rats were fed a high-fat diet for four weeks and were then fed the high-fat diet and intragastrically administered hydroxy citric acid (150 mg/kg. b.wt.) for the next 4 weeks.

After the experimental period, the animals were sacrificed after 12 h of fasting by cervical decapitation. The blood was collected and centrifuged for 5 min at 3000 rpm/min, and the serum was stored at −70°C until various biochemical analysis were analyzed.

The detection of cytochrome P450 2E1 (CYP2E1) enzyme levels in liver was carried out by the immunoblot analysis (western-blotting analysis). The microsomal protein concentration was determined by Coomassie protein assay using bovine serum albumin (BSA) as the standard. Proteins from the microsomal fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and then transferred to nitrocellulose. Immunoblots were stained using affinity-purified anti-dichloroacetyl antiserum. The membranes were then stripped of antibodies according to instructions in Amersham's Enhanced Chemiluminesence (ECL) kit, and re-probed with anti-CYP2E1 antiserum. Trichloroethylene-protein adducts and CYP2E1 were visualized using peroxidase-labeled secondary goat anti-rabbit antibodies with an ECL substrate. Immunoblots were scanned using a laser densitometer and the intensity of CYP2E1 staining for individual animals was quantified using ImageQuant software.

Statistical Analysis

Quantification of CYP2E1 enzyme levels in liver by immunoblot analysis has been performed. The protein levels from individual animals were analyzed by densitometry, and the data is presented as mean intensity in relative arbitrary densitometric units (ADU). Results were expressed as ADU ± SEM with n = 6. Statistical significance is determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. Statistical analysis was performed by using SPSS software package version 13.0 (SPSS Inc., Chicago, IL, USA) and *p* values less than 0.05 was considered as significant.

Results

Histopathological studies revealed the ingestion of the high-fat diet for 8 weeks produces all the prominent characteristics of NASH and the principal histological features of NASH, including steatosis, inflammation, which mimics the NASH in humans[15]. The treatment with drugs alone doesn't cause any deleterious effects. There observed inflammation with no fatty degeneration on treatment with pioglitazone[15] and local hepatocyte necrosis with inflammatory collections was seen on treatment with hydroxy citric acid[15]. But, hepatocytes appear mere normal with no fatty and inflammatory changes on treatment with quercetin[15].

Detection of Cytochrome P450 2E1 (CYP2E1) enzyme levels in liver by immunoblot analysis was depicted in Figure 1. Lane 1 represents the expression of CYP2E1 in control group (group 1). Lane 2 represents the expression of CYP2E1 in experimentally induced NASH group (group 2). Lane 3 represents the expression of CYP2E1 in experimental NASH treated with quercetin (group 7; NASH+quercetin). Lane 4 represents the expression of CYP2E1 in experimental NASH treated with pioglitazone (group 6; NASH+pioglitazone). Lane 5 represents the expression of CYP2E1 in experimental NASH treated with hydroxy citric acid (group 8; NASH+HCA). Lane 6 represents the CYP2E1 standard. CYP2E1 catalytic activity was increased in experimentally induced NASH group (group 2) compared to control group (group 1) as evidenced in Figure 1. Immunoblot analysis revealed the low levels of CYP2E1 in the experimental NASH treated with pioglitazone,

