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Purified Cannabidiol, the main non-psychotropic component of *Cannabis sativa*, alone, counteracts neuronal apoptosis in experimental multiple sclerosis

S. GIACOPPO[1], T. SOUNDARA RAJAN[1], M. GALUPPO[1], F. POLLASTRO[2], G. GRASSI[3], P. BRAMANTI[1], E. MAZZON[1]

[1]Experimental Neurology Laboratory, IRCCS Centre Neurolesi "Bonino-Pulejo", Messina, Italy
[2]Department of Pharmaceutical Sciences, University of Eastern Piedmont, Novara, Italy
[3]Council for Research and Experimentation in Agriculture - Research Centre for Industrial Crops (CRA-CIN), Rovigo, Italy.

Abstract. – OBJECTIVE: Multiple Sclerosis (MS) is a global concern disease leading to a progressive, chronic and demyelinating condition, affecting the central nervous system (CNS). The pathology has an inflammatory/autoimmune origin; nevertheless, neuronal cell death mechanisms are not to be underestimated. The present study was designed to test the effects of intraperitoneal administration of cannabidiol (CBD), the main non-psychotropic cannabinoid of *Cannabis sativa* (CS), in an experimental model of MS. The aim is to evaluate the capability of CBD administration to thwart the cascade of mediators involved in MS-induced apoptosis.

MATERIALS AND METHODS: Experimental Autoimmune Encephalomyelitis (EAE) was induced by immunization with myelin oligodendroglial glycoprotein $(MOG)^{35-55}$ peptide in mice. After immunization, mice were observed daily for signs of EAE and weight loss. Disease signs were evaluated using a standardized scoring system.

RESULTS: Immunohistochemical and Western blot assessments of key apoptotic markers reveal that CBD treatment is able to avoid Fas pathway activation, phospho-ERK p42/44 and cleaved caspase-3 triggering as well as alterations in mitochondrial permeability due to Bax/Bcl-2 unbalance. Moreover, CBD interferes with p53-p21 axis activation. As results, the absence of tissue apobody formation in spinal cord tissues of EAE-mice treated with CBD was established. Most of therapeutic properties of CS are currently ascribed to the psychotropic effects of phenylterpenoid delta-9 tetrahydrocannabinol.

CONCLUSIONS: We have demonstrated that, alone, purified CBD possesses an anti-apoptotic power against the neurodegenerative processes underlying MS development. This represents an interesting new profile of CBD that could lead to its introduction in the clinical management of MS.

Key Words:
Apoptosis, Experimental multiple sclerosis, Non-psychotropic cannabinoid, Cannabidiol, Mitochondrial permeability.

Introduction

Multiple sclerosis (MS) is a demyelinating disease mostly of autoimmune origin that affects and damages CNS, leading to a disabling condition[1]. In particular, myelin sheath loss causes a severe impairment of nerve signal transmission between the brain and spinal cord[2]. Actually, 2.3 million individuals are living with MS. According to data released by National Multiple Sclerosis Society, published in 2013 and relative to the year 2009, there were about 10.400 cases/year with an incidence of 3.6 women/100.000 and 2.0 men/100.000[3]. Currently, pharmacological management of MS is in relationship with the course of the pathology. For the treatment of the so-classified relapsing-remitting MS (RRMS), to date disease modifying therapies (DMT) are adopted. They consist of immunomodulatory drugs, primarily belonging to IFN-β class, specifically IFN-β 1a (Avonex®, Rebif ®) and IFN-β 1b (Betaseron®, Extavia®). Recent trials are aimed at the possible introduction of teriflunomide (Aubagio®) and dimethyl fumarate (Tecfidera®) looking at their efficacy in comparison with another drug prescribed for the treatment of RRMS, the glatiramer acetate (Copaxone®)[4], a synthetic analogue of myelin basic protein[5]. When highly

Corresponding Author: Emanuela Mazzon, Ph.D; e-mail: emazzon.irccs@gmail.com

active MS patients present more and more relapse episodes, the first line drug indicated is the recombinant humanized natalizumab (Tysabri®), a selective adhesion-molecule inhibitor monoclonal antibody against the integrin very late antigen-4 (VLA-4), used as monotherapy to delay progression of disability[6]. Sadly, the risk of contracting the John Cunningham virus (JCV) is high, especially after long treatments[7]. In this scenario, the new frontiers of the pharmacology look at the use of complementary and alternative medicine[8,9], to counteract, if not the disease progression, at least MS symptoms that are based on the affected CNS area and that, for their plurality and variety, can require a multidisciplinary management[10]. Overall, symptoms can include motor control deficit (spasms and spasticity, weakness, impaired coordination, balance and functioning of the arms and legs); altered sensitivity of the limbs; neurological symptoms (vertigo, pins and needles, neuralgia and visual disturbances; neurological bladder (incontinence and constipation); as well as neuropsychological symptoms (memory loss, depression). Moreover, a sense of loss of identity is the direct consequence of physical changes and functional limitations[11].

Medicinal plants are the most ancient resource of the history in the treatment of various diseases[12]. An example is given by *Cannabis sativa* (*CS*) rich in terpenophenolic constituents[12]. *CS* therapeutic use is a controversial open question which goes far beyond mere campaign on the legalization of marijuana[13]. In this regard it is noteworthy to consider the recent introduction of Sativex®, a cannabinoid oromucosal spray containing a 1:1 ratio of the phenylterpenoid delta-9 tetrahydrocannabinol (THC) and cannabidiol (CBD), the two major components of *CS*[14], for the management of symptomatic treatment of chronic pain and spasticity. Despite this combination has been approved and in the current state introduced in several countries under this formulation, over the years the point of view of the scientific community regarding THC and CBD is changed. In particular, the dichotomy between psychotropic and non-psychotropic effects have been stressed and the most fervent supporters for the introduction of *CS* in clinical practice look at the beneficial phytochemicals properties that could derive by CBD isolation rather than to side possible hallucinogenic effects that may result from THC consumption. Ultimately, this is nothing more than the price that CBD, the major non-psychoactive component, pays remaining in the shadow of THC. Several experimental studies have shown that CBD possesses many properties[15], often wrongly attributed by collective imagination just to THC alone and wide experimental evidences demonstrated that isolating non-psychotropic compounds by THC component provide beneficial effects for therapeutic use, mostly for CNS disorders[16]. Actually, all cannabinoids have a wide antioxidant and neuroprotective action[17]. In particular, antitumoral activity of CBD mediated by the triggering of apoptotic mechanisms is not so overly declared, but not even a mystery[18]. CBD exerts its effects via both the interaction with cannabinoids receptor 1 and 2 (CB1 and CB2 receptors, canonical cannabinoid receptor pathway) and activating other receptor-independent channels and by binding with various non-cannabinoid receptors (such as PPARs, transient receptor potential vanilloid type 1 (TRPV1), GPR55, GPR18, GPR119 and 5-hydroxytryptamine receptor subtype 1A (5-HT1A)[19].

