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Protective effect of rimonabant, a cannabinoid receptor 1 antagonist on nonalcoholic fatty liver disease in a rat model by modulating the hepatic expression of activin A and follistatin.

By

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ABSTRACT
Non-alcoholic fatty liver disease (NAFLD) is a major cause of liver morbidity and mortality with no proven effective therapy yet. 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. The present study was designed to investigate the effect of rimonabant (a potent cannabinoid receptor1 (CB1) antagonist) on NAFLD induced by choline deficient (CD) diet in rats, as well as to detect whether it can modulate the hepatic expression of activin A and follistatin or not. Forty rats were divided into 4 groups; control group, rimonabant group (normal rats received rimonabant), CD diet group (NAFLD induced by CD diet), CD diet+ rimonabant group (NAFLD treated with rimonabant). It was found that 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 significant decrease in glutathione peroxidase (GSH-Px) and follistatin mRNA expression in liver tissues. Administration of rimonabant caused significant improvement in all studied parameters compared to CD diet group. The histopathological examination supported these results. We concluded that rimonabant significantly counteracted NAFLD induced by CD diet by decreasing oxidative stress, hepatic expression of TGF-β1 and modulating hepatic expression of activin A and follistatin.

Keywords: Nonalcoholic fatty liver disease, cannabinoid receptor 1, TGF-β, activin A, follistatin, oxidative stress.
Introduction

NAFLD is a worldwide disease considered as the most common cause of abnormal liver function tests and chronic liver disease in both developing and developed countries (Oh et al. 2008). NAFLD is a group of chronic diseases including fatty liver, steatosis, as well as more severe lesions including lobular necroinflammation, steatohepatitis with fibrosis, or 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 accumulation of triglycerides in hepatocytes, which increases the vulnerability of the liver to various possible “second hits” that in turn lead to the inflammation, fibrosis and cellular death characteristics 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 structurally 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 inducing 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 the hepatic cells and have been considered as main regulators of liver biology, liver pathology and liver 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 Bermudez-Silva 2014). The upregulation of CB1 in NAFLD, alcoholic liver disease, autoimmune and viral hepatitis, ischemia/reperfusion and cirrhosis were demonstrated. So ECs are involved in numerous pathophysiological processes in chronic liver diseases (Pisanti et al. 2015).

The liver is identified as a primary site for endocannabinoid-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 is suggested that hepatic CB1 receptors are involved in the progress of fatty liver in diet-induced hepatic steatosis (Li et al.2011; Pagotto et al.2006; Schwabe, 2005). We hypothesized that rimonabant, a potent CB1 antagonist could have a hepatoprotective effect against CD diet induced NAFLD.

Based on this background, this study was designed to investigate the possible protective effect of rimonabant on NAFLD induced by CD diet, as well as to detect its effect on oxidative stress markers and profibrotic cytokine (TGF-β1). Besides to elucidate whether activin A and follistatin may participate in its molecular mechanism or not.

MATERIALS AND METHODS

Chemicals and reagents

Rimonabant was provided by Akros Pharma (Sigma-Chemical .CO, St. Louis, MO, USA). Tween 80 was supplied by Calbiochem, Merck 27 Millipore (Billerica, MA, USA). Beta-A activin, CB 1 receptors, follistatin mRNA and GAPDH antibodies were provided from Applied Biosystems (Foster City, CA). SYBR Green PCR Master Mix was from (Applied Biosystems Inc., USA). AST, ALP, ALT, Albumin and bilirubin estimation kits were supplied by Egyptian company of biotechnology (Egypt). MDA and GSH-Px estimation kits were purchased from Biodiagnostic (Egypt). TGF-β was provided from ABCAM Company (United States).

Composition of the diet used:

Rat Diet was formulated according to NRC (1995) as shown in (table 1) and (table 2)
Animals:

Forty male Sprague–Dawley rats (body weight 200 ± 20 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 12:12 h light/dark cycle and had free access to water and diet. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the 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 divided into 4 groups as follow: group I (control group): Consisted of 10 rats, served as control group and received standard diet for 12 weeks then were given 0.1% Tween 80 in distilled water by oral gavage for two weeks before scarification, group II (rimonabant group): Consisted of 10 rats, received standard diet for 12 weeks then were treated with daily dose of rimonabant (10 mg/kg) by oral gavage for two weeks before scarification (Bojan et al. 2015), group III (CD diet group): Consisted of 10 rats, received only CD diet for 12 weeks, group IV (CD diet + rimonabant group) Consisted of 10 rats, received CD diet for 12 weeks then were treated with daily dose of rimonabant (10 mg/kg) by oral gavage for two weeks before scarification (Bojan et al. 2015).

