Hepatoprotective Effects of Metformin on Fructose Induced Non-Alcoholic Steatohepatitis in Rats

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Abstract: The most known risk factor for nonalcoholic fatty liver disease (NAFLD) is the metabolic syndrome. In this study, we characterized changes in liver pathology, hepatic lipid composition, and plasma biochemistry occurring in rats given fructose-enriched diet 10% (FED). Rats were given FED or standard rat chow for 5 weeks. Rats on FED were divided into 2 groups: One group of rats was fed FED only for 5 weeks and another group of rats was received metformin 50 mg/kg for the last 2 weeks (3 weeks FED + 2 weeks FED and metformin). FED rats had developed hepatic macrovesicular and microvesicular fat deposits, with increase in hepatic triglycerides (+198%) and hepatic cholesterol (+89%), but a decrease in hepatic phospholipids (-36%), hypertriglyceridemia (+223%), and hypertension (+15%). Also, in FED rats there was significant increase in serum cholesterol and serum glucose (100.6±2.5, 9.0±0.4 respectively), and significant increase in hepatic MDA and TNF-α (209.9± 43.9, <12.5, respectively). Metformin reduced blood pressure (-24%), serum triglycerides (-36%), hepatic triglycerides (-51%), hepatic macrovesicular fat (-51%) and increased Hepatic phospholipids (+37%). Also, significant decrease in hepatic MDA and TNF-α (150.1± 27.0, <10.5 respectively). In conclusion: Metformin could reduce most of biochemical and tissue parameters and also improve the histopathological features of liver associated with non alcoholic steatohepatitis in rats.
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1. Introduction

Simple steatosis and nonalcoholic steatohepatitis are similar in terms of excessive accumulation of fatty acids in the hepatocytes. The β-mitochondrial oxidation of these fatty acids is a source of free radicals and produces hydrogen peroxide and reactive oxygen species. Lipid peroxidation, together with cytokines, and other proinflammatory compounds are believed to play a critical role in the transition from steatosis to non-alcoholic steatohepatitis and cirrhosis (McCullough, 2006).

The hallmark of non-alcoholic steatohepatitis is the accumulation of large-droplets of fat in hepatocytes, including fat alone as well as fat with non-specific inflammation. Although several predisposing factors, such as obesity, insulin resistance and type 2 diabetes, are related to the pathogenesis of non-alcoholic steatohepatitis, the exact mechanism of its progression to fibrosis and chronic liver disease is still unclear (Farrell and Larter, 2006).

It has been shown that chronic oxidative stress, generated through the oxidation of cytotoxic free fatty acids, can lead to upregulation of cytokines (Garcia-Ruiz *et al.,* 1995). In addition, enhanced lipid peroxidation leads to the generation of byproducts, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), which have been shown to further stimulate cytokine production (Robino *et al.,* 2000).

Nonalcoholic fatty liver disease (NAFLD) is defined as the accumulation of lipid, primarily triacylglycerols , in individuals who do not consume a large amount of alcohol (McCullough , 2004). Nonalcoholic steatohepatitis (NASH) is a progressive form of NAFLD that is diagnosed by histopathological features (Marchesini *et al.,* 2001). NAFLD and NASH have been readily recognized as hepatic manifestations of metabolic syndrome (Day and Saksena , 2002).

However, fat intake appears to be an important determinant of obesity. Along with an increase in total energy consumption, the consumption of fructose, largely attributable to the intake of high-fructose beverages, is an increasing trend (Havel, 2005). Such consumption of sugar-sweetened drinks has been correlated with childhood obesity and the occurrence of type 2 diabetes in adults (Montonen *et al.,* 2007).

Animals that are fructose loaded for several weeks are widely recognized as good models for metabolic syndrome (Armutcu *et al.,* 2005). NASH diagnosis is based on histopathological features of the liver, thus requiring investigation of hepatic pathology in experimental animals. In this study, we evaluated the degree of steatohepatitis, based on the grading and staging system for NASH.

Metformin is an insulin-sensitizing agent reported to reverse hepatomegaly, steatosis and liver tests abnormalities in a model of fatty liver (Lin *et al.,* 2000). Clinical data reported a controversial metformin effect on liver damage markers (Bugianesi *et al.,* 2005; Marchesini *et al.,* 2001; Nair *et al.,* 2004; Schwimmer *et al.,* 2005). A recent meta-analysis about drugs improving insulin resistance in non-alcoholic

steatohepatitis suggests that further clinical studies are needed to either support or refute the use of these drugs, which are however rated as having a favourable role (Angelico *et al.,* 2007). The aim of this study is to determine whether the medical problems of NASH patients, specifically liver damage, improves when their insulin sensitivity is enhanced with metformin.

