Effect of pioglitazone, quercetin and hydroxy citric acid on inflammatory markers in experimentally induced non alcoholic steatohepatitis (NASH).

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ABSTRACT

Background: Non Alcoholic Fatty Liver Disease (NAFLD) spectrum comprises of diseases ranging from simple steatosis to steatohepatitis. Non-Alcoholic Steatohepatitis (NASH), an asymptomatic disease, characterized by the fatty infiltration with inflammation is a key component of NAFLD spectrum that may proceed to the liver cirrhosis, the end stage liver disease, if not diagnosed and treated properly. Inflammatory mediators have been investigated as potential diagnostic tools to understand the pathogenesis of NASH. In this study, the comparative effect of pioglitazone, quercetin and hydroxy citric acid on the levels of inflammatory markers in experimentally induced NASH has been studied. **Methods:** The experimental protocol consists of 48 Male Wister rats, which were divided into 8 groups. The levels of tumor necrosis factor-α (TNF-α) and myeloperoxidase (MPO) were estimated in experimental NASH. **Results:** A significant increase in the levels of inflammatory markers such as tumor necrosis factor-α (TNF-α) and myeloperoxidase (MPO) was noticed in experimental NASH rats as compared to control group. On treatment with pioglitazone and quercetin, they showed significant decrease in the levels of TNF-α and MPO. Whereas, on treatment with hydroxy citric acid, no significant effect on the levels of inflammatory markers viz. TNF-α and MPO were observed. **Conclusion:** By virtue of our findings, it could be inferred that Quercetin and pioglitazone offers protection against NASH by ameliorating the inflammation (hepatitis), a principle and key feature of NASH, whereas hydroxy citric acid offers very little protection against NASH.

Key words: Pioglitazone, Quercetin, Hydroxy citric acid, TNF- α, myeloperoxidase, Non-Alcoholic Fatty Liver Disease (NAFLD), Non-Alcoholic Steatohepatitis (NASH).

INTRODUCTION

Non Alcoholic Fatty Liver Disease (NAFLD) represents a spectrum of disease ranging from hepatocellular steatosis through steatohepatitis to fibrosis and irreversible cirrhosis. The prevalence of NAFLD has risen rapidly in parallel with the dramatic rise in obesity and diabetes.[1,2] Non-Alcoholic Steatohepatitis (NASH) is a form of metabolic liver disease in which, steatosis is associated with lobular inflammation, hepatocyte injury and/or hepatic fibrosis.[3,4] NASH typically causes no symptoms. When present, clinical features such as fatigue, hepatomegaly and aching hepatic discomfort and most of them are non-specific.[5] In 20–25% of cases, NASH may progress to advanced stages of hepatic fibrosis, cirrhosis and liver failure then becomes the most common cause of death.[6-10] Occasionally, hepatocellular carcinoma (HCC) may also occur. The mechanism of progression from the steatosis of NAFLD to the necro-inflammatory state of NASH is poorly understood. However, inflammatory mediators have also been investigated as potential diagnostic tools.

Submission Date : 27-11-2014
Revision Date : 23-12-2014
Accepted Date : 07-01-2015

DOI: 10.5530/ijper.49.2.12

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It is important to study the tumor necrosis factor alpha (TNF-*a*) and Myeloperoxidase (MPO) levels in NASH, since the imbalance of these important inflammatory markers may play an important role in the development of NASH. TNF-α, a cytokine is a pro-inflammatory mediator and plays a distinct role in hepatocyte inflammation and cell death.[11] Also, it is one of the major pro-inflammatory cytokines involved in the pathogenesis of chronic inflammatory diseases and is modulated by oxidative stress.[12] TNF-α also triggers the cellular release of other cytokines, chemokines, or inflammatory mediators and displays antiviral and antimicrobial effects. MPO possesses potent antimicrobial activity and is an indicator of neutrophilic degranulation and was reported to play a crucial role in tissue injury.[13,14]