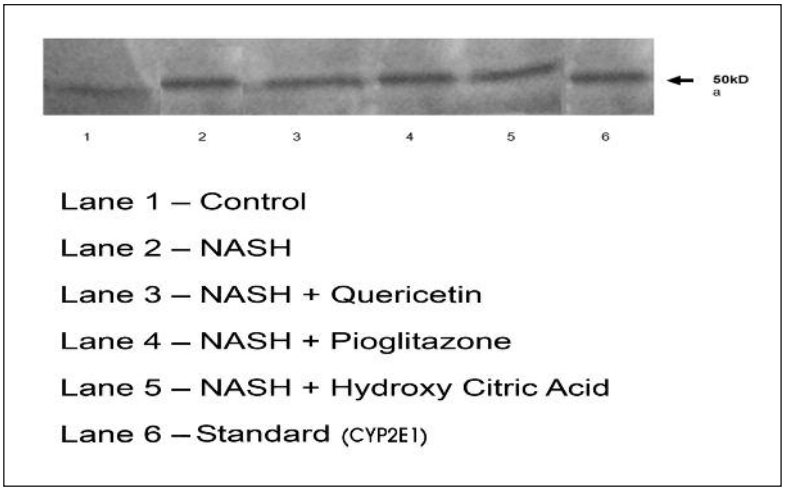

Figure 1. Detection of cytochrome P450 2E1 (CYP2E1) enzyme levels in liver by immunoblot analysis.

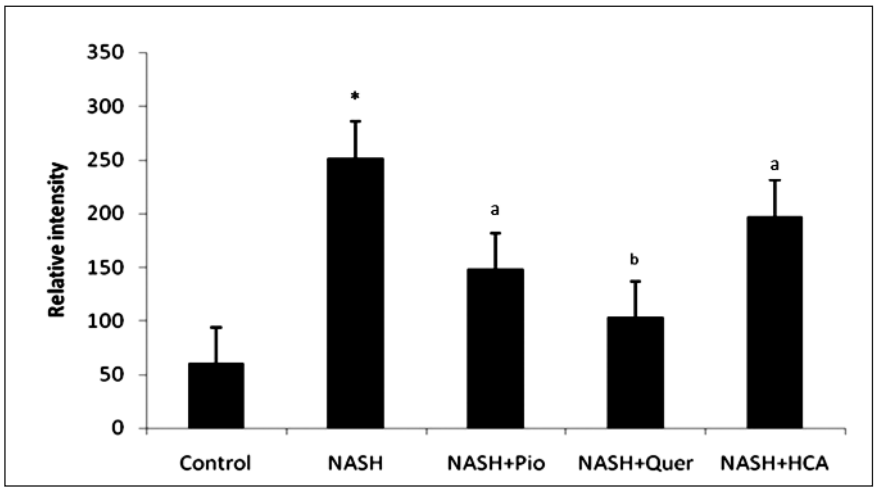

Figure 2. Quantification of cytochrome P450 2E1 (CYP2E1) enzyme levels in liver by immunoblot analysis. Protein levels from individual animals were analyzed by densitometry, and the data is presented as mean intensity in relative arbitrary densitometric units (ADU) ± SEM with n = 6. *$p < 0.001$ compared to control group; [a]$p < 0.05$ compared to NASH group; [b]$p < 0.001$ compared to NASH group.

quercetin and hydroxy citric acid NASH animals (Figure 1). An approximate 2-fold decrease in the level of CYP2E1 have been observed in experimental NASH treated with quercetin (group 7; NASH+quercetin) compared to NASH group (group 2) and mild decrease in the levels of CYP2E1 level was observed in experimental NASH treated with pioglitazone (group 6; NASH+pioglitazone) compared to NASH group (group 2) and also in experimental NASH treated with hydroxy citric acid (group 8; NASH+HCA). The quantification of CYP2E1 paralleled the results of immunoblots as evidenced in Figure 2.

Quantification of CYP2E1 enzyme levels in liver by immunoblot analysis has been performed. The protein levels from individual animals were analyzed by densitometry, and the data is presented as mean intensity in relative arbitrary densitometric units (ADU) ± SEM with n = 6. CYP2E1 levels were significantly increased in NASH group when compared to controls ($p < 0.001$ compared to control group; Figure 2). After treatment with pioglitazone and HCA the CYP2E1 levels were significantly decreased when compared to the NASH group ($p < 0.05$ compared to control group; Figure 2), where as the CYP2E1 levels were significantly decreased and reached to mere normal when treated with quercetin, compared to NASH group ($p < 0.001$ compared to NASH group; Figure 2).