2. Material and Methods:

Drugs and chemicals: Metformin powder (Sigma , Egypt); D- Fructose (Alliance- Bio); Rat TNF- apha ELISA kits (Ray Biotech, Inc.); Insulin ELISA kits (CalBiotech, USA); Reagents for measurement of Serum Glutamic Pyruvic trans-aminase by colorimetric method; Reagents for measurement of Serum Glutamic Oxaloacetic transaminase by colorimetric method. Reagents for measurement of serum and hepatic triglycerides by enzymatic colorimetric method.;Reagents for measurement of serum and hepatic cholesterol by enzymatic colorimetric method. ;Reagents for measurement of serum glucose by enzymatic colorimetric method; Reagents for measurement of hepatic malondialdehyde by colorimetric method and Reagents for measurement of hepatic phospholipids by colorimetric method.

Animals and Procedures:

Fourty male albino rats with initial weight of 110-130 grams were used throughout this study. Animals were kept in cages (maximum of 5 animals per cage). Animals were allowed to acclimatize for at least 3 days prior to start of experiment, water and chow diet were given freely. Animals were kept in fully ventilated room at temperature 26-28^{o}C and on a 12 hour light /dark cycle.

Ten rats given saline orally daily till the end of experiment and used as control group. Thirty rats fed on a fructose enriched diet 10% (FED for 5 weeks) (Zvi Ackermant *et al.,* 2005) , then this group is subdivided into two subgroups: one group received saline for the last 3 weeks (3 weeks FED + 2 weeks FED and saline) and metformin subgroup maintained on FED for 5 weeks and received metformin 50 mg/kg daily for the last 3 weeks (Malgorzata Knaś *et al.,* 2009) (3 weeks FED + 2 weeks FED and metformin).

The fructose enriched diet was made by grinding the chow diet and mixing it with fructose at a ratio of 9 chow diet:1 fructose.

At the end of experimental period, rats were subjected to the following procedures: After the animals have been weighted, blood pressure was measured by rat tail plethysomography (Ultian *et al.,* 1997). Blood samples were collected by the retro-orbital methods 24 hours following the last dose of metformin. Colorimetric estimation of serum glucose (Trinder, 1969), transaminases (Reitman and Frankel, 1957), total cholesterol (Richmond , 1973), triglycerides (Fassati and Prencipe , 1982). Also, serum insulin level was estimated by ELISA kits according to the method of Bowsher *et al.* (1992). Rats were sacrificed by decapitation and the liver was removed, half of the liver was fixed in 10% of neutral buffered and embedded in paraffin for histopathological analysis. The severity of NASH was determined according to grading system described by Brunt *et al.* (1999) and the other half was homogenized and the centrifuged for 10 minutes at 3000 rpm and the supernatant was taken for measurement of triglycerides (Fassati and Prencipe, 1982) , malondialdehyde (Ohkawa *et al.,* 1979) and tissue TNF-α (Bonavida, 1991).

Statistical analysis:

Results are expressed as mean standard error. Comparisons between groups used 1-way analysis of variance with Tukey-Kramer multiple comparisons test. $P \leq 0.05$ was considered statistically significant. The statistical analysis was performed using GraphPad Instant (Version 2.01; Mayo Foundation).

3. Results:

Effects of FED

Baseline parameters did not differ between the rats that were scheduled for FED and the rats that were scheduled for further maintenance on chow diet. After 3 weeks, both groups had similar weight gain. However, the group on FED had 18% higher BP values, 188% higher serum triglyceride levels, and 55% higher serum insulin levels. After 5 weeks, the rats on FED again had similar weights but 15% higher BP, 223% higher serum triglycerides (Tables 1, 2).

No change in liver enzymes was observed between both groups. However, the ratio of alanine to aspartate aminotransferase that was 1.2:1.0 in the chow group increased to 2.0:1.0 in the FED rats , also in FED rats there was significant increase in serum cholesterol and serum glucose (100.6±2.5 , 9.0±0.4, respectively) (Table 2). Also FED rats showed significant increase in hepatic MDA and TNF-α (209.9± 43.9 , <12.5,respectively). The livers of the FED rats had higher concentrations of total lipids (+28%), triglycerides (+198%), and cholesterol (+89%), but lower concentrations of phospholipids (-36%) than the livers of the chow rats (Tables 2 , 3).

