Effect of pioglitazone, quercetin and hydroxy citric acid on extracellular matrix components in experimentally induced non-alcoholic steatohepatitis

Surapaneni Krishna Mohan [1*], Vishnu Priya Veeraraghavan [2], Mallika Jainu [3]

[1] Department of Biochemistry, Saveetha Medical College and Hospital, Faculty of Medicine, Saveetha University, Saveetha Nagar, Thandalam, Chennai – 602 105, Tamilnadu, India
[2] Department of Biochemistry, Saveetha Dental College and Hospital, Saveetha University, 162, P.H.Road, Chennai – 600 077, Tamilnadu, India
[3] Department of Biomedical Engineering, SSN Engineering College, OMR, Klavakkam, Chennai – 603 110, Tamilnadu, India

ARTICLE INFO	ABSTRACT
Article type: Short communication *Article history:* Received: Nov 24, 2014 Accepted: Apr 28, 2015 *Keywords:* Adiponectin Hyaluronic acid Hydroxy citric acid Leptin Non-alcoholic fatty liver – disease (NAFLD) Non-alcoholic– steatohepatitis (NASH) Pioglitazone Quercetin	***Objective(s):*** Non-alcoholic steatohepatitis (NASH), is an important component of Non-alcoholic fatty liver disease (NAFLD) spectrum, which progresses to the end stage liver disease, if not diagnosed and treated properly. The disproportionate production of pro- and anti-inflammatory adipokines secreted from fat contributes to the pathogenesis of NASH. In this study, the comparative effect of pioglitazone, quercetin and hydroxy citric acid on extracellular matrix (ECM) component levels were studied in experimentally induced NASH. ***Materials and Methods:*** The experimental protocol consists of using 48 male Wister rats, which were divided into 8 groups. The levels of hyaluronic acid, leptin and adiponectin were monitored in experimental NASH. ***Results:*** The experimental NASH rats treated with pioglitazone showed significant decrease in the levels of hyaluronic acid and significant increase in adiponectin levels when compared to experimentally induced NASH group, but did not show any effect on the levels of leptin. Contrary to these two drugs, viz. pioglitazone and hydroxy citric acid, the group treated with quercetin showed significant decrease in the levels of hyaluronic acid and leptin and significant decrease in adiponectin levels compared with that of experimentally induced NASH NASH group, offering maximum protection against NASH. ***Conclusion:*** Considering our findings, it could be concluded that quercetin may offer maximum protection against NASH by significantly increasing the levels of adiponectin, when compared to pioglitazone and hydroxy citric acid.

Introduction

Non-alcoholic steatohepatitis (NASH), represented by the accumulation of fat in considerably large amounts, along with inflammation of liver is an asymptomatic disease in the spectrum of non-alcoholic fatty liver disease (NAFLD) and is a severe form of NFLD, which ultimately leads to cirrhosis of the liver (1-4). The mechanism of progression from the steatosis of NAFLD to the necro-inflammatory state of NASH is poorly understood. The increased production of pro-inflammatory mediators might play an important role in the pathogenesis of NASH (5). The disproportionate production of pro- and anti-inflammatory adipokines secreted from fat contributes to the pathogenesis of NASH (6).

Hyaluronic acid (HA) is an extracellular matrix protein, often associated with various diseases that are associated with inflammation and inflammatory responses (7). HA has been considered to promote insulin resistance, which contributes both to oxidative stress and enhanced secretion of inflammatory cytokines (8), and may play a role in liver fibrosis (9).

Leptin has been proved to be a good predictor of the disease with median levels of NASH (10) and is a hormone of the fat cells as a product of the obesity gene and is involved in immunity, bone metabolism, energy balance and body weight regulation (11).

