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COMPARATIVE EFFECT OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID ON THE STATUS OF LIPID PEROXIDATION AND ANTIOXIDANTS IN EXPERIMENTAL NON-ALCOHOLIC STEATOHEPATITIS

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Oxidative stress is one of the key mechanisms responsible for liver damage and disease progression in non-alcoholic steatohepatitis (NASH). Antioxidants try to combat the oxidative stress and minimise the oxidative stress. In this study, the comparative effect of pioglitazone, quercetin and hydroxy citric acid on lipid peroxidation and antioxidants in experimentally induced NASH has been studied. The levels of lipid peroxidation products, malondialdehyde (MDA), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) were estimated in experimental NASH. The levels of lipid peroxidation products (MDA) have been significantly increased in experimentally induced NASH group compared to the control group. The experimental NASH rats treated with pioglitazone, quercetin and with hydroxy citric acid showed significant reduction in MDA levels when compared with that of NASH induced group. Non enzymatic antioxidant such as GSH and antioxidant enzymes such as catalase, SOD, GPx, GR, and GST were decreased significantly in experimental NASH, compared to that of control group. The experimental NASH rats treated with pioglitazone showed marked increase in the levels of GSH, catalase and SOD but no significant effect on the levels of GPx, GR and GST levels when compared to the experimentally induced NASH group. Experimental NASH rats treated with hydroxy citric acid showed marked increase in the levels of GSH and catalase but no significant effect on the levels of SOD, GPx, GR and GST levels when compared to the experimentally induced NASH group. On contrary to these two drugs, the experimental NASH rats treated with quercetin showed significant increase in the levels of antioxidants as follows: GSH, catalase, SOD, GPx, GR and GST when compared with that of NASH induced group. We concluded that the levels of non-enzymatic and enzymatic antioxidants found to be decreased in experimentally induced NASH. The antioxidant property of quercetin could be more beneficial in treatment of NASH when compared to pioglitazone and hydroxy citric acid.

Key words: *pioglitazone, quercetin, hydroxy citric acid, oxidative stress, antioxidants, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis*

INTRODUCTION

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 (1-4). The mechanism of progression from the steatosis of NAFLD to the necro-inflammatory state of NASH is poorly understood (5). However, it is hypothesized that increased production of pro-inflammatory mediators probably plays an important role in the pathogenesis of NASH (6). Reactive oxygen species (ROS) also stimulate tumor necrosis factor (TNF- α) expression and direct lipid peroxidation of mitochondrial membranes (7, 8). ROS formation also increased proportionately for a given level of fatty acid oxidation and worsens the oxidative stress (9). In addition to the formation of excess ROS, increased microsomal activity and peroxisomal fatty acid oxidation further worsens the oxidative stress. This could function as another source of oxidative stress in the individuals with iron overload (10).

From the pathophysiological point of view, insulin resistance, imbalance of adipocytokines, a marker of inflammation (IL-6), a marker of necrosis and apoptosis (M65) play a key role in development of NASH and, therefore, biochemical markers of these events was studied by Grigorescu *et al.* (11). Mitochondria, the main source of reactive oxygen species may trigger steatohepatitis and fibrosis by enhancing the lipid peroxidation and induction of cytokines (12). Since oxidative stress seems to play an essential role in the development and consequences of NASH, the effectiveness of several antioxidant agents such as vitamin E, vitamin C, betaine and melatonin have been evaluated (11, 13). Increased levels of antioxidant may serve a protective role against development of hepatic steatosis (14, 15).

Very little information is known on the role of pioglitazone, quercetin and hydroxy citric acid on oxidative stress and antioxidant status in NASH. This study explores the comparative effect of pioglitazone, quercetin and hydroxy

citric acid on lipid peroxidation and antioxidant status in NASH model has been studied.

MATERIALS AND METHODS

This study conformed to the guiding principles of Institutional Animal Ethical Committee (IAEC), Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Guide for the care and use of laboratory animals (IAEC Approval Number: 001/006/2010 & 01/007/2011).

Animals

The animals were purchased from Tamil Nadu University of Veterinary and Animal Sciences, Chennai. Male Wistar rats weighing approximately 250 g were housed in solid-bottomed polypropylene cages under strict veterinary supervision and maintained in control rooms with 12 hours light/dark cycle. The animals received, standard diet, high-fat diet and water *ad libitum* as per the experimental protocol. The experimental model of NASH in rats by feeding high fat diet for 8 weeks (16) and this model was used to conduct a comparative study of role of pioglitazone, quercetin and hydroxy citric acid on various parameters in experimental model of non-alcoholic steatohepatitis.

