Role of pentoxifylline in non-alcoholic fatty liver disease in high-fat diet-induced obesity in mice

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AIM: To study pentoxifylline effects in liver and adipose tissue inflammation in obese mice induced by high-fat diet (HFD).

METHODOLOGY: Male swiss mice (6-wk old) were fed a high-fat diet (HFD; 60% kcal from fat) or AIN-93 (control diet; 15% kcal from fat) for 12 wk and received pentoxifylline intraperitoneally (100 mg/kg per day) for the last 14 d. Glucose homeostasis was evaluated by measurements of basal glucose blood levels and insulin tolerance test two days before the end of the protocol. Final body weight was assessed. Epididymal adipose tissue was collected and weighed for adiposity evaluation. Liver and adipose tissue biopsies were homogenized in solubilization buffer and cytokines were measured in supernatant by enzyme immunoassay or multiplex kit, respectively. Hepatic histopathologic analyses were performed in sections of paraformaldehyde-fixed, paraffin-embedded liver specimens stained with hematoxylin-eosin by an independent pathologist. Steatosis (macrovesicular and microvesicular), ballooning degeneration and inflammation were histopathologically determined. Triglycerides measurements were performed after lipid extraction in

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) defines a spectrum of hepatic disorders including steatosis or uncomplicated fatty liver and non-alcoholic steato-hepatitis (NASH). NAFLD is frequently associated with metabolic syndrome establishment and it occurs more often in males than in females and primarily affects the middle aged and the elderly[1].

Several factors are associated with the development of fatty liver, and NAFLD diagnosis requires the exclusion of secondary etiologies, including alcohol consumption, drug usage, hepatitis B and C[2]. Although simple steatosis is considered benign, NASH can progress to end-stage liver disease, such as fibrosis, cirrhosis and hepatic cancer[3]. The mechanism of steatosis progression to more severe liver injuries is not fully understood, but it is associated with several risk factors, including elevated serum transaminases, inflammation upon liver biopsy, old age, diabetes mellitus, high body mass index (≥28 kg/m²), presence of ballooning plus Mallory hyaline or fibrosis upon biopsy and increased visceral adipose tissue[2,4].

The growing epidemic of obesity and an aging population have led to an important demand for a medical therapy for NAFLD, but several decades of pharmacological research have resulted in very few options[2]. As NAFLD is considered a hepatic manifestation of a metabolic syndrome, the first treatment approach is a lifestyle change, including dietary alterations and increased physical activity to reduce adiposity and body weight[5]. Therapeutic drugs are an adjunctive approach to lifestyle changes. Statins are used to control dyslipidemias, metformin and glitazones are used to control diabetes mellitus, and angiotensin receptor blockers are used to control inflammatory cell recruitment and hepatic fibrosis development in addition to their antihypertensive effects. In total, these drugs aim to control the symptoms of the metabolic syndrome[2,3-6].

Pentoxifylline is a non-selective phosphodiesterase inhibitor that has been reported to have antioxidant activity and decrease tumor necrosis factor (TNF)-α gene transcription. Pentoxifylline treatment improved the 6-mo survival rate of patients with severe alcoholic hepatitis compared with placebo[7]. Recent studies have shown that pentoxifylline may be a promising drug therapy for NASH treatment[8-10]. Experimental results with pentoxifylline have shown conflicting data depending on the model employed. In NAFLD induced by a choline- and methionine-deficient diet, pentoxifylline treatment was beneficial because it decreased hepatic inflammation and alanine aminotransferase (ALT) levels[11]. However, in a genetic obesity model, pentoxifylline worsened fatty liver in ob/ob mice because it increased intestinal glucose absorption, and thus, hyperglycemia[12].

Considering that obesity is strongly associated with the development of NAFLD and one of the main causes of epidemic obesity is a hyperlipidic and hypercaloric liver tissue.