In our study, we decided to investigate not in the wake of other authors[20], the well known and assessed anti-inflammatory effect of CBD[21,22], but rather its capability to avoid programmed cell death in the spinal cord of animal affected by experimental autoimmune encephalomyelitis (EAE), a model induced by Myelin Oligodendrocyte Glycoprotein $(MOG)_{35\text{-}55}$ peptide injection and validated to reproduce human MS in mice[23]. By examining this profile of CBD, we strongly hope to provide new evidences about the efficacy of the molecule and to contribute into delineating a clearer profile of the compound so that its use should be extended to this as well as other pathologies with similar symptoms.

Materials and Methods

Plant Material

Cannabis sativa L, derived from greenhouse cultivation at CRA-CIN, Rovigo (Italy), was collected in November 2013. The isolation and manipulation of cannabinoids was done in accordance with their legal status (Authorization SP/106 23/05/2013 of the Ministry of Health, Rome, Italy).

Extraction and Isolation of CBD

Pure CBD (>99%) was isolated from an Italian variety of industrial hemp (Carmagnola) according to a standardized method of the cannabinoid purification[24] to avoid any trace of THC that could interfere in the trial or cause legal limitation.

Animals

Male C57BL/6 mice (Harlan Milan, Italy) 12 weeks of age and weighing 20-25g were housed in individually ventilated cages with food and water *ad libitum*. The room was maintained at a constant temperature and humidity on a 12 h/12 h light/dark cycle.

Ethical Approval

This study was carried out in strict accordance with the recommendations in the guide for the care and use of laboratory animals of Italian National Institutes of Health. The protocol was approved by the Ministry of Health "General Direction of animal health and veterinary drug" (Authorization 150/2014-B 28/03/2014). In particular, animal care was in compliance with national regulations on protection of animals used for experimental and other scientific purposes (D.M. 116/92) as well as with the EEC regulations (O.J. of E.C.L 358/1 12/18/1986). In addition, minimized number of animals were used for the experiment and their suffering.

Induction of Experimental Autoimmune Encephalomyelitis (EAE)

After anaesthesia, induced with an anaesthetic cocktail composed of tiletamine plus xylazine (10 ml/kg, i.p.), EAE was actively induced using $(MOG)_{35-55}$ peptide (MEVGWYRSPFSRVVHLYRNGK; % peak area by HPLC ≥ 95, AnaSpec, EGT Corporate Headquarters, Fremont, CA, USA). Mice were immunized subcutaneously with 300 μl/flank of the emulsion consisting of 300 μg of $(MOG)_{35-55}$ in PBS mixed with an equal volume of Complete Freund's Adjuvant (CFA) containing 300 μg heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Sparks, MD, USA). Immediately after $(MOG)_{35-55}$ injection, the animals received 100 μl of *Bordetella pertussis* toxin (Sigma-Aldrich, Milan, Italy) (500 ng/100 μl, i.p), repeated 48 h later. The disease follows a course of progressive degeneration, with visible signs of pathology consisting of flaccidity of the tail and loss of motion of the hind legs.

Experimental Design

Mice were randomly allocated into the following groups (n= 45 total animals):

Naive group (n=10): mice did not receive $(MOG)_{35-55}$ or other treatment.

EAE group (n=20): mice subjected to EAE that received only the vehicle of the pharmacological treatment (1:1:8 EtOH:Tween 20:saline);

EAE + CBD treatment group (n=15): starting from the occurring of first signs of disease (14th day onset), EAE mice were daily subjected to CBD treatment (10 mg/kg i.p.);

CBD-vehicle control group (n=10) mice that did not receive $(MOG)_{35-55}$ but only CBD vehicle as control of the treatment.

At the 28th day from EAE-induction, animals were euthanized with i.p. of Tanax (5 ml/kg body weight). In addition, spinal cord tissues were sampled and processed in order to evaluate parameters of disease.

Clinical Disease Score

The first measurement of clinical disease score was taken on the day of EAE- induction (day zero), the second after 4 days and all subsequent measurements were recorded every 48 hours until sacrifice. Briefly, the signs of EAE were scored using a standardized scoring system[25] as follows: 0=no signs; 1=partial flaccid tail; 2=complete flaccid tail; 3=hind limb hypotonia; 4=partial hind limb paralysis; 5=complete hind limb paralysis; 6=moribund or dead animal. Animals with a score ≥ 5 were sacrificed to avoid animal suffering. The measure of clinical disease score has been expressed as mean ± SEM of all measurements of each experimental group.

Body Weight Evaluation

The first measurement of body weight was taken on the day of EAE-induction (day zero), the second after 4 days and all subsequent measurements were recorded every 48 hours until sacrifice. The daily variation of body weight has been expressed compared to day of EAE induction (day zero), also the value day has been expressed as mean ± SEM of all animals for each experimental group.

Needle Test

The test was aimed to assess mice's responsiveness to a mechanical stimulus. Each animal was subjected to 3 stimuli on the plantar surface of the hindpaw with several seconds between applications of needle. A positive response is a paw withdrawal from the stimulus. The maximum

score has a value of 6. In specific, 7 tests were performed every 48 hours in two weeks from the first administration of CBD. The values are expressed as mean ± SEM of each group.

Immunohistochemical (IHC) Evaluation

After deparaffinization with xylene, sections of spinal cord samples were hydrated. Detection of Fas-Ligand, Bax, Bcl-2 was carried out after boiling in citrate buffer 0.01 M pH 6 for 4 min. Endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min.

Sections were incubated overnight with:

- Anti-FAS-Ligand polyclonal antibody (1:100 in PBS v/v; Abcam, Cambridge, MA, USA)
- Anti-Bax polyclonal antibody (1:100 in PBS v/v; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA);
- Anti-Bcl-2 polyclonal antibody (1:100 in PBS v/v; Santa Cruz Biotechnology, Inc);

Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vectastain ABC kit, Vector, Burlingame, CA, USA). The immunostaining (positivity in brown color) was developed with peroxidase substrate kit DAB (Vector Laboratories, Inc.), while the counterstaining with Mayer's hemalum solution (blue background).

