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Effects of Full-Spectrum Cannabis Oil with a Cannabidiol:Tetrahydrocannabinol 2:1 Ratio on the Mechanisms Involved in Hepatic Steatosis and Oxidative Stress in Rats Fed a Sucrose-Rich Diet

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Keywords
Cannabis oil · Metabolic syndrome · Sucrose-rich diet · Liver · Lipid peroxidation · Oxidative stress

Abstract

Introduction: This study aimed to analyze the effects of cannabis oil (cannabidiol:tetrahydrocannabinol [CBD:THC], 2:1 ratio) on the mechanisms involved in hepatic steatosis and oxidative stress in an experimental model of metabolic syndrome (MS) induced by a sucrose-rich diet (SRD). We hypothesized that noninvasive oral cannabis oil administration improves hepatic steatosis through a lower activity of lipogenic enzymes and an increase in carnitine palmitoyltransferase-1 (CPT-1) enzyme activity involved in the mitochondrial oxidation of fatty acids. Furthermore, cannabis oil ameliorates liver oxidative stress through the regulation of the main regulatory factors involved, nuclear factor erythroid 2 (NrF2) and nuclear factor-kB (NF-κB) p65. For testing this hypothesize, a relevant experimental model of MS was induced by feeding rats with a SRD for 3 weeks. ***Methods:*** Male Wistar rats were fed the following diets for 3 weeks: reference diet: standard commercial laboratory diet, SRD, and SRD + cannabis oil: noninvasive oral administration of 1 mg/kg body weight cannabis oil daily. The full-spectrum cannabis oil presents a total cannabinoid CBD:THC 2:1 ratio. Serum glucose, triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol, uric acid, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase (AP), N-arachidonoylethanolamine or anandamide and 2-arachidonoylglycerol endocannabinoids levels, thiobarbituric acid reactive substance (TBARS) levels, and nonenzymatic antioxidant capacity (ferric ion-reducing antioxidant power [FRAP]) were evaluated. In the liver tissue: histology, nonalcoholic fatty liver disease activity score (NAS), triglycerides and cholesterol content, lipogenic enzyme activities (fatty acid synthase, acetyl-CoA carboxylase, malic enzyme, and glucose-6-phosphate dehydrogenase), enzyme related to mitochondrial fatty acid oxidation (CPT-1), reactive oxygen species, TBARS, FRAP, glutathione,

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catalase, glutathione peroxidase, and glutathione reductase enzyme activities. 4-hydroxynonenal, NrF2, and NF-κB p65 levels were analyzed by immunohistochemistry. ***Results:*** The results showed that SRD-fed rats developed dyslipidemia, liver damage, hepatic steatosis (increase of key enzymes related to the novo fatty acid synthesis and decrease of key enzyme related to mitochondrial fatty acid oxidation), lipid peroxidation, and oxidative stress. Hepatic NrF2 expression was significantly decreased and NF-κB p65 expression was increased. Cannabis oil administration improved dyslipidemia, liver damage, hepatic steatosis, lipid peroxidation (improving enzymes involved in lipid metabolism), and oxidative stress. In the liver tissue, NrF2 expression increased, and NF-κB p65 expression was reduced. ***Conclusion:*** The present study revealed new aspects of liver damage and steatosis, lipid peroxidation, and oxidative stress in dyslipidemic insulin-resistant SRD-fed rats. We demonstrated new properties and molecular mechanisms of cannabis oil (CBD:THC, 2:1 ratio) on lipotoxicity and hepatic oxidative stress in an experimental model of MS.

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Introduction

Oxidative stress results in the activation of pathological pathways and cellular damage. This stress can occur as a secondary reaction to a preexisting disease condition, or it can play a role in the pathophysiology of inflammatory chronic liver diseases, such as nonalcoholic fatty liver disease (NAFLD) and other metabolic disorders present in the development of the metabolic syndrome (MS) such as obesity, systemic arterial hypertension, atherosclerosis, and type 2 diabetes mellitus [1–3]. In addition, it is defined as an imbalance in the interaction between the production of reactive oxygen species (ROS) and the antioxidant-mediated defense mechanism that our body has to protect against damage. The latter consists of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), and nonenzymatic components that include small molecules such as glutathione (GSH) [1, 2, 4].

Studies have shown that fat accumulation in liver cells, oxidative stress, and low-grade inflammation, stimulates the production of a greater quantity of ROS. These highly reactive compounds promote different biochemical reactions such as peroxidation of the polyunsaturated fatty acids through interaction with membrane lipids, leading to an elevation in 4-hydroxynonenal content (4-HNE). Thiobarbituric acid reactive substances (TBARS) and 4-HNE have been used as biomarkers of lipid peroxidation [5, 6].

Oxidative stress plays an important role in the functioning of redox-sensitive transcription factors such as nuclear factor erythroid 2 (NrF2) and nuclear factor-kB (NF-kB). The increase in ROS levels leads to the activation of NF-kB, a redox-sensitive transcription factor that plays important roles in immune and stress responses, inflammation, and apoptosis, promoting the formation of a vicious cycle of oxidative stress. NF-κB activation is associated with decreased NrF2 expression. Nrf2 directly affects ROS homeostasis by regulating the antioxidant defense systems [1, 7, 8].

Experimentally, our group has shown that rats fed a sucrose-rich diet (SRD) for a short and long term, exhibit metabolic and physiological characteristics that mimic several aspects of human MS. The animals develop insulin resistance, dyslipidemia, hypertension, ectopic lipid accumulation, steatosis and liver fibrosis, hypercoagulable basal state, and systemic oxidative stress [9–13]. Currently, there are only a few specific nutraceuticals strategies available to treat NAFLD and a variety of others are being evaluated. In this context, greater interest has been addressed to identify new natural products that are capable of acting on some of the targets including in the MS [14].

The plant *Cannabis sativa L.*, has been widely used for millennia for both recreational and medicinal purposes and has generated great expectations in society due to the therapeutic properties of its chemical compounds. More than 150 phytocannabinoids have been isolated from the plant, tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most commonly studied and are abundant [15, 16]. The synergy between phytocannabinoids, suggests the concept of the *entourage effect*, which refers to the enhanced therapeutic response of full-spectrum cannabis extracts compared to individual molecules [17–19]. In recent years, numerous studies have suggested that the major bioactive components of the plant, in specific ratios, may be considered as potential treatments for decreasing liver fat accumulation and other metabolic diseases, including MS [20–25]. Our group has previously reported that cannabis oil (CBD:THC, 2:1 ratio) improved systolic and diastolic blood pressure, dyslipidemia, liver damage, and liver fat accumulation in male Wistar rats fed a SRD [13]. There is evidence that cannabinoids are associated with antioxidant functions [26], but more studies are needed on the molecular mechanisms and the doses and types of cannabinoids used to prevent these metabolic disorders. In this study, we investigated the effects of orally administered full-

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spectrum cannabis oil with a CBD:THC 2:1 ratio on liver damage, liver steatosis, lipid peroxidation, oxidative stress, and new molecular mechanisms associated with an experimental model of MS induced by SRD-fed rats for 3 weeks.