Composition of the diet

Experimental NASH was established according to the model of Rivera *et al.* (17) with slight modifications. Male Wistar rats, which were individually housed and fed either a standard diet with protein 20%; fat 5%; carbohydrates 5%; fiber 5% and a high-fat diet with 20% of energy derived from protein; 15% from corn oil; 50% from sucrose; 5% from fiber. The standard diet has the same fat content as the average "normal" diet available commercially purchased from Biomed scientific Products, India. The overall compositions of the standard and high fat diets are shown in *Table 1*.

The Male wistar rats selected for the study were divided into 8 groups as follows (18, 19):

Group 1: controls (n=6); control rats received regular standard diet for 8 weeks.

Group 2: NASH (n=6); rats induced with NASH by feeding high fat diet for 8 weeks.

Group 3: pioglitazone control (n=6); rats fed with standard diet for 4 weeks, then feeding them with standard diet

simultaneously with intra gastric administration of pioglitazone (4 mg/kg. b.wt) (0.5% methyl cellulose w/v) for further 4 weeks.

Group 4: quercetin control (n=6); rats fed with standard diet for 4 weeks, then feeding them with standard diet simultaneously with intra gastric administration of quercetin (20 mg/kg. b.wt) dissolved in 1% DMSO v/v for further 4 weeks.

Group 5: hydroxy citric acid control (n=6); rats fed with standard diet for 4 weeks, then feeding them with standard diet simultaneously with intra gastric administration of hydroxy citric acid (150 mg/kg. b.wt) for another 4 weeks.

Group 6: NASH + pioglitazone (n=6); rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intra gastric administration of pioglitazone (4 mg/kg. b.wt) (0.5% methyl cellulose w/v) for another 4 weeks.

Group 7: NASH + quercetin (n=6); rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intra gastric administration of quercetin (20 mg/kg. b.wt) dissolved in 1% DMSO v/v for another 4 weeks.

Group 8: NASH + hydroxy citric acid (HCA) (n=6); rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intra gastric administration of hydroxy citric acid (150 mg/kg. b.wt) for another 4 weeks.

After the experimental period, the animals were sacrificed after 12 hours of fasting by giving over dose of anesthetic drug (0.5% sodium pentobarbitone i.p.) and the liver tissues of all the experimental groups were dissected out and fixed in 10% buffered neutral formalin solution for histopathological studies for the assessment of the development of NASH. Lipid peroxide content in liver tissues was determined by thiobarbituric acid reaction as described by Okhawa *et al.*, (20). The levels of reduced glutathione (GSH) were measured according to the method of Moron *et al.*, (21). The activity of catalase was assayed by the method of Sinha (22). The activity of superoxide dismutase (SOD) was assayed by the method of Misra and Fridovich (23). The activity of glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al.*, with some modifications (24). The activity of glutathione reductase (GR) was assayed by the method of Carlberg (25). The activity of glutathione-S-transferase (GST) was determined by the method of Habig and Jackobyus (26).

Statistical analysis

All data are presented as means \pm standard errors of the mean (S.E.M.). Statistical analysis among groups was performed by one-way analysis of variance (ANOVA) followed by Dunnett's T3 comparison post-hoc test. Differences were considered statistically significant if $p < 0.05$.

Table 1. The overall compositions of the standard and high fat diets.

Standard diet (g/kg)	Composition	High fat diet (g/kg)
200	Casein	200
50	Sucrose	500
50	Dextrin	50
2.5	Cornoil	150
3	L-methionine	3
2	Choline	2
10	Cyanocobalamine	10
2.5	NaCl	2.5
5	Fibre	5
50	Fructose	5
0	Cholesterol	2

RESULTS

The results of histopathological changes in all the experimental groups have been shown in *Fig. 1A-1H*. *Fig. 1B* clearly shows that 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. *Fig. 1C-1E* showed that the treatment with drugs alone doesn't cause any deleterious effects. There observed inflammation with no fatty degeneration on treatment with pioglitazone (*Fig. 1F*) and local

hepatocyte necrosis with inflammatory collections on treatment with hydroxy citric acid (*Fig. 1H*). But, hepatocytes appear mere normal with no obvious fatty and inflammatory changes on treatment with quercetin as evident in *Fig. 1G*.

