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Pentoxifylline and melatonin in combination with pioglitazone ameliorate experimental non-alcoholic fatty liver disease

Sawsan Zaitone [a,*], Neven Hassan [b], Naglaa El-Orabi [a], El-Sayed El-Awady [a]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt
[b] Directorate of Health and Population, Port Said, Egypt

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ABSTRACT

Insulin resistance, oxidative stress and cytokine imbalance are key pathophysiological mechanisms in non-alcoholic fatty liver disease (NAFLD). This study aimed at evaluating the effect of treatment with the insulin sensitizer, pioglitazone, the tumor necrosis factor-α inhibitor, pentoxifylline, and the antioxidant, melatonin and their combinations in rats with NAFLD. Rats were fed a high-fat diet (HFD) for eight weeks to induce NAFLD. For an additional eight weeks, rats were fed the HFD along with pioglitazone, pentoxifylline, melatonin alone or in combination. Liver index and insulin resistance index were calculated. Serum liver enzyme activities, total cholesterol, triglycerides and tumor necrosis factor-α (TNF-α) were determined. Tissue triglycerides, malondialdehyde and reduced glutathione were measured and liver injury was evaluated by histopathological examination. HFD induced severe hepatic steatosis, inflammation and fibrosis. In addition, liver index, insulin resistance index, activities of liver enzymes and serum level of total cholesterol, triglycerides and TNF-α were elevated. This was coupled with an increase in tissue triglycerides, malondialdehyde and depletion of reduced glutathione. Pioglitazone, pentoxifylline and melatonin, alone or in combination; reduced the insulin resistance index, activities of liver enzymes, hepatic malondialdehyde and increased hepatic reduced glutathione level. Pentoxifylline led to a decrease in serum TNF-α level, however, pioglitazone and melatonin reduced serum total cholesterol and triglycerides. In conclusion, data in this study indicate that pentoxifylline and melatonin can be used as promising adjuvant therapies to pioglitazone in the clinical management of NAFLD.

1. Introduction

Non-alcoholic fatty liver disease is the most common cause of liver diseases in childhood and adolescence initially reported in adults by Ludwig in 1980 (Ludwig et al., 1980). NAFLD encompasses a spectrum of liver pathology from isolated steatosis to non-alcoholic steatohepatitis, where fat accumulation is associated with inflammation and/or evidence of cellular injury and cirrhosis (Elizabeth et al., 2010). The biological mechanism underlying steatosis occurrence and progression to non-alcoholic steatohepatitis is not entirely understood. A two-hit hypothesis has been proposed since 1998 (Day and James, 1998); in which a first hit is able to induce liver fat accumulation and a second hit prompts steatosis progression to non-alcoholic steatohepatitis.

The most widely supported theory implicates insulin resistance as the key mechanism in primary NAFLD, leading to hepatic steatosis. The presumed factors initiating second hits are oxidative stress and subsequent lipid peroxidation, proinflammatory cytokines (principally TNF-α) and hormones derived from adipose tissue (adipocytokines) (Duvnjak et al., 2007).

The medical therapy of NAFLD and non-alcoholic steatohepatitis is an arena of significant research. Currently, there is no approved therapy for NAFLD. There are many proposed agents being evaluated, each targeting a different step in the pathogenesis of development of hepatic steatosis or its progression to steatohepatitis (Oh et al., 2008).

Thiozolidinediones are high-affinity ligands of peroxisome proliferator-activated receptor-γ (Yki-Jarvinen, 2004), stimulating the storage of free fatty acids in subcutaneous adipocytes, thereby improving insulin sensitivity (Shulman, 2000). This class also has anti-inflammatory properties, as demonstrated by a decrease in nuclear factor kappa-B levels and an increase in adiponectin levels (Lutchman et al., 2006).

The modulation of cytokines associated with hepatic steatosis and its associated inflammation and fibrosis has become a recent focus of research. Circulating levels of TNF-α have been demonstrated to be elevated in patients with non-alcoholic steatohepatitis compared to control patients (Kugelmas et al., 2003). Therefore, therapeutic options targeting TNF-α appear as rational treatments for non-alcoholic steatohepatitis.

* Corresponding author. Tel.: +20 002 011 1690365; fax: +20 002 064 3230741.
E-mail addresses: Sawsan_zaytoon@pharm.suez.edu.eg, sawsanzaitune@hotmail.com (S. Zaitone), nhassan_pharm@yahoo.com (N. Hassan), nfelorabi@yahoo.com (N. El-Orabi), sayedawady@yahoo.com (E.-S. El-Awady).

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Melatonin, a secretory product of the pineal gland, is a powerful endogenous antioxidant. Exogenous application of this molecule leads to a remarkable decline in oxidative stress by directly neutralizing the hydroxyl radicals. In addition, melatonin is also indirectly effective by enhancing the levels of potential antioxidants such as glutathione peroxidase, superoxide dismutase and glutathione (Hoyos et al., 2000; Tahan et al., 2004).

The aim of the present study was to further our knowledge of the pathogenesis of NAFLD. In addition, we aimed to evaluate the protective effect of pioglitazone, an insulin sensitizer, pentoxifylline, a TNF-α inhibitor and melatonin as an antioxidant in an experimental model of NAFLD. Further, the work was extended to examine the benefits of the combination of pioglitazone with either pentoxifylline or melatonin.