Adiponectin is an important adipokine secreted specifically by adipocytes (12). It is produced outside the liver and appears to protect the liver (12). These molecules reveal modulatory functions in systemic and hepatic inflammation (13, 14). In our previous studies, we have reported the effect of pioglitazone, quercetin, and hydroxy citric acid on the hepatic bio-markers, lipid

*Corresponding author: Surapaneni Krishna Mohan. Department of Biochemistry, Saveetha Medical College and Hospital, Faculty of Medicine, Saveetha University, Saveetha Nagar, Thandalam, Chennai – 602 105, Tamilnadu, India. email: krishnamohan.surapaneni@gmail.com

profile and lipoproteins in NASH, induced experimentally (15-17).

We have previously studied the comparative effect of pioglitazone, quercetin and hydroxy citric acid on the status of lipid peroxidation and antioxidants in experimental NASH (18). By virtue of this present study, we tried to explore the comparative effects of pioglitazone, quercetin, and hydroxy citric acid on the levels of HA, leptin and adiponectin, the major components of extracellular matrix (ECM) 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 (18, 19), and this model was used to conduct a comparative study of the effects of the three drugs namely, pioglitazone, quercetin, and hydroxy citric acid (HCA) on numerous parameters in NASH. Male Wistar rats weighing around 250 g were housed in solid-bottomed polypropylene cages under strict veterinary supervision and maintained in control rooms with a 12-hr light/12-hr dark cycle. The animals received a commercial rat diet, standard diet, or high-fat diet and water *ad libitum* according to the approved experimental protocol. This study was conducted complying 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).

Male Wistar rats were chosen for the study and the selected rats were divided into eight groups (6 each) as follows (18, 19, 20):

Group 1, Controls: The control rats received the regular standard diet for eight weeks.

Group 2, NASH: These rats were fed a high-fat diet for eight weeks to induce NASH.

Group 3, pioglitazone control: 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) dissolved in 0.5% methyl cellulose solution for the next four weeks.

Group 4, quercetin control: These rats were fed the standard diet for four weeks and were then fed the standard diet and intragastrically administered quercetin, 20 mg/kg. b.wt dissolved in 1% DMSO v/v for the next four weeks.

Group 5, hydroxy citric acid Control: These rats were fed the standard diet for four weeks and were then fed the standard diet and intragastrically administered hydroxy citric acid (dose of 150 mg/kg. b.wt) for the next four weeks.

Group 6, NASH + pioglitazone: 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 in 0.5%methyl cellulose solution) for the next four weeks.

Group 7, NASH + quercetin: 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% DMSO v/v for the next four weeks.

Group 8, NASH + hydroxy citric acid: 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 following 12 hr of fasting by cervical decapitation method. The collected blood was centrifuged and the serum was stored at −70 °C until various biochemical analyses were conducted.

HA levels were assayed (measured) according to Stryer *et al* (21). The levels of Leptin were assayed (measured) by the method of Maffei *et al* (22) and the Adiponectin levels were measured by the method of Tsao *et al* (23).

Results

Histopathological studies after the ingestion of the high-fat diet for 8 weeks revealed all the prominent characteristic features of NASH in humans (18). Treatment with the selected drugs alone did not cause any deleterious effects and did not alter the normal metabolism. Inflammation with no fatty degeneration was observed on treatment with pioglitazone (18) and local hepatocyte necrosis with inflammatory collections was seen on treatment with hydroxy citric acid (18). But, hepatocytes appear normal (without any fatty infiltration and inflammatory changes) upon treatment with quercetin (18).

The levels of HA and leptin were increased significantly in experimentally induced NASH group (group 2) compared to control group (group 1); whereas adiponectin, an important adipokine decreased significantly in experimentally induced NASH group compared to control group (group 1). The patterns of HA, leptin and adiponectin were shown in Table 1. The experimental NASH rats treated with pioglitazone (group 6; NASH+pioglitazone) showed significant decrease in the levels of HA and significant increase in adiponectin levels when compared to experimentally induced NASH group (group 2) but did not show any change in leptin levels, compared to experimentally induced NASH group (group 2). The experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) did not show any change in any of the three extracellular components viz. Leptin, HA and adiponectin.