The results of oxidative stress and antioxidant levels in experimentally induced and treated NASH were shown in *Figs. 2-8*. Non enzymatic antioxidant such as GSH and antioxidant enzymes such as catalase, SOD, GPx, GR, and GST were decreased significantly in group 2 rats, compared to that of control group as shown in *Figs. 2-8*. The group 6 (NASH + pioglitazone) rats showed marked increase in the levels of GSH,

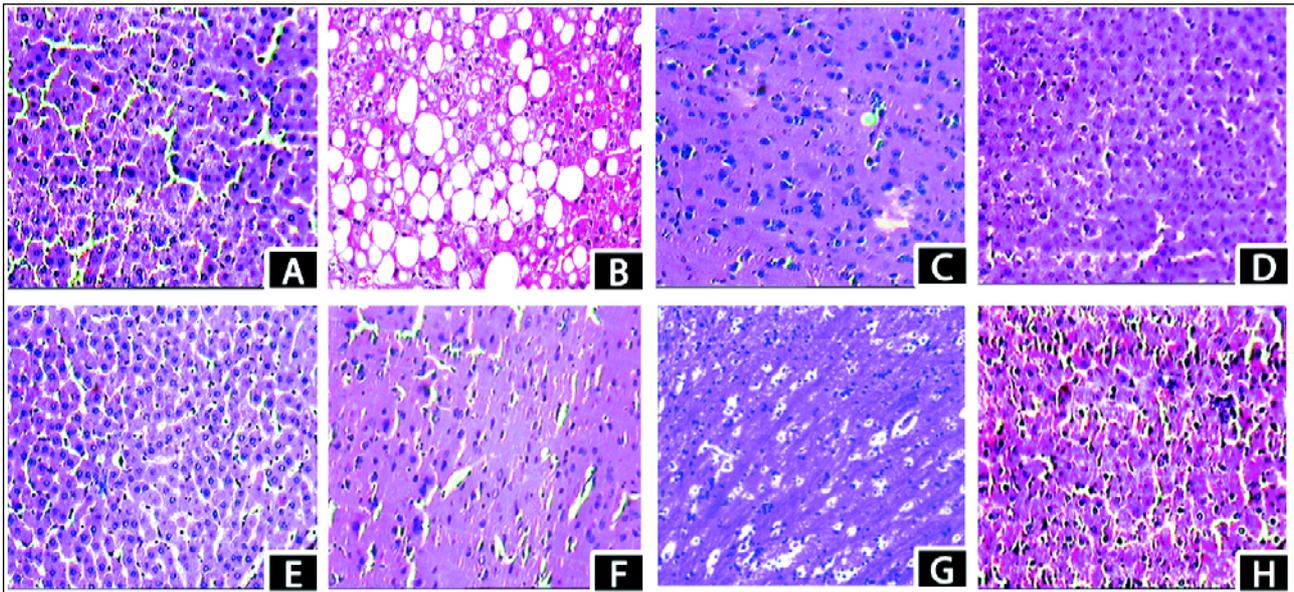


Fig. 1. Results of histopathological examination of all experimental groups.

(A): Group 1: (control with standard diet) - showed normal hepatocytes. (B): Group 2: (NASH with high fat diet) - showed steatosis, ballooning hepatocytes and diffused fatty infiltration of hepatocytes. (C): Group 3: (pioglitazone control) - showed normal hepatocytes, no pathological changes observed. (D): Group 4: (quercetin control) - showed normal hepatocytes, no pathological changes observed. (E): Group 5: (HCA control) - showed normal hepatocytes, no pathological changes observed. (F): Group 6: (NASH + pioglitazone) - showed no fatty degeneration, but inflammation was seen. (G): Group 7: (NASH + quercetin) - showed hepatocytes appear normal, no obvious fatty and inflammatory changes were seen. (H): Group 8: (NASH + HCA) - showed local hepatocyte necrosis with inflammatory collections were seen.