Methods

Cannabis Oil Preparation and Characterization

Cannabis oil was obtained from dried inflorescences of the *Cannabis sativa* CAT1 variety grown at the environmental research center (CIM-CONICET-UNLP) (RESOL-2021-3236-APN-MS). Briefly, in order to obtain neutral cannabinoids, the inflorescences were first decarboxylated in oven (145°C) for 7 min. After that, an alcoholic extraction (10 mL ethanol at 96° per gram of inflorescence) was carried out and subsequently the ethanol was evaporated with Rotavapor (Buchi R 3000). The resulting resin was diluted in corn oil and the cannabinoids in the oil were quantified by HPLC/UV-DAD techniques. Cannabis oil contains 0.60 mg/mL CBD and 0.43 mg/mL THC, with a CBD:THC ratio of 2:1. Finally, the adequate dilution was carried out to obtain the working oil with a concentration of 1 mg/mL.

For cannabinoid HPLC/UV-DAD analyses, the extraction was performed with 96° ethanol (Purocol) using 20 mL/g oil and shaking in vortex (10 min) to favor efficient contact and extraction. The obtained solution was then centrifuged for 10 min at 5,000 rpm (Rolco Centrifuge) to separate the alcohol extract from insoluble residues. Finally, the alcoholic phase was filtered using Osmonics 45 lm filters, to further analyze by HPLC/UV-DAD.

Analytical determination of cannabinoids: cannabinoid profiles were studied by HPLC/UV-DAD (Shimadzu LC-20A), employing a Thermo Hypersil BDS C18 column (150 × 4.6 mm, 5 lm) according to the analytical technique described by De Backer et al. [27] with slight modifications [28]. The mobile phase consisted of A: methanol and B: 25 mM ammonium acetate solution. The gradient was 75% A: 1 min, 75–95% A in 15 min, 95% A: 2 min, 95–75% A in 2 min, and 75% A: 5 min. Total run time was 25 min, flow was 1 mL/min, and detection was at 205 nm. Cannabinoid analytical standards were purchased from Cerilliant Corporation.

Animals and Diets

Male Wistar rats (n = 18) were purchased from the Veterinary Sciences Institute of Litoral (ICIVET-Litoral) – Faculty of Veterinary Sciences, National University of Litoral (Esperanza, Santa Fe, Argentina) and were maintained with unrestricted access to water and food under controlled temperature (22 ± 1°C), humidity, and air flow conditions, with a fixed 12-h light/dark cycle (light on 07:00 a.m. to 7:00 p.m.). Adequate measures were taken to minimize pain or discomfort in the rats, and the smallest number of animals possible was used. This study was performed in strict accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Ethics Committee of the Faculty of Biochemistry and Biological Sciences.

The animals were initially fed a standard powdered commercial rodent diet (GEPSA FEED, Buenos Aires, Argentina). Rats' weight was 180–190 g (young adults) and were randomly divided into three experimental groups for 3 weeks: (1) rats fed a standard powdered rodent commercial diet (reference diet, RD, n = 6), (2) rats fed a semisynthetic SRD (n = 6), (3) rats fed SRD plus orally administered cannabis oil (SRD + Ca, n = 6). Rats received noninvasive oral cannabis oil (CBD:THC, 2:1 ratio – SRD + Ca) or vehicle control (corn oil – RD and SRD). Cannabis oil was administered at a dose of 1 mg/kg body weight daily during the experimental protocol (3 weeks). The diet compositions are detailed in a study by Degrave et al. [13]. Individual body weight was recorded daily. Food intake of the animals in each group was assessed twice a week throughout the experimental period (3 weeks). The experimental protocol was evaluated and approved by the Institutional Ethics Committee of the Faculty of Biochemistry and Biological Sciences (UNL, Santa Fe, Argentina – Acta 03/21).

At the end, the food was removed at 07:00 a.m. and experiments were performed between 07:00 and 09:00 a.m. Animals were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg body weight). Blood samples were collected from the inferior vena cava and rapidly centrifuged, and serum was either immediately assayed or stored at −20°C until use. The liver of each rat was totally removed, weighed, and sectioned for different subsequent assays. Liver samples were fixed in 10% (v/v) buffered formalin for 24 h at room temperature and embedded in paraffin for histology and immunohistochemistry analysis or frozen and stored at the temperature of liquid N_2. The animals were euthanized by removal of vital organ (the heart).

Analytical Methods

Serum glucose, uric acid, triglyceride, total cholesterol, HDL-cholesterol (HDL-c), and LDL-cholesterol (LDL-c) levels were measured by spectrophotometric methods using commercial enzymatic kits according to the manufacturer's protocols (Wiener Lab., Argentina). Serum activities of aspartate aminotransferase, alanine aminotransferase (ALT), and alkaline phosphatase (AP) enzymes were measured by spectrophotometric methods using commercial enzymatic kits according to the manufacturer's protocols (Wiener Lab., Argentina). Serum 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine or anandamide (AEA) endocannabinoid levels were measured with the method described by Thieme et al. [29]. A 0.5-mL aliquot of serum samples was prepared for analysis by adding the deuterated internal standards such as AEA-d4 (0.1 ng/mL), 2-AG-d3 (0.5 ng/mL) each. Following the addition of 2 mL ethyl acetate: hexane (2:1), samples were vortex-mixed for 1 min. After phase separation by centrifuging for 5 min the organic layer was evaporated to dryness, reconstituted with 1,000 µL acetonitrile. All MS analyses were carried out using a Waters 2695 LC system coupled to triple quadrupole mass spectrometer (Quattro Premier XE, Micromass technology). Chromatographic separation was achieved using an Ultisil C18 4.6 mm × 150 mm column with 5 µm particle size. The mobile phase consisted of (A) water with 5 mM ammonium formate and (B) methanol containing 5 mM ammonium formate buffer and 0.01% (v/v) formic acid. The flow rate was set at 0.5 mL/min with an injection volume of 50 µL. Detection was performed in ESI-positive mode, with precursor ions at 348 m/z and 379 m/z for anandamide and 2-AG, respectively.

Triglycerides and total cholesterol contents in the liver were extracted with chloroform-methanol (2:1) mixture. Aliquots were evaporated and total cholesterol and triglycerides were analyzed using the enzymatic methods mentioned previously. Lipid peroxidation was estimated by measuring TBARS in the serum and liver according to D'Alessandro et al. [10]. Intracellular ROS in the liver was measured as previously described [30].