2. Methods

2.1. Chemicals and drugs

Pioglitazone and pentoxifylline powders were kindly provided by Medical Union Pharmaceuticals (MUP, Ismailia, Egypt). Melatonin powder was purchased from Bio Basic Inc. (Ontario, Canada). Pioglitazone and melatonin were dissolved in 2% Tween-80 solution; whereas pentoxifylline was dissolved in distilled water. Freshly prepared melatonin solution was stored in a dark bottle covered with aluminum foil as it is photosensitive. Cholesterol was purchased from GFS chemicals & reagents (Texas, USA) and bile salts were purchased from SAS Chemicals Co. (Mumbai, India).

2.2. Experimental animals

Seventy male Wistar rats, obtained from the Egyptian Organization for Biological Products and Vaccines (Cairo, Egypt), were used in the current study. Rats had an initial body weight in the range of 120–150 g. Rats were housed in stainless steel cages in groups of four, with free access to food and water for sixteen weeks. Rats were kept under controlled laboratory conditions of normal light-dark cycle and temperature between 25 ± 3 °C. All experimental protocols were approved by the Animal Care and Use Committee at the Faculty of Pharmacy, Suez Canal University.

2.3. Experimental design

Rats were randomly divided into seven groups; 10 rats each. Group 1 served as a normal group and was maintained on normal rat chow diet throughout the experiment (sixteen weeks). The remaining six groups were maintained on a HFD containing 87.7% standard diet (w/w), 10% pork fat (w/w), 2% cholesterol (w/w) and 0.3% bile salts (w/w) (Pan et al., 2006) for eight weeks. For an additional eight weeks, HFD was given in addition to the following treatment regimens, group 2 (NAFLD group) received distilled water (1 ml/kg/day, p.o.), group 3 received pioglitazone (4 mg/kg/day, p.o.), group 4 received pentoxifylline (50 mg/kg/day, p.o.), group 5 received melatonin (10 mg/kg/day, p.o.), group 6 received a combination of pioglitazone and pentoxifylline (combination 1) in the same aforementioned doses, and group 7 received a combination of pioglitazone and melatonin (combination 2) in the same aforementioned doses. At the end of the experiment, the final body weight of each animal was recorded.

2.4. Blood glucose determination and processing of the liver

After the last drug treatment, rats were fasted overnight. Fasting blood glucose was determined with an automatic blood glucose meter (Super Glucocard, Japan) using blood samples from the tail tip. Rats were anesthetized by injection of ketamine (80 mg/kg, i.p.) and blood samples were collected by cardiac puncture. The collected samples were centrifuged for 3 min at 1000×*g* to obtain the serum, which was then stored at −80 °C.

The liver was rapidly dissected and washed free of blood with ice-cold 0.9% NaCl solution. The liver was weighed and the liver index was calculated (liver weight/body weight×100). One part of the liver (0.3 g) was then blotted and finally kept at −80 °C. This liver tissue was homogenized either in phosphate buffer pH 7.4 (supernatant A) for determination of reduced glutathione or in Tris. hydrochloride buffer pH 7.4 (supernatant B) for malondialdehyde assay, and then centrifuged at 3000×*g* for 15 min at 4 °C. Liver samples were taken 5 mm away from the edge of the largest hepatic lobe, fixed with 10% (v/v) formaldehyde, embedded in paraffin wax, stained with hematoxylin and eosin (H&E) and Masson's trichrome stain.

2.5. Measurement of serum biochemical parameters

Serum activity of alanine transaminase (ALT) and aspartic transaminase (AST) were measured spectrophotometrically using commercial kits (Biocon Diagnostic, Germany). Serum total cholesterol and triglycerides were determined using colorimetric kits (Biodignostic, Cairo, Egypt) using a UV-visible spectrophotometer (UV-1601PC, Shimadzu, Japan). Serum levels of TNF-α were determined using a rat TNF-α ELISA kit (Ray Biotech Inc., Norcross, USA) and fasting insulin was determined using ultra sensitive rat insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL 60515, USA).

Insulin resistance was determined using the homeostasis model assessment index for insulin resistance (HOMA-IR) using the following formula: HOMA-IR index = [fasting glucose (mmol/L)×fasting insulin (μU/ml)]/22.5) (Mathews et al., 1985).

2.6. Measurement of hepatic triglycerides, malondialdehyde and reduced glutathione

Hepatic triglyceride level was measured following the method of Foster and Dunn (1950) after extraction of tissue lipids according to the method of Folch and Stanely (1957). Hepatic malondialdehyde (MDA) was determined by the reaction with thiobarbituric acid according to the method of Ohkawa et al. (1979). Hepatic reduced glutathione (GSH) was determined using the spectrophotometric method of Ellman (1959).

2.7. Histopathological examination

All histological examinations were performed by an experienced pathologist who was blinded to the experiment groups. Histopathological changes were assessed by a semiquantitative method according to standards proposed by Dixon et al. (2004).