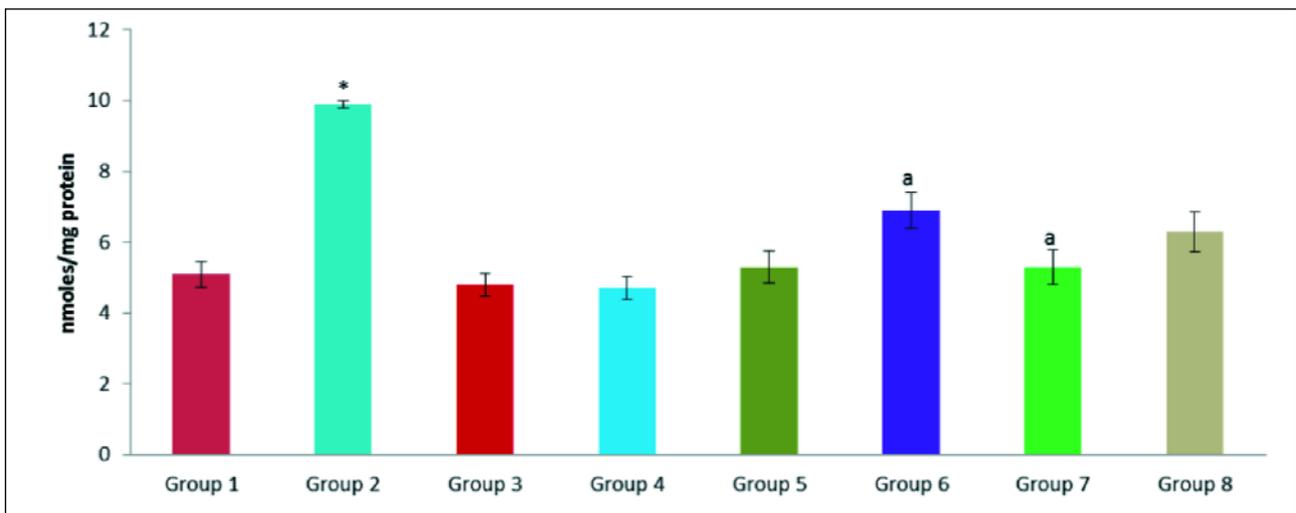


Fig. 2. Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of malondialdehyde (MDA), an index of lipid peroxidation in experimental NASH.

* $P < 0.001$ compared to control group.

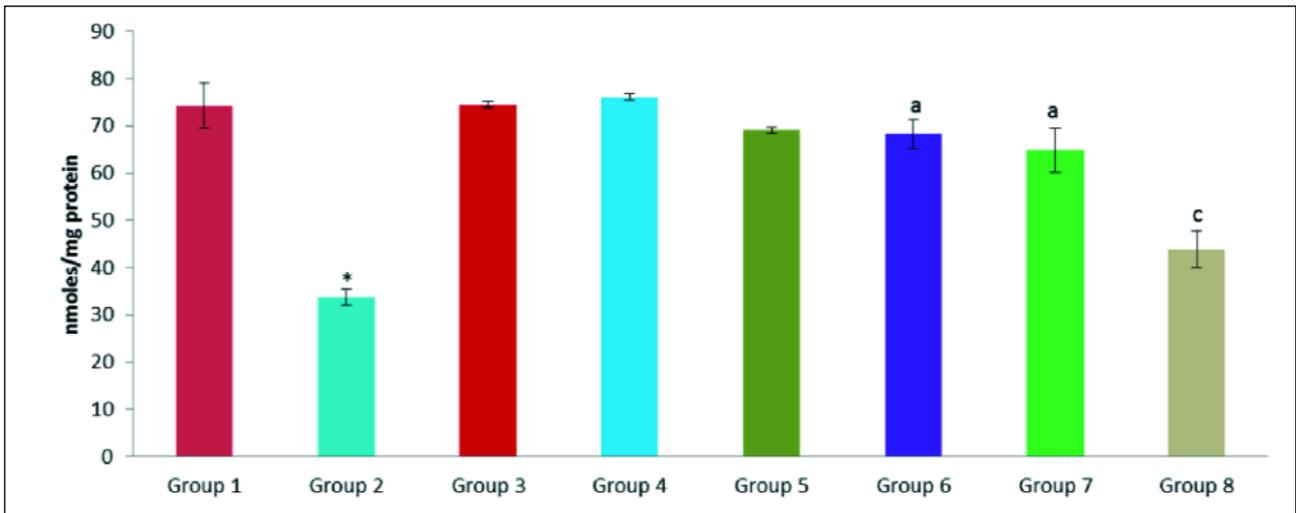


Fig. 3. Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of reduced glutathione (GSH) in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^cP<0.05 compared to NASH group.

