



## Beneficial effect of the non-psychotropic plant cannabinoid cannabigerol on experimental inflammatory bowel disease

Francesca Borrelli<sup>a,1,\*</sup>, Ines Fasolino<sup>a</sup>, Barbara Romano<sup>a</sup>, Raffaele Capasso<sup>a,1</sup>, Francesco Maiello<sup>b</sup>, Diana Coppola<sup>b</sup>, Pierangelo Orlando<sup>c,1</sup>, Giovanni Battista<sup>b</sup>, Ester Pagano<sup>a</sup>, Vincenzo Di Marzo<sup>d,1</sup>, Angelo A. Izzo<sup>a,1,\*</sup>

<sup>a</sup> Department of Pharmacy, University of Naples Federico II, via D Montesano 49, 80131 Naples, Italy

<sup>b</sup> Ospedale dei Pellegrini, Department of Diagnostic Services (Anatomy and Pathologic Histology Service), ASL 1, Naples, Italy

<sup>c</sup> Institute of Protein Biochemistry, National Research Council, Naples, Italy

<sup>d</sup> Institute of Biomolecular Chemistry, National Research Council, Pozzuoli (NA), Italy

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### ABSTRACT

Inflammatory bowel disease (IBD) is an incurable disease which affects millions of people in industrialized countries. Anecdotal and scientific evidence suggests that Cannabis use may have a positive impact in IBD patients. Here, we investigated the effect of cannabigerol (CBG), a non-psychotropic Cannabis-derived cannabinoid, in a murine model of colitis. Colitis was induced in mice by intracolonic administration of dinitrobenzene sulphonic acid (DNBS). Inflammation was assessed by evaluating inflammatory markers/parameters (colon weight/colon length *ratio* and myeloperoxidase activity), by histological analysis and immunohistochemistry; interleukin-1 $\beta$ , interleukin-10 and interferon- $\gamma$  levels by ELISA, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) by western blot and RT-PCR; CuZn-superoxide dismutase (SOD) activity by a colorimetric assay. Murine macrophages and intestinal epithelial cells were used to evaluate the effect of CBG on nitric oxide production and oxidative stress, respectively. CBG reduced colon weight/colon length *ratio*, myeloperoxidase activity, and iNOS expression, increased SOD activity and normalized interleukin-1 $\beta$ , interleukin-10 and interferon- $\gamma$  changes associated to DNBS administration. In macrophages, CBG reduced nitric oxide production and iNOS protein (but not mRNA) expression. Rimobant (a CB<sub>1</sub> receptor antagonist) did not change the effect of CBG on nitric oxide production, while SR144528 (a CB<sub>2</sub> receptor antagonist) further increased the inhibitory effect of CBG on nitric oxide production. In conclusion, CBG attenuated murine colitis, reduced nitric oxide production in macrophages (effect being modulated by the CB<sub>2</sub> receptor) and reduced ROS formation in intestinal epithelial cells. CBG could be considered for clinical experimentation in IBD patients.

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**Abbreviations:** CB, cannabinoid; CBD, cannabidiol; CBG, cannabigerol; CD, Crohn's disease; COX-2, cyclooxygenase-2; DNBS, 2,4,6-dinitrobenzene sulphonic acid; H2DCF-DA, 2',7'-dichlorofluorescein-diacetate; IBD, Inflammatory bowel disease; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; UC, ulcerative colitis.

\* Corresponding authors. Tel.: +39 081 678665; fax: +39 081 678403.

E-mail addresses: [franborr@unina.it](mailto:franborr@unina.it) (F. Borrelli), [inesfasolino@gmail.com](mailto:inesfasolino@gmail.com)

(I. Fasolino), [barbara.romano@unina.it](mailto:barbara.romano@unina.it) (B. Romano), [rafcapas@unina.it](mailto:rafcapas@unina.it) (R. Capasso), [framai@iol.it](mailto:framai@iol.it) (F. Maiello), [diana.coppola@virgilio.it](mailto:diana.coppola@virgilio.it) (D. Coppola), [p.orlando@ibp.cnr.it](mailto:p.orlando@ibp.cnr.it) (P. Orlando), [giovanni.battista.90@hotmail.it](mailto:giovanni.battista.90@hotmail.it) (G. Battista), [ester.pagano@unina.it](mailto:ester.pagano@unina.it) (E. Pagano), [vdimarzo@icmib.na.cnr.it](mailto:vdimarzo@icmib.na.cnr.it) (V. Di Marzo), [aizzo@unina.it](mailto:aizzo@unina.it) (A.A. Izzo).