The livers of the chow group had no signs of macrovesicular steatosis or evidence of fibrosis. Minimal microvesicular steatosis and minimal lobular and portal inflammatory changes were present. The livers of the FED rats showed evidence of mild to moderate deposition of macrovesicular and microvesicular fat with minimal signs of perisinusoidal fibrosis (Figure 1).

Effects of metformin

Metformin caused a reduction in BP measurements in rats (-24%) (Table 1), the alanine aminotransferase levels were the highest observed (Table 2). Serum triglyceride levels decreased (-36%) as well as hepatic triglycerides (-51%). Hepatic phospholipids concentrations also increased (+37%) (Table 3).

Histopathological analysis of liver tissue :

Formalin fixed liver tissue was processed, and 5-um- thick paraffin sections were stained with hematoxylin and eosin (H&E) for histological analysis. Steatosis was evaluated to the experimental groups and sections was given a score from 0 to 4 as follows according the percentages of lipid-loden hepatocytes: 0, no steatosis; 1, fatty hepatocytes less than 10% of the parenchyma; 2, between 10 and 30%; 3, between 30 and 60% and 4, > 60% of the parenchyma.

Table 1 : Body Weight, Blood Pressure, Triglyceride, and Insulin Levels In the Various Study Groups

Groups Parameters	Chow	FED Only	FED and Metformin
Body weight, grams	309.0±11.0	307.0±6.0	296.0±6.0
Blood pressure, mm Hg	124.0±2.0*	142.0±2.0	108.0±3.0**
Triglycerides, mg/dL	107.0±20.0*	346.0±27.0	223.0±34.0*
Insulin, U/mL	23.0±1.7*	43.0±4.6	34.2±2.3

FED indicates fructose-enriched diet. *P< 0.01 (vs FED only).

Table 2 : Liver-Related Parameters, Plasma Biochemistry In the Various Study Groups

Groups Parameters	Chow	FED Only	FED and Metformin
Liver weight, gram	9.6±0.04	10.1±0.4	8.7±0.3
Alanine amino-transferase, IU/L	39.5±1.7	41.2±1.6	74.8±9.0*
Aspartate amino-transferase, IU/L	33.9±5.0	20.3±1.4	18.3±12.3
Cholesterol, mg/dL	85.9±4.5	100.6±2.5	105.3±9.8
Glucose, mmol/L	7.9±0.3	9.0±0.4	8.7±0.3
MDA, µmol/g protein	3.8 ±1.7	209.9± 43.9*	150.1 ± 27.0
TNF-α_ pg/mL	< 8.5	< 12.5	< 10.5

TNF-α, tumor necrosis factor alpha. *P<0.05 (vs FED).

Table 3 : Hepatic Lipids Composition In the Various Study Groups

Groups Parameters	Chow	FED Only	FED and Metformin
Total lipids, mg/gram liver	23.1±0.4*	29.5±0.4	32.5±0.5*
Phospholipids, mmol/gram liver	14.6±0.1*	9.3±0.2	12.7±0.1*
Triglycerides, mmol/gram liver	4.1±0.2*	12.2±0.8	6.0±0.4*
Cholesterol, mg/gram liver	0.9±0.05*	1.7±0.05	1.8±0.06