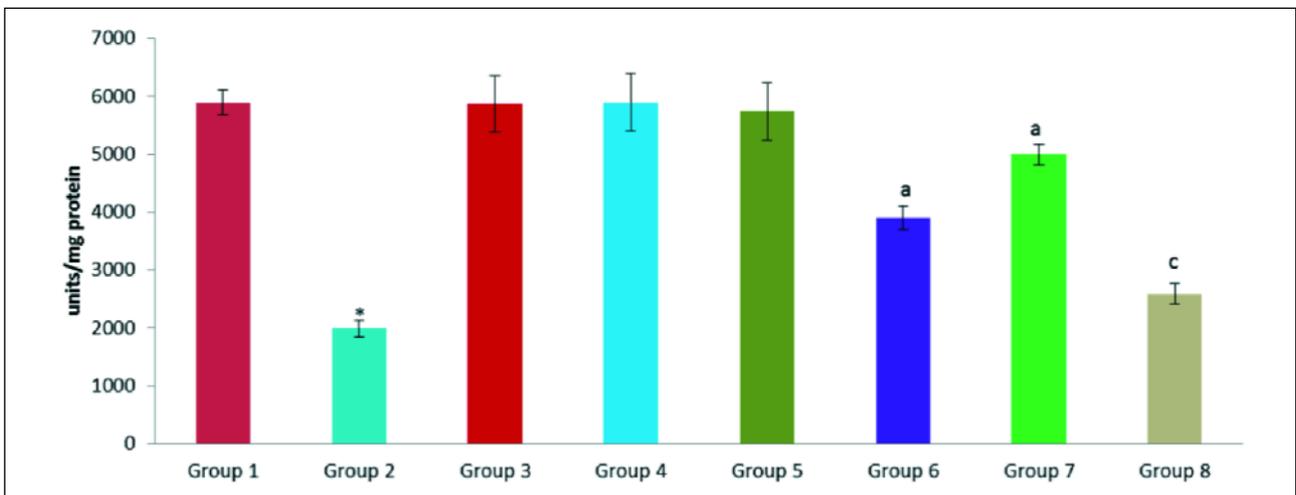


Fig. 4. Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of catalase (CAT) in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group; ^cP<0.05 compared to NASH group.

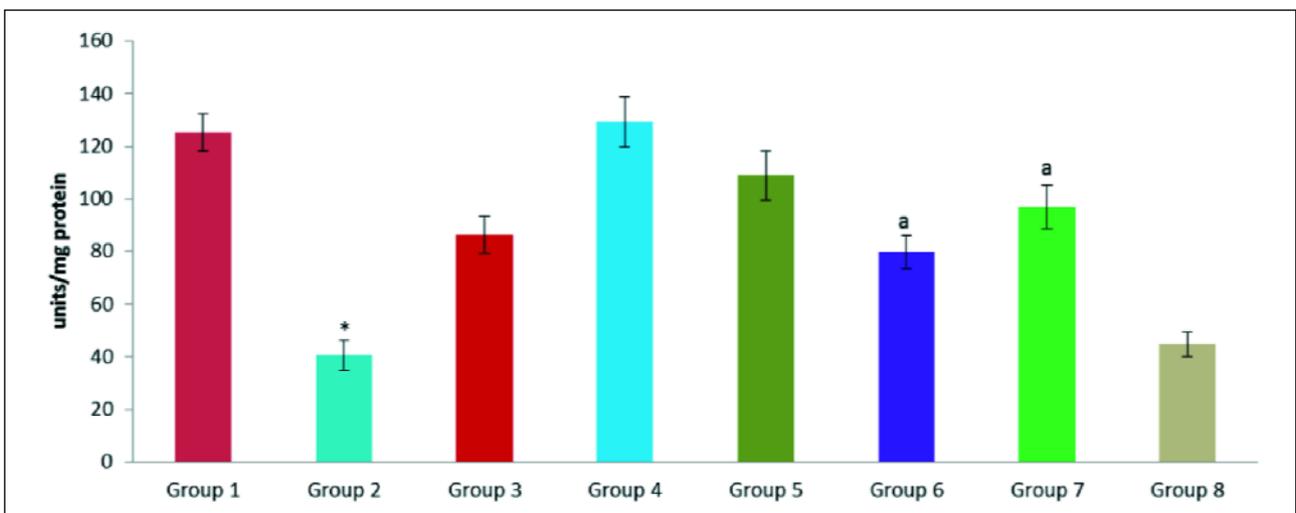


Fig. 5. Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of superoxide dismutase (SOD) in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group.