<sup>1</sup> Endocannabinoid Research Group.

### 1. Introduction

Inflammatory bowel disease (IBD) comprises the chronic relapsing inflammatory disorders Crohn's disease (CD) and ulcerative colitis (UC) [1]. It is characterized by abdominal pain, diarrhoea, bleeding and malabsorption [2]. Its incidence is increasing worldwide, and the disease remains incurable [3,4]. The incidence and prevalence of IBD has increased in the past 50 years, up to 8–14/100,000 and 120–200/100,000 cases, respectively, for UC and 6–15/100,000 and 50–200/100,000 cases, respectively, for CD [3]. Conventional therapies for IBD include aminosaliclates, corticosteroids, thiopurines, methotrexate, and anti-tumour necrosis factor agents [5]. Although these drugs may

be effective, their long-term use can induce severe side effects that have detrimental impact on life quality of patients [6]. Hence, it is required to develop new approaches with fewer side effects for the treatment of IBD.

Anecdotal reports suggest that IBD patients experience relief by smoking marijuana [7,8]. Recent retrospective observational studies, by showing that Cannabis use is common in patients with IBD for symptom relief, have confirmed such reports [9,10]. Also, a pilot prospective study found that treatment with inhaled Cannabis improved quality of life in patients with long-standing CD and UC [11]. In Israel, inhaled Cannabis has been legally registered for palliative treatment of both CD and UC.  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC), the main Cannabis psychotropic ingredient which activates cannabinoid (CB<sub>1</sub> and CB<sub>2</sub>) receptors, and cannabidiol (CBD), the best studied among the so-called non-psychotropic cannabinoids, have been previously shown to ameliorate experimental colitis in rodents [12–14].

Cannabigerol (CBG) is a non-psychotropic cannabinoid – obtained in 1964 by Gaoni and Mechoulam when they separated a hexane extract of hashish on Florisil [15] – which does not induce  $\Delta^9$ -THC-like effects *in vivo* [16]. Relatively few studies have sought to investigate the pharmacological actions of this compound [17,18]. CBG was shown to exert antiproliferative [19], antibacterial [20] and anti-glaucoma [21] actions and to antagonize the anti-nausea effect of CBD [22]. Potential targets of CBG actions include transient receptor potential (TRP) channels [23], cyclooxygenase (COX-1 and COX-2) enzymes [24], as well as cannabinoid, 5-HT<sub>1A</sub> and  $\alpha_2$  adrenergic receptors [25].

In the present study, we investigated the effect of CBG in an experimental model of murine colitis. To further characterize CBG action, we evaluated the effect of this phytocannabinoid in peritoneal macrophages and in intestinal epithelial cells.

## 2. Materials and methods

### 2.1. Drugs and reagents

CBG [purity by high-performance liquid chromatography (HPLC), 99.0%] was kindly supplied by GW Pharmaceuticals (Porton Down, Wiltshire, UK). Rimonabant (5-(p-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-piperidinopyrazole-3-carboxamide hydrochloride) and SR144528 (N-[1S-endo-1,3,3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methyl benzyl)-pyrazole-3-carboxamide) were a kind gift from Drs Madaleine Mosse and Francis Barth (SANOFI Recherche, Montpellier, France). Dinitrobenzene sulphonic acid (DNBS), neutral red solution, myeloperoxidase from human leucocytes, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), FeCl<sub>2</sub>·4H<sub>2</sub>O, 2',7'-dichlorofluorescein-diacetate (H<sub>2</sub>DCF-DA), lipopolysaccharide (LPS, from *Escherichia coli* serotype O111:B4), thioglycollate medium, cadmium, 2,3-diaminonaphtalene (DAN), 2,6-di-tert-butyl-4-methylphenol (BHT), fluorescein isothiocyanate (FITC)-conjugated dextran (molecular mass 3–5 kDa), streptavidin, 3,3'-diaminobenzidine tetrahydrochloride, Mayer's haematoxylin solution, N,N,N',N'-tetramethylbenzidine were purchased from Sigma Aldrich S.r.l. (Milan, Italy). All reagents for cell culture and western blot analysis were obtained from Sigma Aldrich S.r.l. (Milan, Italy), Amersham Biosciences Inc. (UK), Bio-Rad Laboratories (USA) and Microtech S.r.l. (Naples, Italy). All chemicals and reagents employed in this study were of analytical grade.

