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ARTICLE

Protective effect of rimonabant, a canabinoid receptor 1 antagonist, on nonalcoholic fatty liver disease in a rat model through modulation of the hepatic expression of activin A and follistatin

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Abstract: Non-alcoholic fatty liver disease (NAFLD) is a major cause of liver morbidity and mortality, and there is still no proven effective therapy. The endocannabinoid system plays an important role in various liver diseases. Activin A is a member of the transforming growth factor beta (TGF-β) superfamily and inhibits hepatocyte growth. Follistatin antagonizes the biological actions of activin A. This study was designed to investigate the effect of rimonabant (a potent cannabinoid receptor1 (CB1) antagonist) on NAFLD induced with a choline-deficient (CD) diet in rats, as well as to detect whether it can alter the hepatic expression of activin A and follistatin. Forty rats were distributed among 4 groups: the control group, the rimonabant treatment group (normal rats that received rimonabant); the CD diet group (NAFLD induced with a CD diet); and the CD diet + rimonabant group (NAFLD treated with rimonabant). It was found that the CD diet caused significant increase in liver index, serum levels of liver enzymes, malondialdehyde (MDA), TGF-β1, activin A, and CB1 expression in liver tissue, with a significant decrease in glutathione peroxidase (GSH-Px) and follistatin mRNA expression in liver tissues. The administration of rimonabant significantly improved all of the studied parameters compared with the group fed the CD diet alone. Histopathological examination supported these results. We concluded that rimonabant significantly counteracted NAFLD induced with the CD diet by decreasing oxidative stress and hepatic expression of TGF-β1, and modulating the hepatic expression of activin A and follistatin.

Key words: nonalcoholic fatty liver disease, canabinoid receptor 1, TGF-β, activin A, follistatin, oxidative stress.

Résumé : La stéatose hépatique non alcoolique (SHNA) est une cause majeure de morbidité et de mortalité hépatiques, qui ne sont pas encore contenues par un traitement efficace appuyé par des données probantes. Le système des endocannabinoïdes joue un rôle important dans diverses maladies hépatiques. L'activine A est un membre de la superfamille du facteur de croissance transformant bêta (TGF-β), laquelle entraîne une inhibition de la croissance des hépatocytes. Pour sa part, la follistatine inhibe les actions biologiques de l'activine A. La présente étude a été conçue en vue d'évaluer l'effet du rimonabant (un puissant antagoniste du récepteur 1 des cannabinoïdes [CB1]) sur la SHNA provoquée par un régime alimentaire sans choline (RSC) chez le rat, ainsi que pour établir si cette substance peut entraîner ou non la modulation de l'expression de l'activine A et de la follistatine dans le foie. Nous avons réparti 40 rats dans 4 groupes : témoin, rimonabant (des rats normaux ont reçu du rimonabant), RSC (SHNA provoquée par un RSC), RSC + rimonabant (rats SHNA exposés au rimonabant). Nous avons observé que le RSC entraînait une augmentation notable de l'index hépatique, des taux sériques d'enzymes hépatiques, de malondialdéhyde (MDA), de TGF-β1 et d'activine A, ainsi que de l'expression des CB1 dans le tissu hépatique, avec une diminution notable de la glutathion peroxydase (GSH-Px) et de l'expression de l'ARNm de la follistatine dans les tissus hépatiques. L'administration de rimonabant a permis d'améliorer nettement tous les paramètres étudiés par rapport au groupe RSC. L'examen histopathologique est venu appuyer ces résultats. Nous en sommes venus à la conclusion que le rimonabant permet de contrecarrer de façon notable la SHNA provoquée par le RSC en entraînant une diminution du stress oxydatif et de l'expression du TGF-β1 dans le foie, ainsi que la modulation de l'expression hépatique de l'activine A et de la follistatine. [Traduit par la Rédaction]

Mots-clés : stéatose hépatique non alcoolique, récepteur 1 des cannabinoïdes, TGF-β, activine A, follistatine, stress oxydatif.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is considered the most common cause of abnormal liver function and chronic liver disease in both developing and developed countries worldwide (Oh et al. 2008). NAFLD is a group of chronic diseases including fatty liver disease and steatosis, as well as more severe lesions, including lobular necroinflammation, steatohepatitis with fibrosis, and cirrhosis. NAFLD-related cirrhosis can lead to end-stage liver disease and hepatocellular carcinoma (Bieghs and Trautwein 2014).

The exact reasons and mechanisms by which NAFLD advances from one stage to the next are not known. NAFLD has been considered a condition with a "two-hit" process of pathogenesis. Basically, the first hit is the development of hepatic steatosis via the accumulation of triglycerides in hepatocytes, which increases the vulnerability of the liver to various possible "second hits" that in

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turn lead to the inflammation, fibrosis, and cellular death that are characteristic of steatohepatitis. Hormonal imbalances, oxidative stress, and mitochondrial abnormalities are possible causes for this "second hit" phenomenon (Tziomalos et al. 2012).

Fibrosis is the main process in the development of NAFLD from the start to the end. An animal model of "fibrosing steatohepatitis" that resembles the histologic features of human non-alcoholic steatohepatitis (NASH) explains the series of steatosis, inflammatory cell injury, and fibrogenesis, mediated by hepatic stellate cells via up-regulation of TGF-β1 (George et al. 2003).