To verify the binding specificity, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these cases no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out.

All sections were obtained using light microscopy (Leica DM 2000 combined with Leica ICC50 HD camera, Wetzlar, Germany). Leica Application Suite V4.2.0 software was used as image computer program to acquire IHC pictures.

Western Blot Analysis

All the extraction procedures were performed on ice using ice-cold reagents. In brief, spinal cord tissues were suspended in extraction buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN_3, 10 mM 2-mercaptoethanol, 50 mM NaF, protease inhibitor tablets (Roche Applied Science, Monza, Italy), and they were homogenized at the highest setting for 2 min. The homogenates were chilled on ice for 15 min and then centrifuged at 1000g for 10 min at 4°C, and the supernatant (cytosol extract) was collected to evaluate content of citoplasmatic proteins. The pellets were suspended in the supplied complete lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA protease inhibitors (Roche), and then were centrifuged for 30 min at 15.000 g at 4°C. Then, supernatant containing nuclear extract was collected to evaluate the content of nuclear proteins. Supernatants were stored at -80°C until use. Protein concentration in homogenate was estimated by Bio-Rad Protein Assay (Bio-Rad, Segrate, Italy) using bovine serum albumine (BSA) as standard, and 20 μg of cytosol and nuclear extract from each sample were analyzed.

Proteins were separated on sodium dodecyl sulfate-polyacrylamide minigels and transferred onto PVDF membranes (Immobilon-P Transfer membrane, Millipore), blocked with PBS containing 5% nonfat dried milk (PM) for 45 min at room temperature, and subsequently probed at 4°C overnight with specific antibodies for phospho-ERK p42/44 (1:1000; Cell Signaling Technology, Boston, MA, USA), ERK2 (1:2000; Cell Signaling Technology), cleaved-caspase 3 (1:500; Cell Signaling Technology), caspase-3 (1:500; Cell Signaling Technology), p21 (1:1000; Merck Millipore) and p53 (1:2000; Abcam) in 1x phosphate buffered saline (PBS), 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT). HRP-conjugated goat anti-rabbit IgG was incubated as secondary antibody (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated with antibody for GAPDH HRP Conjugated (1:1000; Cell Signaling Technology) and beta-actin (1:1000; Santa Cruz Biotechnology, Inc). The relative expression of protein bands has been visualized using an enhanced chemiluminescence system (Luminata Western HRP Substrates, Millipore, Billerica, MA, USA) and protein bands were acquired and quantified with ChemiDoc™ MP System (Bio-Rad) and a computer program (ImageJ software) respectively.

Blots are representative of three separate and reproducible experiments. The statistical analysis was carried out on three repeated blots performed on separate experiments.

TUNEL Assay

To test whether in EAE model spinal cord was associated with cell death by apoptosis, we measured TUNEL-like staining in the perilesional spinal cord tissue. TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Apotag, HRP kit DBA, Milan, Italy). Sections were incubated with 15 mg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP, incubated in a humid atmosphere at 37°C for 1 h, and then washed with PBS. Sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine (DAB) and controstained with nuclear fast blue.

Statistical Analysis

GraphPad Prism version 6.0 program (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis of the data. The results were statistically analyzed using one-way ANOVA followed by a Bonferroni *post hoc* test for multiple comparisons. A p value less than or equal to 0.05 was considered significant. Results are expressed as the mean ± SEM of n experiments.

Results

CBD Treatment Ameliorates General Wellness in EAE-Affected Mice

Body weight measurement (Figure 1 a) as well as Clinical Disease Score (Figure 1 b) evaluation were assessed as parameters of disease. In both cases, CBD-treated EAE-affected mice show a trend of recovery over time compared to untreated EAE mice, in particular following the disease onset (fifth measurement) and until sacrifice. Mice belonging to vehicle CBD and naive group have a normal increase in body weight as well as absence of motor deficit.

Needle test to Evaluate paw Sensibility

EAE development leads untreated mice to a constant loss of paw sensibility measured (Figure 2) since the disease onset until sacrifice. Conversely, CBD-treatment preserves a degree of paw sensibility in EAE-affected mice with significant score levels.

CBD Treatment is Able to Modulate Apoptotic Pathway Triggering via Fas Pathway Modulation

To evaluate upstream-enhanced apoptotic mechanisms we looked at processes mediated by the signal cascade triggered by FAS-ligand. IHC localization of spinal cord sections for FAS-Ligand has shown a marked immunopositivity in untreated EAE mice (Figure 3 c) comparing to animal that under the same conditions received the intraperitoneal CBD treatment (Figure 3 d). Naïve mice (Figure 3 b) and mice that were administrated with the CBD-vehicle alone (Figure 3 a) resulted negative for FAS-ligand. Moreover, statistical analysis established significant differences between EAE group and all other experimental groups (see densitometric analysis, Figure 3 e).

CBD Treatment Controls Cascade of Mediators Involved in Apoptosis

Western blot analysis for phospho-ERK p42/44 revealed that Mitogen-Activated Protein Kinases (MAPK) signaling pathway is strongly activated following EAE-induction while CBD treatment reduces the expression levels of this marker (Figure 4 a, see densitometric analysis b).

Since ERK activity has been clearly and widely implicated in the releasing of classical markers of apoptosis execution, such as effector caspase-3[26], we evaluated the degree of caspase 3 activation via detection of cleaved-caspase 3 expression levels. Treatment with CBD inhibits this marker that, conversely, results highly expressed in untreated EAE mice (Figure 4 c, see densitometric analysis d).

CBD Treatment Avoids Bax/Bcl-2 Unbalance.