CBG was dissolved in ethanol/Tween20/saline (1:1:8; for *in vivo* experiments) or ethanol (for *in vitro* experiments). Rimonabant and SR144528 were dissolved in dimethyl sulphoxide (DMSO). DNBS was dissolved in 50% ethanol (0.15 ml/mouse). The CBG vehicles (60  $\mu$ l/mouse *in vivo* or 0.01% ethanol *in vitro*) had no significant effects on the responses under study.

### 2.2. Animals

Male ICR mice, weighing 30–35 g, were supplied by Harlan Italy (Corezzana, Milan, Italy). All animals, used after 1 week of acclimation (temperature, 23  $\pm$  2 °C; humidity, 60%), had free access to water and food. Mice were fed *ad libitum* with standard food, except for the 24-h period immediately preceding the administration of DNBS. All experiments complied with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

### 2.3. Induction of experimental colitis and pharmacological treatment

Colitis was induced by the intracolonic administration of DNBS [12]. Briefly, mice were anesthetized with inhaled 5% isoflurane (Centro Agrovete Campania, Scafati, SA, Italy) and subsequently DNBS (150 mg/kg) was inserted into the colon using a polyethylene catheter (1 mm in diameter) *via* the rectum (4.5 cm from the anus). Three days after DNBS administration, all animals were euthanized by asphyxiation with CO<sub>2</sub>, the mice abdomen was opened by a midline incision and the colon removed, isolated from surrounding tissues, opened along the antimesenteric border, rinsed, weighed and length measured [in order to determine the colon weight/colon length ratio (mg/cm) used as an indirect marker of inflammation]. All measurements were performed by operators which were unaware of the particular treatment (blinded evaluation). For biochemistry analysis, tissues were kept at –80 °C until use, while for histological examination tissues were fixed in 10% formaldehyde.

The dose of DNBS was selected on the basis of preliminary experiments showing a remarkable colonic damage associated to high reproducibility and low mortality for the 150 mg/kg dose. The time point of damage evaluation (*i.e.*, 3 days after DNBS administration) was chosen because maximal DNBS-induced inflammation has been reported in mice after 3 days [26]. Furthermore, previous studies have shown that 3 days after intracolonic DNBS administration in mice, the inflammatory response may be modulated by administration of cannabinoid drugs such as direct cannabinoid receptor agonists or antagonists [12,26].

In the preventive protocol CBG (1–30 mg/kg) was given once a day for six consecutive days starting 3 days before DNBS administration, while in the curative protocol CBG (1–30 mg/kg) was injected for two consecutive days starting 24-h after DNBS administration.

### 2.4. Histology and immunohistochemistry

Histological and immunochemistry evaluations, performed 3 days after DNBS administration, were assessed on a segment of 1 cm of colon located 4 cm above the anal canal by blinded examiners. After fixation for 24 h in saline 10% formaldehyde, samples were dehydrated in graded ethanol and embedded in paraffin. Thereafter, 5- $\mu$ m sections were deparaffinized with xylene, stained with haematoxylin–eosin, and observed in a DM 4000 B Leica microscope (Leica Microsystems, Milan, Italy). For microscopic scoring we used a modified version of the scoring system reported by D'Argenio and colleagues [27]. Briefly, colon was scored considering (1) the submucosal infiltration (0, none; 1, mild; 2–3, moderate; 4–5 severe), (2) the crypt abscesses (0, none; 1–2 rare; 3–5, diffuse) and (3) the mucosal erosion (0, absent; 1, focus; 2–3, extended until the middle of the visible surface; 4–5, extended until the entire visible surface).