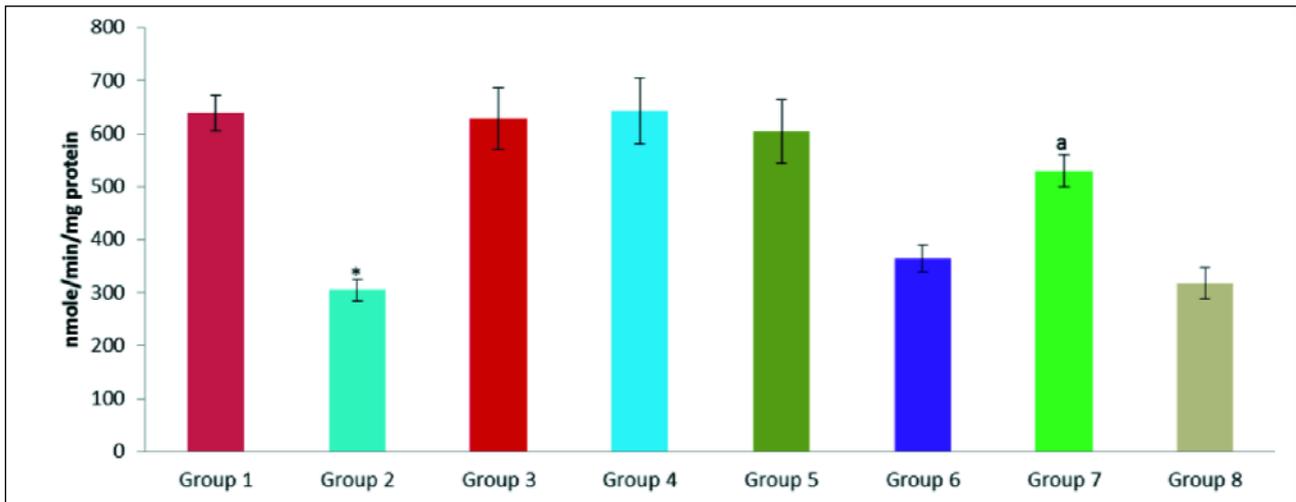


Fig. 6. Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of glutathione peroxidase (GPx) in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group.

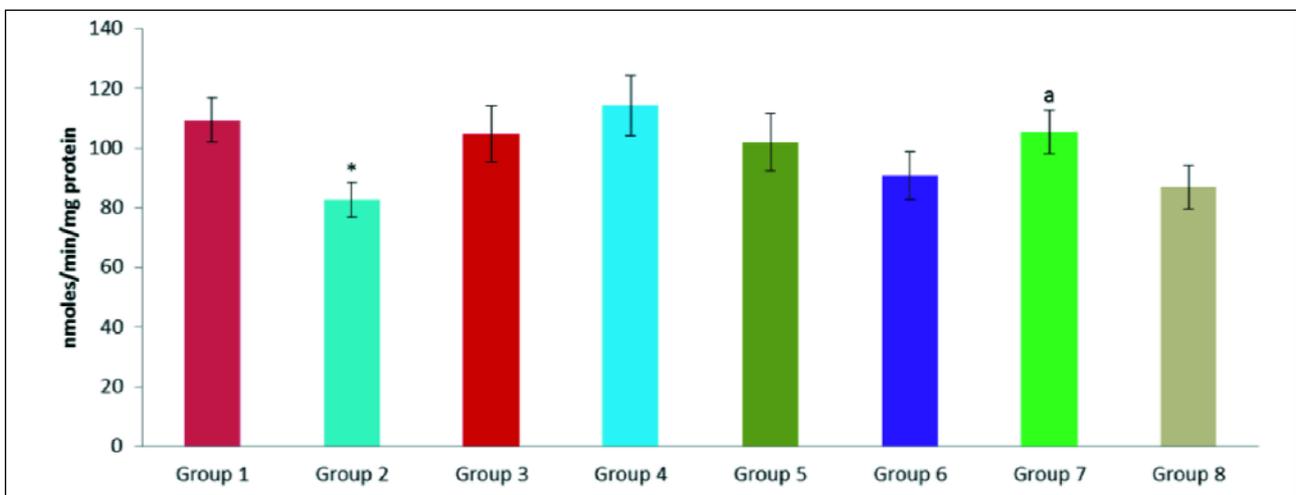


Fig. 7. Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of glutathione reductase (GR) in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group.