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Liver Histology

A semi-automatic rotary microtome (Leica ®M2255) was used to obtain paraffin-embedded cross-sections (5 μm thickness) that were stained with hematoxylin-eosin to provide an overall view of the tissue. The images of the stained sections were taken under a bright field microscope (Olympus BH2, Tokyo, Japan) with ×20 magnification. Analysis of images was performed using the software Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD, USA). The degree of liver steatosis was assessed semiquantitatively as the percentage of hepatocytes involved. The NAFLD activity score (NAS) was performed as described in Lackner [31]: steatosis 0 (<5%), 1 (5–33%), 2 (34–66%), 3 (>66%); hepatocellular ballooning 0 (none), 1 (few ballooned cells), 2 (many cells/prominent ballooning lobular inflammation); inflammatory foci 0 (none), 1 (<2 foci/×20 field), 2 (2–4 foci/×20 field), 3 (>4 foci/×20 filed). A score greater than or equal to five was considered NASH.

Enzymatic Activity Assays

Acetyl-CoA carboxylase (ACC) was assayed as previously described [32]. The cytosolic fraction was obtained after centrifugation of the supernatant at 30,000 rpm for 1 h at 4°C. ACC was measured by an NADH-linked assay. Fatty acid synthase (FAS) activity was assayed on the cytosolic fraction of the liver tissue by measuring malonyl CoA-dependent oxidation of nicotinamide adenine dinucleotide phosphate at 37°C, as previously described [31, 32]. Liver tissue glucose-6-phosphate dehydrogenase (G-6-PDH), malic enzyme (ME), and carnitine palmitoyltransferase-1 (CPT-1) were determined as previously described [33].

Antioxidant Defense System

The antioxidant capacity of the serum and liver was measured using the ferric ion-reducing antioxidant power (FRAP) assay according to Vega Joubert et al. [12]. Liver GSH, a non-enzymatic antioxidant of the hepatic antioxidant defense system, was assayed according to Tietze [34]. The activities of different antioxidant enzymes, namely, CAT, GR, and GPx, were estimated in liver tissue homogenates and were carried out following the methodology previously described by Vega Joubert et al. [12].

Immunohistochemical Analysis

A standard immunohistochemical technique, following protocols previously described [12, 35], was performed. Paraffin-embedded (5 μm thickness) liver cross-sections were used to evaluate the protein expression of 4-HNE, NrF2, and NF-κB p65. The sections were mounted on 3-aminopropyltriethoxysilane (Sigma-Aldrich, Buenos Aires, Argentina)-coated slides and a subsequent microwave pretreatment for antigen retrieval was performed. The samples were incubated in a humid chamber first with a specific primary antibody for 4-HNE, NrF2, and NF-κB p65 (for 14–16 h at 4°C) and then with biotin-conjugated secondary antibody (anti-mouse, 1:100 dilution, Sigma) for 30 min at room temperature. The reactions were developed using the streptavidin-biotin peroxidase method and diaminobenzidine (Sigma) as a chromogenic substrate. Each immune-histochemical run included positive and negative controls. The expression of 4-HNE, NrF2, and NF-κB p65 was evaluated by image analysis using the Image Pro-Plus 5.0.2.9 system (Media Cybernetics, Silver Spring, MD, USA). Immunostained images were captured with a dPlan ×40 magnification (numerical aperture, 0.65; Olympus) attached to a Spot Insight V3.5 color video camera. Quantification was performed on at least 10 randomly selected fields per section. After converting each image into a gray scale, the integrated optical density was measured as a linear combination of the average gray intensity and the relative area occupied by positive cells, as was previously described by Ingaramo et al. [36].

Statistical Analysis

Results were expressed as mean ± SEM. Statistical comparisons were made transversely between different dietary groups. Data were tested for variance using Levene's test and normality by Shapiro-Wilk's test. The statistical difference between groups (RD, SRD, and SRD + Ca) was determined by one-way ANOVA followed by post hoc Newman-Keuls test. Correlation analyses were done by Pearson's correlation method. p values lower than 0.05 were considered to be statistically significant (SPSS 17.0 for Windows, SPSS Inc., Chicago, Illinois).

Results

Body Weight, Food Intake, and Serum Metabolite Levels

There was no significant difference ($p < 0.05$) in the initial and final body weights of the animals in the different experimental groups (Table 1). Final food intake did not differ between the groups. Serum triglyceride, total cholesterol, LDL-c, uric acid, ASL, ALT, AP, and TBARS levels were significantly higher in SRD-fed rats compared to RD-fed rats. Serum HDL-c and antioxidant capacity (FRAP) were decreased by a SRD. Cannabis oil administration (SRD + Ca) significantly decreased ($p < 0.05$) serum triglyceride, total cholesterol, LDL-c, ASL, ALT, uric acid, and TBARS levels, reaching the reference values. AP enzyme was significantly reduced ($p < 0.05$) in SRD + Ca group, although the values were still higher than RD group. Cannabis oil administration increased serum HDL-c and FRAP, reaching values similar to those of the RD group. No changes in serum glucose levels were observed among the three dietary groups.

Endocannabinoids Serum Levels, Histological Analysis, NAS Score, and Lipid Content of the Liver Tissue

Figure 1A, B show increased serum AEA and 2-AG endocannabinoid levels in the SRD group. Figure 1C depicts a high positive correlation (Pearson $r = 0.9538$, $p < 0.05$) between serum triglyceride and 2-AG levels. The results showed that in SRD-fed rats, increased serum triglyceride levels were correlated with increased serum 2-AG levels. Figure 1D shows an abnormal accumulation of lipid droplets within the cytoplasm of hepatocytes and infiltration of inflammatory cells in the histological

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Table 1. Body weight, food intake, and serum parameters in male rats fed a reference diet (RD), sucrose-rich diet (SRD), or SRD with cannabis oil (SRD + Ca)

	RD	SRD	SRD + Ca
Initial body weight, g	178.0±2.5	180.3±4.3	179.7±2.3
Final body weight, g	269.92±4.85	267.3±5.9	272.2±4.1
Food intake, g/day	16.82±0.35	16.87±0.27	16.90±0.43
Glucose, mM	8.51±0.15	8.44±0.16	8.42±0.19
Uric acid, μM	63.97±6.12^{b}	120.30±6.07^{a}	77.55±3.75^{b}
Triglyceride, mM	1.32±0.09^{b}	3.08±0.16^{a}	1.23±0.07^{b}
Total cholesterol, mM	2.02±0.10^{b}	2.64±0.09^{a}	2.12±0.18^{b}
HDL-c, mM	1.11±0.04^{a}	0.70±0.12^{b}	1.02±0.09^{a}
LDL-c, mM	0.28±0.3^{b}	0.39±0.02^{a}	0.26±0.01^{b}
AST, U/L	19.56±0.75^{b}	27.31±0.82^{a}	21.54±1.35^{b}
ALT, U/L	20.93±1.39^{b}	29.92±0.73^{a}	21.57±1.97^{b}
AP, U/L	672.42±54.61^{c}	1,183.01±25.07^{a}	1,032.03±11.95^{b}
TBARS, μM	2.92±0.11^{b}	3.94±0.11^{a}	2.99±0.13^{b}
FRAP, μM	531.62±20.25^{a}	451.72±18.49^{b}	506.02±13.26^{a}

Values are expressed as mean ± SEM, $n = 6$. Values in a line that do not share the same superscript letter (a, b, c) are significantly different ($p < 0.05$) when one variable at a time was compared by one-way ANOVA followed by a Newman-Keuls test. AST, aspartate aminotransferase.