2.8. Statistical analysis

Results were expressed as mean ± S.E.M and analyzed using the Statistical Package of Social Sciences (SPSS) program version 16, (Chicago, IL, USA). Quantitative variables were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Qualitative variables were analyzed by rank-sum test. Differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Body weight and liver index

A significant increase in the body weight and the liver index was observed in the HFD-fed rats as compared to normal rats ($P \leq 0.05$, Table 1).

The % increase in the body weight was significantly reduced in pioglitazone group, pentoxifylline group and the combination 1 group

Table 1
Effect of pioglitazone (4 mg/kg, p.o.), pentoxifylline (50 mg/kg, p.o.), melatonin (10 mg/kg, p.o.) and their combinations on body weight, and liver index in the experimental groups.

Groups	% increase in body weight (g)	Liver index (%)
Normal	1.07 ± 0.1 (233–323 g)	3.1 ± 0.13 (28–37)
NAFLD	1.3 ± 0.1[a] (283–378 g)	6.2 ± 0.3[a] (39–66)
Pioglitazone	0.97 ± 0.1[b] (235–341 g)	5 ± 0.24[a,b] (39–60)
Pentoxifylline	1.09 ± 0.1[b] (220–320 g)	5.4 ± 0.5[a] (39–65)
Melatonin	1.1 ± 0.1[c] (217–325 g)	5.6 ± 0.18[a] (45–62)
Pioglitazone + pentoxifylline	1.08 ± 0.1[b] (270–325 g)	4.1 ± 0.3[b] (38–52)
Pioglitazone + melatonin	1.05 ± 0.1 (262–327 g)	4.4 ± 0.2[a,b] (39–54)

NAFLD: nonalcoholic fatty liver disease. Results are expressed as mean ± S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons.
[a] $P \leq 0.05$ versus normal group.
[b] $P \leq 0.05$ versus NAFLD group.
[c] $P \leq 0.05$ versus pioglitazone group, n = 10.

(pioglitazone plus pentoxifylline) as compared to NAFLD group ($P \leq 0.05$). Liver index was improved in pioglitazone group and in the combination 1 group (pioglitazone plus pentoxifylline) and in combination 2 group (pioglitazone plus melatonin) as compared to NAFLD group ($P \leq 0.05$, Table 1).

3.2. Liver enzyme activities (AST and ALT)

Feeding with a HFD for sixteen weeks induced a significant increase in serum activities of AST and ALT in rats as compared to the normal group ($P \leq 0.05$, Table 2). All the treatment regimens significantly decreased the elevated activities of AST and ALT as compared to NAFLD group ($P \leq 0.05$, Table 2).

3.3. Serum total cholesterol and triglycerides and hepatic triglycerides

Table 2 shows a significant elevation in serum total cholesterol and triglyceride levels in HFD-fed rats as compared to normal rats ($P \leq 0.05$). The elevation in serum total cholesterol was significantly ameliorated only by treatment with melatonin and its combination with pioglitazone (combination 2) ($P \leq 0.05$). The elevation in serum triglycerides level was markedly attenuated by treatment with pioglitazone, melatonin and their combination (combination 2) ($P \leq 0.05$). In contrast, pentoxifylline did not influence the elevated serum levels of total cholesterol and triglycerides ($P \leq 0.05$, Table 2).

Table 2
Effect of pioglitazone (4 mg/kg, p.o.), pentoxifylline (50 mg/kg, p.o.), melatonin (10 mg/kg, p.o.) and their combinations on serum liver enzyme activities (AST and ALT), total cholesterol, triglycerides and hepatic triglycerides level in the experimental groups.

Group	AST (U/L)	ALT (U/L)	TC (mg/dl)	Serum TG (mg/dl)	Hepatic TG (mg/g)
Normal	45 ± 4	31 ± 5	68 ± 5	151 ± 10	9 ± 0.3
NAFLD	94 ± 7[a]	74 ± 5[a]	113 ± 8[a]	216 ± 18[a]	15 ± 1[a]
Pioglitazone	51 ± 8[b]	26 ± 3[b]	91 ± 4	138 ± 6[b]	12 ± 0.3[b]
Pentoxifylline	51 ± 9[b]	38 ± 8[b]	97 ± 7[a]	157 ± 12	13 ± 0.6
Melatonin	59 ± 5[b]	38 ± 5[b]	87 ± 6[b]	151 ± 14[b]	11 ± 0.3[b]
Pioglitazone + pentoxifylline	48 ± 7[b]	32 ± 6[b]	91 ± 5	158 ± 13	10 ± 0.5[b]
Pioglitazone + melatonin	57 ± 6[b]	33 ± 7[b]	85 ± 5[b]	144 ± 19[b]	9.4 ± 0.4[bc]

NAFLD: nonalcoholic fatty liver disease, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, TC: total cholesterol. TG: triglycerides. Results are expressed as mean ± S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons.
[a] $P \leq 0.05$ versus normal group.
[b] $P \leq 0.05$ versus NAFLD group.
[c] $P \leq 0.05$ versus pioglitazone group, n = 10.