Discussion

Detection of Cytochrome P450 2E1 (CYP2E1) enzyme levels in liver by immunoblot analysis was depicted in Figures 1 and 2. CYP2E1 catalytic activity was increased in experimentally induced NASH group (group 2) compared to control group (group 1) as evidenced in Figures 1 and 2. Quantification of CYP2E1 enzyme levels in liver by immunoblot analysis has been performed. The protein levels from individual animals were analyzed and quantified by densitometry.

CYP2E1 plays a key role in the pathogenesis of liver injury by virtue of its capacity to generate ROS and lipid peroxides[18]. CYP2E1 over expression occurs in animals and humans with non-alcoholic steatohepatitis[19,20]. The significant increased activity of CYP2E1 in our present study in experimentally induced NASH is concordance with these studies[19,20].

Oxidative stress mediated by over expression of CYP2E1 has been shown to promote liver injury in both alcoholic and non alcoholic fatty liver disease[21,22]. It is hypothesized that CYP2E1-induced oxidative stress may act to sensitize hepatocytes to death. The present study supported this hypothesis by demonstrating over expression of CYP2E1 in NASH induced rat hepatocytes. CYP2E1 catalyze "leaky" redox cycles, which can also produce ROS during fatty acid metabolism in the endoplasmic reticulum or even in the absence of substrate[23,24]. In this investigation, the

activity of CYP2E1 was significantly increased in experimentally induced NASH, which agrees with the previous report[25,26].

Immunoblot analysis revealed the low levels of CYP2E1 in the experimental NASH treated with pioglitazone, quercetin and hydroxy citric acid NASH animals (Figure 1). Mild decrease in the levels of CYP2E1 level was observed in experimental NASH treated with pioglitazone (group 6; NASH+pioglitazone) compared to NASH group (group 2). This could be due to the reduction of insulin resistance in pioglitazone treated rats and this observation of our present work was supported by the other reports[27]. Insulin resistance and CYP2E1 expression may be interrelated through the ability of CYP2E1-induced oxidant stress to impair hepatic insulin signaling[8]. Hydroxy citric acid also showed mild decrease in the levels of CYP2E1 in experimental NASH treated with hydroxy citric acid (group 8; NASH+HCA). This effect could be attributed to the little antioxidant property of the hydroxy citric acid[28].

On contrary to the action of pioglitazone and hydroxy citric acid, quercetin showed an approximate 2-fold decrease in the level of CYP2E1 in experimental NASH treated with quercetin (group 7; NASH+quercetin) compared to NASH group (group 2). The present findings were supported by various other researches on quercetin's ability to reduce CYP2E1 levels[29]. The CYP2E1 levels were significantly increased in NASH group when compared to controls ($p < 0.001$ compared to control group; Figure 2). After treatment with pioglitazone and HCA the CYP2E1 levels were significantly decreased when compared to the NASH group ($p < 0.05$ compared to control group; Figure 2), where as the CYP2E1 levels were significantly decreased and reached to mere normal when treated with quercetin, compared to NASH group ($p < 0.001$ compared to NASH group; Figure 2), confirming the fact that quercetin offers maximum protection against NASH when compared to pioglitazone and hydroxyl citric acid (HCA).

This significant effect of quercetin on the levels of CYP2E1 could be attributed to the powerful antioxidant property of the quercetin, which achieves this effect by reducing the lipid peroxidation and, increasing activities of the antioxidant system on oxidative stress[30-33]. Quercetin's chemical structure enables it to scavenge oxygen-centered free radicals, the reactive oxygen species (ROS) in the body that participate in oxidative reactions that cause cell damage[30,34,35].

Conclusions

Being a powerful antioxidant, quercetin offers absolute protection to liver against NASH by reducing the levels of CYP2E1 and thereby reducing CYP2E1 mediated oxidative stress, which is believed to be the one of the key factor in the pathogenesis of NASH. On the other hand, pioglitazone and quercetin exerted limited effect on the levels of CYP2E1.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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