Spinal cord tissues sampled by untreated EAE mice display high positivity for Bax and negative staining for Bcl-2 leading to believe that mechanism of mitochondrial altered permeability are primed in these animals (Figures 5 c and 6 c, respectively). Conversely, intraperitoneal administration of CBD inhibits the above cited alterations. In fact, IHC localization for Bax/Bcl-2 reveals that in CBD-treated EAE mice these markers are more targeted towards anti-apoptotic mechanisms showing higher Bcl-2 (Figure 6 d) than Bax immunopositivity (Figure 5 d) as well

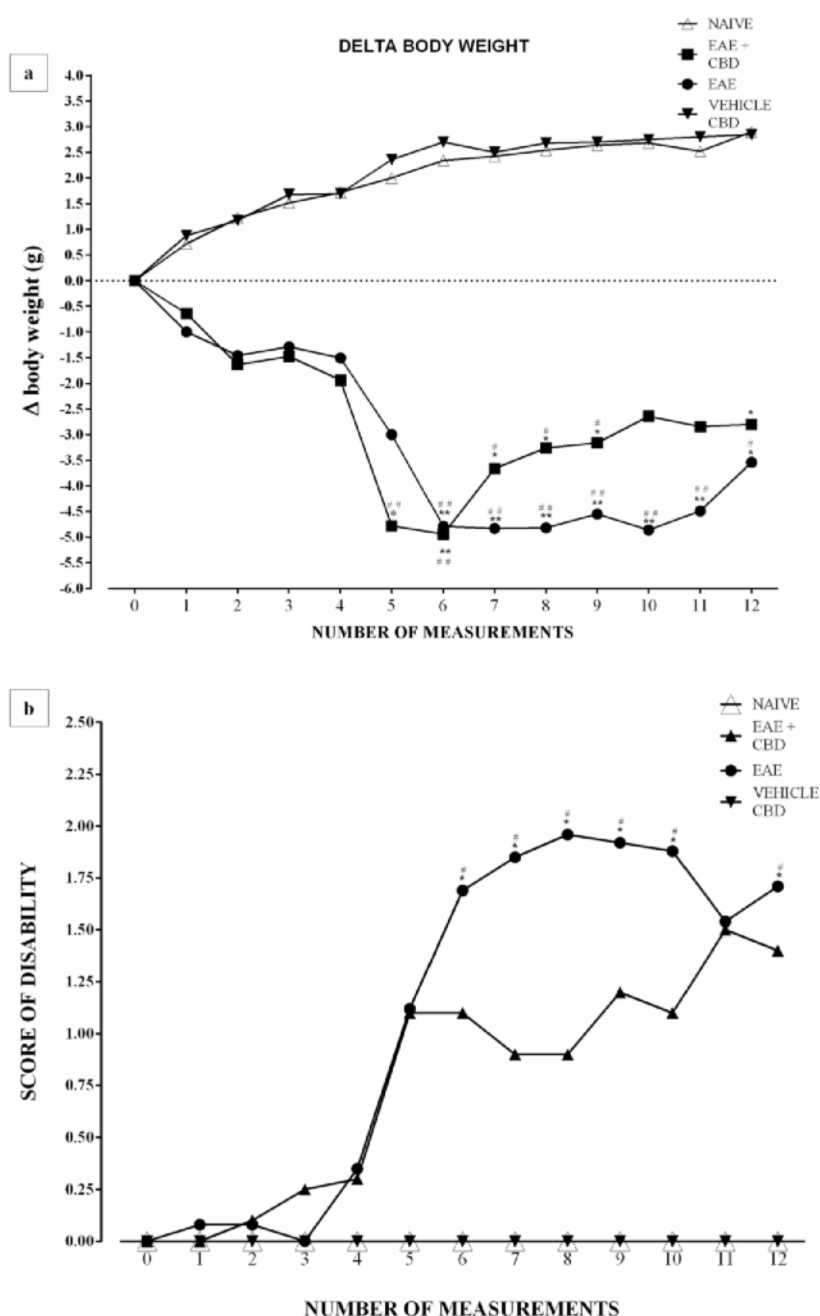

Figure 1. CBD-treated EAE-injured mice display body weight and motor function recovery. A measure of disease degree is the evaluation of body weight ***(a)*** and of the disability score ***(b)***. For both parameters CBD-treated mice show high significant recovery when compared with untreated EAE mice, in particular following the disease onset (fifth measurement) and until sacrifice. A *p* value < 0.05 was considered significant. vs. NAIVE; #vs. VEHICLE CTR.

as in vehicle-control (Figures 6 a and 5 a, respectively) and naïve groups (Figures 6 b and 5 b, respectively). For both Bax and Bcl-2, a quantitative analysis of the immunopositivity was performed in all experimental groups as showed in Figures 5 e and 6 e, respectively.

CBD Regulates p53-p21 Axis Activation

About downstream nuclear mechanisms of apoptosis Western blot analysis revealed that there are very high expression levels of the transcription factor p53 in untreated EAE mice (Figure 7 a, see densitometric analysis b), that,

in turn, activates and stimulates the overexpression of p21 (Figure 7 c, see densitometric analysis d). CBD treatment in EAE mice reverses this panel decreasing p53 and p21 at levels comparable with vehicle-control (Figure 7 a, see densitometric analysis b and c, see densitometric analysis d, respectively) and naïve groups (Figure 7 a, see densitometric analysis b and c, see densitometric analysis d respectively).

CBD Treatment, Interfering with the Activation of Several Mediators Thwarts Neuronal Cell Death

Finally, TUNEL assay revealed that all displayed cellular mechanisms of apoptosis are translated in a marked presence of apobodies (mean of ±337.5 positive nuclei/field, positive staining control Figure 8 e) in untreated EAE mice (Figure 8 c) that, conversely are wholly ab-