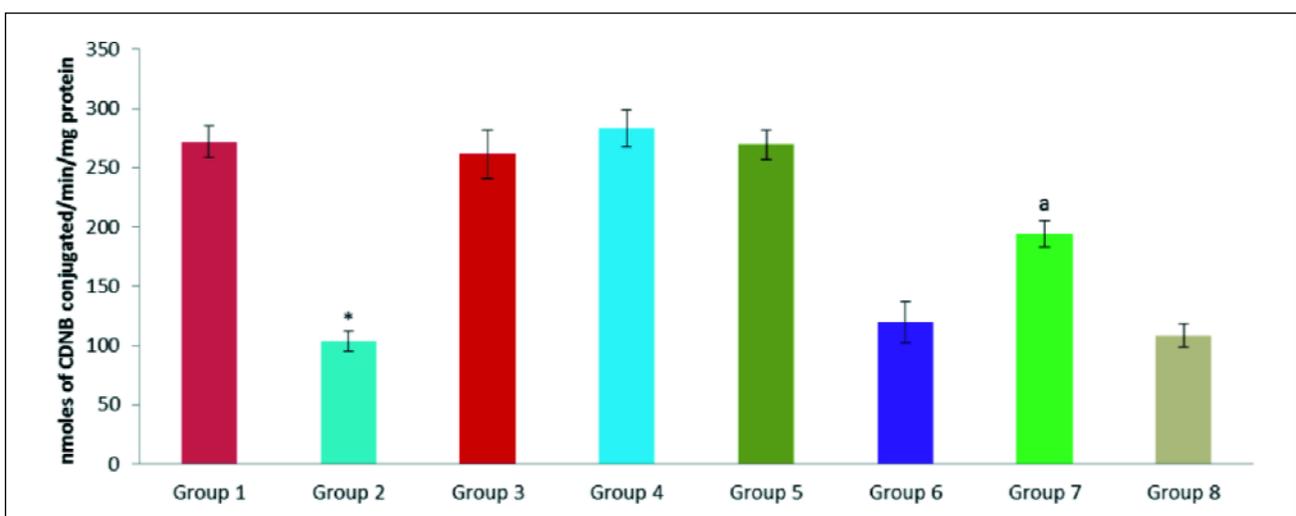


Fig. 8. Effect of pioglitazone, quercetin and hydroxy citric acid on the levels of glutathione-S-transferase (GST) in experimental NASH. *P<0.001 compared to control group; ^aP<0.001 compared to NASH group.

catalase and SOD but does not show a significant effect on the levels of GPx, GR and GST levels when compared to group 2. Similarly, experimental NASH rats treated with hydroxy citric acid (group 8: NASH + HCA) showed marked increase in the levels of GSH and catalase but does not show a significant effect on the levels of SOD, GPx, GR and GST levels when compared to the experimentally induced NASH group (group 2). On contrary to pioglitazone and hydroxy citric acid, the experimental NASH rats treated with quercetin (group 7: NASH + quercetin) showed significant increase in the levels of antioxidants viz. GSH, catalase, SOD, GPx, GR and GST when compared with that of NASH induced group (group 2) showing maximum protective effect against NASH. The levels of lipid peroxidation products have been significantly increased in experimentally induced NASH group (group 2) compared to the control group (group 1). The experimental NASH rats treated with pioglitazone (group 6: NASH + pioglitazone), with quercetin (group 7: NASH + quercetin) and with hydroxy citric acid (group 8: NASH + HCA) showed significant reduction in malondialdehyde levels when compared with that of NASH induced group (group 2). Rats fed with standard diet simultaneously with pioglitazone (group 3: pioglitazone control), with quercetin (group 4: quercetin control) showed no significant effect on the levels of lipid peroxidation products (MDA) and non-enzymatic (GSH) and enzymatic (catalase, SOD, GPx, GR and GST) antioxidants when compared to control group (group 1). On the other hand, rats fed with standard diet simultaneously with hydroxy citric acid (group 5: HCA control) does not show any significant effect on the levels of lipid peroxidation products (MDA) and GSH, GPx, GR and GST but showed significant decrease in the levels of catalase and SOD when compared to control group (group 1).

DISCUSSION

Insulin resistance may be an important factor in accumulation of hepatocellular fat, whereas genetic predisposition, apoptosis, adenosine triphosphate depletion, elevated dietary fructose intake, altered intestinal motility, serotonin degradation by monoamine oxidase A as an important source of reactive oxygen species, mitochondrial dysfunction, impaired innate immunity regulation and endoplasmic reticulum stress may be important causes of hepatocellular injuries in the steatotic liver (27). It has been proposed that the vulnerable fatty liver is injured by reactive oxygen species generated from microsomal, mitochondrial, and/or other hepatocellular pro-oxidant pathways. Increased lipid peroxidation has been demonstrated in both animal models of fatty liver (28-30) and patients with NASH. Free fatty acids (FFAs) are the likely source of oxidative stress within the liver in these patients. NASH patients have increased lipolysis and increased delivery of FFAs to the liver (31, 32). The products of FFA oxidation (hydrogen peroxide, superoxide, and lipid peroxides) are capable of generating oxidative stress and subsequent lipid peroxidation (33-36).

In response to oxidative stress, there is usually an increased synthesis of antioxidants and ROS scavengers. However, changes in the activities of the serum antioxidant enzymes and their relationships to oxidative stress have inadequately been studied in patients with NASH. The body protects itself from oxygen free radical toxicity by enzymatic antioxidant mechanisms (*e.g.* glutathione peroxidase (GSH-Px), glutathione reductase (GR), superoxide dismutase (SOD), and catalase) and by non-enzymatic antioxidants (*e.g.*, vitamins, uric acid, albumin, bilirubin, and many others) (36, 37). The emerging ROS in NASH condition are successfully neutralized by a cell

through both antioxidant enzyme systems such as superoxide dismutase glutathione peroxidase catalase and low molecular compounds such as glutathione, melatonin and others (38).