RESULTS: Pentoxifylline treatment reduced micro-steatosis and tumor necrosis factor (TNF)-α in liver (156.3 ± 17.2 and 62.6 ± 7.6 pg/mL of TNF-α for non-treated and treated obese mice, respectively; P < 0.05). Serum aspartate aminotransferase levels were also reduced (23.2 ± 6.9 and 12.1 ± 1.6 U/L for non-treated and treated obese mice, respectively; P < 0.05) but had no effect on glucose homeostasis. In obese adipose tissue, pentoxifylline reduced TNF-α (106.1 ± 17.6 and 51.1 ± 9.6 pg/mL for non-treated and treated obese mice, respectively; P < 0.05) and interleukin-6 (340.8 ± 51.3 and 166.6 ± 22.5 pg/mL for non-treated and treated obese mice, respectively; P < 0.05) levels; however, leptin (8.1 ± 0.7 and 23.1 ± 2.9 ng/mL for non-treated and treated lean mice, respectively; P < 0.05) levels increased in lean adipose tissue. TNF-α level in the liver of lean mice also increased (29.6 ± 6.6 and 75.4 ± 12.6 pg/mL for non-treated and treated lean mice, respectively; P < 0.05) while triglycerides presented a tendency to reduction.

CONCLUSION: Pentoxifylline was beneficial in obese mice improving liver and adipose tissue inflammation. Unexpectedly, pentoxifylline increased pro-inflammatory markers in the liver and adipose tissue of lean mice.

Key words: Pentoxifylline; Steatosis; Obesity; Adipose tissue; Adipokine; Tumor necrosis factor-α
diet, our study evaluated the effects of pentoxifylline in a high-fat diet (HFD)-induced obesity model. Metabolic parameters, hepatic inflammation and adipose tissue alteration were studied after 2 wk of pentoxifylline treatment in mice after 12 wk of a HFD.

MATERIALS AND METHODS

Animals, diets and treatment
Specific pathogen-free, 4-wk-old male Swiss mice were obtained from CEMIB (State University of Campinas, Campinas, São Paulo, Brazil). All experiments were performed in accordance with the principles outlined by the National Council for the Control of Animal Experimentation (CONCEA, Brazil) and received approval from the Ethics Committee of São Francisco University, Bragança Paulista, SP, Brazil (Protocol CEA/USF 00.02.11). The animal protocol was designed to minimize pain or discomfort to the animals.

The animals were individually housed and acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation. After random selection, 6-wk-old mice were introduced to control AIN-93 or HFD ad libitum for 12 wk (Table 1). The mice received 100 mg/kg per day intraperitoneal pentoxifylline (Sigma Aldrich, Co. - Saint Louis, Missouri, United States) diluted in 0.9% NaCl for further analysis.

Table 1: Diet composition

| Diet Composition | Control (AIN-93) | HFD |
|------------------|----------------|-----|
|                  | g/kg | kcal/kg | g/kg | kcal/kg |
| Cornstarch (QSP) | 397.5 | 1590 | 1155 | 462 |
| Casein           | 200  | 800   | 200  | 800   |
| Sucrose          | 100  | 400   | 100  | 400   |
| Dextrin starch   | 132  | 528   | 132  | 528   |
| Soybean oil      | 70   | 630   | 40   | 360   |
| Lard             | -    | -     | 312  | 2808  |
| Cellulose        | 50   | -     | 50   | -     |
| Mineral mix      | 35   | -     | 35   | -     |
| Vitamin mix      | 10   | -     | 10   | -     |
| L-cystine        | 3    | -     | 3    | -     |
| Choline          | 2.5  | -     | 2.5  | -     |
| Total            | 1000 | 3948  | 1000 | 5358  |

HFD: High-fat diet.

Blood glucose levels and insulin tolerance tests
Twenty-four hours before the end of the protocol, mice were fasted for 6 h, and blood samples were collected from the tails. Glucose was measured using the glucose oxidase method. Insulin (1.5 U/kg) was administered by intraperitoneal injection, and blood samples were collected for serum glucose determination at 0, 10, 15, 20 and 30 min. The rate constant for glucose disappearance during an insulin tolerance test (kITT) was calculated using the formula 0.693/t1/2. The glucose t1/2 was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase.