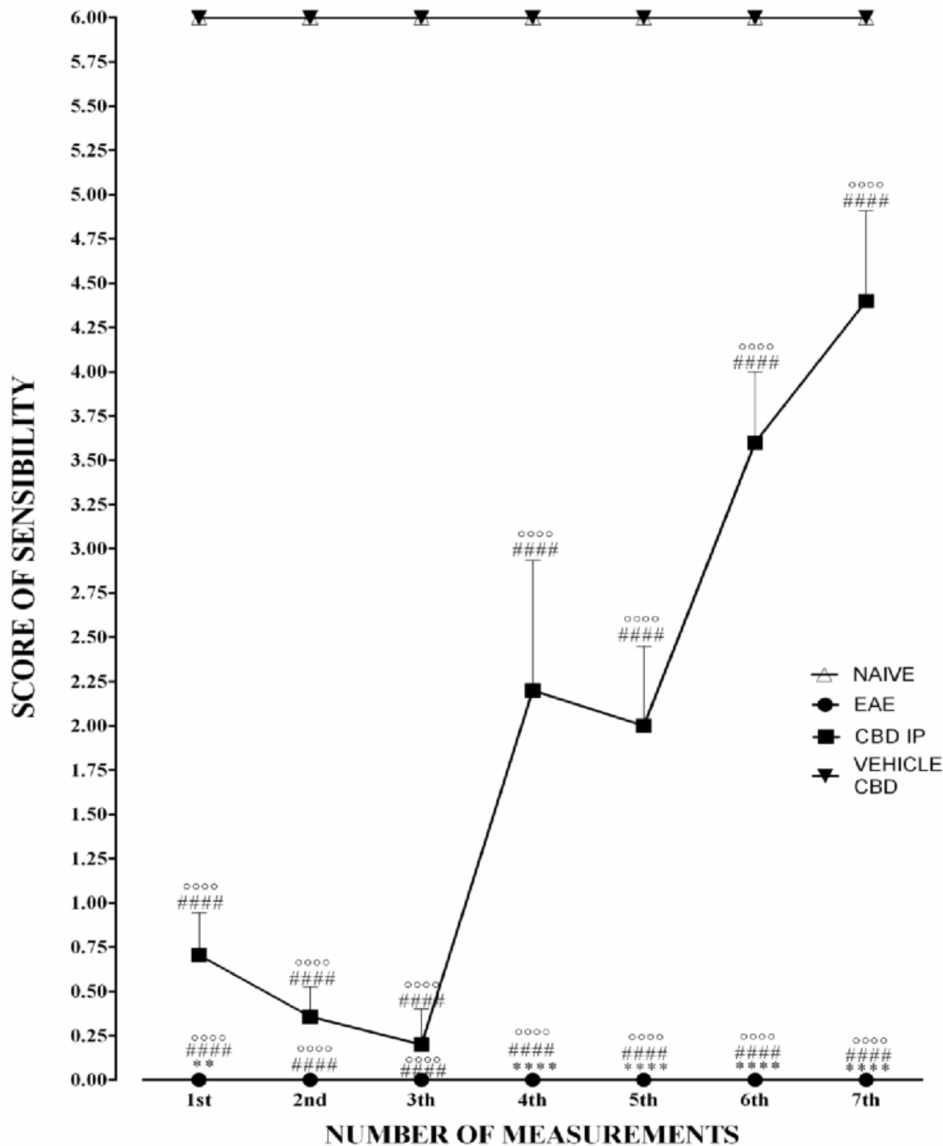

Figure 2. CBD treatment preserves paw sensibility. Behavioral test aimed to evaluate the loss of paw sensibility caused by disease development. Needle test clearly shows that CBD-treated EAE mice keep a higher and significant sensitivity of the lower limbs when compared with untreated CBD mice that following the disease onset (14th from disease induction, first test measurement) display a total loss of response to any needle solicitation. A p value < 0.05 was considered significant. *vs*. CBD IP; #*vs*. NAIVE; °*vs*. VEHICLE CTR.

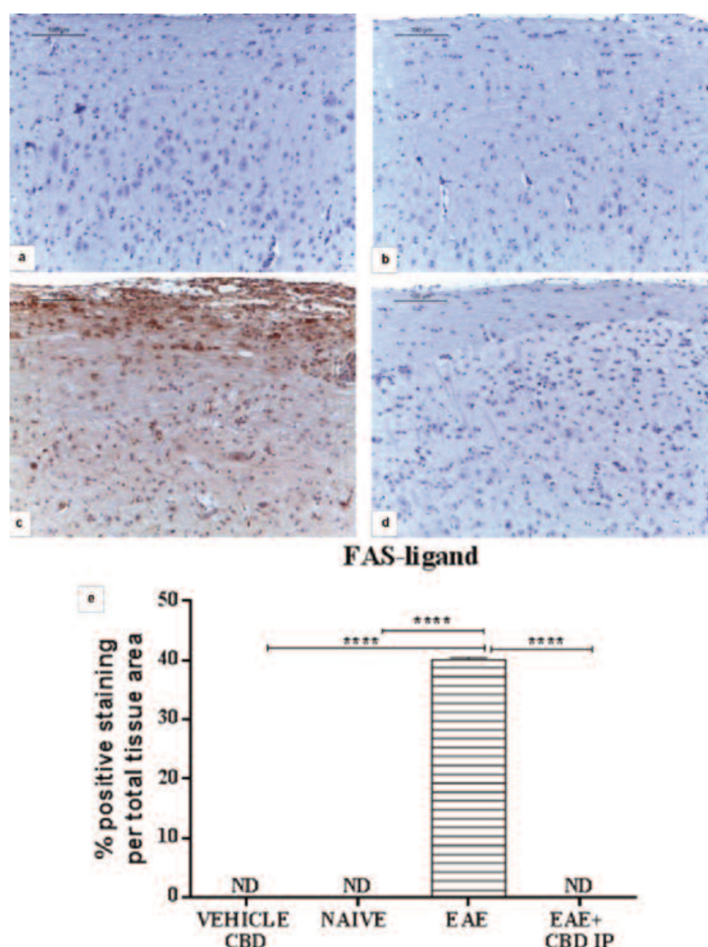

Figure 3. CBD-treatment interferes with Fas pathway. IHC evaluation of apoptotic pathways modulated by CBD- treatment display that untreated EAE mice result highly positive to Fas-Ligand staining ***(c)***. Conversely, sections sampled by CBD-treated EAE mice ***(d)***, vehicle CBD ***(a)*** as well as naive ***(b)*** groups result completely negative for this marker. A densitometric analysis of the result is provided in e. A p value * < 0.05 was considered significant. (20x).

sent in CBD-treated EAE mice (Figure 8 d) as well as in vehicle-control (Figure 8 a) and naïve groups (Figure 8 b).