Activins are members of the TGF-β superfamily that are formed of bioactive dimeric proteins composed of two beta subunits. Activin A, a homodimer composed of two beta-A subunits, is involved in the pathogenesis of several liver disorders, including NAFLD and liver fibrosis (Yndestad et al. 2011). Activin A inhibits the replication of hepatocytes and induces apoptosis, so it is considered to be a negative regulator of liver growth. Follistatin, a glycoprotein that binds activin A with high affinity and blocks activin A signaling, counteracts the biological actions of activin (Ooe et al. 2012). Both activin A and follistatin are expressed on hepatic cells, and have been considered as the main regulators of liver biology, pathology, and regeneration (Rodgarkia-Dara et al. 2006).

Endocannabinoids (ECs) are endogenous arachidonic-acid-derived mediators synthesized on demand from membrane phospholipids. They are released from cells immediately after production and activate CB1 to elicit a biological response, after which they are inactivated through reuptake (Romero-Zerbo and Bermúdez-Silva 2014). The upregulation of CB1 in NAFLD, alcoholic liver disease, autoimmune and viral hepatitis, ischemia–reperfusion, and cirrhosis have demonstrated. Thus, ECs are involved in numerous pathophysiological processes associated with chronic liver diseases (Pisanti et al. 2015).

The liver is identified as a primary site for EC-mediated modulation of lipogenesis. Actually, the activation of the CB1 receptor increases the expression of lipogenic genes in the liver, which is the major source of de novo fatty acid synthesis in the body. It has been suggested that hepatic CB1 receptors are involved in the progress of fatty liver disease in diet-induced hepatic steatosis (Schwabe 2005; Pagotto et al. 2006; Li et al. 2011). We hypothesized that rimonabant, a potent CB1 antagonist could have a hepatoprotective effect against NAFLD induced with a choline-deficient (C) diet.

Based on this background, this study was designed to investigate the possible protective effects of rimonabant on NAFLD induced with a CD diet, as well as to evaluate the effect of rimonabant on oxidative stress markers and profibrotic cytokine (TGF-β1). Further, to determine whether activin A and follistatin participate in its molecular mechanisms.

Materials and methods

Chemicals and reagents

Rimonabant was provided by Akros Pharma (Sigma Chemical Co., St. Louis, Missouri, USA). Tween 80 was supplied by Calbiochem, (Millipore–Sigma, Billerica, Massachusetts, USA). Activin$_{\beta A}$, CB1 receptors, follistatin mRNA, and GAPDH antibodies were provided from Applied Biosystems (Foster City, California, USA). SYBR Green PCR Master Mix was from Applied Biosystems. Kits for measuring aspartate transferase (AST), alkaline phosphatase (ALP), alanine transferase (ALT), albumin, and bilirubin were supplied by the Egyptian Company for Biotechnology. Kits for measuring malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) were purchased from Biodiagnostics. TGF-β was provided by Abcam.

Composition of the diets

The rat diets were formulated according to NRC (1995) as shown in (Tables 1 and 2).

Table 1. Ingredients in the experimental feed as a percentage of the standard diet.

Feed ingredients	Standard diet (%)
Sunflower oil	15.00
Concentrate mixture[a]	10.00
Yellow corn	49.00
Soybean meal (44%)	11.00
Wheat bran	10.00
Molasses	03.00
Common salt	00.50
Ground limestone	00.20
Dicalcium phosphate	00.10
Lysine	00.20
DL-Methionine	00.70
Mineral–vitamin premix[b]	00.30
Total	**100%**

[a]The concentrate mixture was composed of the following: corn gluten, dicalcium phosphate, soybean meal, fish meal, limestone, broiler premix, L-lysine HCl, DL-methionine, and common salt in different concentrations.

[b]Each 3 kg of feed contained: 12 000 000 IU vit. A; 2 000 000 IU vit. D3; 10 000 mg vit. E; 1000 mg vit. K3; 1000 mg vit. B1; 5000 mg vit. B2; 1500 mg vit. B6; 10 mg vit. B12; 50 mg biotin; 10 000 mg pantothenic acid; 30 000 mg nicotinic acid; 1000 mg folic acid; 60 000 mg manganese; 50 000 mg zinc; 30 000 mg iron; 4000 mg copper; 300 mg iodine; 100 mg selenium; 100 mg cobalt; up to 3 kg carrier ($CaCO_3$).

Table 2. Choline deficient diet composition.

Ingredients	Choline deficient diet (%)
Corn	58.275
Soya	21.9
Oil	15.0
Limestone	1.2
Sucrose	3.0
Lysine	0.2
Common salts	0.125
Premix choline free	0.3
Total	**100%**

Animals

Forty male Sprague–Dawley rats weighing 180–220 g were used. They were obtained from the Experimental Animal Unit of Moshtohor, Faculty of Agriculture, Benha University. The animals were acclimatized to the laboratory conditions for 10 days prior to the initiation of the experiment. They were housed in the animal room at controlled temperatures in a 12 h : 12 h light/dark cycle, and had free access to water and food. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH publication No. 85-23, revised 1996). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine, Benha University.

Experimental design

The animals used were randomly distributed among 4 groups as follows: group I (control group), consisted of 10 rats, served as the control group, and received the standard diet for 12 weeks, then were given 0.1% Tween 80 in distilled water by oral gavage for 2 weeks before scarification; group II (rimonabant group), consisted of 10 rats, received the standard diet for 12 weeks, then were treated daily with a dose of rimonabant (10 mg/kg body mass) by oral gavage for 2 weeks before scarification (Jorgačević et al. 2015);

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group III (CD diet group), consisted of 10 rats, received the CD diet (only) for 12 weeks; group IV (CD diet + rimonabant group), consisted of 10 rats, received CD diet for 12 weeks, then were treated daily with a dose of rimonabant (10 mg/kg body mass) by oral gavage for 2 weeks before scarification (Jorgačević et al. 2015). Rimonabant was dissolved in 0.1% Tween 80 in distilled water, and then sonificated on ice for 20 s using a digital Branson sonificator before administration. Blood samples were obtained from retro-orbital venous plexus to measure liver enzymes before administering the CD diet, and again at the end of the 10th week. The rats had a non-significant difference in liver enzymes between the beginning of the CD diet and week 10 were excluded from the study, while those had significant difference were included in the study and treated with rimonabant.