For immunohistochemical detection of Ki-67, paraffin-embedded slides were immersed in a Tris/ethylenediaminetetraacetic acid buffer (pH 9.0), heated in a decloaking chamber at 125 °C for

3 min and cooled at room temperature for 20 min. Sections were incubated for 10 min with 3% hydrogen peroxide, washed with Tris-buffered saline Tween-20 (pH 7.6) and incubated with rabbit monoclonal antibody to Ki-67 (1:100, Ventana Medical systems, Tucson, Arizona). ( ) for 30 min at room temperature. The slides were washed three times with Tris-buffered saline Tween-20 and incubated with secondary antibody for 30 min. After, the slides were reacted with streptavidin for 20 min and 3,3'-diaminobenzidine tetrahydrochloride for 5 min. Finally, the slides were counterstained with Mayer's haematoxylin. The intensity and localization of immunoreactivities against the primary antibody used were examined on all sections with a microscope (Leica Microsystems, Milan, Italy). To ensure specificity of the immunohistochemical procedure, some tissues were processed without primary antibody to Ki-67.

### 2.5. Intestinal permeability assay

Intestinal permeability was examined using a fluorescein isothiocyanate (FITC)-labelled-dextran method, as described previously [28]. Briefly, 2 days after DNBS administration, mice were gavaged with 600 mg/kg body weight of fluorescein isothiocyanate (FITC)-conjugated dextran (molecular mass 3–5 kDa). One day later, blood was collected by cardiac puncture, and the serum was immediately analysed for FITC-derived fluorescence using a fluorescent microplate reader with an excitation–emission wavelengths of 485–520 nm (LS55 Luminescence Spectrometer, PerkinElmer Instruments). Preliminary experiments showed that FITC-dextran was stable after 24 h from its preparation. Serial-diluted FITC-dextran was used to generate a standard curve. Intestinal permeability was expressed as FITC nM found in the serum.

### 2.6. Myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined as previously described [29]. Full-thickness colons were homogenized in an appropriate lysis buffer (0.5% hexadecyltrimethylammonium bromide in MOPS 10 mM) in ratio 50 mg tissue/1 ml MOPS. The samples were then centrifuged for 20 minutes at 15,000 × g at 4 °C. An aliquot of the supernatant was then incubated with NaPP (sodium phosphate buffer pH 5.5) e tetra-methylbenzidine 16 mM. After 5 minutes, H<sub>2</sub>O<sub>2</sub> (9.8 M) in NaPP was added and the reaction stopped adding acetic acid. The rate of exchange in absorbance was measured by a spectrophotometer at 650 nm. Different dilutions of human MPO enzyme of known concentration were used to obtain a standard curve. MPO activity was expressed as unit (U)/ml.

### 2.7. Superoxide dismutase (SOD) assay

A modified version of the Kuthan and colleagues method was used to detect SOD activity [30]. Full-thickness colons from control and DNBS-treated mice (treated or not with CBG 30 mg/kg) were homogenized in PBS 1X. Homogenates were centrifuged at 25,000 × g for 15 min at 4 °C. Extraction of Cu-Zn SOD was obtained treating the cytosolic lysates with ethanol (1:1) and chloroform (1:0.6) at 25 °C for 15 min. After centrifugation (15,000 × g, 15 min, 4 °C), 125 µl of the supernatant was incubated (for 20 min) with 613 µl of a reaction mixture containing 0.12 mM xanthine, 48 mM Na<sub>2</sub>CO<sub>3</sub>, 0.094 mM EDTA, 60 mg/l BSA, 0.03 mM nitro blue tetrazolium (NBT), 0.006 U/ml xanthine oxidase (all substances were purchased from Sigma Aldrich S.r.l., Milan, Italy). Finally, CuCl<sub>2</sub> (0.8 mM) was added to stop the reaction. Absorbance readings at 560 nm were recorded using a Beckman DU62 spectrophotometer. Superoxide radical scavenging capacity of

CBG (30 mg/kg) at the end of 30 min were expressed as ng SOD/mg tissues contained in the lysates.