In addition, hepatic triglyceride level was significantly higher in NAFLD group as compared to normal group ($P \leq 0.05$, Table 2). Treatment with pioglitazone, melatonin, combination 1 (pioglitazone plus pentoxifylline) and combination 2 (pioglitazone plus melatonin) significantly reduced the high hepatic triglyceride level as compared to NAFLD group. The hepatic triglyceride level in the combination 2 group was significantly lower than that observed in pioglitazone group ($P \leq 0.05$, Table 2).

3.4. HOMR-IR index

A significant increase in fasting blood glucose, fasting insulin and HOMA-IR index was observed in rats with NAFLD as compared to normal rats ($P \leq 0.05$, Table 3). Pioglitazone was the sole treatment that could decrease the elevated fasting blood glucose significantly as compared to the NAFLD group. However, fasting insulin was reduced significantly only in the combination 2 group (pioglitazone plus melatonin) as compared to NAFLD group. All the implemented pharmacological agents significantly reduced the elevated HOMA-IR index as compared to NAFLD group ($P \leq 0.05$, Table 3).

3.5. The inflammatory cytokine, TNF-α

Serum TNF-α level was significantly increased in NAFLD rats as compared to normal rats (2965 ± 215 versus 1621 ± 234, $P \leq 0.05$, Fig. 1). Pentoxifylline and its combination with pioglitazone (combination 1) could significantly attenuate this increase ($P \leq 0.05$, Fig. 1).

3.6. Oxidative stress markers

3.6.1. Lipid peroxidation

NAFLD group showed a significant increase in MDA content in the liver homogenate of rats as compared to normal rats (422 ± 13 versus 232 ± 26, $P \leq 0.05$, Fig. 2). All treatment regimens significantly prevented this increase. Further, melatonin group showed statistically lower MDA level in comparison with pioglitazone group ($P \leq 0.05$, Fig. 2).

3.6.2. Hepatic reduced glutathione content

Feeding with a HFD for sixteen weeks induced a significant decrease in reduced GSH content in liver homogenate of rats as compared to rats fed a normal diet (30 ± 2.5 versus 52.6 ± 3, $P \leq 0.05$). All pharmacological treatments significantly increased the level of GSH as compared to NAFLD group ($P \leq 0.05$, Fig. 3).

Table 3
Effect of pioglitazone (4 mg/kg, p.o.), pentoxifylline (50 mg/kg, p.o.), melatonin (10 mg/kg, p.o.) and their combinations on fasting blood glucose, fasting insulin and HOMA-IR index in the experimental groups.

Groups	Fasting blood glucose (mg/dl)	Fasting insulin (μU/ml)	HOMA-IR index
Normal	71 ± 2	13 ± 1	2.2 ± 0.17
NAFLD	90 ± 4[a]	21 ± 1.3[a]	4.6 ± 0.5[a]
Pioglitazone	73 ± 4[b]	12 ± 2.4	2 ± 0.4[b]
Pentoxifylline	77.5 ± 3	14.3 ± 1.5	2.7 ± 0.2[b]
Melatonin	78 ± 2.5	14 ± 2	2.7 ± 0.3[b]
Pioglitazone + pentoxifylline	79 ± 2	12 ± 2.6	2.3 ± 0.5[b]
Pioglitazone + melatonin	79 ± 2	11 ± 1.7[b]	2.2 ± 1[b]

NAFLD: nonalcoholic fatty liver disease, HOMA-IR index: Homeostatic Model Assessment–Insulin Resistance index. HOMA-IR index = [fasting glucose (mMol/L) × fasting insulin (μU/ml)]/22.5). Results are expressed as mean ± S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons.
[a] $P \leq 0.05$ versus normal group.
[b] $P \leq 0.05$ versus NAFLD group.
[c] $P \leq 0.05$ versus pioglitazone group, n = 10.

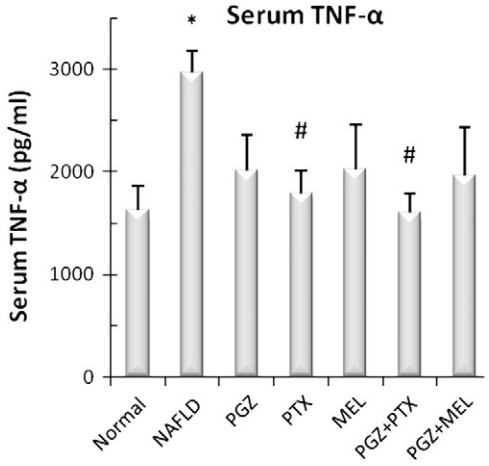

Fig. 1. Effect of pentoxifylline (PTX, 50 mg/kg, p.o.), melatonin (MEL, 10 mg/kg, p.o.) and their combination with pioglitazone (PGZ, 4 mg/kg, p.o.) on serum TNF-α level in the experimental groups. NAFLD: nonalcoholic fatty liver disease, TNF-α: tumor necrosis factor-α. Results are expressed as mean ± S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons. $^{*}P \leq 0.05$ versus normal group, $^{\#}P \leq 0.05$ versus NAFLD group, $P \leq 0.05$ versus pioglitazone group, n = 10.