At the end of the 12th week after an overnight fasting, the animals were anesthetized with ketamine (100 mg/kg body mass by intraperitoneal injection (i.p.)). The animals were fixed on the operating table and the blood samples and liver biopsies were taken as follow: A cranio-caudal incision of about 2 cm was made for blood sample collection, parallel with and slightly to the left of the sternum through the skin and pectoral muscles to expose the ribs. A blunt curved forceps was then inserted between the 5th and 6th ribs, through the intercostal muscles. The gap was then widened with the forceps so that the rapidly beating heart became visible, then the blood samples were taken from the right ventricle. The previous incision was continued through the animal's anterior abdominal wall, then, the abdominal cavity was entered by cutting the muscles and peritoneum. The liver was exposed then freed from the surrounding tissues and was gently pulled out through the incision (Corbin and Minuk 2003). The liver was then was immediately isolated, washed with ice-cold saline and weighed. Then the liver was divided into 2 halves: the first one was rapidly frozen and stored in liquid nitrogen −70 °C and was later used to measure oxidative stress markers and for real-time PCR analysis; the second half was kept in formaldehyde to be prepared for histopathological examination with hematoxylin and eosin (H&E) to determine any histopathological signs of NAFLD, and immunohistochemical examination to assess TGF-β1 expression.

Liver mass index calculation

The liver mass index was calculated according to Iwo et al. (2017) as follows: (liver mass/body mass × 100).

Assessment of hepatic function

Serum levels of AST, ALT, ALP, total bilirubin, and albumin were measured using the commercial assay kits mentioned above, according to the manufacturer's instructions.

Assessment of oxidative stress in hepatic tissue

The frozen liver samples were cut and homogenized using a mixer mill (MM400; Retsch, Haan, Germany) to measure GSH-Px according to the methods ofTappel (1978), and the results are expressed in units per milligram of tissue. Also lipid peroxidation contents in the form of MDA levels were measured by a modified method of Ohkawa et al. (1979) and the results are expressed in nanomoles per gram of liver tissue.

RNA extraction and quantitative real-time PCR

The frozen liver samples were cut and homogenized using a mixer mill to isolate the mRNA. Total RNA was isolated from 40 mg tissue using a total RNA purification kit (Jena Bioscience). The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and 280 nm. The amount of activin$_{\beta A}$ and follistatin mRNA was determined with an ABI Prism 7900HT quantitative real-time PCR (Applied Biosystems). The primer sequences used were as follows: activin$_{\beta A}$ (forward primer) 5′-ATGGACCTAACTCTCAGCCAGA-3′; (reverse primer) 5′-CTCTCCCCCTTCAAGCCCAT-3′; follistatin (forward primer) 5′-GGCGTACTGCTTGAAGTGAA-3′; (reverse primer) 5′-GGGAAGCTGTAGTCCTGGTC-3′; canabinoid-1 receptor (forward primer) 5′-ACCTACCTGATGTTCTGGATTGGG-3′; (reverse primer) 5′-CGTGTGGATGATGATGCTCTTCTG-3′; GAPDH (forward primer) 5′-GATGCTGGTGCTGAGTATGTCG-3′; (reverse primer) 5′-GTGGTGCAGGATGCATTGCTGA-3′. For real-time PCR, 20 ng cDNA and 0.4 μmol/L of each primer were used in a 25 μL reaction volume containing SYBR Green PCR Master Mix (Applied Biosystems). The temperature program was as follows: inactivation of reverse transcriptase at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. The specificity of the PCR results was confirmed by dissociation curve analysis. According to the RQ manager program ABI SDS software (ABI 7900), the data are produced as sigmoid-shaped amplification plots in which the number of cycles is plotted against fluorescence (when using a linear scale). The threshold cycle (C_T) serves as a tool for calculating the quantity of starting template in each sample. Because the samples from the control group and also samples from the treatment groups were used for calibration, the expression levels are set to 1. Because the relative quantities of activin$_{\beta A}$ and the follistatin gene are normalized against the relative quantities of the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene fold-expression changes are calculated using the equation $2^{-\Delta\Delta C_T}$ (Al Husseini et al. 2010).

Morphometric analysis

The non-frozen liver samples were fixed in 10% neutral buffered formalin and embedded in paraffin. This was followed by dehydration of the fixed tissue in various grades of alcohol (100%, 90%, 80%, 70% *v*/*v*); the tissue samples were then cleared in benzene. To evaluate liver injury, section 5 μm thick were cut from the paraffin blocks using a microtome, and stained with H&E. Liver biopsies were evaluated, following a blind protocol, using the NASH Clinical Research Network Histological Scoring System (Kleiner 2006). The NAFLD activity score is a sum of three histological scores, including steatosis (0–3), lobular inflammation (0–2), hepatocellular ballooning (0–2), where 0 = absent; 1 = mild; 2 = moderate; 3 = severe. The mean area% of TGF-β immunoreaction in hepatocytes was quantified in 10 images of high-power magnification (×400) for each group using Image-Pro Plus version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA) in the Pathology Department of the Faculty of Medicine, Benha University.