Rimonabant was dissolved into 0.1% Tween 80 in distilled water and for 20 s sonificated on ice using a digital Branson sonificator before administration. Retro-orbital blood samples were obtained from retro-orbital venous plexus to measure liver enzymes before receiving the CD diet and then at the end of the tenth week. The rats had non-significant difference in liver enzymes were excluded from the study, while those had significant difference 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 intraperitoneally /i.p). The animals were fixed on operating table and the blood samples and liver biopsies were taken as follow: A cranio-caudal incision of about 2 cm is made for blood sample collection, parallel and with slightly
to the left of the sternum through the skin and pectoral muscles to expose the ribs. A blunt curved forceps is then binged between the 5th and 6th ribs, through the intercostals muscles. The gap is widened so that the rapidly beating heart becomes visible, then the blood samples were taken from the right ventricle. The previous incision was continued through the animal's anterior abdominal wall, the abdominal cavity was entered by cutting the muscles and peritoneum. The liver was exposed then freed from the surrounding tissues and is pulled out of the incision gently (Corbin and Minuk 2003). Then it was immediately isolated, washed with ice-cold saline and weighted. Then the liver was divided into two halves the first one was rapidly frozen and stored at liquid nitrogen −70 °C for measurement of oxidative stress markers and real-time PCR study. The second one was kept in formaldehyde to be prepared for histopathological examination with Hematoxylin and Eosin (H&E) for detection of the histopathological signs of NAFLD and immunohistochemical examination for assessment of TGF-β1 expression.

Liver weight index calculation:

Liver weight index was calculated according to Iwo et al. (2017) as follow: (liver weight/body weight x 100).

Assessment of hepatic function

Serum levels of aspartate transferase (AST), alanine transferase (ALT), alkaline phosphatase (ALP), total bilirubin and albumin were measured using commercial assay kits according to the manufacturer’s instructions.

Assessment of Oxidative Stress in hepatic tissue

Frozen liver samples were cut and homogenized using a Mixer Mill MM400 (Retsch, Germany) to measure GSH-Px according to Tappel (1978) and the results were expressed as units/mg. Also lipid Peroxidation contents (LPO) in the form of MDA level were measured by a modified method of Ohkawa et al. (1979) and the results were expressed as nmoles/gm liver tissue.

RNA extraction and quantitative real-time PCR

Frozen liver samples were cut and homogenized using a Mixer Mill MM400 (Retsch, Germany) to isolate the mRNA. Total RNA was isolated from 40 mg tissue using total RNA purification kit Jena Bioscience Germany. The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and 280 nm. The amount of
beta-A activin and follistatin mRNA was determined with ABI Prism 7900HT quantitative real-time PCR (Applied Biosystems, Foster City, CA). The primer sequences used were: beta-A activin (forward primer: 5′-ATGGACCTAACTTCTCAGCCAGA-3′; reverse primer: 5′-CTCTCCCCCTTCAAGCCCATT-3′); follistatin (forward primer: 5′-GGCGTACTTGCTTAAGTGAA-3′; reverse primer: 5′-GGGAAGCCTGTAGTCTCGGTG-3′); cannabinoid-1 receptor (forward primer: 5′-ACCTACCTGTGATTCTGGATGGG-3′; reverse primer: 5′-CGTGTGGATGATGATGCTCTTG-3′); GAPDH (forward primer: 5′-GATGCTGGTCTGAGATGATGTCG-3′; reverse primer: 5′-GTGGTGAGTGCATTGCTGA-3′); For real-time PCR, 20 ng cDNA and 0.4 μM of each primer were used in a 25 μL reaction volume containing SYBR Green PCR Master Mix (Applied Biosystems Inc., USA). The temperature program was as follow: 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 cycle is plotted against fluorescence (when using linear scale). The Threshold Cycle (CT) serves as a tool for calculation of the starting template amount in each sample. Because the samples of control group and also samples of treated group are used as calibrators, the expression levels are set to 1. Because the relative quantities of the beta-A activin and 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-ΔΔct (Alhusseini et al. 2010).