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sections of the SRD group. However, the NAS score (table insert in Fig. 1D) was higher in the SRD group (score = 4). These results were accompanied by a significant increase ($p < 0.05$) in triglycerides and cholesterol content in the liver tissue (Fig. 1E, F). Cannabis oil administration reduced the levels of both serum endocannabinoids and remarkably improved the abnormalities of the histological sections. In the SRD + Ca group, NAS decreased significantly ($p < 0.05$) (table insert in Fig. 1D). The triglycerides content decreased significantly ($p < 0.05$), although the values were still higher than those in the RD group, and the cholesterol content was similar to the values of the RD group.

Hepatic Enzyme Activities Involved in Lipid Metabolism

As shown in Figure 2, ACC (A), ME (B), FAS (C), and G-6-PDH (D) activities (key enzymes related to the novo fatty acids synthesis) were increased in the liver of the SRD-fed rats compared with those of the RD-fed rats. However, cannabis oil administration for 3 weeks prevented these alterations. Furthermore, the activity of ACC and ME decreased significantly ($p < 0.05$) and returned to values similar to those recorded in RD-fed rats. In addition, a significant reduction in FAS and G-6-PDH activities was observed, although the values were still higher than those in the RD group.

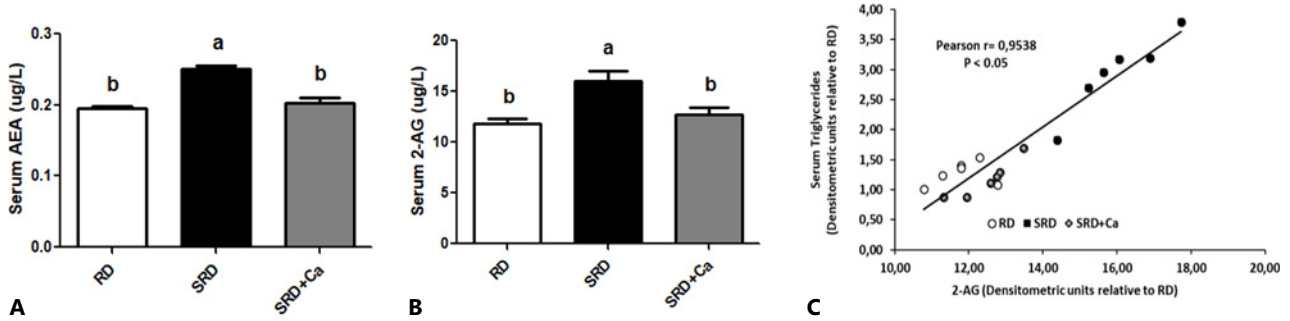
On the other hand, Figure 2E shows the hepatic activities of key enzyme related to mitochondrial (CPT-1) fatty acid oxidation in the three dietary groups. Cannabis oil administration prevented the decreased activity of CPT-1 observed in SRD-fed rats for 3 weeks, reaching values similar to those recorded in the RD.

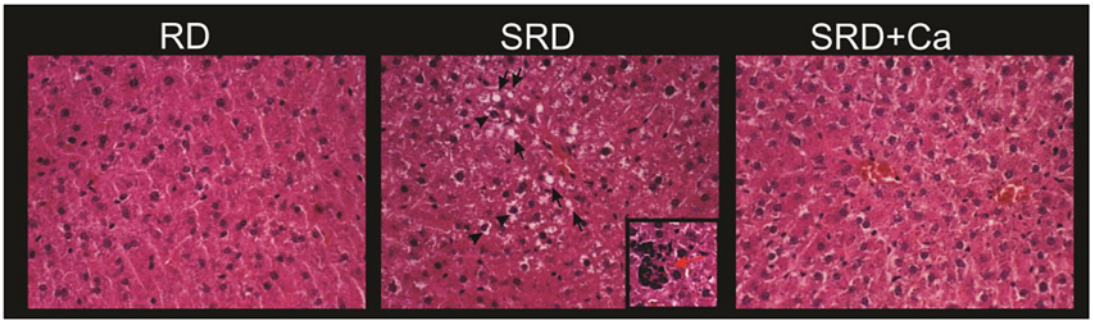
Lipid Peroxidation

Figure 3A shows a significant increase ($p < 0.05$) in the TBARS hepatic content in the SRD group compared to the RD group. In the SRD + Ca group, this parameter decreased significantly ($p < 0.05$), reaching similar values to those of the RD group. Figure 3B, C show representative photomicrographs and quantitative analysis of 4-HNE expression in the liver. The SRD-group presented a higher 4-HNE protein expression ($p < 0.05$) than the RD group. The expression protein showed both cytoplasmic and nuclear localization. Cannabis oil administration decreased the upregulation of 4-HNE expression ($p < 0.05$), although the values were still higher than those in the RD group.

Liver Oxidative Stress Biomarkers

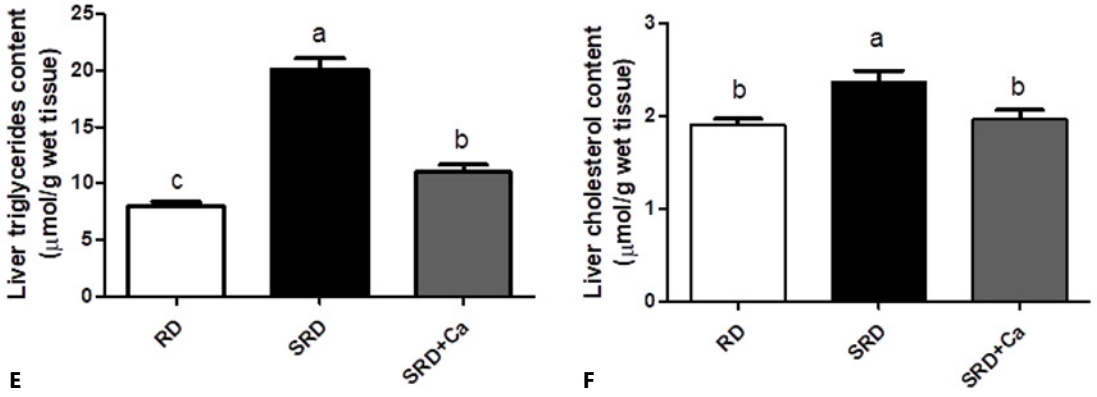
Table 2 shows that liver ROS was significantly increased ($p < 0.05$) in the SRD group compared to the RD group. When cannabis oil was administered in the SRD group, this parameter decreased significantly ($p < 0.05$), reaching similar values to those of the RD group. Liver antioxidant capacity (FRAP) was decreased ($p < 0.05$) in SRD group. Cannabis oil administration increased the liver antioxidant capacity, reaching values similar to those of the RD group. In addition, the decrease in the GSH content in the liver of the SRD group was increased





D

	RD		SRD		SRD+Ca	
	%	scoring	%	scoring	%	scoring
Steatosis	<5%	0	53%	2	<5	0
Hepatocelullar ballooning	none	0	few balloned cells	1	none	1
Lobular inflammation	none	0	<2foci/20x field	1	none	0
NAFLD activity score (NAS)		0		4		1



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($p < 0.05$) in the SRD + Ca group, reaching values similar to those of the RD group. Moreover, a significant decrease in CAT, GPx, and GR activities was observed in the SRD group ($p < 0.05$). Cannabis oil increased the CAT activity, although the values were still lower than those in the RD group. GPx and GR activities were increased ($p < 0.05$) in SRD + Ca group, reaching values similar to those of the RD group.