Statistical analysis

All the data presented are the mean ± SD. Evaluation of the differences between groups was performed using one-way ANOVA followed with a post-hoc test (Fisher's LSD) between groups, using SPSS 19.0 software. The correlations between CB1 receptor gene expression, activin$_{\beta A}$ mRNA, and follistatin mRNA levels were analyzed using Pearson's correlation coefficient (r) 2-tailed test. A p value of less than 0.05 was considered statistically significant.

Results

Effect of rimonabant on liver index and liver function in all of the experimental groups (Table 3)

Serum ALT, AST, ALP, liver mass, and liver mass index were significantly higher ($p < 0.001$) in the CD diet group compared with the control and the rimonabant group. There was no statistically significant difference in body mass, albumin levels, and bilirubin levels among all of the experimental groups. Treatment with rimonabant induced a significant decrease ($p < 0.001$) in serum ALT, AST, ALP, liver mass, and liver mass index in the CD diet + rimonabant group compared with the CD diet (only) group. In contrast, there was no statistically significant difference in ALT, AST, ALP, liver mass, body mass, and liver mass index between the rimonabant group and the control group.

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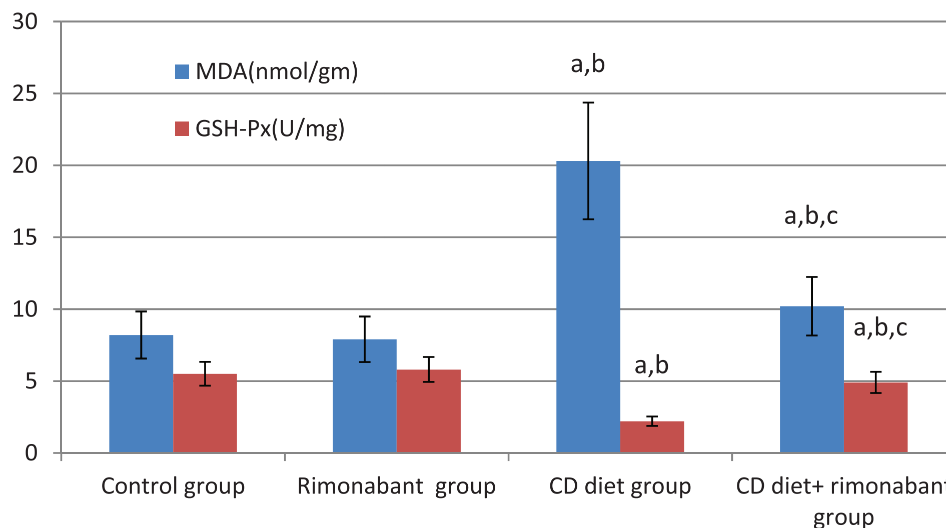
Table 3. Effect of rimonabant on liver index and liver function in normal rats and rats fed the choline-deficient (CD) diet (mean ± SD).

	Control group	Rimonabant group	CD diet group	CD diet + rimonabant group
Body mass (g)	204.7±10.8	200.3±11.9	196.9±10.4	198.7±10.6
Liver mass (g)	6.3±0.22	6.2±0.2	10.6±0.7^{ab}	6.8±0.33^{abc}
Liver mass index%	3.05±0.1	3.1±0.13	5.1±0.14^{ab}	3.5±0.14^{abc}
ALT (U/L)	76.3±1.09	77.8±1.3	115.8±4.1^{ab}	91.4±1.8^{abc}
AST (U/L)	98.5±1.58	98.8±1.6	139.3±1.64^{ab}	112±2.05^{abc}
ALP (U/L)	133.3±1.5	132.9±1.2	162.2±1.99^{ab}	142.6±2^{abc}
Albumin (g/dL)	3.54±0.33	3.73±0.42	3.77±0.43	3.66±0.23
Bilirubin (mg/dL)	0.87±0.16	0.89±0.12	0.85±0.22	90±0.12

Note: ALT, alanine transferase; AST, aspartate transferase; ALP, alkaline phosphatase; *a*, $p < 0.001$ compared with the control group; *b*, $p < 0.001$ compared with rimonabant group; *c*, $p < 0.001$ compared with CD diet group.

Fig. 1. Effect of rimonabant on oxidative stress in all experimental groups; *a*, $p < 0.001$ compared with the control group; *b*, $p < 0.001$ compared with rimonabant only treatment group; *c*, $p < 0.001$ compared with choline-deficient (CD) diet group; MDA, malondialdehyde; GSH-Px, glutathione peroxidase. [Colour online.]



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Effect of rimonabant on oxidative stress in all of the experimental groups (Fig. 1)

MDA concentration was significantly increased ($p < 0.001$) and GSH-Px activity was significantly decreased ($p < 0.001$) in the CD diet (only) group when compared with the control group. By comparison, rimonabant treatment induced a significant decrease in MDA concentration and a significant increase in GSH-Px activity ($p < 0.001$) in the CD diet + rimonabant group when compared with the CD diet (only) group. An effect was also observed in the rimonabant group when compared with the control group, but the effect was non-significant.

Effect of rimonabant on activin$_{\beta A}$ mRNA expression, follistatin mRNA expression, activin$_{\beta A}$: follistatin mRNA ratio, and CB1 receptor gene expression in rat liver (Table 4)

Activin$_{\beta A}$ mRNA expression, activin$_{\beta A}$: follistatin mRNA ratio, and CB1 receptor gene expression were significantly higher ($p < 0.001$) in the CD diet (only) group compared with the control group and rimonabant group. By comparison, follistatin mRNA expression was significantly decreased in the CD diet (only) group compared with the control group and rimonabant group. Treatment with rimonabant induced a significant decrease ($p < 0.001$) in activin$_{\beta A}$ mRNA expression, activin$_{\beta A}$: follistatin mRNA ratio, and CB1 receptor gene expression, with a parallel significant increase ($p < 0.05$) in follistatin mRNA expression compared with the CD diet (only) group. Moreover Pearson's correlation analysis revealed a positive correlation for CB1 receptor gene expression with activin$_{\beta A}$ mRNA levels ($r = 0.989$; $p < 0.01$). A negative correlation between CB1 receptor gene expression and mRNA levels of follistatin ($r = -0.992$; $p < 0.01$) was also observed (Figs. 2A and 2B).