Contrary to these two drugs, viz. pioglitazone and hydroxy citric acid, the experimental NASH rats

Table 1. Effects of pioglitazone, quercetin and hydroxy citric acid on the levels of extracellular matrix (ECM) components in experimental nonalcoholic steatohepatitis (NASH) rats

Parameter	Group 1: Control	Group 2: NASH	Group 3: Pioglitazone Control	Group 4: Quercetin Control	Group 5: HCA Control	Group 6: NASH+ Pioglitazone	Group 7: NASH+ Quercetin	Group 8: NASH + HCA
Hyaluronic acid (ng/ml)	43.6±2.9	82.8±7.1[*]	49.5±3.4	42.1±2.6	70.4±5.8[#]	60.9±4.4[c]	57.4±2.8[b]	80.4±6.7
Leptin (ng/ml)	34.6±2.2	48.5±3.7[*]	40.8±2.9	33.9±3.1	34.1±2.4	47.1±2.9	37.3±3.3[c]	40.3±2.6
Adiponectin (mg/l)	9.77±0.55	4.13±0.23[*]	10.1±0.91	10.5±0.85	9.6±0.74	7.9±0.71[a]	8.3±0.63[a]	4.37±0.39

Values are mean ± SEM for 6 animals in each group. [*]$P<0.001$ compared to control group; [a]$P<0.001$ compared to NASH group; [b]$P<0.01$ compared to NASH group; [c]$P<0.05$ compared to NASH group; [#]$P<0.001$ as compared to control group; HCA: hydroxy citric acid; NASH: Non-alcoholic steatohepatitis

treated with quercetin (group 7; NASH+quercetin) showed significant decrease in the levels of HA and leptin compared with that of NASH induced group (group 2) and significant decrease in adiponectin levels compared with that of NASH induced group (group 2), providing maximum protection against NASH, compared to the effects of pioglitazone and hydroxyl citric acid. The animals that were fed with the standard diet with accompanying pioglitazone (group 3; pioglitazone control) or with quercetin (group 4; quercetin control) did not show any significant change in the levels of ECM components viz. hyaluronic acid, leptin and adiponectin compared to the control group (group 1). On the other hand, rats fed with the standard diet and simultaneously with hydroxy citric acid (group 5; HCA control) did not show any significant change in the levels of leptin and adiponectin but showed significant increase in HA levels when compared to the control group (group 1).

Discussion

Fibrosis is a dynamic process that may result in increased levels of circulating components of ECM. This hypothesis formed the basis for several researchers to develop different diagnostic tests using individual ECM components or a composite of ECM components. Suzuki *et al* (2005) determined the reliability of serum HA to predict the severity of hepatic fibrosis in patients with histologically confirmed NAFLD (24). Fibrosis assessment is crucial in NASH because it represents an advanced stage of liver injury. Transforming growth factor β (TGF- β), HA, metalloproteinase inhibitors, and other matrix components were evaluated by several researchers in several studies (25).

HA and tissue inhibitor of metalloproteinase-1 (TIMP-1) are reliable markers of liver fibrosis and are closely linked to the proinflammatory status (26). HA is one of the important inflammatory markers, shown to be elevated in patients with NASH (27). Evaluation of the HA levels was found to be useful for predicting severe fibrosis in patients with NASH (24).

In obese or NASH subjects, serum leptin level is significantly increased. Conversely, the leptin receptor (Ob-Re) level is down-regulated, thus leading to resistance of leptin (28). Leptin was shown to have its involvement in the etiology and pathogenesis of NAFLD, due to its close relationship with adipose tissue metabolism and body's fat stores (29). Leptin has a pro-inflammatory role and is considered to be an essential mediator of liver fibrosis (30).