***P< 0.01 (vs FED)**

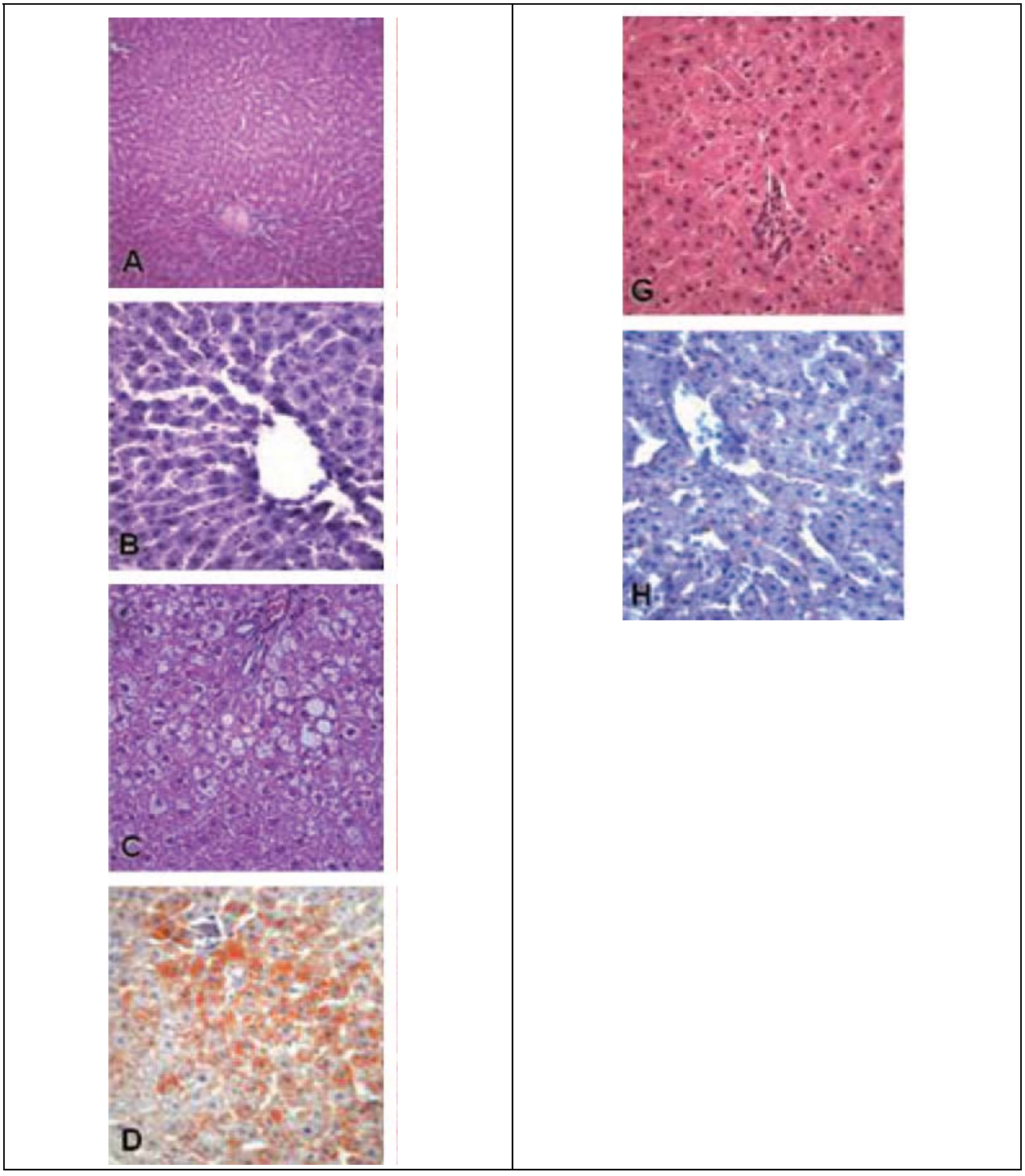

Fig. 1: **Liver pathology in the various study** groups. Photomicrographs of liver samples stained with hematoxylin & eosin, in rats given standard rat chow diet (A, B), in rats on fructose-enriched diet (FED) (C, D) , in rats on FED and metformin (G, H).

4. Discussion:

Non-alcoholic steatohepatitis is an increasingly recognized condition that may eventually progress to an end stage liver disease. A net retention of lipids within hepatocytes is a prerequisite for the development of the disease. Increased intrahepatic

levels of fatty acids provide a source of oxidative stress which may in large part be responsible for the progression from steatosis, to steatohepatitis, to cirrhosis (Bugianesi *et al.,* 2005).

The presumed factors initiating the progression of non-alcoholic steatohepatitis are insulin resistance (Larter and Farrell, 2006), an increased oxidative status with subsequent lipid peroxidation, pro-inflammatory cytokines (principally TNF-α), and hormones derived from adipose tissue (adipocytokines) (Albano *et al.,* 2005; Duvnjak *et al.,* 2007; Kojima *et al.,* 2007).

Experimental rats fed fructose-enriched diets are good models for metabolic syndrome. Characterized changes in liver pathology occurring in male rats given a fructose-enriched (10%) diet. we used the scoring system for NASH (Brunt *et al.,* 1999) and found that rats fed the fructose-enriched diet had significantly higher degrees of macrovesicular and microvesicular steatosis than rats fed a chow diet. (Armutcu *et al.,* 2005).