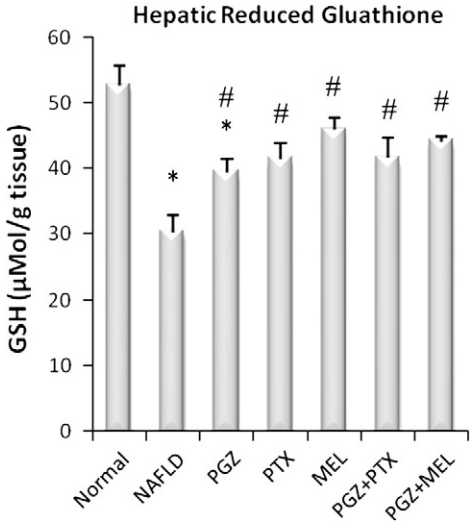

Fig. 3. Effect of pentoxifylline (PTX, 50 mg/kg, p.o.), melatonin (MEL, 10 mg/kg, p.o.) and their combination with pioglitazone (PGZ, 4 mg/kg, p.o.) on hepatic level of reduced glutathione in the experimental groups. NAFLD: non-alcoholic fatty liver disease, GSH: reduced glutathione. Results are expressed as mean ± S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons. *P ≤ 0.05 versus normal group, #P ≤ 0.05 versus NAFLD group, P ≤ 0.05 versus pioglitazone group, n = 10.

3.7. Histopathological examination

In the present study, the NAFLD group showed significant changes in the liver histology. Liver samples from the NAFLD group stained with H&E or Masson's trichrome stain showed diffuse macrovesicular steatosis and multifocal portal inflammation as well as hepatocellular fibrosis. Liver samples from the pioglitazone group showed moderate portal fibrosis. The liver in the pentoxifylline group showed only mild fatty change with few inflammatory cell infiltrations along with mild portal fibrosis. Liver samples from the melatonin group showed mild steatosis and moderate portal fibrosis (Fig. 4). The combination of pioglitazone with pentoxyfylline or melatonin resulted in moderate steatosis and mild portal inflammation.

The degree of steatosis, lobular inflammation and fibrosis in the NAFLD group was significantly higher than in the normal group ($P \leq 0.05$, Table 4). All the treatment regimens, with the exception of pioglitazone, significantly reduced the degree of steatosis; the steatosis score in pentoxifylline group and melatonin group was significantly lower than that observed in pioglitazone group ($P \leq 0.05$, Table 4). However, the combination 1 group (pioglitazone plus pentoxifylline) was the sole group that showed a significant decrease in the score of lobular inflammation. Finally, fibrosis scores were attenuated in all treatment groups, except the melatonin group. The fibrosis score in pentoxifylline group was significantly lower than that recorded in pioglitazone group ($P \leq 0.05$, Table 4).

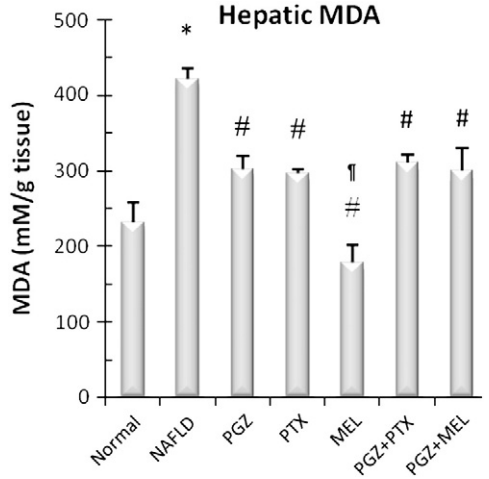

Fig. 2. Effect of pentoxifylline (PTX, 50 mg/kg, p.o.), melatonin (MEL, 10 mg/kg, p.o.) and their combination with pioglitazone (PGZ, 4 mg/kg, p.o.) on hepatic MDA content in the experimental groups. NAFLD: nonalcoholic fatty liver disease, MDA: malondialdehyde. Results are expressed as mean ± S.E.M. and analyzed using one-way ANOVA followed by Bonferroni's test for multiple comparisons. *P ≤ 0.05 versus normal group, #P ≤ 0.05 versus NAFLD group, ¶P ≤ 0.05 versus pioglitazone group, n = 10.

4. Discussion

In the present study, sixteen weeks of HFD feeding in rats induced severe steatosis and portal inflammation accompanied by liver fibrosis. Consistently, the liver of rats fed a HFD for twelve weeks (Gao et al., 2005) or six weeks (Yalniz et al., 2007) showed moderate to severe steatosis, lobular inflammation and developed typical histopathologic non-alcoholic steatohepatitis lesions.

The present results revealed that HFD feeding induced a marked elevation in the liver index and activities of AST and ALT. Similar results were observed previously (Pan et al., 2006; Yalniz et al., 2007). In addition, significant elevations in total cholesterol and triglycerides in the sera of rats were observed in the current study. In agreement, a significant elevation in serum triglycerides (Sebokova et al., 2002) or both serum total cholesterol and triglycerides was observed after HFD feeding (Hsiao et al., 2008; Pan et al., 2006). Further, the current study revealed that NAFLD rats showed high hepatic triglycerides level and this extends findings of others (Gomathy et al., 1989).