Morphometric analysis

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

All the data are presented as mean ± standard deviation (SD). Evaluation of differences between groups was performed using one-way ANOVA with post hoc test (LSD) between groups with SPSS 19.0 software. The correlation between CB-1 receptor gene expression, Activin β 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 experimental groups (table 3)

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

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

MDA concentration was significantly increased (p<0.001) and GSH-Px activity was significantly decreased (p<0.001) in CD diet group when compared to control group. On the other hand, rimonabant treatment induced a significant decrease in MDA concentration and significant increase in GSH-Px activity (p<0.001) in CD diet+ rimonabant group when compared to CD diet group. Non-significant effect was also observed in rimonabant group compared to control group.

Effect of Rimonabant on Activin β A mRNA expression, Follistatin mRNA expression, Activin β A/ Follistatin mRNA ratio and CB-1 receptor gene expression in rat liver (table 4).

Activin β A mRNA expression, Activin β A/Follistatin mRNA ratio and CB-1 receptor gene expression were significantly higher (P<0.001) in CD diet group compared to control group.
and rimonabant group. On the other hand, Follistatin mRNA expression was significantly decreased in CD diet group compared to control group and rimonabant group. Treatment with rimonabant induced a significant decrease ($P<0.001$) in Activin β A mRNA expression, Activin β A/Follistatin mRNA ratio and CB -1 receptor gene expression with a parallel significant increase ($P<0.05$) in Follistatin mRNA expression compared to CD diet group. Moreover Pearson’s correlation analysis revealed a positive correlation of CB-1 receptor gene expression with Activin β A mRNA level ($r = 0.989; p < 0.01$). A negative correlation of CB-1 receptor gene expression with Follistatin mRNA level ($r = -0.992; p < 0.01$) was also observed Fig.2(A,B).

Effect of Rimonabant on NAFLD score by H & E and the area% of TGF-β immunoreaction of hepatocytes (Table 5) , Fig.3(A,B,C,D) and Fig.4(A,B,C,D)

CD diet group showed typical features of NAFLD, such as macrovesicular Steatosis and foci of lobular inflammation and ballooning degeneration Fig.3(C). The NAFLD scores was significantly higher ($P<0.001$) in CD diet group compared to control Fig.3(A) and rimonabant Fig. 3 (B) groups. Treatment with rimonabant in (CD diet + rimonabant group) as in Fig.3 (D) induced a significant decrease ($P<0.001$) in NAFLD scores compared to CD diet group.

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

Discussion:

Despite the high prevalence of NAFLD and its potential for serious sequelae, the underlying etiologic factors that determine disease progression remain poorly understood; therefore, effective therapeutic strategies need to be further explored. The mechanisms that underlying 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 (Claudia et al. 2015).

In the current study we chose a CD diet as 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 CD diet rather than high fat diet as it manipulates liver fat content without affecting adipose fat stores (Peter et al. 2015). Moreover CD diet reaches the 2nd hit of NAFLD model in shorter duration (10-12 weeks) than that of high fat diet (minimum 24 weeks) (Masahiko et al. 2013).

This study has shown that CD diet administration for 12 weeks resulted in development of NAFLD, manifested by significant increase in serum liver biomarkers ALT, ALP and AST when compared to control group. The mechanism by which CD diet induces fatty liver was explained by Noga and Vance (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 Han et al. (2015) and Cheung and Sanyal, (2010) who reported that 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 oxidation of fatty acids and oxidative phosphorylation. The 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 CD diet caused NAFLD through endoplasmic reticulum stress, that inhibits 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 level of albumin and bilirubin, there was non-significant effect of CD diet in comparison to control group. These results were in agreement with 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 weight and liver index with a non significant effect on the body weight in CD diet group when compared to control group. These results were in agreement with Kitson et al. (2016) and Han et al. (2015) who stated that there was no body weight 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 affection of enzymes involved in denovo lipogenesis, So the liver weight increases without change in body weight.