### 2.8. Immunoblotting (COX-2 and iNOS expression)

Full-thickness colons from control and DNBS-treated mice (treated or not with CBG 30 mg/kg) were homogenized in lysis buffer (1:2, w/v) containing 0.5 M β-glycerophosphate, 20 mM MgCl<sub>2</sub>, 10 mM ethylene glycol tetraacetic acid, and supplemented with 100 mM dithiothreitol and protease/phosphatase inhibitors (100 mM dimethylsulphonyl fluoride, 2 mg/ml aprotinin, 2 mM leupeptin, and 10 mM Na<sub>3</sub>VO<sub>4</sub>). Homogenates were centrifuged at 600 × g for 5 min at 4 °C; the supernatants were collected and centrifuged at 16,200 × g for 10 min at 4 °C. Proteins (50 µg, determined with the Bradford method) were subjected to electrophoresis on a sodium dodecyl sulphate 10% polyacrylamide gel and electrophoretically transferred to a nitrocellulose transfer membrane (Protran, Schleicher & Schuell, Germany). After blocking in 5% non-fat dry milk buffer, membranes were incubated with mouse anti-COX-2 (BD Bioscience, Belgium) and anti-iNOS (Cayman Chemical, USA) (1:1000 dilution for both antibodies), and subsequently with mouse or rabbit anti-peroxidase-conjugated goat IgG (Jackson ImmunoResearch from LiStarFish, Italy). The signal was visualized by enhanced chemiluminescence using ImageQuant 400 equipped with software ImageQuant Capture (GE Healthcare, Milan, Italy) and analysed using Quantity One Software version 4.6.3. The membranes were probed with an anti β-actin antibody to normalize the results, which were expressed as a ratio of densitometric analysis of COX-2/β-actin and iNOS/β-actin bands.

### 2.9. Enzyme-linked immunosorbent assay

Interleukin-1β (IL-1β), interleukin-10 (IL-10) and interferon-γ (INF-γ) levels in homogenate obtained from full-thickness colonic tissues were quantified using commercial ELISA kits (Tema Ricerca Srl, Bologna) according to the manufacturer's instructions.

### 2.10. Cell culture

Peritoneal macrophages and a conditionally immortalized colonic epithelial cell line (from a Ptk6 null mouse) were used. Peritoneal macrophages were obtained from mice as previously described by Aviello and colleagues [31]. Briefly, to evoke the production of peritoneal exudates rich in macrophages, mice were injected intraperitoneally (i.p.) with 1 ml of 10% sterile thioglycollate medium. After 4 days, mice were killed and the peritoneal macrophages were collected and seeded in appropriate plates for performing *in vitro* experiments. Peritoneal macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum. Ptk6 null colonic epithelial cells, supplied by Dr R.H. Whitehead at the Ludwig Institute for Cancer Research (Melbourne Branch) [32], were cultured in RPMI-1640 medium (GIBCO) containing 10% foetal bovine serum (FBS), 80 U/ml penicillin and 80 µg/ml streptomycin. Cell viability was evaluated by trypan blue staining. The inflammatory response in peritoneal macrophages was induced by LPS from *Escherichia coli* serotype O111:B4 (1 µg/ml). The acute inflammatory response in macrophages required an LPS incubation time of 18 h. The oxidative stress in Ptk6 null colonic epithelial cells was induced by Fenton's reagent (H<sub>2</sub>O<sub>2</sub>/Fe<sup>2+</sup> 2 mM, time of incubation 3 h).

### 2.11. Cytotoxicity assay

The effect of CBG on cell (murine macrophages and Ptk6 null colonic epithelial cells) survival was measured using the neutral red assay [31]. After incubation with CBG (0.001–10 µM) for 24 h,

macrophages ( $1 \times 10^5$  cells per well seeded in a 96-well plate) or Ptk6 null colonic epithelial cells ( $3 \times 10^4$  cells per well seeded in a 96-well plate) were incubated with neutral red dye solution (50  $\mu\text{g/ml}$ ) for 3 h, and then lysed by adding 1% acetic acid. The absorbance was read at 532 nm (iMark™ Microplate Assorbance Reader, BioRad). Treatments were compared with a positive control, i.e., DMSO 20% (v/v).

### 2.12. Nitrites measurement

Nitrites, stable metabolites of NO, were measured in macrophages medium as previously described [31]. Mouse peritoneal macrophages ( $5 \times 10^5$  cells per well seeded in a 24-well plate) were incubated with CBG (0.001–1  $\mu\text{M}$ ) for 30 min and subsequently with LPS (1  $\mu\text{g/ml}$ ) for 18 h. After reduction of nitrates to nitrites by cadmium, cell supernatants were incubated with DAN (50  $\mu\text{g/ml}$ ) for 7 min. After stopping the reaction with 2.8 N NaOH, nitrite levels were measured using a fluorescent microplate reader (LS55 Luminescence Spectrometer, PerkinElmer Instruments, excitation–emission wavelengths of 365–450 nm). In a subsequent set of experiments, rimonabant (0.1  $\mu\text{M}$ , CB<sub>1</sub> receptor antagonist) and SR144528 (0.1  $\mu\text{M}$ , CB<sub>2</sub> receptor antagonist) were incubated 30 min before CBG (1  $\mu\text{M}$ ).