Necropsy and sample collection
At the end of protocol, mice were fasted for 12 h and euthanized by xylazine/ketamine overdose (0.1 mL/30 g body weight of 1:1 v/v of 2% xylazine and 10% ketamine), and blood samples were collected in tubes by portal vein or cardiac puncture. Liver was perfused with 15 mL phosphate buffered saline (PBS), collected and weighed. Samples were immediately processed or stored at -80 °C for further analysis.

Hepatic enzyme analysis
Aspartate aminotransferase (AST) and ALT serum levels were determined using a commercial kit (LABORLAB, São Paulo, Brazil).

Cytokine and chemokine analysis in the liver and adipose tissue
Liver and adipose tissue biopsies were homogenized in solubilization buffer containing 100 mmol/L Tris (pH = 7.6), 1% Triton X-100, 150 mmol/L NaCl, 0.1 mg aprotinin, 35 mg/mL PMSF, 10 mmol/L NaVO4, 100 mmol/L NaF, 10 mmol/L Na-p-O4 and 4 mmol/L EDTA to extract total protein. Liver and adipose tissue extract supernatants were collected and used in ELISA kits (R and D Systems, Inc, Minneapolis, MN, United States) or Multiplex Assay kits (Millipore, Billerica, MA, United States), respectively, according to the manufacturer’s protocol.

Liver histology
Hydrated 4.0 mm sections of paraformaldehyde-fixed, paraffin-embedded liver specimens were stained with hematoxylin-eosin to evaluate liver histology. Additional sections were stained with Masson’s trichrome for fibrosis analysis. For each group, six to nine mouse livers were prepared and stained. An expert pathologist evaluated the stained samples in a blinded fashion. Steatosis, ballooning degeneration and inflammation were histopathologically determined. The percentage of steatotic cells (macrovesicular and microvesicular) was determined and graded as follows: (1) 0: absent; (2) 1: < 25%; (3) 2: 26%-50%; (4) 3: 51%-75%; or (5) 4: > 75% of the parenchyma. Hyperemia, inflammation and fibrosis were evaluated as either present or absent.

Measurement of triglycerides in the liver
Liver tissues were homogenized with in chloroform and methanol (2:1 v/v) and an aqueous solution of NaCl was added[13]. The chloroform layer was dried under N2, the total extract resuspended in PBS and triglycerides were determined using commercial enzymatic kit (LaborClin, Pinhais, PR, Brazil).

Statistical analysis
Data are expressed as the mean ± SEM. Comparisons...
among groups of data were made using a one-way ANOVA test followed by the Dunnett multiple comparisons test. Non-parametric data (scores) are expressed as the median (range) and were analyzed using the Mann-Whitney test. An associated probability (P value) of 5% was considered statistically significant.

RESULTS

Metabolic and anthropometric parameters after pentoxifylline treatment

Animals on a HFD for 12 wk presented significant alterations in body weight. However, pentoxifylline-treated mice showed no change in body weight compared with the matched controls. Pentoxifylline treatment decreased liver weight in obese mice, but the depot of visceral adipose tissue significantly increased. We evaluated blood glucose levels and insulin tolerance at the end of the treatment and did not find any differences between treated animals and untreated animals. AST levels decreased after pentoxifylline treatment, but ALT levels did not change (Table 2).

Liver histological analysis and triglycerides content

The livers from HFD mice presented pronounced macrosteatosis, microsteatosis, hyperemia and inflammation, features that were not observed in lean mice. We did not observe fibrosis in any of the groups. Pentoxifylline treatment did not alter the livers of lean mice, but it reduced inflammation, and we observed a trend to reduce microsteatosis and hyperemia in HFD mice (Figure 1 and Table 3). However, triglycerides measurement revealed a tendency to reduction in livers from lean mice but not from obese mice (Figure 1).

Inflammatory markers in liver and adipose tissue

We evaluated TNF-α, interleukin (IL)-10 and monocyte chemoattractant protein (MCP)-1 protein levels in the livers of untreated obese mice or obese mice treated with pentoxifylline. A high-fat diet increased TNF-α and MCP-1 levels but did not affect IL-10 expression. Pentoxifylline treatment reduced TNF-α level but did not modify hepatic MCP-1 or IL-10 levels (Table 4). Adipose tissue analysis revealed that total plasminogen activator inhibitor (PAI)-1, MCP-1 and leptin levels increased in obese mice. Pentoxifylline treatment significantly decreased TNF-α and IL-6 levels in obese adipose tissue, but increased leptin and PAI-1 in lean adipose tissue (Table 5).