In the current study particular attention is paid to oxidative stress and its role in the development and progression of NAFLD and its sequelae such as fibrosis. As our results demonstrated that CD diet enhanced oxidative stress state in liver tissue evidenced by reduction in antioxidant enzyme as GSH-Px and increase in marker of lipid peroxidation (MDA). In agreement with these finding Juliana et al.(2015) and Rolo et al.(2012) demonstrated that lipid accumulation in liver causes alteration in electron transport chain and causes an increase of reactive oxygen species (ROS) production. Moreover, 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 cell and organelle membranes producing lipid peroxidation metabolites like MDA (Rolo et al.2012).

We have also considered the consequences of CD diet on activin- β A, Follistatin mRNA and Activin β A/Follistatin mRNA ratio and the results showed significant increase in activin- β A, Activin β A/Follistatin mRNA ratio and significant decrease in follistatin mRNA expression in liver tissues of CD diet group when compared to control group. These results were parallel to that of Yndestad et al. (2009) who demonstrated that serum levels of activin A and activin A/follistatin mRNA ratio in liver are increased in patients with NAFLD, potentially reflecting increased activin A bioactivity. Follistatin antagonizes the biological actions of activin A and blocks its signaling (Refaat et al. 2015).

The author's attention was also focused on immunohistochemical examination of TGFβ1 that showed a strong positive expression of TGFβ1 in CD diet group when compared to that of control group. These results were in agreement with Yang et al. (2014) and Vincent et al. (2012) who reported that TGF-β1 plays a pivotal role in hepatic fibrosis by mediating the activation of stellate cells and their production of extracellular matrix proteins. Indeed, Kupffer and stellate cells produce TGF-β1 that increases synthesis and deposition of type I collagen ending by transformation of resting stellate cells to myofibroblasts. Moreover, this finding parallel to that of Tarantino et al. (2008) who revealed that enhanced serum TGF-β1
concentrations could represent a marker of early activation of mesenchymal hepatic stellate cells and 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 cells infiltration in CD diet group. These results were in agreement with Han et al. (2015) who stated that CD diet causes macrovesicular steatosis, ballooning degeneration and foci of lobular inflammation. Moreover Juliana et al. (2015) explained the morphofunctional alterations in rats having NAFLD by lipid peroxidation of polyunsaturated fatty acids in mitochondrial membrane which is associated with apolipoprotein B proteolysis, and this reduces VLDL secretion, promoting triacylglycerol accumulation in liver.

Regarding hepatic CB1 receptor expression, we showed significant increase of hepatic CB1 receptor gene expression in CD diet group when compared to control group. This finding agree with a clinical study on human, which showed that CB1 mRNA expression was significantly high in NASH (Teresa et al.2011). Also van der 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 we analyzed that it was positively correlated with activin-A and negatively correlated with follistatin hepatic expression suggesting a deleterious role of CB1 in NAFLD.

On studying the effect of rimonabant on rats receiving normal standard diet, there was non significant effect on all parameters when compared to control group .This can be explained by Jeong et al.(2008) who stated that in 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 has come only recently. However, during liver pathology, endocannabinoid system is activated, and CB1 receptors undergo marked up-regulation.

We have also considered that rimonabant caused significant decrease in liver enzymes, liver weight and liver index and exhibited minimal activation of kupffer cells with very minimal hydrobic degeneration of some hepatocytes in CD diet+ rimonabant group when compared to CD diet group. These results coincide with Chanda et al. (2011); Mallat et al. (2011) and Tam et al. (2012) who explained that the major pathway is CB1 activation of lipoprotein lipase in adipose tissue, resulting in increased fatty-acid release and transfer to
liver. 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 expression of the lipogenic transcription factor sterol regulatory element-binding protein and its target enzymes: Acetyl coenzyme-A carboxylase-1 and fatty acid synthase (Joseph et al. 2011).

Another mechanism explained by Osei-Hyiaman et al. (2008) who revealed 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 GSH-Px level was significantly higher and lipid peroxidation was significantly reduced in CD diet fed rats treated with rimonabant compared to untreated CD diet fed rat. In consistence with these findings only one study, to our knowledge examined the effect of rimonabant on oxidative stress markers in rat model of NAFLD and found significant rise in GSH-Px level with significant decrease in MDA and explained that by an adaptive response of hepatocytes to increased ROS production. It also suggested that CB1 receptor blockade could have a beneficial effect on the redox state in hepatocytes (Bojan et al. 2015).