Liver NF-κB p65 and NrF2

Figure 4 shows the representative photomicrographs (A) and quantitative analysis (B) of NF-κB p65 expression in the liver. In the SRD group an increase of NF-κB p65 protein expression was observed ($p < 0.05$) compared to the RD group. The expression of NF-κB p65 showed both nuclear and cytoplasmic localization. In the SRD + Ca group, the protein expression was decreased ($p < 0.05$) compared to the SRD group, reaching similar values to those of the RD group.

Figure 5 shows representative photomicrographs (A) and quantitative analysis (B) of NrF2 expression in the liver. In the SRD group, there was a decrease NrF2 protein expression ($p < 0.05$) compared to the RD group. NrF2 showed nuclear and cytoplasmic expression. In the SRD + Ca group, the expression protein was increased ($p < 0.05$) compared to the SRD group, although the values were still lower than those in the RD group.

Discussion

Oxidative stress is associated with many metabolic disorders present in MS, such as liver diseases, which can be caused by unhealthy dietary patterns, including high-fat and fructose-rich diets [37]. While various pharmacological approaches are being evaluated for NAFLD treatment, there is an increasing emphasis on finding therapeutic strategies to address metabolic dysfunctions. In this context, the bioactive compounds present in the plant *Cannabis sativa L.* are the subject of numerous studies, as they are considered potential treatments for a wide range of metabolic processes and diseases [1, 38, 39]. In previous study of our group [13], we demonstrated that cannabis oil administration did not affect body weight, food intake, and liver weight, but significantly reduced diastolic and systolic blood pressure in SRD-fed rats for 3 weeks. Additionally, cannabis oil significantly increased analgesia and decreased locomotion, which were elevated in the SRD group, without significant differences in body temperature and catalepsy.

Another key component in the development of MS is dysfunction of lipid metabolism in the liver, a key tissue involved in fatty acid beta-oxidation and de novo lipogenesis [40]. The main endogenous ligands of the endocannabinoid system are AEA and 2-arachidonoylglycerol (2-AG). Some studies have shown that the stimulation of this system is associated with several metabolic pathologies, such as obesity, diabetes, and NAFLD, highlighting its participation in alterations of lipid metabolism, such as the stimulation of the synthesis of fatty acids and the decrease in beta oxidation [41, 42]. In the present study, we observed that SRD-fed rats show dyslipidemia, increased serum endocannabinoid levels, liver damage, and hepatic steatosis. This is accompanied by a significant increase in key enzymes involved in the synthesis of the novo fatty acids and a decrease in key enzymes involved in the oxidation of mitochondrial fatty acids. This suggests that sucrose induces a change in the metabolic fate of lipids (synthesis vs. oxidation), which could be one of the mechanisms involved in increasing triglycerides storage within liver cells, leading to hepatic steatosis in SRD-fed rats. In this line, different studies, including those from our group, have shown that rats fed a diet rich in sucrose or fructose for a short period (3–5 weeks) present hyperlipidemia, accompanied by a significant increase in lipid storage within of the liver tissue. The induction of gene expression of several lipogenic enzymes, including ACC and FAS, among others, plays a central role in this increase. In contrast, the activities of key fatty acid oxidation enzymes were significantly decreased in rats receiving a high sucrose/fructose diet [32, 43–45].

In recent years, the study of the hepatoprotective effects of various components of *Cannabis sativa L.* has

Fig. 1. Endocannabinoids serum levels, histological analysis, NAS score, and triglycerides and cholesterol content in liver tissue in rats fed a reference diet (RD), sucrose-rich diet (SRD), or SRD with cannabis oil (SRD + Ca). **A** Serum AEA levels. **B** Serum 2-AG levels. **C** Correlation between serum triglycerides and 2-AG levels. **D** Representative photomicrograph of histological abnormalities observed in liver sections. The accumulation of lipid droplets (black arrow), ballooning cells (black arrowhead), and inflammatory foci (inset, red arrow) are observed in the liver section H&E-stained. ×400 magnification. Table insert: histologic scoring system for activity grade of nonalcoholic fatty liver disease (NAS) in liver sections. **E** Liver triglycerides content. **F** Liver cholesterol content. Values are expressed as mean ± SEM, $n = 6$. Bars that do not share the same letter are significantly different ($p < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls test. H&E, hematoxylin-eosin.

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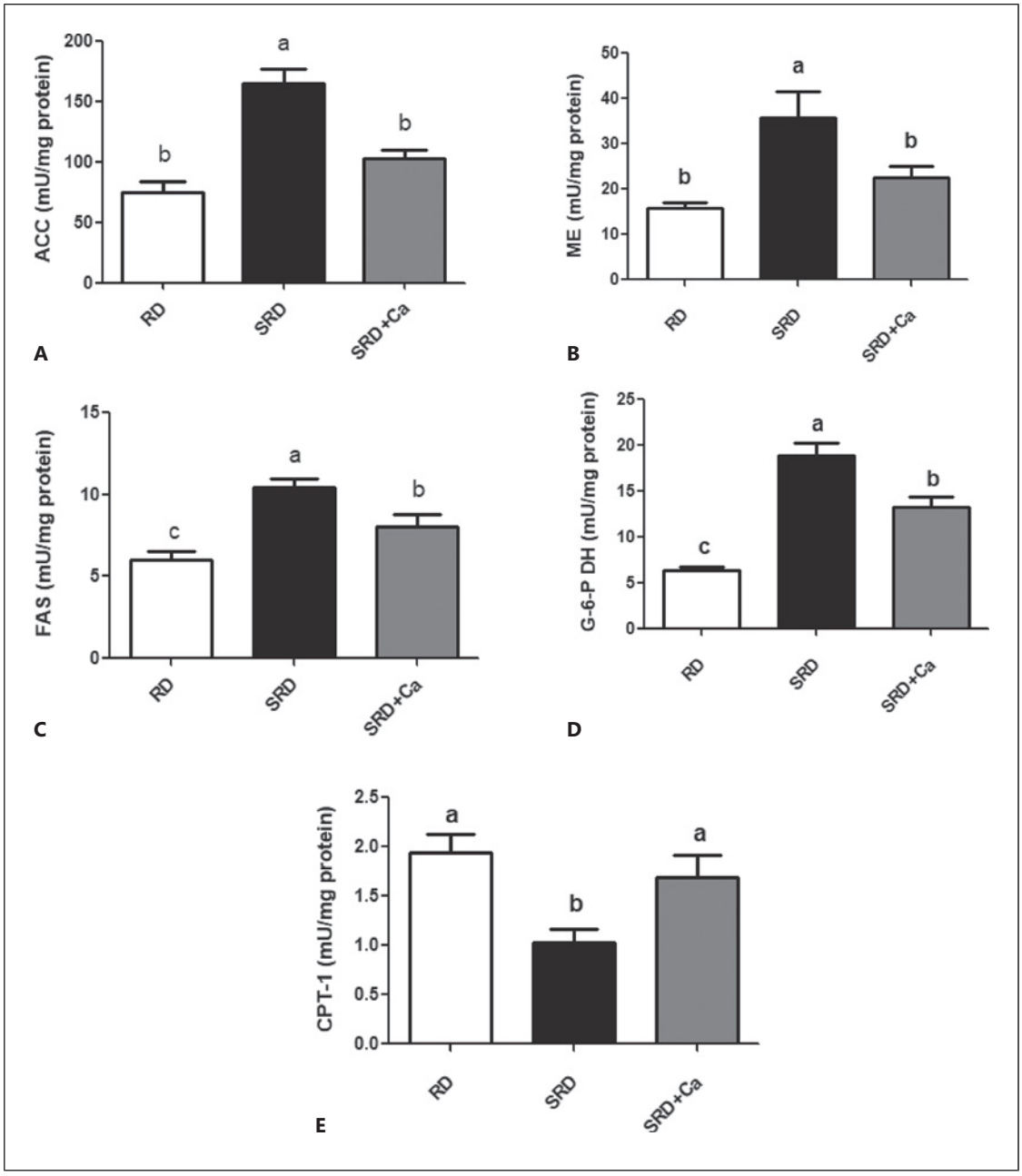