Further, a significant increase in HOMA-IR index was observed in NAFLD group as compared to the normal group. These results are supported by previous studies (Sebokova et al., 2002; Xu et al., 2006). This indicates that rats with NAFLD suffer from high insulin resistance and thus, insulin resistance plays an important role in the development of fatty liver.

In the current study, serum level of TNF-α was significantly increased by HFD feeding and this finding came on line with those obtained previously (Yalniz et al., 2007). Moreover, an elevation in tissue MDA and a suppression of tissue GSH were detected in NFALD group; this seems to be consistent with previous reports (Hsiao et al.,

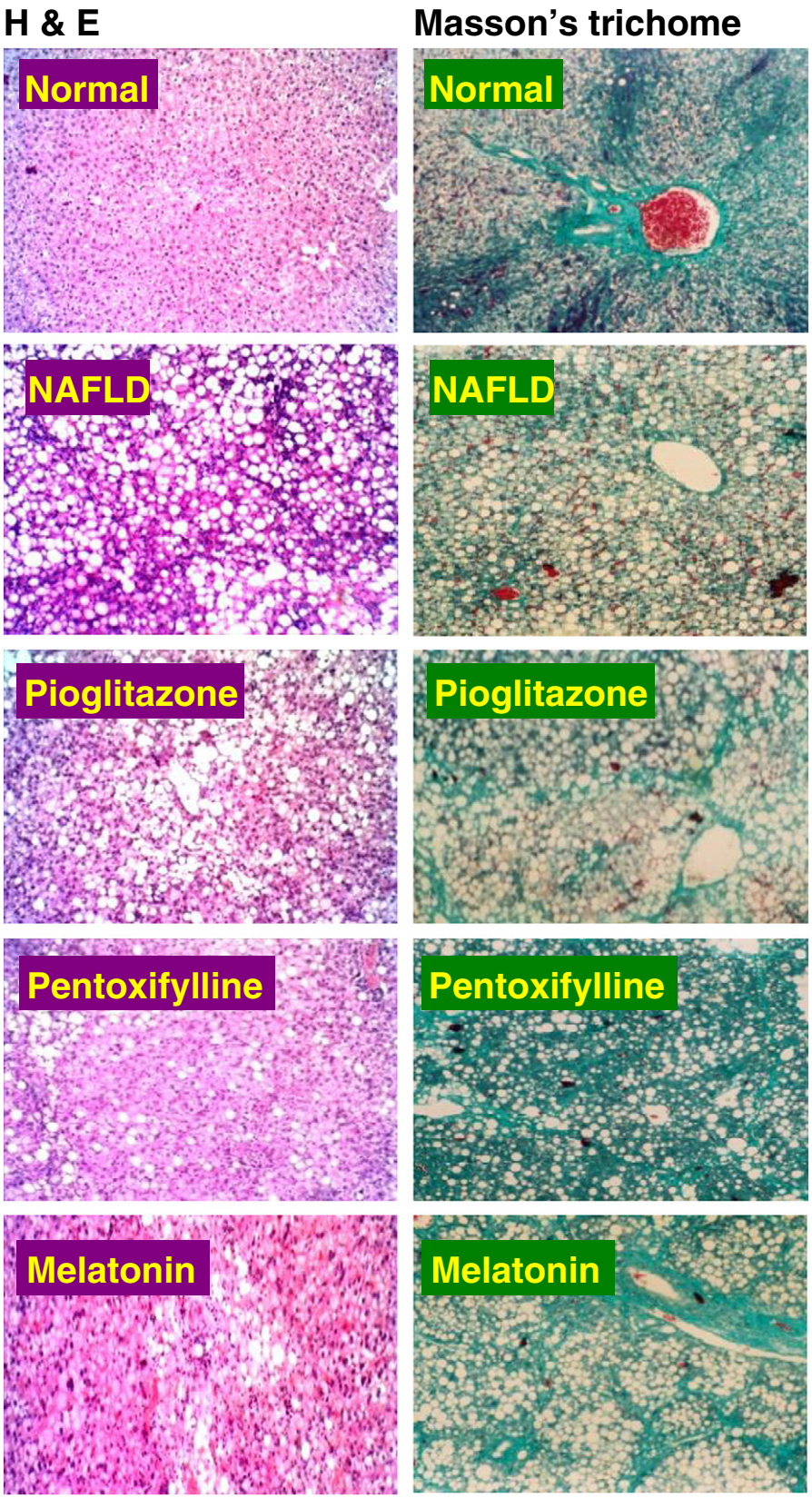

Fig. 4. Histology of the liver sections stained with hematoxylin and eosin (H&E) and Masson's trichrome stain. Microscopic picture of sections of liver from normal group showing normal liver tissues with normal hepatic cords, portal area and hepatic lobules. Liver sections of NAFLD group show diffuse marked macrovesicular steatosis, multifocal portal inflammation and marked fibrosis. Liver sections from pioglitazone group show moderate local extensive steatosis with mild ballooning degeneration. Liver sections from pentoxifylline group show moderate fatty change, few inflammatory cells infiltrations together with mild portal fibrosis. Liver sections from melatonin group show mild steatosis, moderate steatohepatitis (H&E and Masson's trichrome stain X100).