By feeding rats with fructose enriched diet, the hepatic lesions of NASH were apparent within 5 weeks. Histopathological examination showed macrovesicular steatosis, hepatocyte ballooning, mallory bodies, and mild to moderate inflammation. In this condition, the liver failed to synthesize apolipoprotein that is required for packaging and exporting fat from the liver, triglycerides (TG) thus accumulate in the liver (Benzie , 1996). β-oxidation of FFA in hepatocytes produces reactive oxygen species (ROS) which activate lipid peroxidation. ROS and lipid peroxidation cause direct damage to hepatocytes by disrupting membranes, protein, and DNA (de Knegt , 2002) hepatocyte damage and lipid peroxidation products induce an inflammatory response.

AST and ALT are useful screening tests for detecting liver injury (Fan *et al.,* 2003). They are found in hepatocytes and can not diffuse out of the cells in the physiological condition. When the hepatocyte is injured, plasma membrane can be disrupted and the leakage through extracellular fluid of the enzyme occurs where they can be detected at abnormal levels in the serum (Kirsch *et al.,* 2003). AST and ALT activities have been found to be increased in NASH rats (George *et al.,* 2003).

In this study, we demonstrated that rats given FED are a suitable model for non-obese rats with NAFLD that present some aspects of the metabolic syndrome (Marchesini *et al.,* 2003) such as hypertension, insulin resistance, and hypertriglyceridemia. Treatment modalities have been directed toward reduction of weight, improvement of insulin resistance, lipid-lowering agents, and hepato-protective drugs (Angulo, 2002; Sanyal, 2002). In this study, we investigated the hepatic effects of medications with known beneficial effects on few of the manifestations of NASH , like hypertension and hypertriglyceridemia.

In this study, metformin reduced plasma and hepatic triglycerides, which was accompanied by a significant reduction in macrovesicular steatosis score, without a significant increase in microvesicular steatosis score. Moreover, a decrease in BP and insulin resistance was also observed. Administration of metformin to humans with the metabolic syndrome caused amelioration of many aspects of the metabolic syndrome (Kim *et al.,* 2003). The levels of plasma tumor necrosis factor-α was increased in FED rats in relation to chow diet rats (Nagai *et al.,* 2002).

In this experimental study, was reported a crucial and significant alteration of liver parameters (liver weight, transaminases, trygliceride content), induced by FED. Moreover, we demonstrated that in FED rat this diet induced a significant increase in the malonyldialdehyde content in the liver. Malonyldialdehyde and other substances, such as 4-hydroxynonenal, 8-isoprostane, and 3-nitrotyrosine are produced by lipid peroxidation and the level in the plasma of several oxidative stress markers was found to have increased in non-alcoholic steatohepatitis rats (Loguercio *et al.,* 2001; Sumida *et al.,* 2003).

In this model we used a new therapeutic tool i.e., metformin, as insulin sensitizers. Metformin was reported to reverse hepathomegaly, steatosis and liver tests abnormalities in a model of fatty liver (Lin *et al.,* 2000). Several clinical studies confirm the improvement in liver tests, insulin sensitivity, and loss of body weight (Marchesini et al., 2001; Angulo and Lindor, 2002; Schwimmer *et al.,* 2005) induced by metformin treatment, even if some other results showed no effect (Nair *et al.,* 2004).

Although an amelioration of aminotransferase level and liver histology was evidenced, on the other hand, no statistically significant change in the severity of liver inflammation or fibrosis after metformin treatment was reported (Uygun *et al.,* 2004).

Moreover, metformin reduced body weight gain and fat mass in comparison to high fat diet fed animals. It did not significantly reduce liver weight, but ameliorates transaminase profile and reduced significantly liver triglyceride content. Although having a slight effect on lipid peroxidation (MDA content), metformin was found markedly reduce nitrotyrosylation of hepatic proteins. This anti-inflammatory effect was confirmed by inhibition of TNF-α content in liver tissue (Mohanty *et al.,* 2004; Lutchman *et al.,* 2006).

Finally, among the major potential therapeutic options, insulin sensitizers, such as metformin, and antioxidant vitamin E (Bugianesi *et al.,* 2006; Mishra and Younossi, 2007) are surely efficacious and well-tolerated, but their long-term clinical efficacy either alone or in combination has not yet been confirmed.

Although experimental and clinical studies suggest for these drugs a favourable role in improving non-alcoholic fatty liver disease, further investigations and large randomized trials will be needed.

In conclusion: Metformin could reduce most of biochemical and tissue parameters and also improve the histopathological features of liver asscociated with non alcoholic steatohepatitis in rats.

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4/18/12