In our previous studies we have reported the effect of pioglitazone, quercetin, and hydroxy citric acid (HCA) on the hepatic bio markers, lipid profile and lipoproteins, cytochrome P450 2E1 (CYP2E1) in experimentally induced non-alcoholic steatohepatitis (NASH).[15-17]

We have studied the comparative effect of pioglitazone, quercetin and hydroxy citric acid on the status of lipid peroxidation and antioxidants in experimental non-alcoholic steatohepatitis.[18] This study explores the comparative effects of pioglitazone, quercetin, and hydroxy citric acid on the levels of TNF-α and MPO, the major inflammatory markers 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 role of pioglitazone, quercetin, and hydroxy citric acid on various inflammatory marker 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:[18-20]

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% 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% 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 conducted.

The levels of tumor necrosis factor - α (TNF-α) was assayed by the method as described by Konturek et al[21] as described by Konturek *et al* and The myeloperoxidase (MPO) activity was assayed by the method as described by Krawisz *et al.*[22]

RESULTS

Histopathological studies after the ingestion of the high-fat diet for 8 weeks revealed all the prominent characteristic features of NASH including steatosis, inflammation, which mimics the NASH in humans.[18] The treatment with the selected drugs of choice alone doesn't 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

Table 1: Effect of Pioglitazone, Quercetin and Hydroxy Citric Acid on the Levels of Inflammatory Markers in Experimental nash.

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
TNF-alpha (ng/ml)	8.75±0.51	12.14±1.0*	8.83±0.71	8.51±0.69	9.01±0.82	9.14±0.8[c]	8.02±0.5[b]	9.64±0.7
Plasma MPO (ng/ml)	40.6±3.8	59.3±4.3*	39.2±2.6	41.5±3.8	43.6±4.1	46.9±3.5[c]	44.3±3.2[b]	53.2±4.9

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

acid.[18] But, hepatocytes appear mere normal with no obvious fatty and inflammatory changes on treatment with quercetin.[18]

Inflammatory mediators have also been investigated as potential diagnostic tools. NASH was associated with an increase in tumor necrosis factor alpha (TNF-α) and MPO levels and the imbalance may play an important role in the development of NASH. The pattern of inflammatory markers has been depicted in the Table-1 and Figure 1-2. A significant increase in the levels of inflammatory markers such as tumor necrosis factor-α (TNF-α) and myeloperoxidase (MPO) was noticed in experimental NASH rats (group 2) as compared to control group (group 1). The experimental NASH rats treated with pioglitazone (group 6; NASH+pioglitazone) and rats treated with quercetin (group 7; NASH+quercetin) showed significant decrease in the levels of TNF-α and MPO. Whereas, the experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) does not show any significant effect on the levels of inflammatory markers viz. TNF-α and MPO. Rats fed with standard diet simultaneously with pioglitazone (group 3; pioglitazone control), with quercetin (group 4; quercetin control) and with hydroxy citric acid (group 5; HCA control) does not show any significant effect on the inflammatory markers compared to control group (group 1).

DISCUSSION

A significant increase in the levels of inflammatory markers such as tumor necrosis factor-α (TNF-α) and myeloperoxidase (MPO) was noticed in experimental NASH rats (group 2) as compared to control group (group 1). The enhanced levels of TNF-α in NASH in turn activates specific redox sensitive kinases.[23] These activated specific redox sensitive kinases up regulate the pro-inflammatory pathways resulting in the enhanced insulin resistance which is believed to play a key role in the pathogenesis of NASH.[23]

Cytokine production is increased in nonalcoholic steatohepatitis and is believed to play a role in its pathogenesis. In the liver, tumor necrosis factor-alpha can contribute to oxidative stress[24,25] and may contribute