DISCUSSION

NAFLD is currently considered a consequence of obesity, and its prevalence in obese subjects is very high. Sedentary life style and consumption of foods with high-fat and high-caloric content are the main contributing factors to NAFLD.
factors to obesity\(^{[14]}\). The reduction of body weight and lifestyle changes are the primary recommendations to

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**Table 5** Adipokine profile of lean (control) and obese (high-fat diet) mice treated with pentoxifylline

|                | Control        | HFD            |
|----------------|----------------|----------------|
|                | NT  | PTX  | NT  | PTX  |
| **TNF-α (pg/mL)** | 87.2 ± 2.8 | 75.5 ± 3.9  | 106.1 ± 17.6 | 51.1 ± 9.6\(^a\) |
| **IL-6 (pg/mL)**  | 227.9 ± 23.3 | 178.5 ± 30.9 | 340.8 ± 51.3 | 166.6 ± 22.5\(^a\) |
| **PAI-1 (pg/mL)** | 600.2 ± 32.3 | 1508.6 ± 210.4 | 2646.3 ± 755.1\(^c\) | 4434.5 ± 1400.1|
| **MCP-1 (pg/mL)** | 287.8 ± 17.1 | 253.8 ± 13.3 | 271.5 ± 112.8\(^a\) | 563.8 ± 109.7|
| **Leptin (ng/mL)**  | 8.1 ± 0.7  | 23.1 ± 2.9\(^c\) | 26.6 ± 3.0\(^c\) | 20.5 ± 3.7|
| **Adiponectin (ng/mL)** | 109.1 ± 0.5 | 102.8 ± 0.6 | 106.8 ± 1.7 | 102.8 ± 5.9|

\(^{a}\)P < 0.05 vs control NT group; \(^{c}\)P < 0.05 vs the matched NT group. HFD: High-fat diet; NT: Non-treated; PTX: Pentoxifylline; TNF-α: Tumor necrosis factor α; IL-6: Interleukin-6; MCP: Monocyte chemoattractant protein-1; PAI: Plasminogen activator inhibitor-1.
control NAFLD, and pharmacological interventions aim to induce weight loss (e.g., orlistat and sibutramine), to improve the antioxidant response (e.g., vitamin E and C, ursodeoxycholic acid) or to ameliorate insulin resistance and glucose and lipid metabolism (e.g., metformin, thiazolidinediones)\[15\]. Pentoxifylline has been considered an alternative treatment to control NAFLD, as it has been recommended in alcoholic fatty liver disease by acting as an anti-inflammatory drug\[16\]. Pentoxifylline is a methylxanthine derivative that acts as a nonspecific phosphodiesterase inhibitor to promote an increase in cyclic AMP levels and inhibit TNF-α gene transcription\[17,18\].

A high-fat diet obesity model is suitable to study metabolic and liver disease associated with adipose tissue expansion and to study potential therapeutics to control obesity. Our results show that Swiss mice fed a HFD for 12 wk present increased body weight, increased adiposity, adipose tissue inflammation, insulin resistance, hyperglycemia, steatosis, inflammation and increased TNF-α and MCP-1 levels in the liver. Pentoxifylline treatment did not change the final body weight but did decrease the liver weight. Visceral (epididymal) adipose tissue increased after pentoxifylline treatment, which may explain why we did not observe a decrease in body weight. Pentoxifylline treatment did not improve glucose homeostasis, but some NAFLD features improved, such as hepatic steatosis, inflammation, TNF-α levels, and serum AST levels. Our results are consistent with a previous study, where Sprague-Dawley rats fed a HFD for 16 wk were treated with pentoxifylline for 4 wk (16 mg/kg per day). The results showed decreased AST levels but not ALT, and improvements in basal glucose but not HOMAIR index\[19\]. Additionally, in a previous study of Sprague-Dawley fed HFD for 10 wk and treated with pentoxifylline for 6 wk (50 mg/kg per day), hepatic steatosis and plasma levels of TNF-α were reduced\[20\]. In our work, we evaluated both hepatic and adipose tissue TNF-α levels and found that pentoxifylline treatment reduced both. However, glucose homeostasis did not improve, but TNF-α and IL-6 levels decreased. A balance between leptin and adiponectin have been suggested to have a role in metabolic syndrome and type 2 diabetes\[21\]. Pentoxifylline treatment could not reverse the alterations in the obesity-induced leptin/adiponectin ratio.