Effect of rimonabant on NAFLD score as determined by H&E staining, and the area% of TGF-β immunoreaction in hepatocytes (Table 5; Figs. 3A–3D, and 4A–4D)

The CD diet (only) group showed typical features of NAFLD, such as macrovesicular steatosis and foci of lobular inflammation and ballooning degeneration (Fig. 3C). The NAFLD scores were significantly higher ($p < 0.001$) in the CD diet (only) group compared with the control (Fig. 3A) and the rimonabant treatment groups (Fig. 3B). Treatment with rimonabant (CD diet + rimonabant group; as shown in Fig. 3D) induced a significant decrease ($p < 0.001$) in NAFLD scores compared with the CD diet (only) group.

Similar to the NAFLD score, the area% of TGF-β immunoreaction of hepatocytes was significantly higher ($p < 0.001$) in the CD diet (only) group (Fig. 4C) compared with the control (Fig. 4A) and the rimonabant group (Fig. 4B). Additionally, as shown in Fig. 4D), treatment with rimonabant in the CD diet + rimonabant group induced a significant decrease ($p < 0.001$) in area% of TGF-β immunoreaction compared with the CD diet (only) group.

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Table 4. Effect of rimonabant on activin$_{\beta A}$, follistatin mRNA expression, activin$_{\beta A}$: follistatin mRNA ratio, and CB-1 receptor gene expression in normal rats and rats fed a choline deficient (CD) diet.

	Control group	Rimonabant group	CD diet group	CD diet + rimonabant group
Activin$_{\beta A}$ mRNA level	1.24±0.18	1.2±0.19	6.27±0.22^{ab}	3.28±0.23^{abc}
Follistatin mRNA level	1.47±0.14	1.39±0.12	0.60±0.05^{ab}	0.96±0.04^{abc}
Activin$_{\beta A}$: follistatin mRNA	0.84±0.12	0.86±0.2	10.45±0.4^{ab}	3.4±0.6^{abc}
CB-1 receptor gene expression	0.84±0.14	0.83±0.12	7.2±0.19^{ab}	4.3±0.25^{abc}

Note: mRNA is expressed in log10 relative units of relative quantitation (mean ± SD). *a*, $p < 0.001$ compared with control group; *b*, $p < 0.001$ compared with the rimonabant group; *c*, $p < 0.001$ compared with CD diet group.

Fig. 2. (A) Correlation between CB1 receptor gene expression and mRNA levels of follistatin. (B) Correlation between CB1 receptor gene expression and mRNA levels of activin$_{\beta A}$. [Colour online.]

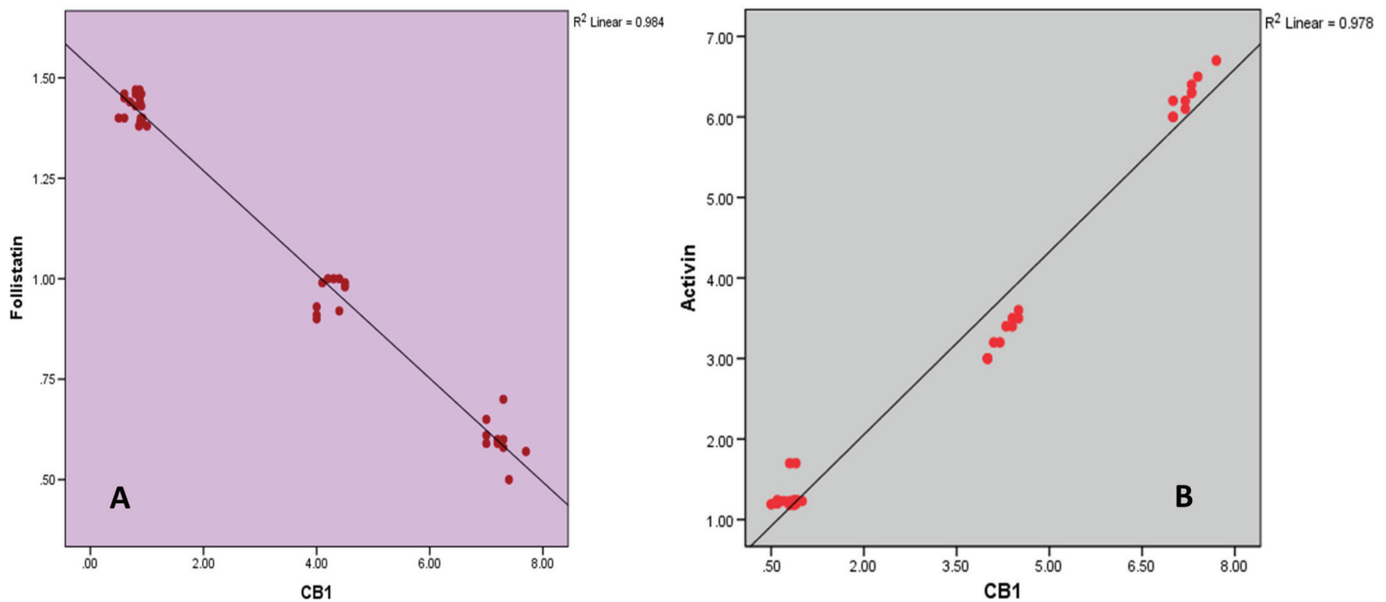

Table 5. Effect of rimonabant on the non-alcoholic fatty liver disease (NAFLD) score, as determined by staining with hematoxylin and eosin, and the area% of TGF-β immunoreaction of hepatocytes in normal rats and rats fed a choline-deficient (CD) diet (mean ± SD).