To the best of our knowledge, this is the first study to report the effect of rimonabant on liver expression of activin-A and follistatin mRNA levels in experimental animal model of NAFLD. Our results demonstrated a significant decrease in activin-A and significant increase in follistatin hepatic expression in CD diet group treated with rimonabant compared to CD diet untreated group; additionally, there was positive correlation between CB-1 receptor gene expression and Activin β A mRNA level. Moreover there was negative correlation between CB-1 receptor gene expression and Follistatin mRNA level indicating that activin and follistatin may be involved in the beneficial effects of CB 1 receptor antagonist on NAFLD.

Activin A seems to have multiple roles in NAFLD through different mechanisms as what 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 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 study on primary rat hepatocytes done by Gressner et al. (2008) showed that activin A may induce expression of connective tissue growth factor which would promote fibrogenesis.

Furthermore another mechanism reported by Yndestad et al. (2011) who 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 opposes the anti-inflammatory cytokines.

On the other hand, the immunohistochemical results of the current study showed that the immunostaining intensity of the TGF-β1 decreased in the CD diet + rimonabant treated group when compared to CD diet untreated group. These results runs parallel with DeLeve et al. (2008) who showed that 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 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 cannabinoid receptor1 (CB1) antagonist, rimonabant has a potential therapeutic effect on NAFLD induced by 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 effect on NAFLD by modulating hepatic expression of activin A and follistatin.

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**Conflict of interest:**
The authors declare that they have no conflict of interest.

**References:**


Chanda, D., Kim, DK., Li, T., Kim, YH., Koo, SH., Lee, CH., et al. 2011. Cannabinoid receptor type 1 (CB1R) signaling regulates hepatic gluconeogenesis via induction of


Pisanti, S., Picardi, P., Pallottini, V., Martini, C., Petrosino, S. and Proto, MC. 2015. Anandamide drives cell cycle progression through CB1 receptors in a rat model of


https://mc06.manuscriptcentral.com/cjpp-pubs
Schwabe, R. F., 2005 “Endocannabinoids promote hepatic lipogenesis and steatosis through CB1 receptors,” Hepatology, 42. 4:959–961. doi: 10.1002/hep.20900


Figure Caption:

Figure 1:
Effect of rimonabant on oxidative stress in all experimental groups
a: Significant difference ($p<0.001$) compared with control group; b: Significant difference ($p<0.001$) compared with rimonabant group. c: Significant difference ($p<0.001$) compared with CD diet group.

Figure 2 (A & B):
(A): Correlation of CB-1 receptor gene expression with follistatin mRNA level.
(B): Correlation of CB-1 receptor gene expression with Activin $\beta$ A mRNA level.

Figure 3 (A, B, C, D):
Histological changes of rat liver stained with H&E. A. Control group showing normal histological structure of hepatic lobule. B. Rimonabant group showing normal histological structure of hepatic lobule. C. CD diet group showing Steatosis (S) of hepatocytes and focal inflammatory cells infiltration (IC). D. CD diet + Rimonabant group showing slight hydropic degeneration of hepatocytes and minimal activation of kupffer cells (H & E X 400).

Figure 4 (A, B, C, D):
Immunostaining reaction for TGF-β. Photomicrographs of TGF-β immune-stain reaction stained liver sections. A. Immunohistochemical staining of TGF-β in liver of rat from control group showing no expression of TGF-β (negative immunohistochemical reaction). B. Immunohistochemical staining of TGF-β in liver of rat from Rimonabant group showing no expression of TGF-β (negative immunohistochemical reaction). C. Immunohistochemical staining of TGF-β in liver of rat from CD diet group showing strong positive expression of TGF-β (dark brown colour). C. Immunohistochemical staining of TGF-β in liver of rat from CD diet + Rimonabant group showing weak positive expression of TGF-β (X 400).
Table (1): Ingredients % of the control diet

<table>
<thead>
<tr>
<th>Feed ingredients</th>
<th>Control diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower oil</td>
<td>15.00</td>
</tr>
<tr>
<td>Concentrate mixture (45%)</td>
<td>10.00</td>
</tr>
<tr>
<td>Yellow corn</td>
<td>49.00</td>
</tr>
<tr>
<td>Soybean meal (44%)</td>
<td>11.00</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>10.00</td>
</tr>
<tr>
<td>Molasses</td>
<td>03.00</td>
</tr>
<tr>
<td>Common salt</td>
<td>00.50</td>
</tr>
<tr>
<td>Ground limestone</td>
<td>00.20</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>00.10</td>
</tr>
<tr>
<td>Lysine</td>
<td>00.20</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>00.70</td>
</tr>
<tr>
<td>Mineral-vitamin premix</td>
<td>00.30</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
</tr>
</tbody>
</table>

\(^1\)Concentrate mixture composed of corn gluten 60\%, dicalcium phosphate, soybean meal 48\%, fish meal (65\%), limestone, broiler premix, L-lysine HCl, DL-methionine, and common salt.