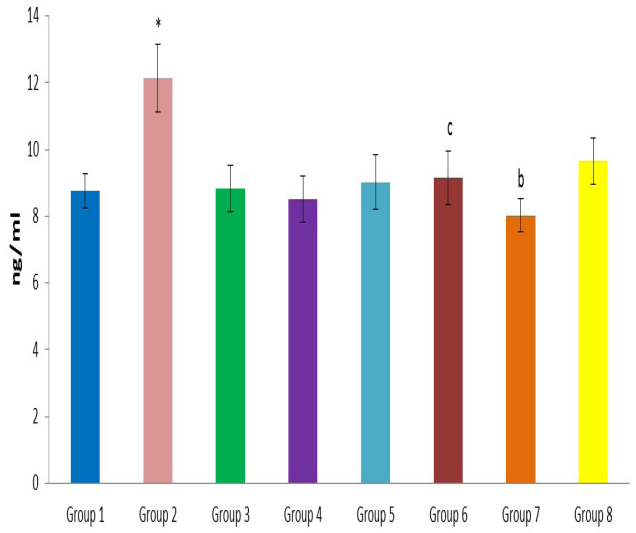

Figure 1: Effect of Pioglitazone, Quercetin and Hydroxy Citric Acid on the Levels of Tumor Necrosis Factor-Alpha (Tnf-α) In Experimental NASH.

*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

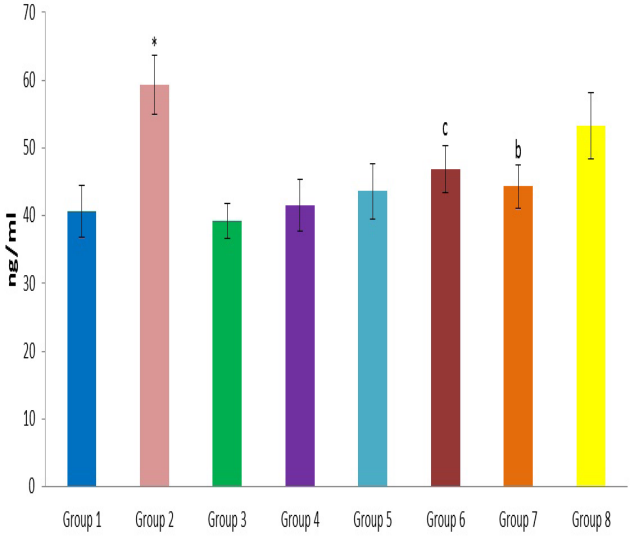

Figure 2: Effect of Pioglitazone, Quercetin and Hydroxy Citric Acid on the Levels of Plasma Myeloperoxidase (MPO) In Experimental NASH.

*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

to insulin resistance through activation of the inhibitor of kappa kinase beta.[23] It is suggested that the cytokine TNF-α can be inhibited by quercetin, which may be of clinical significance in host defense mechanisms against various infections.[26]

Myeloperoxidase (MPO) is an important neutrophil enzyme that can generate aggressive oxidants.[27] One of the principal molecules released after recruitment and activation of phagocytes is myeloperoxidase (MPO), an important enzyme involved in the generation of reactive oxygen species.[13]

Inflammation and oxidative stress are considered critical factors in the progression of nonalcoholic fatty liver disease.[17,18,28] In the presence of physiological chloride concentrations, MPO reacts with hydrogen peroxide (H_2O_2, formed by the respiratory burst) to catalyze formation of hypochlorous acid/ hypochlorite and other oxidizing species.[13,27] These oxidants may contribute to host tissue damage at sites of inflammation through reactions with a wide range of biological substrates, including DNA, lipids, and protein amino groups.[29] In the absence of physiological chloride concentrations, the MPO-H_2O_2 system can also form reactive nitrogen species[30] that may initiate lipid peroxidation. Interestingly, macrophages are known to generate high amounts of reactive oxygen and nitrogen species. Therefore, MPO-containing macrophages/ Kupffer cells could play a role in NASH.[31] MPO activity might be a driving factor underlying the progression of human NASH.[32,33]

To analyze the effect of pioglitazone, quercetin and hydroxy citric acid alone on the liver and on normal metabolic activities, we have chosen to have three drug control groups. Rats fed with standard diet simultaneously with pioglitazone (group 3; pioglitazone control), with quercetin (group 4; quercetin control) and with hydroxy citric acid (group 5; HCA control) does not show any significant effect on the inflammatory markers compared to control group (group 1). All the three drugs did not produce any significant alterations in the levels of inflammatory markers as evidenced by the Table-1 and Figure 1-2.