	Steatosis	Lobular inflammation	Hepato-cellular ballooning	The mean area% of TGF-β immunoreaction
Control group	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Rimonabant group	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CD diet group	3.00±0.00^{ab}	2.5±0.2^{ab}	2.7±0.3^{ab}	6.6±0.17^{ab}
CD diet + rimonabant group	1.9±0.42^{abc}	1.3±0.3^{abc}	1.2±0.5^{abc}	1.2±0.13^{abc}

Note: *a*, $p < 0.001$ compared with the control group; *b*, $p < 0.001$ compared with the rimonabant group; *c*, $p < 0.001$ compared with the CD diet group.

Discussion

Despite the high prevalence of NAFLD and its potential for serious sequelae, the underlying etiological factors that determine disease progression remain poorly understood; therefore, effective therapeutic strategies need to be further explored. The mechanisms that underline hepatic fat accumulation and triggering of hepatocyte injury and hepatic fibrosis in NASH are still largely unknown. In particular, little is known about the mediators that could trigger the extensive hepatic fibrogenic response in certain individuals with NAFLD, leading to advanced NASH (Filozof et al. 2015).

In this study we chose a CD diet because it induces a comprehensive histological and dysmetabolic phenotype resembling human NASH (Li et al. 2011). This phenotype is characterized by fatty liver, inflammation, fibrosis, cirrhosis, and even hepatocellular carcinoma (Pagotto et al. 2006). We preferred using a CD diet rather than high-fat diet because it manipulates liver fat content without affecting adipose fat stores (Raubenheimer et al. 2006). Moreover, the CD diet reaches the second hit of NAFLD model in more quickly (10–12 weeks) than that of the high-fat diet (minimum of 24 weeks) (Matsumoto et al. 2013).

This study has shown that CD diet administration for 12 weeks resulted in the development of NAFLD, manifested by significant increases in the serum liver biomarkers ALT, ALP, and AST when compared with the control group. The mechanism by which the CD diet induces fatty liver was explained by Noga and Vance

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Fig. 3. (A–D) Histological changes of rat liver stained with hematoxylin and eosin (H&E). (A) Control group showing normal histological structure of the hepatic lobule. (B) Rimonabant-treated group showing normal histological structure of the hepatic lobule. (C) Tissue from the liver of a rat fed the choline-deficient (CD) diet, showing steatosis (S) of the hepatocytes and focal inflammatory cell infiltration (IC). (D) Tissue of the liver from a rat in the CD diet + rimonabant treatment group, showing slight hydropic degeneration of hepatocytes and minimal activation of Kupffer cells (magnif. ×400). [Colour online.]

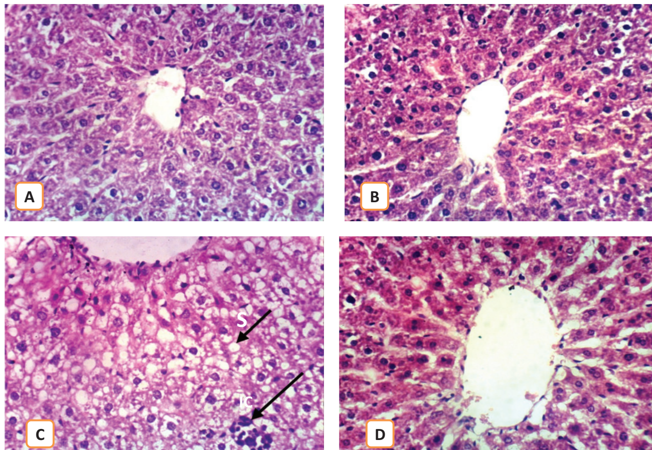
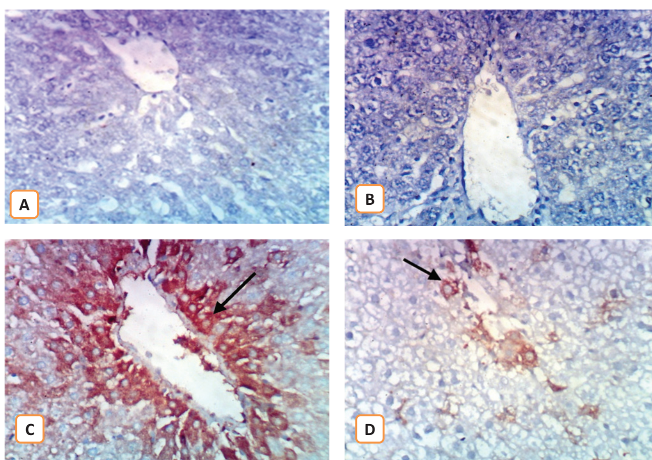


Fig. 4. (A–D) Immunostaining reaction for TGF-β. Photomicrographs of liver tissue stained with TGF-β immune-stain reaction. (A) Immunohistochemical staining of TGF-β in the liver of a rat from the control group showing no expression of TGF-β (negative immunohistochemical reaction). (B) Immunohistochemical staining of TGF-β in liver of a rat from the rimonabant group, showing no expression of TGF-β (negative immunohistochemical reaction). (C) Immunohistochemical staining of TGF-β in liver of rat from the group fed the choline-deficient (CD) diet showing strong positive expression of TGF-β (dark brown colour). (C) Immunohistochemical staining of TGF-β in liver of a rat from the CD diet + rimonabant treatment group, showing weakly positive expression of TGF-β (magnif. ×400). [Colour online.]