Discussion

MS is a complex, chronic, progressive neurodegenerative disease with a wide range of outcome and symptoms classified as primary, secondary, or tertiary[27]. In this regard, *CS* is experimentally demonstrated to limit neurodegeneration that leads to progressive disability[28] as well as cannabinoids are commercially available and currently introduced in the clinical practice in different percentage and formulations with THC (i.e. Sativex® as well as Bedrocan®, Bedrobinol®, Bediol®) to treat MS symptoms. For example, experimental studies reveal that activation of cannabinoid CB2 receptors by JWH-133, a synthetic cannabinoid, reduces hyperalgesia in EAE-affected mice[29]. These effects can be the result of the specific and selective CB1/CB2 receptors activation by cannabinoids (natural and synthetic) or non-classic receptor ligands[30] (one receptor class, many agonists) as well as the effect of cannabinoid binding to the receptors other than CB1/CB2 receptors (one agonist class/many receptor classes). The present work was designed to define a new profile of CBD, the main non psychotropic compound present in *CS,* whose intrinsic potential as a molecule with a therapeutic effect has not yet completely understood. In fact, it is

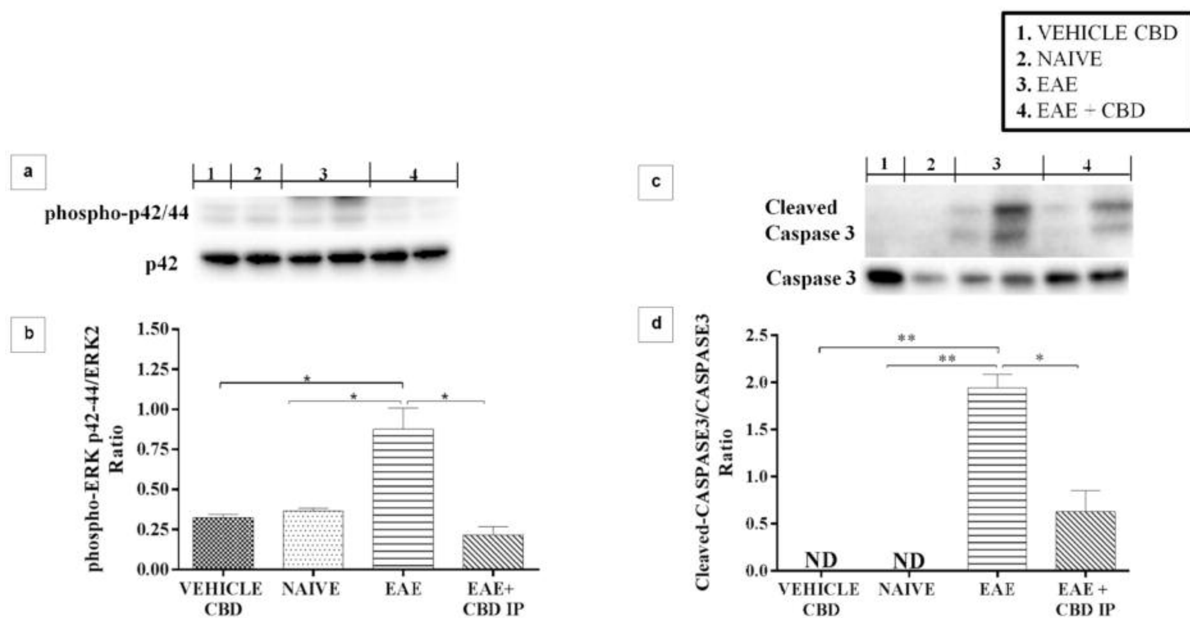

Figure 4. CBD-treatment modulates phospho-ERK p42/44 and cleaved-caspase 3 expression. Western blot analysis to evaluate phospho-ERK p42/44 level expression (a, densitometric analysis b) displays that untreated EAE-affected mice have the highest protein levels. Conversely, CBD-treated EAE mice, vehicle CBD as well as naive groups show significant lower phospho-ERK p42/44 expression levels. ERK p42 was used to normalize the signal. A *p* value *< 0.05 was considered significant. Parallel, cleaved-caspase 3 detection (c, densitometric analysis d) reveals that tissue homogenates sampled by untreated EAE-affected mice contain high levels of this marker. Conversely, CBD-treated EAE mice, vehicle CBD as well as naive groups show significant cleaved-caspase 3 lower expression levels. Caspase 3 was used to normalize the signal. A *p* value *< 0.05 was considered significant.

well known that CBD exerts anti-inflammatory properties, reducing the generation of pro-inflammatory cytokines (such as TNF-α and IL-1β) as well as reacting oxygen species[19], nevertheless, to date little or nothing has been deepened about its anti-apoptotic power. In particular, our study was aimed to highlight these underestimated properties of CBD. In this perspective, our present data are encouraging.

Verified the high presence of apobodies following EAE degeneration, we have further investigated the processes leading to CBD neuronal protection. The mechanism by which CBD counteracts neuronal death is not fully known. However, by evaluating the main mediators of both intrinsic and extrinsic apoptotic pathway, we can summarize with some confidence that CBD can interfere, modulating them, processes underlying the programmed cell death mechanism. Fas cell surface receptor, belonging to TNF receptor superfamily, has been widely recognized to have a role in transducing apoptotic signals in multiple sclerosis[31]. An indirect measure of Fas activation has been here provided by assessment of Fas-Ligand detection. This step was considered by us as the first evidence about the possible anti-apoptotic power of CBD that has proved able to avoid the releasing of this key-mediator. Moreover, since the Fas pathway is closely linked to the intrinsic pathway of apoptosis, it was interesting to investigate the Bcl-2 family proteins, regulating the integrity of the outer mitochondrial membrane permeability[32]. Mitochondria are the powerhouse of all cells and their integrity is the core for the regulation of cell survival[33], directing cell balance towards survival or death signals. Moreover, Rimmerman et al[34] showed that CBD directly modulates the outer mitochondrial membrane channel as an agonist of voltage-dependent anion channel 1 (VDAC1), involved in functioning of cell energy, metabolic homeostasis and apoptosis.

Clinical studies[35] showed that RRMS patients display aberrant expression analysis of apoptosis-related genes. Nevertheless, our achieved re-