Fig. 2. Hepatic enzyme activities involved in lipid metabolism in rats fed a reference diet (RD), sucrose-rich diet (SRD), or SRD with cannabis oil (SRD + Ca). **A** Acetyl-CoA carboxylase (ACC). **B** Malic enzyme (ME). **C** Fatty acid synthase (FAS). **D** Glucose-6-phosphate dehydrogenase (G-6-PDH). **E** Carnitine palmitoyltransferase-1 (CPT-1) activities. Values are expressed as mean ± SEM, $n = 6$. Bars that do not share the same letter are significantly different ($p < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls test.

gained great importance. In the present study, after 3 weeks of cannabis oil administration, favorable changes were evident in the lipid profile and markers of liver damage and prevented the increase in serum endocannabinoid levels in SRD-fed rats. Furthermore, we observed an improvement in the hepatic lipid content, NAS, and enzyme activities involved in de novo lipogenesis and oxidation of fatty acids, highlighting the beneficial effects of the full-spectrum cannabis extract used in dyslipidemia, liver damage, hepatic steatosis, and

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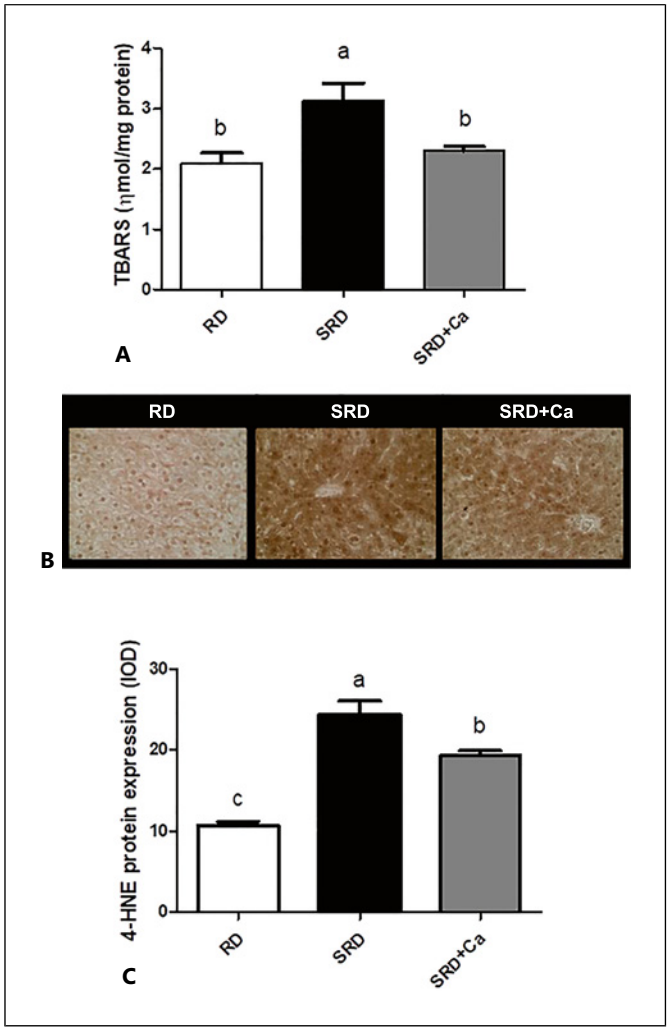


Fig. 3. Lipid peroxidation in rats fed a reference diet (RD), sucrose-rich diet (SRD), or SRD with cannabis oil (SRD + Ca). **A** Thiobarbituric acid reactive substance (TBARS) in the liver. Values are mean ± SEM (n = 6). Bars that do not share the same letter are significantly different ($p < 0.05$). **B** Representative photomicrographs of 4-HNE expression in liver sections of rats. An increase of hepatocytes with nuclear and cytoplasmic positive markers is observed in the SRD group. ×400 magnification. **C** Quantitative immunohistochemical analysis of expression of 4-HNE in the liver. Values are expressed as mean ± SEM, n = 6. Bars that do not share the same letter are significantly different ($p < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls test.

possible mechanisms involved in lipid metabolism. Some studies have suggested that Δ9-THC and/or CBD cannabinoids increased HDL-c concentration, reduced lipid serum and hepatic triglyceride accumulation, and liver damage in other animal models [46–48]. Similarly, Silvestri et al. [49] revealed the positive influence of CBD on the liver, namely, the reduction of intracellular lipid content in an in vitro hepatosteatosis model. Wang et al. [48] showed that CBD (injected i.p.) attenuates alcohol-induced liver steatosis and dysregulation of numerous key genes of fatty acid biosynthetic and oxidation pathways and transcription factor PPARα, implicated in development of alcohol-induced steatohepatitis in female mice. Eitan et al. [50] showed that prolonged non-invasive oral administration of pure THC to C57BL/6 male mice fed with a high-fat diet improved markers of lipid metabolism in the liver, but pure CBD administration had no effect on liver steatosis markers; conversely, however, THC treatment improved liver steatosis. Bielawiec et al. [51] demonstrated that CBD administration significantly reduced the intramuscular fatty acids accumulation and inhibited de novo lipogenesis in the gastrocnemius muscle of male Wistar rat fed with a high-fat diet and daily injections with synthetic CBD (10 mg/kg). However, none of these studies were performed in male Wistar rats fed with SRD. This is the first study to evaluate the effects of noninvasive full-spectrum cannabis oil (CBD:THC, 2:1 ratio) oral administration on liver steatosis and the possible mechanisms involved (lipogenesis vs. oxidation) in SRD-fed rats.