### 2.13. Quantitative (real-time) RT-PCR analysis (cannabinoid receptors and iNOS mRNA expression)

Peritoneal macrophages (treated or not with CBG 30 min before LPS) were collected in RNA later

(Invitrogen, Carlsbad, CA, USA) and homogenized in 1.0 mL of Trizol® (Invitrogen). Total RNA was extracted according to the manufacturer's recommendations and further purified and DNA digested by the Micro RNA purification system (Invitrogen). Total RNA eluted from spin cartridge was UV-quantified by a Bio-Photometer® (Eppendorf, Santa Clara, CA, USA), and purity of RNA samples was evaluated by the RNA-6000-Nano® microchip assay using a 2100 Bioanalyzer® equipped with a 2100 Expert Software® (Agilent, Santa Clara, CA, USA) following the manufacturer's instructions.

For all samples tested, the RNA integrity number was greater than 8 relative to a 0–10 scale. One microgram of total RNA, as evaluated by the 2100 Bioanalyzer, was reverse transcribed in cDNA by the SuperScript III SuperMix (Invitrogen).

The reaction mixture was incubated in a thermocycler iCycler-iQ5® (Bio-Rad, Hercules, CA, USA) for a 5 min at 60 °C step, followed by a rapid chilling for 2 min at 4 °C. The protocol was stopped at this step and the reverse transcriptase was added to the samples, except the negative controls (–RT). The incubation was resumed with two thermal steps: 10 min at 25 °C followed by 40 min at 50 °C. Finally, the reaction was terminated by heating at 95 °C for 10 min. Quantitative realtime PCR was performed by an iCycler-iQ5® in a 20 mL reaction mixture containing 1 X SYBR green supermix (Bio-Rad), 10 ng of cDNA (calculated on the basis of the retro-transcribed RNA) and 330 nM for each primer. Primer sequences and optimum annealing temperature (TaOpt) were designed by the AlleleID software (PremierBiosoft). The amplification profile consisted of an initial denaturation of 2 min at 94 °C and 40 cycles of 30 s at 94 °C, annealing for 30 s at TaOpt and elongation for 45 s at 68 °C. Fluorescence data were collected during the elongation step. A final melt-curve data analysis was also included in the thermal protocol. Assays were performed in quadruplicate (maximum Ct of replicate samples <0.5), and a standard curve from consecutive fivefold dilutions (100 to 0.16 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Relative normalized expression was evaluated as previously described [33].

### 2.14. Intracellular ROS measurement in Ptk6 null colonic epithelial cells

The generation of intracellular reactive oxygen species (ROS) was estimated using the fluorescence probe 2',7'-dichlorofluorescein-diacetate (H2DCF-DA) (12). For the experiments, cells were plated in 96-multiwell black plates (Corning, USA) at the density of  $3 \times 10^4$  cells per well. Ptk6 null colonic epithelial cells were incubated for 24 h at 37 °C with CBG (0.1–10  $\mu\text{M}$ ). After washing, cells were incubated for 1 h with 200  $\mu\text{l}$  of 100  $\mu\text{M}$  H2DCF-DA in HBSS containing 1% FBS. Finally, cells were rinsed and incubated with the Fenton's reagent ( $\text{H}_2\text{O}_2/\text{Fe}^{2+}$  2 mM) for 3 h at 37 °C. The DCF fluorescence intensity was detected using a fluorescent microplate reader (excitation 485 nm and emission 538 nm; Perkin-Elmer Instruments). The intracellular ROS levels were expressed as fluorescence intensity (picogreen).

### 2.15. Statistical analysis

Data are expressed as the mean  $\pm$  SEM of n experiments. To determine statistical significance, Student's t test was used for comparing a single treatment mean with a control mean, and a one-way analysis of variance followed by a Tukey–Kramer multiple comparisons test was used for the analysis of multiple treatment means. Values of p less than 0.05 were considered significant.