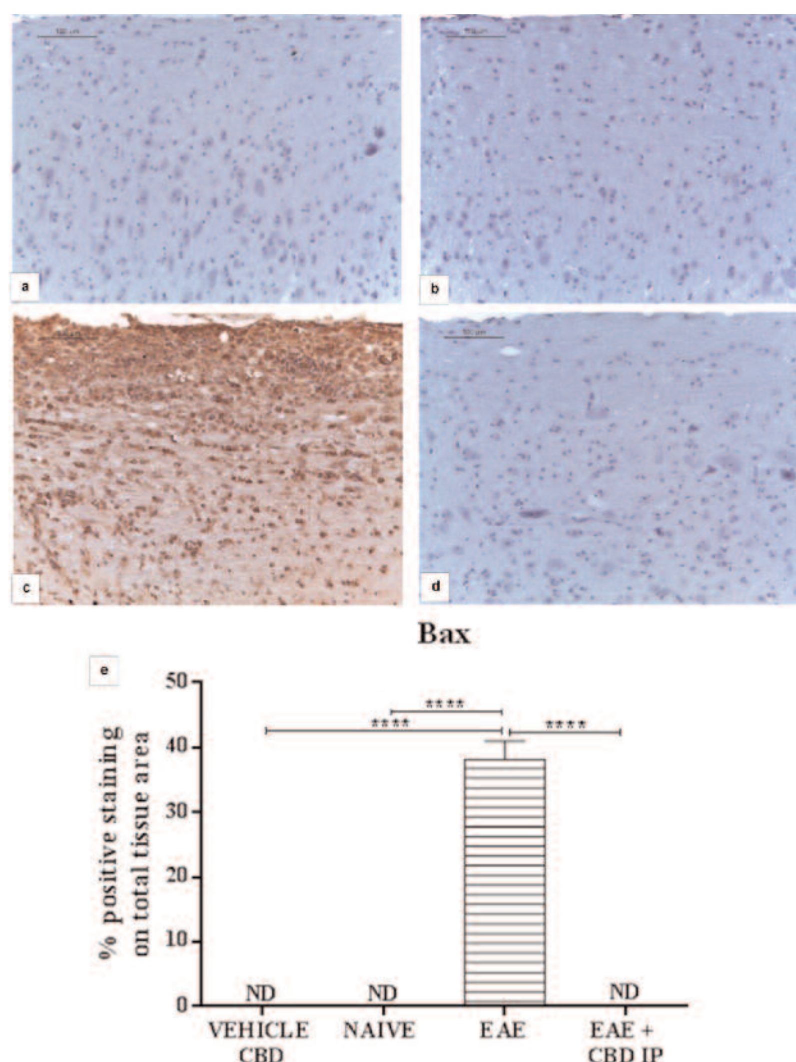

Figure 5. CBD-treatment counteracts alteration of mitochondrial permeability avoiding Bax overexpression. IHC staining for Bax localization displays a high immunopositivity in sections sampled by untreated EAE-affected mice ***(c)***, when compare with CBD-treated EAE mice ***(d)***, vehicle CBD ***(a)*** as well as naive groups ***(b)***. A densitometric analysis of the result is provided in e. A *p* value $^{*}< 0.05$ was considered significant. (20x).

sults indicate that mitochondrial maintenance was preserved by CBD treated EAE-mice with a greater tendency to release anti-apoptotic (Bcl-2) than pro-apoptotic factors (Bax). Parallel, looking at the extrinsic pathway of apoptosis, we received a further confirm about the preservation of cell survival mediated by CBD administration. In this, a central role is covered by the lacking of cleaved-caspase 3 activation, a classic hallmark of apoptosis-induction of particular importance for oligodendrocyte death in multiple sclerosis[36].

This data has been an object of study for other authors in a paper where they did not attribute the caspase 3 cleavage to the involvement of CB1, CB2, TRPV1 or PPARγ receptors, but rather to and activity independent by classical and alternative cannabinoid receptors[37]. CBD has demonstrated to avoid upstream triggering of MAPK pathway, serine/threonine protein kinases, which play pivotal roles regulating many cell functions in different cell types[38]. Finally, we investigated the possible role of p53, an important transcription factor of genes such as p21, strongly involved in processes both of cell proliferation and death[39]. Corroborating p53 role promoting p21 transactivation, EAE-affected mice display

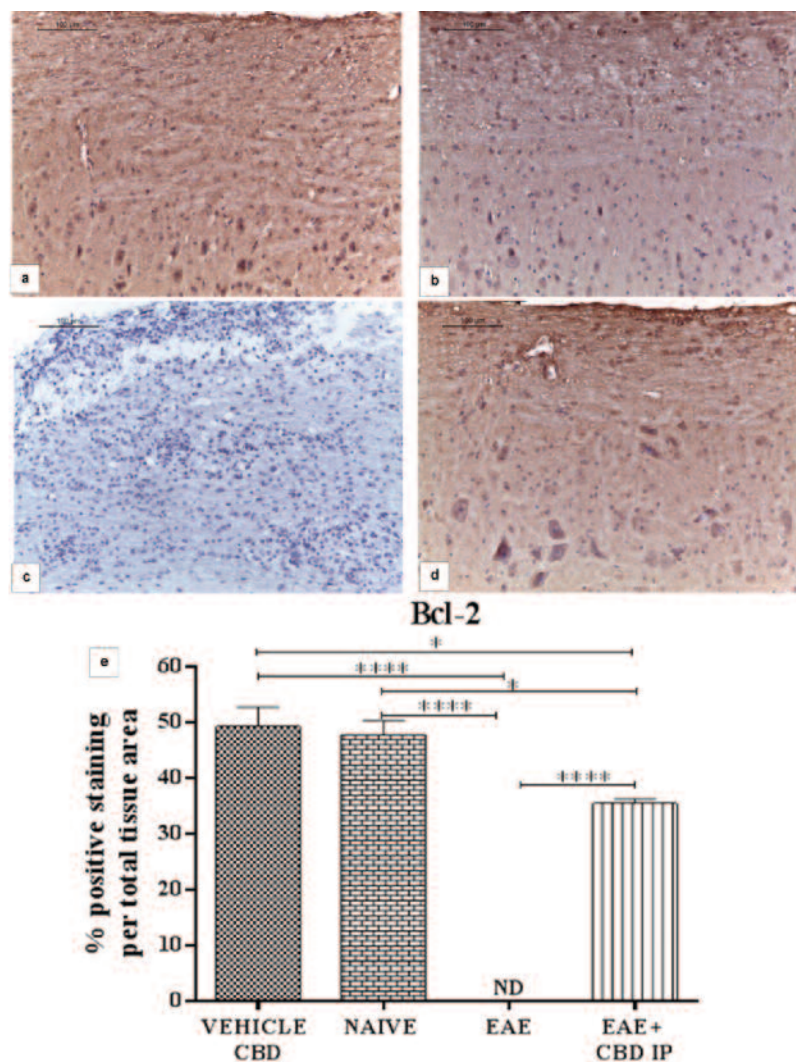

Figure 6. CBD-treatment counteracts alteration of mitochondrial permeability avoiding Bcl-2 unbalance. IHC localization for Bcl-2 displays a negative staining in sections sampled by untreated EAE-affected mice ***(c)***. Conversely, CBD-treated EAE mice ***(d)***, vehicle CBD ***(a)*** as well as naive groups ***(b)*** result positive for this marker. A densitometric analysis of the result is provided in e. A *p* value $^{*}< 0.05$ was considered significant. (20x).

high expression levels of these markers that, conversely, result downregulated following CBD treatment.