Evidence of lipid peroxidation in the form of increased MDA production, a marker of oxidative stress, has been noted in previous studies and serum levels of MDA have been correlated with the severity of chronic hepatitis (39, 40). In the present study, serum MDA levels were significantly increased in experimental NASH, indicating increased oxidative stress. The defence against free radical-mediated injury includes enzymatic deactivation and direct reaction with free radicals (41). SOD, the first line of defense against oxygen derived free radicals, converts superoxide anion into H₂O₂, forming as neutral products O₂ and H₂O. GSH-Px catalyses reductive destruction of hydrogen and lipid hydroperoxides, using glutathione as an electron donor (42).

The balance between oxidative stress and antioxidant defense mechanisms may be impaired by depletion of enzymatic antioxidants and decreased serum levels of MDA and NO in patients with NASH. The present study demonstrated that failure of antioxidant defense mechanisms against oxidative stress may be an important factor in the pathogenesis of NASH.

Decreased glutathione levels have been reported in NASH patients (43). Therefore, patients with NASH have an impaired ability to produce sufficient antioxidants. This may be related to the recently observed decrease in three genes involved in ROS sequestration (Cu/Zn superoxide dismutase, glutathione peroxidase, and catalase) in cirrhosis secondary to NASH (44). GST has an important role in antioxidant defense system at the cellular level and is a valuable marker of oxidative stress in NASH (45).

Malondialdehyde, lipid peroxidation product is an index of lipid peroxidation (11, 43). The experimental NASH rats treated with pioglitazone (group 6: NASH + pioglitazone), with quercetin (group 7: NASH + quercetin) and with hydroxy citric acid (group 8: NASH + HCA) showed significant reduction in malondialdehyde levels when compared with that of NASH induced group (group 2).

However, The experimental NASH rats treated with pioglitazone (group 6: NASH + pioglitazone) showed marked increase in the levels of GSH, catalase and SOD but does not show a significant effect on the levels of GPx, GR and GST levels when compared to the experimentally induced NASH group (group 2). Pioglitazone showed limited positive effect on the antioxidant parameters (46).

Similarly, experimental NASH rats treated with hydroxy citric acid (group 8: NASH + HCA) showed marked increase in the levels of GSH and catalase but does not show a significant effect on the levels of SOD, GPx, GR and GST levels when compared to the experimentally induced NASH group (group 2). Hydroxy citric acid also showed a limited positive effect on the the antioxidant parameters (47) and this could be attributed to the limited antioxidant properties of the HCA. Hydroxy citric acid may improve non-alcoholic steatohepatitis because it protects cellular structures against damage from oxygen free radicals and reactive products of lipid peroxidation. Previous reports suggested that the involvement of oxidative stress in the pathogenesis of NASH, suggests that antioxidants might have beneficial effects in the treatment of NASH patients (48-50). Moreover HCA attenuates the increase in oxidative stress in brain tissue during obesity condition (51). These findings consistent with our data where there were reduction in MDA level in HFD + HCA compared with HFD group indicating the antioxidant effect of HCA.

On contrary to pioglitazone and hydroxy citric acid, the experimental NASH rats treated with quercetin (group 7: NASH + quercetin) showed significant increase in the levels of

antioxidants namely GSH, catalase, SOD, GPx, GR and GST when compared with that of NASH induced group (group 2) showing maximum protective effect against NASH. Quercetin demonstrates these protective effects on liver damage by increasing the antioxidant (enzymatic and non enzymatic) activity and decreasing prooxidant effect (52-54). It has been documented that the structure of quercetin plays an important role in its antioxidant property. The O-dihydroxy structure in the B-ring of quercetin has been recognized to accord higher stability to the radical form there by enabling it to participate in the delocalization of electrons (55). Quercetin offers protective effect against NASH by attenuating lipid peroxidation, by scavenging free radicals that were generated by the excessive oxidative stress and by increasing the levels of glutathione and enhancing the activity of antioxidant enzymes, which in turn detoxify free radicals (52-54). Quercetin has beneficial effects on liver fibrosis in rats by enhancing antioxidant enzyme activity and decreasing the pro-oxidant effect (53). The quercetin showed a significant decrease in hepatic damage enzymes, lipoperoxidation, DNA damage and a lower degree of macrovesicular steatosis, ballooning and inflammatory process. These findings suggest that quercetin may have protective effects by improving liver integrity in NASH which was evidenced in the previous study (56) is concordant with the present report.

It can be concluded that quercetin offers maximum protection against NASH by showing positive effect on all antioxidant parameters as follows GSH, catalase, SOD, GPx, GR, GST and by decreasing the lipid peroxidation unlike the action of pioglitazone and hydroxy citric acid.

Conflict of interests: None declared.

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