The experimental NASH rats treated with pioglitazone (group 6; NASH+pioglitazone) and rats treated with quercetin (group 7; NASH+quercetin) showed significant decrease in the levels of TNF-α and MPO. Pioglitazone stimulate maturation of visceral fat, and hence change the adipocytokine profile secreted by adipose tissue. Pioglitazone leads to an increase in adiponectin levels, which counteracts pro-inflammatory cytokines such as tumor necrosis factor-α (TNF α) and promotes beta oxidation of fatty acids via adenosine monophosphate-activated protein kinase (AMP-K) activation.[34,35] The increase in beta oxidation, immature adipose tissue in conjunction with a reduction in de novo lipogenesis, decreases gluconeogenesis.[36,37] Quercetin is an effective inhibitor of human myeloperoxidase (MPO) activity, both with purified enzyme and in a system using stimulated human neutrophils. Moreover, quercetin is directly able to scavenge hypochlorous acid (HOCl), a chlorinated species generated by the, MPO/H2O2/Cl– system.[38]

In this present study, quercetin showed protective effect against NASH by significant reduction in inflammatory markers due to its anti-inflammatory activity. Quercetin inhibits TNF-α and IL-6, simultaneously induces IL-10 release, and thus evokes the anti-inflammatory effect.[39] Quercetin also inhibits STAT-1 and NF-κB and thus possesses the anti-inflammatory effect.[40] Quercetin's anti-inflammatory activity may also have a beneficial effect on the muscle damage experienced by athletes after intense exercise.[41] It has been demonstrated that quercetin can inhibit nuclear factor-kappa B (NF-κB),[41] a chemical in the body which has been shown to play a central role in regulating the immune response to inflammation. Cell culture studies and in vivo (animal) studies have provided evidence supporting quercetin's anti-inflammatory effects.[41]

Whereas, the experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) does not show any significant effect on the levels of inflammatory markers viz. TNF-α and MPO. Hydroxy citric acid which is a phytoconstituent present in the *Garcinia Indica* or in general *Garcinia species*, and is a proven anti obesity agent with the lipid lowering actions.[42-44] It's been well established as a lipid lowering agent and Hypocholesterolemic agent.[42-44] Recent research showed that it also possesses little anti-inflammatory activity, apart from these two properties[45]. But, in the present study the experimental NASH rats treated with hydroxy citric acid (group 8; NASH+HCA) does not show any significant effect on the levels of inflammatory markers, showing that HCA does not possess any anti-inflammatory action. Due to this, HCA can confer very less protection against NASH, since inflammation of the liver (hepatitis) is one of the principle and key features of NASH.

CONCLUSION

TNF-α and MPO have long been recognized for their pro-inflammatory properties and their role in NASH progression was clearly established. TNF-α and MPO were highly expressed in experimentally induced NASH group, compared to control group. Quercetin and pioglitazone offers protection against NASH by ameliorating the inflammation (hepatitis), a principle and key feature of NASH, whereas hydroxy citric acid offers very little protection against NASH.

CONFLICTS OF INTEREST

Authors declared no conflicts of interest

ACKNOWLEDGEMENTS

The Authors are very much thankful to Dr.S.Porchelvan, M.Sc., M.B.A., PGDCA.,PhD., Professor in Biostatistics, Saveetha Medical College & Hospital, Saveetha University for assisting us in performing the statistical analyses.

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