## 3. Results

### 3.1. Colon weight/colon length ratio

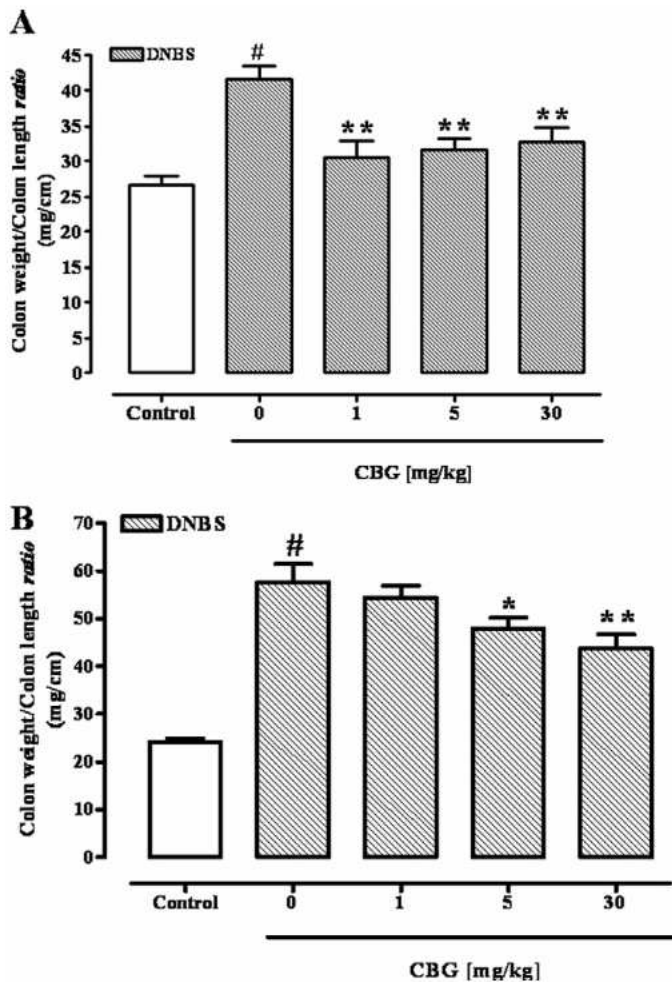
DNBS administration caused a significant increase in colon weight/colon length ratio, a simple and reliable marker of intestinal inflammation/damage [34] (Fig. 1A and 1B). CBG (1–30 mg/kg) given before (preventive protocol, Fig. 1A) or after (curative protocol, Fig. 1B) the inflammatory insult, significantly reduced the effects of DNBS on colon weight/colon length ratio. Significant protection was achieved starting from the 1 mg/kg (preventive protocol) and 5 mg/kg (curative protocol) doses. To confirm the anti-inflammatory curative activity of CBG we performed histological analysis, immunohistochemistry and measured intestinal permeability, MPO and SOD activities as detailed below. The selected CBG dose was 30 mg/kg

### 3.2. Histological analysis

Histological evaluations of colonic mucosa of healthy control animals showed normal appearance with intact epithelium (Fig. 2A). In the DNBS group, colons showed tissue injury which was mainly characterized by necrosis involving the full thickness of the mucosa, infiltrations of granulocytes into the mucosa/submucosa and oedema of submucosa (Fig. 2B). CBG (30 mg/kg, given after the inflammatory insult) reduced the signs of colon injury (microscopic score: control,  $0.50 \pm 0.22$ ; DNBS,  $9.0 \pm 0.45^{\#}$ ; CBG 30 mg/kg,  $6.0 \pm 0.45^*$ ,  $n = 4$ ,  $^{\#}p < 0.001$  vs control and  $^*p < 0.01$  vs DNBS alone). In the colon of CBG (30 mg/kg)-treated animals, the glands were regenerating, the oedema in submucosa was reduced, and the erosion area was superficial (Fig. 2 C).