Conclusions

Summarizing the CBD activity on the apoptotic pathway, we strongly hope to have provided a, short but significant roundup of evidences that are useful to better characterize the efficacy as well as the molecular pathways modulated by the molecule. Currently, there is an extended literature about the overall role of cannabinoids in the treatment of neurodegenerative diseases[19,40]. In our view, considering its evident therapeutic benefits, the barriers, largely mental, about the introduction of *CS*, and especially of CBD formulation, in clinical practice, should be torn down.

Of course, further studies should be performed to isolate and characterize the non-psychotropic component of the plant, among all mostly the CBD, that, as we showed in the present work, alone, possesses very interesting pharmacological properties into counteracting the cascade of mediators leading to neuronal cell death in MS.

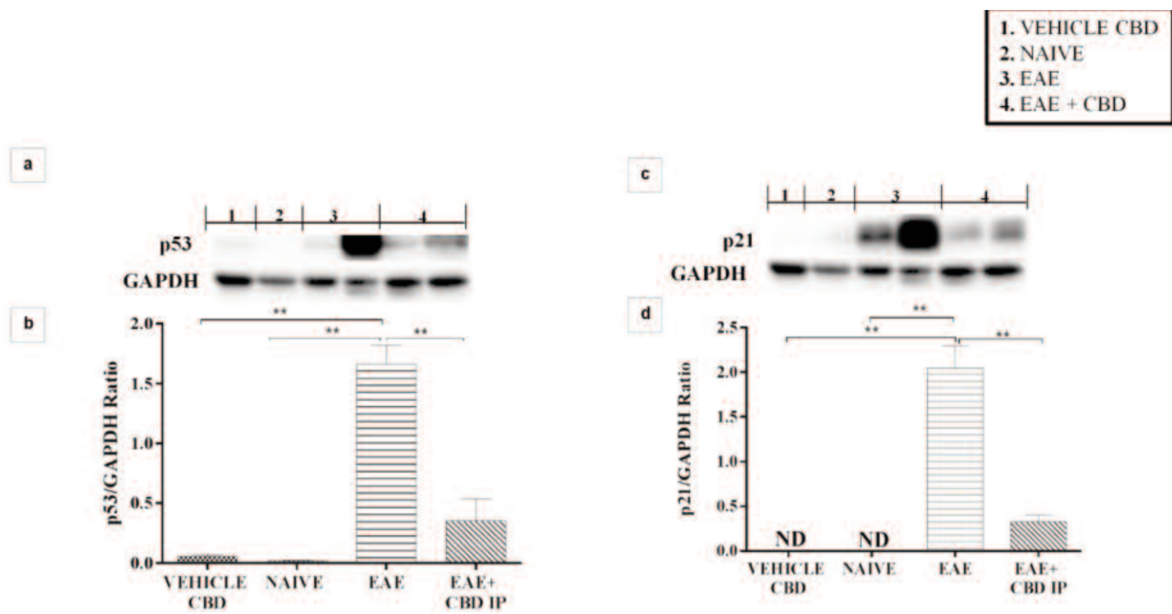

Figure 7. CBD-treatment inhibits p53/p21 activation. Western blot analysis to evaluate p53 level expression (a, densitometric analysis b) displays that untreated EAE-affected mice have the highest protein levels. Conversely, CBD-treated EAE mice, vehicle CBD as well as naive groups show significant lower p53 expression levels. A *p* value *< 0.05 was considered significant. Parallel, p21 detection (c, densitometric analysis d) reveals that tissue homogenates sampled by untreated EAE-affected mice contain high levels of this marker. Conversely, CBD-treated EAE mice show significant lower expression levels. Vehicle CBD as well as naive groups have totally negative p21 expression levels. A *p* value *< 0.05 was considered significant.

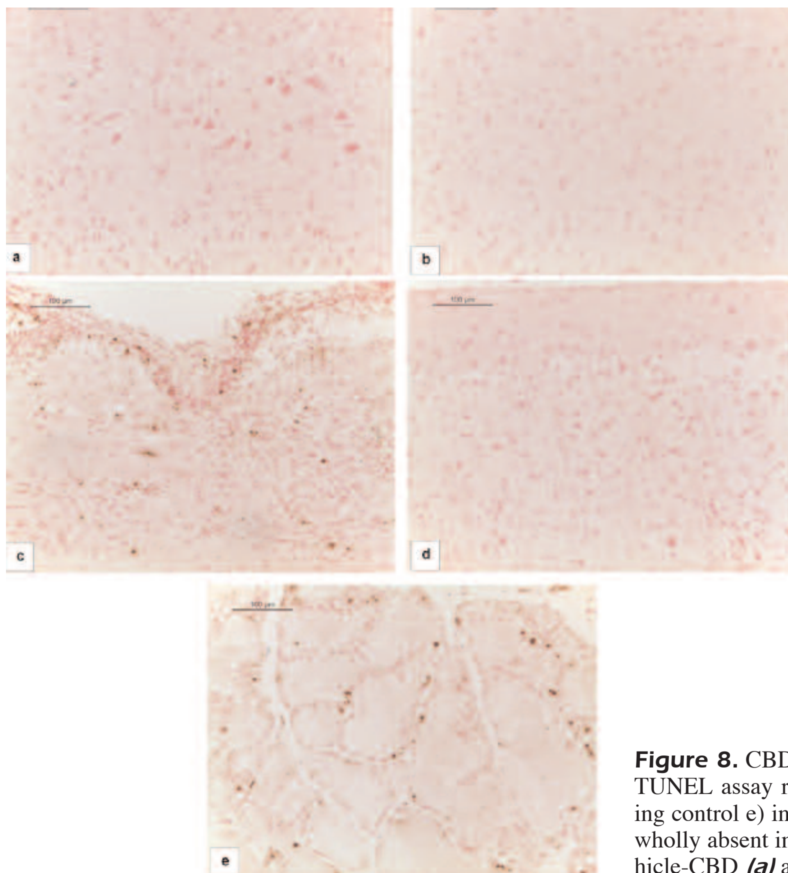

Figure 8. CBD avoids the formation of neuronal apobodies. TUNEL assay reveals presence of apobodies (positive staining control e) in untreated EAE mice ***(c)*** that, conversely, are wholly absent in CBD-treated EAE mice ***(d)*** as well as in vehicle-CBD ***(a)*** and naive groups ***(b)***. (20x).

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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