Hyperlipidemia, characterized by elevated levels of cholesterol and/or triglycerides, is often associated with oxidative stress [36]. When ROS levels increase, they can react with biological molecules such as lipids, leading to lipid peroxidation, which includes hepatocyte damage and liver injury [52, 53]. Jiang et al. [39] suggested that high-carbohydrate diets are associated with oxidative stress by elevating lipid peroxidation products levels. In previous work of our group, we found that SRD-fed rats for a short term developed dyslipidemia and an imbalance between a significant increase in TBARS in adipose tissue and plasma and decreased the activities of key enzymes involved in antioxidant defense in adipose tissue [10, 13, 32, 54]. In the present study, these results were also associated with a significant increase in lipid peroxidation markers (TBARS and 4-HNE) and ROS levels in the liver, accompanied by a significant reduction in hepatic antioxidant capacity (FRAP). This was reflected in the antioxidant system of these animals, which showed a significant decrease in hepatic GSH content, as well as in the activity of antioxidant enzymes, such as CAT, GPx, and GR. Other studies have shown that short-term administration (3–4 weeks) of high-carbohydrate diets in rats promotes elevated hepatic lipid content,

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Table 2. Liver oxidative stress biomarkers in male rats fed a reference diet (RD), sucrose-rich diet (SRD), or SRD with cannabis oil (SRD + Ca)

	RD	SRD	SRD + Ca
ROS arbitrary units, relative to RD	0.96±0.04[b]	2.46±0.31[a]	1.45±0.12[b]
FRAP, µmol/mg protein	465.7±5.61[a]	395.9±8.53[b]	467.3±16.63[a]
GSH, ηmol/mg protein	55.71±2.58[a]	44.73±2.53[b]	60.97±2.79[a]
CAT, U/mg protein	338.6±18.2[a]	225.1±36.9[b]	287.8±11.8 [ab]
GPx, mU/mg protein	154.5±10.5[a]	84.9±5.8[b]	146.8±18.3[a]
GR, mU/mg protein	47.39±3.5[a]	28.50±2.7[b]	44.07±3.2[a]

Values are expressed as mean ± SEM, $n = 6$. Values in a line that do not share the same superscript letter (a, b) are significantly different ($p < 0.05$) when one variable at a time was compared by one-way ANOVA followed by a Newman-Keuls test.

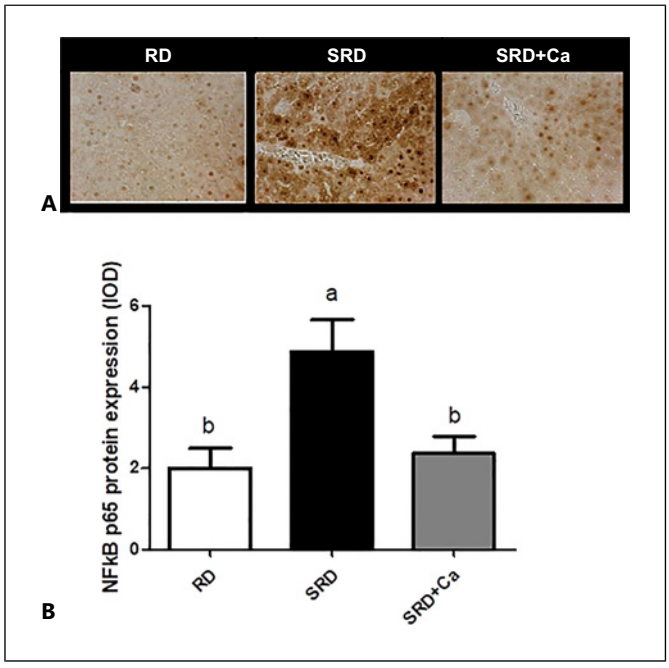


Fig. 4. Liver NF-κB p65 expression in rats fed a reference diet (RD), sucrose-rich diet (SRD), or SRD with cannabis oil (SRD + Ca). **A** Representative photomicrographs of immunocytochemical staining NF-κB in the liver sections of rats. Increased levels of nuclear and cytoplasmic positive markers are observed in the SRD group. ×400 magnification. **B** Quantitative immunohistochemical analysis of NF-κB p65 expression in the liver. Values (mean ± SEM, $n = 6$) are expressed as the integrated optical density (IOD). Values are expressed as mean ± SEM, $n = 6$. Bars that do not share the same letter are significantly different ($p < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls test.

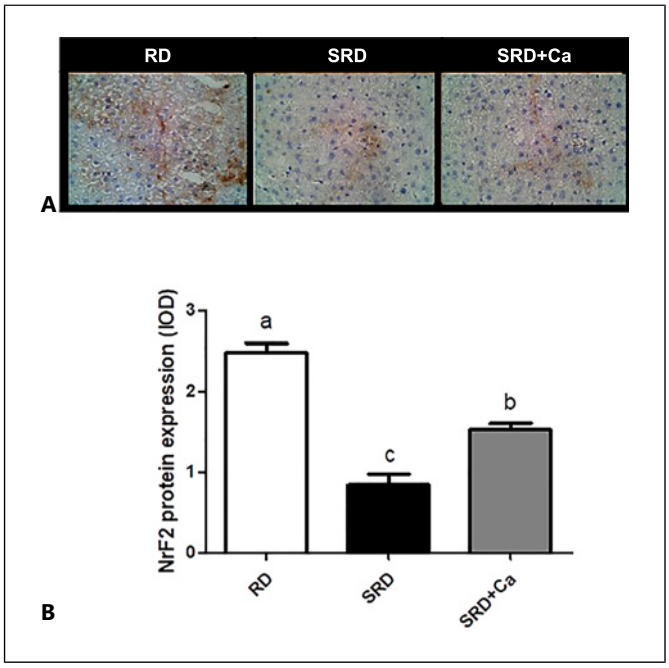


Fig. 5. Liver NrF2 expression in rats fed a reference diet (RD), sucrose-rich diet (SRD), or SRD with cannabis oil (SRD + Ca). **A** Representative photomicrographs of immunocytochemical staining of NrF2 in the liver sections of rats. Decreased levels of nuclear and cytoplasmic positive markers are observed in the SRD group. ×400 magnification. **B** Quantitative immunohistochemical analysis of NrF2 expression in the liver. Values (mean ± SEM, $n = 6$) are expressed as the integrated optical density (IOD). Values are expressed as mean ± SEM, $n = 6$. Bars that do not share the same letter are significantly different ($p < 0.05$) when one variable at a time was compared by one-way ANOVA followed by Newman-Keuls test.

cholesterol, and triglycerides [55, 56]. Busserolles et al. [57] found higher serum triglycerides and TBARS levels in rats fed a sucrose diet for 2 weeks compared to the control group, although there were no significant differences in hepatic TBARS levels. Similarly, Francini et al. [55] showed a significant decrease in the total GSH content and CAT activity in the liver of rats fed a fructose-rich diet for 3 weeks.