(2003), who revealed that choline is an important element for formation of phosphatidylcholine, which is a critical component of very-low-density lipoproteins (VLDL), which are responsible for transporting triglycerides out of the liver.

Also, our results were in agreement with the findings of Han et al. (2015) and Cheung and Sanyal (2010), who reported that a CD diet induces NAFLD by causing mitochondrial dysfunction, which is a central mechanism in the pathogenesis of NAFLD. Mitochondria play an important role in hepatocyte metabolism, being the primary site for the oxidation of fatty acids and oxidative phosphorylation. Mitochondrial dysfunction decreases the capacity to oxidize fatty acids, increases the delivery and transport of free fatty acids into the liver, and augments hepatic fatty acid synthesis, which is likely to play a significant role in the pathogenesis of NAFLD (Pessayre and Fromenty 2005). Another mechanism, explained by Malhi and Kaufman (2011), who showed that a CD diet caused NAFLD through endoplasmic reticulum stress, is inhibiting the activity of insulin sensitizing kinase, which mediates insulin signaling in hepatocytes leading to insulin resistance, which can promote hepatic steatosis (Ron and Walter 2007).

Regarding the serum levels of albumin and bilirubin, there was a non-significant effect from the CD diet by comparison with the control group. These results were in agreement with the findings of Arora and Sharma (2012) and Smith and Adams (2011), who stated that total bilirubin and albumin are usually normal in NASH and NAFLD, and only increase in cases associated with cirrhosis that developed in the end-stage of the disease.

The results of our study also indicated that there was a significant increase in liver mass and liver mass index, with a non-significant effect on the body mass in the CD diet (only) group when compared with the control group. These results were in agreement with Kitson et al. (2016) and Han et al. (2015), who stated that there was no body mass gain in the CD-diet-induced NAFLD models, also Raubenheimer et al. (2006) who reported that low choline leads to reduced secretion of liver triglyceride as VLDL, resulting in accumulation of liver triglycerides without affecting the enzymes involved in de-novo lipogenesis, so liver mass increases without change in body mass.

In this study, particular attention is paid to oxidative stress and its role in the development and progression of NAFLD and its sequelae such as fibrosis. Our results demonstrated that the CD diet enhanced the oxidative stress state in liver tissue, as evidenced by reductions in antioxidant enzymes such as GSH-Px and increases in markers of lipid peroxidation (MDA). In agreement with these findings Santos et al. (2015) and Rolo et al. (2012) demonstrated that lipid accumulation in the liver alters the electron transport chain and causes an increase in the production of reactive oxygen species (ROS). Moreover, the activation of Kupffer cells and other inflammatory cells also generates ROS through nicotinamide adenine dinucleotide phosphate oxidase (Gornicka et al. 2011). These species can oxidize polyunsaturated fatty acids present in cells and organelle membranes, producing lipid peroxidation metabolites like MDA (Rolo et al. 2012).

We have also considered the consequences of a CD diet on activin$_{\beta A}$, follistatin mRNA, and the activin$_{\beta A}$: follistatin mRNA ratio, and the results showed significant increases in activin$_{\beta A}$ and the activin$_{\beta A}$: follistatin mRNA ratio, and significant decrease in follistatin mRNA expression in liver tissues from the CD diet (only) group when compared with the control group. These results were similar to those of Yndestad et al. (2009), who demonstrated that serum levels of activin A and the activin A : follistatin mRNA ratio in liver are increased in patients with NAFLD, potentially reflecting increased bioactivity of activin A. Follistatin antagonizes the biological actions of activin A, and blocks its signaling (Refaat et al. 2015).

The authors' attention was also focused on immunohistochemical examination of TGF-β_1, which showed a strong positive expression of TGF-β1 in the CD diet (only) group when compared with that of the control group. These results were in agreement with those of Yang et al. (2014) and Braunersreuther et al. (2012), who reported that TGF-β_1 plays a pivotal role in hepatic fibrosis by mediating the activation of stellate cells and their production of proteins for the extra-cellular matrix. Indeed, Kupffer and stellate cells produce TGF-β1, which increases the synthesis and deposition of type I collagen, ending with the transformation of resting

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stellate cells to myofibroblasts. Moreover, this findings agree with those of Tarantino et al. (2008), who revealed that enhanced serum concentrations of TGF-β1 could represent a marker for early activation of mesenchymal hepatic stellate cells, ultimately leading to liver damage.

Our results were supported with histopathological examination of the liver tissues, and the results showed fatty liver changes in the form of severe steatosis of hepatocytes and inflammatory cell infiltration in the CD diet (only) group. These results were in agreement with those of Han et al. (2015), who stated that a CD diet causes macrovesicular steatosis, ballooning degeneration, and foci of lobular inflammation. Moreover Santos et al. (2015) explained the morphofunctional changes in rats having NAFLD, as lipid peroxidation of polyunsaturated fatty acids in the mitochondrial membrane, which is associated with proteolysis of apolipoprotein B, and this reduces VLDL secretion, promoting triacylglycerol accumulation in liver.

Regarding hepatic CB1 receptor expression, we showed significant increases in hepatic CB1 receptor gene expression in the CD diet (only) group when compared with the control group. This finding agrees with the results from a clinical study on humans, which showed that mRNA expression of CB1 was significantly elevated in NASH (Auguet et al. 2014). Also, van der Poorten et al. (2010) observed that hepatic CB1 expression correlated with the extent of steatosis, and was significantly up-regulated in those with increased steatosis grade, suggesting CB1 receptor activation and signaling. Additionally, our analyses showed that CB1 was positively correlated with activin A and negatively correlated with the hepatic expression of follistatin, suggesting a deleterious role of CB1 in NAFLD.