\(^2\)Each 3 kg contains: vit. A 12000000 IU, vit. D3 2000000 IU, vit. E 10000 mg, vit. K3 1000 mg, vit. B1 1000 mg, vit. B2 5000 mg, vit. B6 1500 mg, vit. B12 10 mg, biotin 50 mg, pantothenic acid 10000 mg, nicotinic acid 30000 mg, folic acid 1000 mg, manganese 60000 mg, zinc 50000 mg, iron 30000 mg, copper 4000 mg, iodine 300 mg, selenium 100 mg, cobalt 100 mg, carrier (CaCo3) up to 3 kg.
Table (2): Choline deficient diet composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Choline deficient diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>58.275</td>
</tr>
<tr>
<td>Soya 46%</td>
<td>21.9</td>
</tr>
<tr>
<td>Oil</td>
<td>15</td>
</tr>
<tr>
<td>Lime stone</td>
<td>1.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.2</td>
</tr>
<tr>
<td>Common salts</td>
<td>0.125</td>
</tr>
<tr>
<td>Premix choline free</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

Table (3): Effect of rimonabant on liver index and liver function in normal and CD diet ingested rats. (mean±SD).

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

a: Significant difference (p<0.001) compared with control group; b: Significant difference (p<0.001) compared with rimonabant group. c: Significant difference (p<0.001) compared with CD diet group.
Table (4): Effect of rimonabant on Activin β A, Follistatin mRNA expression, Activin β A/Follistatin mRNA ratio and CB-1 receptor gene expression in normal and CD diet ingested rats. The mRNA expressed by Log10 relative units of relative quantitation (RQ) (mean+SD).

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Rimonabant group</th>
<th>CD diet group</th>
<th>CD diet+ rimonabant group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activin β A mRNA level</td>
<td>1.24±0.18</td>
<td>1.2±0.19</td>
<td>6.27±0.22</td>
<td>3.28±0.23</td>
</tr>
<tr>
<td>Follistatin mRNA level</td>
<td>1.47±0.14</td>
<td>1.39±0.12</td>
<td>0.60±0.05</td>
<td>0.96±0.04</td>
</tr>
<tr>
<td>Activin β A/Follistatin mRNA</td>
<td>0.84±0.12</td>
<td>0.86±0.2</td>
<td>10.45±0.4</td>
<td>3.4±0.6</td>
</tr>
<tr>
<td>CB-1 receptor gene expression</td>
<td>0.84±0.14</td>
<td>0.83±0.12</td>
<td>7.2±0.19</td>
<td>4.3±0.25</td>
</tr>
</tbody>
</table>

a: Significant difference (p<0.001) compared with control group; b: Significant difference (p<0.001) compared with rimonabant group. c: Significant difference (p<0.001) compared with CD diet group.

Table 5: Effect of rimonabant on NAFLD score by H & E and the area% of TGF-β immunoreaction of hepatocytes in normal and CD diet ingested rats (mean+SD).

<table>
<thead>
<tr>
<th></th>
<th>steatosis</th>
<th>Lobular inflammation</th>
<th>hepatocellular ballooning</th>
<th>The mean area % of TGF-β immunoreaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Rimonabant group</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>CD diet group</td>
<td>3.00±0.00</td>
<td>2.5±0.2</td>
<td>2.7±0.3</td>
<td>6.6±0.17</td>
</tr>
<tr>
<td>CD diet+ rimonabant group</td>
<td>1.9±0.42</td>
<td>1.3±0.3</td>
<td>1.2±0.5</td>
<td>1.2±0.13</td>
</tr>
</tbody>
</table>

a: Significant difference (p<0.001) compared with control group; b: Significant difference (p<0.001) compared with rimonabant group. c: Significant difference (p<0.001) compared with CD diet group.
Figure 1
Figure 2(A,B)
Figure 3(A,B,C,D)