Interestingly, we observed increased TNF-α in the liver and increased PAI-1 and leptin in adipose tissue of lean mice after pentoxifylline treatment. A systematic review of pentoxifylline data in patients with NAFLD revealed that AST and ALT plasma levels and liver histological scores were improved in several studies using pentoxifylline. However, pentoxifylline treatment did not inhibit plasma cytokines levels such as IL-6 in all studies\[21\]. Although pentoxifylline has been shown to inhibit TNF-α, Zein et al\[22\] reported that pentoxifylline treatment did not inhibit TNF-α plasma levels in NASH patients. The authors suggested that TNF-α plasma levels may not be related to hepatic levels of this cytokine because they observed histological improvement. Both the adipose tissue and liver of lean mice increased pro-inflammatory cytokine production in response to pentoxifylline treatment, an unexpected result that should be further studied. Interestingly, triglycerides levels presented a tendency to reduction in liver of lean mice. Several findings suggested that triglycerides per se are not toxic, on contrary; they protect liver from lipotoxicity by buffering the accumulation of fatty acids. Triglycerides synthesis inhibition improves steatosis but stimulates oxidizing systems that increase hepatic oxidative stress and liver damage\[23\]. Although, pentoxifylline is able to decrease oxidative stress and to inhibit lipid peroxidation in patients with NASH\[24\], we did not rule out the possibility that lean mice had an increase in oxidative response due pentoxifylline treatment. We hypothesize that metabolic status, liver metabolism, adiposity or inflammation degree can interfere with the pentoxifylline response, which could explain the controversial data obtained in different clinical studies of NAFLD patients. In this line of reasoning, pentoxifylline is effective in states of hyperinflammation because relevant anti-inflammatory effects can be achieved only in the presence of sufficient adenosine concentrations\[25\]. Metabolic stress, hypoxia and inflammation are conditions related to increase adenosine extracellular concentrations\[26\], and thus, could interfere with the pentoxifylline response.

In conclusion, our results showed that pentoxifylline was beneficial in an obesity-associated NAFLD model by improving liver inflammation and adipose tissue inflammation, but it was not able to improve obesity-induced metabolic disturbances. Unexpectedly, pentoxifylline treatment increased pro-inflammatory markers in the liver and adipose tissue of lean mice.

**COMMENTS**

**Background**

Non-alcoholic fatty liver disease (NAFLD) defines a spectrum of hepatic disorders including steatosis and non-alcoholic steatohepatitis that can progress to end-stage liver disease, such as fibrosis, cirrhosis and hepatic cancer. NAFLD is frequently associated with metabolic syndrome establishment and obesity. Several decades of pharmacological research have resulted in very few options for NAFLD management; therefore, new therapeutic approaches need to be researched.

**Research frontiers**

Pentoxifylline is a non-selective phosphodiesterase inhibitor that has been reported to have antioxidant activity and decrease tumor necrosis factor (TNF-α) gene transcription. Pentoxifylline is prescribed to patients with severe alcoholic hepatitis, which suggest that this drug could also be beneficial to NAFLD. In this study, authors described pentoxifylline effects upon NAFLD using an experimental model of high-fat diet induced obesity in mice. Pentoxifylline was beneficial in obesity-associated NAFLD reducing liver microsteatosis and TNF-α, as well as, serum aspartate aminotransferase levels. However, pentoxifylline treatment in lean mice resulted in pro-inflammatory cytokine production in the liver and adipose tissue, suggesting that pentoxifylline effects could be dependent of additional conditions, as metabolic status, liver metabolism, adiposity or inflammation degree.

**Innovations and breakthroughs**

NAFLD has reached epidemic proportions nowadays. The current therapy is
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disease (NAFLD).

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