Cannabis sativa-derived compounds, such as THC and CBD, have been extensively studied to investigate their

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numerous biological effects, including their antioxidant effects. Some studies have suggested that these compounds can regulate the generation of ROS by interrupting free radical chain reactions or the activity of the antioxidant system [26, 58, 59]. In present study, we observed that cannabis oil administration, CBD:THC 2:1 ratio, reduced lipid peroxidation and decreased hepatic TBARS levels and expression of 4-HNE. In addition, we showed that daily administration of cannabis oil for 3 weeks resulted in an improvement in hepatic oxidative stress biomarkers. This was evidenced by a reduction in ROS levels and an increase in the antioxidant capacity in the liver. Both enzymatic (CAT, GR, and GPx) and non-enzymatic (GSH content) hepatic antioxidant components were improved by daily administration of cannabis oil. In this line, Comelli et al. [46] showed that *Cannabis sativa* extract rich in THC and CBD at a dose of 15 or 30 mg/kg for 8 days provided protection against lipid peroxidation and oxidative damage due to its antioxidant activity in streptozotocin-induced diabetic rats. Yang et al. [60] demonstrated that CBD treatment reversed lipid accumulation and liver damage, and attenuated the increase in mouse liver 4-HNE induced by acute ethanol administration. Erukainure et al. [61] demonstrated that treatment with *Cannabis sativa* extracts significantly improved oxidative injury and increase in GSH levels and CAT activity in liver tissues treated with FeSO4 in vitro. This is the first study to investigate hepatic lipid peroxidation and antioxidant activity in SRD-fed rats treated with full-spectrum cannabis oil (CBD:THC, 2:1 ratio).

Oxidative stress may contribute to the development of metabolic disorders through key transcription factors such as NrF2 and NF-kB, which regulate the balance of cellular redox status and responses to stress. The reduction of NrF2 is associated with increased oxidative stress because NF-κB is readily activated in an oxidative environment [37, 62]. In our study, we observed that NrF2 protein expression was significantly decreased, and NF-κB protein expression was significantly increased in the hepatic tissue of SRD-fed rats for 3 weeks. We recently demonstrated in long-term SRD-fed rats that liver injury and oxidative stress are accompanied by decreased NrF2 expression and increased NF-κB p65 protein expression in the liver [12]. In this line, Pengnet et al. [63] showed that NF-κB expression was increased and NrF2 expression was decreased in the liver of fructose-fed rats during 12 weeks. Hernandez-Rodas et al. [64] demonstrated significantly decreased hepatic NrF2 DNA binding and increased hepatic NF-κB DNA binding in male C57BL/6J mice fed with a high-fat diet for 12 weeks. Beydogan et al. [65] showed that the number of NF-κB immunopositive cells increased in Sprague-Dawley rats that received 10% fructose in drinking water for 12 weeks.

Some molecular effects of cannabinoids depend on NrF2-NF-κB crosstalk. Some authors have suggested that cannabinoids may decrease ROS production via NrF2 [57, 66]. The most studied cannabinoid to date in this aspect is CBD, suggesting that CBD-mediated alterations in NrF2-NF-κB are one of the key points in the modulation of intracellular redox homeostasis and determination of the cellular response under oxidative stress conditions. The antioxidant activity of CBD begins at the protein level by activating the redox-sensitive transcription factor, NrF2, which is responsible for the transcription of antioxidant genes [26, 67]. In our study, cannabis oil administration induced liver NrF2 expression, and decreased NF-κB p65 expression. The effects of full-spectrum cannabis oil on the molecular mechanisms of hepatic NrF2 and NF-κB p65 in in vivo animal models have been poorly investigated. Some authors have studied the effects of cannabinoids in in vivo animal models using isolated cannabinoids. Jiang et al. [39] demonstrated that CBD treatment (CBD with a purity ≥98%, 5 mg/kg body weight of CBD solution per day for 8 weeks) suppressed NF-κB activation in a model induced by ethanol combined with a high-fat diet for 8 weeks. Huang et al. [68] demonstrated that CBD (CBD solution as 5 mg/kg body weight per day for 8 weeks) significantly inhibited the NF-κB p65 nuclear translocation in liver fed high-fat/cholesterol mice for 8 weeks. This is the first study to investigate the effects of a full-spectrum cannabis oil (CBD:THC, 2:1 ratio) on the transcription factors NrF2 and NF-kB in hepatic oxidative stress in an experimental model that was nutritionally induced by short-term administration of an SRD that mimics human MS.

Conclusions

The present study demonstrates that dyslipidemia, hepatic damage, liver steatosis, lipid peroxidation, and oxidative stress are accompanied by changes in NrF2 and NF-κB expression in the liver of SRD-fed rats in the short term. In addition, full-spectrum cannabis oil (CBD:THC 2:1 ratio) showed new mechanisms of action by mitigating dyslipidemia, hepatic damage, and liver steatosis induced by SRD. These effects are related to a lower activity of lipogenic enzymes and an increase in CPT-1 enzyme activity involved in the mitochondrial oxidation of fatty acids. Furthermore, cannabis oil could ameliorate lipid peroxidation and oxidative stress through the regulation of the main regulatory factors involved, NrF2 and NF-κB p65. The activation of the transcription factor

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NrF2, concomitant with the inactivation of NF-κB. Furthermore, our results suggest that full-spectrum cannabis oil with a CBD:THC 2:1 ratio may serve as a natural nutraceutical agent to prevent metabolic disorders related to hepatic steatosis, oxidative stress, and NASH. We cannot rule out the possibility that other components of cannabis oil, such as terpenes, flavonoids, and alkaloids, may also contribute to the beneficial effects found in the present study. Finally, although promising, these results warrant further investigation before extrapolating the use of full-spectrum cannabis oil as a complementary nutraceutical that could help in the treatment of some metabolic disorders in humans.

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Statement of Ethics

This study was performed in strict accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Ethics Committee of the Faculty of Biochemistry and Biological Sciences (UNL, Santa Fe, Argentina – Acta 03/21).

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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Author Contributions

Valentina Degrave and Paola Ingaramo: investigation, data curation, formal analysis, and writing the manuscript. Michelle Vega Joubert: investigation, methodology, data curation, formal analysis, and writing the manuscript. Daniela Sedan and Dario Andrinolo: investigation, data curation, and formal analysis. María Eugenia D'Alessandro: conceptualization, methodology, data curation, formal analysis, and writing the manuscript. María Eugenia Oliva: funding acquisition, conceptualization, investigation, methodology, data curation, formal analysis, supervision, and writing the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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