Table 4
Effect of pioglitazone (4 mg/kg, p.o.), pentoxifylline (50 mg/kg, p.o.), melatonin (10 mg/kg, p.o.) and their combinations on histological picture of the liver tissues of the experimental groups.

Groups	Steatosis	Lobular inflammation	Fibrosis
Normal	0.7 ± 0.2	0	0
NAFLD	3.5 ± 0.3[a]	2.8 ± 0.2[a]	3.8 ± 0.2[a]
Pioglitazone	2.8 ± 0.2	1.5 ± 0.5	2.2 ± 0.2[b]
Pentoxifylline	1.7 ± 0.2[bc]	1.25 ± 0.2	1.2 ± 0.2[bc]
Melatonin	1.7 ± 0.5[bc]	1.25 ± 0.2	2.7 ± 0.2
Pioglitazone + pentoxifylline	2.3 ± 0.3[b]	1.17 ± 0.2[b]	1.3 ± 0.2[b]
Pioglitazone + melatonin	2.1 ± 0.2[b]	2.2 ± 0.5	2.5 ± 0.2[b]

NAFLD: nonalcoholic fatty liver disease. Results are expressed as mean ± S.E.M.
[a] $P \leq 0.05$ versus normal group.
[b] $P \leq 0.05$ versus NAFLD group.
[c] $P \leq 0.05$ versus pioglitazone group, n = 10.

2008; Yalniz et al., 2007). These results highlight that inflammation and oxidative stress play important roles in NAFLD pathogenesis.

Data in this study showed that pioglitazone, alone or in combination with pentoxifylline or melatonin, induced a significant reduction in liver index and activities of AST and ALT. Consistently, ALT activity was reduced by pioglitazone in rats maintained on a choline-deficient, l-amino acid-defined diet-induced non-alcoholic steatohepatitis (Fujita et al., 2007) and HFD-fed rats (Xu et al., 2006). Pioglitazone alone and in combination with melatonin significantly lowered HFD-induced hypertriglyceridemia; whereas, the combination of two drugs significantly lessened the hypercholesterolemia. These results agreed with those reported by Ding et al. (2005) and Yoshiuchi et al. (2009).

Pioglitazone, alone or in combination with pentoxifylline or melatonin, significantly decreased the HOMA-IR index. Consistently, the HOMA-IR index decreased significantly in rats with HFD-induced NAFLD (Xu et al., 2006) and in obese rats (Ding et al., 2005) after treatment with pioglitazone. Recently, pioglitazone was shown to significantly reduce plasma insulin and the HOMA-IR index in fructose-drinking rats (Liu et al., 2010) and diabetic mice (Yoshiuchi et al., 2009).

Pioglitazone and its combination with pentoxifylline or melatonin significantly decreased the elevated hepatic MDA and increased the GSH content. In a previous study, HFD-induced oxidative DNA damage in mice was attenuated by pioglitazone. The authors highlighted that the hepatoprotective mechanisms of pioglitazone was mediated by retrieving oxidative DNA repair, which in turn blocked the vicious cycle of reactive oxygen species production, improved insulin sensitivity and halted proinflammatory signaling transduction (Hsiao et al., 2008). In agreement, pioglitazone attenuated MDA concentrations in white adipose tissue in db/db mice (Sugimoto et al., 2009), in liver and kidney in alloxan-diabetic rats (Chaudhry et al., 2007) and in streptozotocin-diabetic rats (Majithiya et al., 2005).

In the present study, pioglitazone in combination with pentoxifylline significantly decreased the elevated serum TNF-α, however, pioglitazone *per se* could not exert a similar effect. In contrast, other research groups found that the level of TNF-α in rats treated with pioglitazone was significantly lower than in HFD-fed rats with NAFLD (Xu et al., 2006), choline-deficient, l-amino acid-defined diet-fed rats (Fujita et al., 2007) and in ethanol and lipopolysaccharide-induced acute liver injury in rats (Ohata et al., 2004). The authors suggested that the therapeutic effect of pioglitazone on fatty liver may be associated with the regulation of TNF-α.

Pioglitazone in combination with pentoxifylline or melatonin induced a moderate improvement in the liver histology. Consistently, an improvement of hepatic steatosis, inflammation and liver cell ballooning was produced by pioglitazone (Fujita et al., 2007; Hsiao et al., 2008; Xu et al., 2006).

In the current study, pentoxifylline significantly reduced serum aminotranferase activities as compared to the NAFLD group. In agreement, pentoxifylline induced a significant reduction in serum ALT activity in rats maintained on a methionine and choline deficient diet with non-alcoholic steatohepatitis (Koppe et al., 2004). A similar improvement was observed in patients with non-alcoholic steatohepatitis after one month (Mihaila et al., 2009) and three months of pentoxifylline therapy (Lee et al., 2008).

Pentoxifylline had no effect on serum total cholesterol or triglycerides levels. Similarly, serum total cholesterol and triglycerides were unaffected by pentoxifylline in rats fed methionine and choline deficient diet (Koppe et al., 2004). In accordance, pentoxifylline reduced the development of hypercholesterolemic atherosclerosis in rabbits without changes in serum lipids (Prasad and Lee, 2007). More recently, pentoxifylline did not affect the lipid profile in patients of occlusive peripheral arterial disease (Singh et al., 2009).