On studying the effect of rimonabant on rats receiving the standard diet, there was a non-significant effect on all parameters when compared with the control group. This was explained by Jeong et al. (2008), who stated that in a normal liver, the expression of CB1 receptors is modest, which probably explains why the focus of research on the role of CB1 receptors in the liver pathophysiology is so recent. However, when there are pathological processes occurring in the liver the endocanabinoid system is activated, and CB1 receptors undergo marked up-regulation.

We have also considered that rimonabant may have caused significant decreases in liver enzymes, liver mass, and liver mass index, and exhibited minimal activation of Kupffer cells with very minimal hydrobic degeneration of some hepatocytes in the CD diet + rimonabant group when compared with the CD diet (only) group. These results agree with those of Chanda et al. (2011), Mallat et al. (2011), and Tam et al. (2012), who explained that the major pathway for increased fatty-acid release and transfer to liver is CB1 activation of lipoprotein lipase in adipose tissue. Additional mechanisms mediated via hepatic CB1 receptors include increased de-novo hepatic lipogenesis, decreased fatty acid oxidation, and decreased secretion of triglyceride-rich VLDL by increasing the expression of the lipogenic transcription factor sterol regulatory element-binding protein and its target enzymes: acetyl coenzyme-A carboxylase-1 and fatty acid synthase (Tam et al. 2011).

Another mechanism explained by Osei-Hyiaman et al. (2008), who stated that the activity of hepatic carnitine palmitoyltransferase 1 (the rate-limiting enzyme in mitochondrial fatty acid β-oxidation) is suppressed by treatment with a CB1 agonist, and is prevented by rimonabant. Additionally, Migrenne et al. (2009) reported that CB1 blockade increases plasma adiponectin, which is a key stimulator of fatty acid β-oxidation.

The results of our study showed that liver levels of GSH-Px were significantly higher and lipid peroxidation was significantly reduced in rats fed a CD diet and treated with rimonabant, compared with untreated CD diet fed rats. Consistent with these findings, only one study, to our knowledge examined the effect of rimonabant on oxidative stress markers in a rat model of NAFLD, and found significant increases in GSH-Px levels with significant decreases in MDA, and explained that via an adaptive response of hepatocytes to increased ROS production. It has also been suggested that CB1 receptor blockade could have a beneficial effect on the redox state in hepatocytes (Jorgačević et al. 2015).

To the best of our knowledge, this is the first study to report the effect of rimonabant on the liver expression of activin A and follistatin mRNA levels in an experimental animal model of NAFLD. Our results demonstrated a significant decrease in activin A and significant increase in the hepatic expression of follistatin in the CD diet rats treated with rimonabant, compared with the untreated rats fed the CD diet; additionally, there was positive correlation between CB1 receptor gene expression and $\text{activin}_{\beta A}$ mRNA levels. Moreover, there was negative correlation between CB1 receptor gene expression and follistatin mRNA levels, indicating that activin and follistatin may be involved in the beneficial effects of CB1 receptor antagonist on NAFLD.

Activin A seems to have multiple roles in NAFLD through different mechanisms, as was reported by Yeh and Brunt (2007) who showed that activin A induces hepatocyte apoptosis, which potentially represents an important mechanism for the loss of hepatocytes that occurs during the progression of NAFLD to NASH, and further to cirrhosis (Tarantino et al. 2011). Furthermore Patella et al. (2006) reported that administration of the activin-binding protein follistatin in an animal model of liver fibrosis has been shown to reduce fibrosis development, at least partly due to inhibited hepatocyte apoptosis.

Another mechanism was explained by Yndestad et al. (2011), who reported a role for activin A in the promotion of fibrogenesis in NAFLD by increasing the release of TGF- β from hepatocytes and by activating hepatic stellate cells. Also, in-vitro studies on primary rat hepatocytes performed by Gressner et al. (2008) showed that activin A induces the expression of connective tissue growth factors, which would promote fibrogenesis.

Yet another mechanism, reported by Yndestad et al. (2011), showed that activin A has a role in increasing inflammation in NAFLD, as activin A has been shown to potently stimulate the production of proinflammatory cytokines and oppose anti-inflammatory cytokines.

On the other hand, the immunohistochemical results of this study showed that the immunostaining intensity of the TGF-β1 decreased in the CD diet + rimonabant treatment group when compared with the CD diet (only) group. These results runs support the findings of DeLeve et al. (2008), who showed that the administration of rimonabant to wild-type mice or genetic inactivation of CB1 receptors were both associated with a significant reduction in fibrosis progression. Rimonabant-treated or CB1 knock-out mice also displayed reduced hepatic expression of the TGF-β1, and a decrease in the number of fibrogenic cells. Antifibrogenic properties of the CB1-selective antagonist were ascribed to the antiproliferative property of rimonabant in hepatic myofibroblasts (Domenicali et al. 2009).

Conclusions

In light of the results of this study, it can be concluded that the cannabinoid receptor1 (CB1) antagonist rimonabant has a potential therapeutic effect on NAFLD induced with a CD diet. This might be due to suppression of oxidative stress and hepatic expression of TGF-β1. The study also demonstrated for the first time that rimonabant has hepatoprotective effects in the context of NAFLD by modulating the hepatic expression of activin A and follistatin.

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pathological examination. Conflict of interest statement: The authors declare that they have no conflict of interest asscoiated with this study.

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