Adiponectin is an antidiabetic and antiathero-genic polypeptide which is strongly correlated with systemic insulin sensitivity in humans (31). Adiponectin increases fatty acid beta oxidation in muscle (32), improves post-absorptive insulin mediated suppression of hepatic glucose output by enhancing hepatic insulin action (33) and decreases lipid accumulation in macrophages. Adiponectin also has direct anti-inflammatory effects, attenuates oxidative stress and pro-inflammatory cytokine production and ameliorates liver fibrosis via suppression of activated hepatic stellate cell function (34). Therefore, low levels of circulating adiponectin may be associated with the development of NASH in patients with steatosis and in the progression of NASH towards cirrhosis.

To analyze the effect of the drugs alone (pioglitazone, quercetin and hydroxyl citric acid) on the liver and on normal metabolic activities, we have chosen three groups as "drug controls". The rats in these drug control groups, that were fed with standard diet and simultaneously with pioglitazone (group 3; pioglitazone control) or with quercetin (group 4; quercetin control) did not show any significant change in the levels of ECM components viz. hyaluronic acid, leptin and adiponectin compared to control group (group 1). On the other hand, rats fed with standard diet and simultaneously with hydroxy citric acid (group 5; HCA control) did not show any significant effect on the levels of leptin and adiponectin but showed significant increase in HA levels when compared to controls (group 1). No significant metabolic alterations in ECM were observed in rats that were present in these three drug control groups. All these three drugs viz. pioglitazone, quercetin and hydroxyl citric acid did

not produce any significant alterations in the levels of ECM components as evidenced by Table 1.

The experimental NASH rats treated with pioglitazone (group 6; NASH+pioglitazone) showed significant decrease in the levels of HA and significant increase in the levels of adiponectin levels when compared to experimentally induced NASH group (group 2) but did not show any change in the leptin levels, when compared to the NASH group (group 2). The reduction in hepatic steatosis after pioglitazone treatment is inversely correlated with the elevated levels of adiponectin in plasma (35). Adiponectin was established as a major insulin-sensitizing adipokine (36) and is a plausible candidate for one of the adipokines that may mediate the pioglitazone induced amelioration of insulin resistance (36).

Pioglitazone can improve blood glucose and lipid levels without the involvement of Tumour Necrosis Factor-α (TNFα) (37). Thiazolidinediones have been shown to up-regulate adiponectin expression in white adipose tissue and levels of adiponectin in plasma (37, 38). These mechanisms were put forward as the major mechanisms of the amelioration of insulin resistance induced by thiazolidinedione (37, 38). In this study, we have successfully demonstrated the amelioration of insulin resistance induced by pioglitazone.

The experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) did not show any change in any of the three extracellular components (adiponectin, HA and leptin). The disproportionate production of pro- and anti-inflammatory adipokines secreted from fat contributes to the pathogenesis of NASH. But hydroxy citric acid did not show any effect on the decreased levels of adiponectin. This clearly indicates that hydroxy citric acid offers less protection against NASH.

Contrary to these two drugs viz. pioglitazone and hydroxy citric acid, the experimental NASH rats treated with quercetin (group 7; NASH+quercetin) showed significant decrease in the levels of HA and leptin compared with the NASH induced group (group 2) and significant decrease in adiponectin levels compared with the NASH group (group 2), conferring maximum protection against NASH, when compared with pioglitazone and hydroxy citric acid. Quercetin may increase adiponectin levels possibly by improving the secretion of adiponectin from adipose tissue, which is known to inhibit hepatic fatty acid synthesis, gluconeogenesis and de novo synthesis of lipids to improve insulin resistance. Adiponectin offers protection to the liver through the inhibition of steatogenesis and fibro genesis at multiple levels viz. attenuation of oxidative stress and production of pro-inflammatory cytokines (28, 39, 40).

Conclusion

Based on our findings in the present study, it could be inferred that quercetin may offer maximum protection against NASH by virtue of increasing the levels of adiponectin significantly, when compared to pioglitazone and hydroxy citric acid.

Acknowledgment

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