In the current study, a significant reduction in the HOMA-IR index was observed after pentoxifylline treatment. Clinically, a significant reduction in HOMA-IR index in patients with non-alcoholic steatohepatitis was observed after six (Satapathy et al., 2004) and twelve months of pentoxifylline therapy (Satapathy et al., 2007). The improvement of the HOMA-IR index could be due to the down regulation of TNF-α by pentoxifylline. This cytokine is an important mediator of insulin resistance due to its ability to influence the tyrosine kinase activity of the insulin receptor (Hotamisligil et al., 1996). TNF-α is known to inhibit the propagation of insulin receptor-initiated signals in hepatocytes. Thus, modulation of insulin resistance by pentoxifylline could be a potential mechanism. In the present study, pentoxifylline reduced hepatic MDA and increased hepatic GSH contents significantly as compared to the NAFLD group. It has been reported that rats treated with pentoxifylline had significantly higher GSH levels than rats fed a methionine and choline deficient diet (Koppe et al., 2004) and a significant decrease in hepatic MDA in NAFLD rats (Yalniz et al., 2007). More recently, pentoxifylline was found to protect against cerulein-induced liver damage (Batcioglu et al., 2009) and D-galactosamine-induced reduction of antioxidant enzyme activities and elevation in MDA level in hepatic tissue (Taye et al., 2009).

Pentoxifylline induced a significant improvement in liver histology in the NAFLD group. A similar improvement was observed using a methionine and choline deficient diet model of non-alcoholic steatohepatitis (Koppe et al., 2004). In contrast, pentoxifylline in a dose of (9 mg/kg) did not influence either liver injury or early profibrogenic events in the choline-deficient diet model of non-alcoholic steatohepatitis (Vial et al., 2006).

Data presented here demonstrated that melatonin reduced the elevated activities of AST and ALT induced by HFD feeding. Similar results were obtained by melatonin in α-naphthylisothiocyanate-induced hepatotoxicity (Ohta et al., 2006), carbon tetrachloride-induced liver fibrosis (Shaker et al., 2009) and methionine and choline deficient diet-induced non-alcoholic steatohepatitis in rats (Tahan et al., 2009).

Further, melatonin exerted a significant antihyperlipidemic effect. Similar results were observed in α-naphthylisothiocyanate-induced hepatotoxicity (Ohta et al., 2006) and in adriamycin-induced hyperlipidemia in rats (Túnez et al., 2002). The hypolipidemic effect of melatonin may be related to the enhancement of the catabolism of cholesterol to bile acids (Chan and Tang, 1995), inhibition of cholesterol synthesis and LDL receptor activity (Muller-Wieland et al., 1994) and actions directly on the adipose tissue through specific melatonin receptors (MT_1 and MT_2) (Alonso-Vale et al., 2005; Brydon et al., 2001).

Melatonin significantly improved the HOMA-IR index as compared to the NAFLD group; these findings are consistent with previous reports (Mazepa et al., 2000; Shieh et al., 2009). In addition, melatonin decreased hepatic MDA and increased hepatic GSH content

significantly in comparison to the NAFLD group. These findings are supported by recent reports (Bekyarova et al., 2009; El-Sokkary et al., 2010; Hong et al., 2009). The elevation in liver GSH concentration after melatonin treatment could be linked to the stimulation of γ-glutamylcysteine synthase, a rate-limiting enzyme in GSH synthesis (Urata et al., 1999). Melatonin activity includes up-regulation of antioxidant enzymes and down-regulation of prooxidant enzymes (Reiter et al., 2007; Tan et al., 2007).

In the current study, melatonin (10 mg/kg) alone was unable to improve serum TNF-α level. In agreement, a previous work has shown that the combination of melatonin (10 mg/kg) and dexamethasone (0.025 mg/kg) significantly reduced the levels of TNF-α in brain tissue using an intracerebral hemorrhage model in rats, while each drug alone was unable to produce this effect (Li et al., 2009). Nevertheless, melatonin (50 mg/kg) reduced the increased TNF-α level significantly in a methionine and choline deficient diet model of non-alcoholic steatohepatitis (Tahan et al., 2009).

In the current study, the liver histology showed moderate improvement after melatonin treatment. In agreement, melatonin improved histopathologic abnormalities in the liver of HFD-fed rats (Pan et al., 2006) and methionine and choline deficient diet-fed rats (Tahan et al., 2009).

5. Conclusions

In conclusion, the current study improved our knowledge of the pathogenesis of NAFLD and highlighted that HFD-induced NAFLD in rats is a promising and reproducible model, bearing almost all biochemical, histopathological aspects of human NAFLD. Insulin resistance, oxidative stress and proinflammatory cytokines seem to be the major mechanisms in NAFLD development. Many of these abnormalities were successfully ameliorated by pioglitazone, pentoxifylline and melatonin treatments alone or in combination. However, the combination of pioglitazone with either pentoxifylline or melatonin provided minor improvements with respect to pioglitazone alone. Further investigations are needed to ensure the benefits of these combinations and to determine long-term consequences of these medications in patients with NAFLD.

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