### 3.3. Immunohistochemical detection of Ki-67

The curative action of CBG was further confirmed by immunohistochemistry. In normal colonic mucosa, the predominant area of cell proliferation is localized to the lower part of the crypts as revealed by Ki-67 distribution (Fig. 3A). In the colon from DNBS-treated mice, total necrosis with Ki-67 immunoreactivity on inflammatory cells and in a few remaining surface elements was



**Fig. 1.** Dinitrobenzene sulfonic acid (DNBS)-induced colitis in mice. Colon weight/length ratio (mg/cm) of colons from untreated and DNBS-treated mice in the presence or absence of cannabigerol (CBG). Tissues were analysed 3 days after vehicle or DNBS (150 mg/kg, intracolonic) administration. CBG (1–30 mg/kg) was administered (i.p.) once a day for six consecutive days starting 3 days before DNBS (preventive protocol, A) or for two consecutive days starting 24-h after the inflammatory insult (curative protocol, B). Bars are mean  $\pm$  SEM of 12–15 mice for each experimental group. # $p < 0.001$  vs control, \* $p < 0.05$  and \*\* $p < 0.01$  vs DNBS alone.

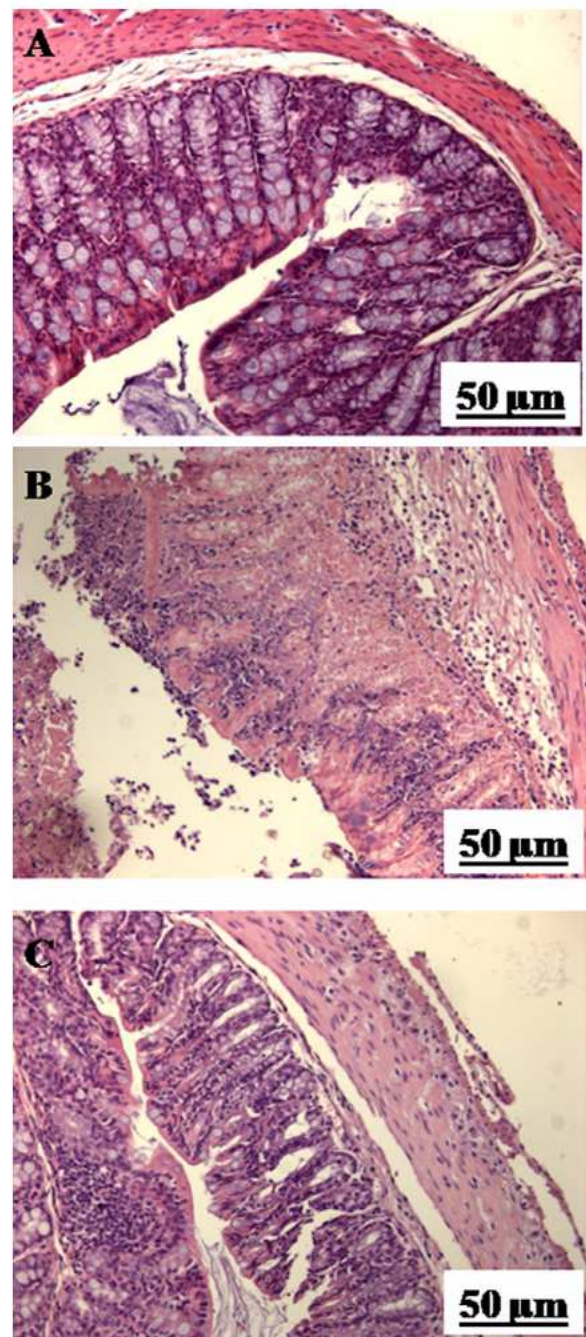
observed (Fig. 3B). CBG (30 mg/kg, given after the inflammatory insult) partially counteracted the effect of DNBS on cell proliferation, its mitotic activity being restricted to the lower half of the mucosa (i.e. the mature superficial cells were not in a proliferative state) (Fig. 3C).

### 3.4. Intestinal permeability

FITC- conjugated dextran presence was not detected in the serum of healthy control animals, which is suggestive of intestinal membrane integrity (Fig. 4A). The administration of DNBS induced a significant FITC-conjugated dextran appearance in the serum, indicating disruption of intestinal membrane integrity (Fig. 4A). CBG treatment (30 mg/kg) completely abolished DNBS-induced increased intestinal permeability (Fig. 4A).

### 3.5. Myeloperoxidase activity

MPO activity is considered to be an index of neutrophil infiltration (because MPO is predominantly found in these cells) and it is largely used to quantify intestinal inflammation [35]. DNBS-induced colitis was associated with significantly increased neutro-

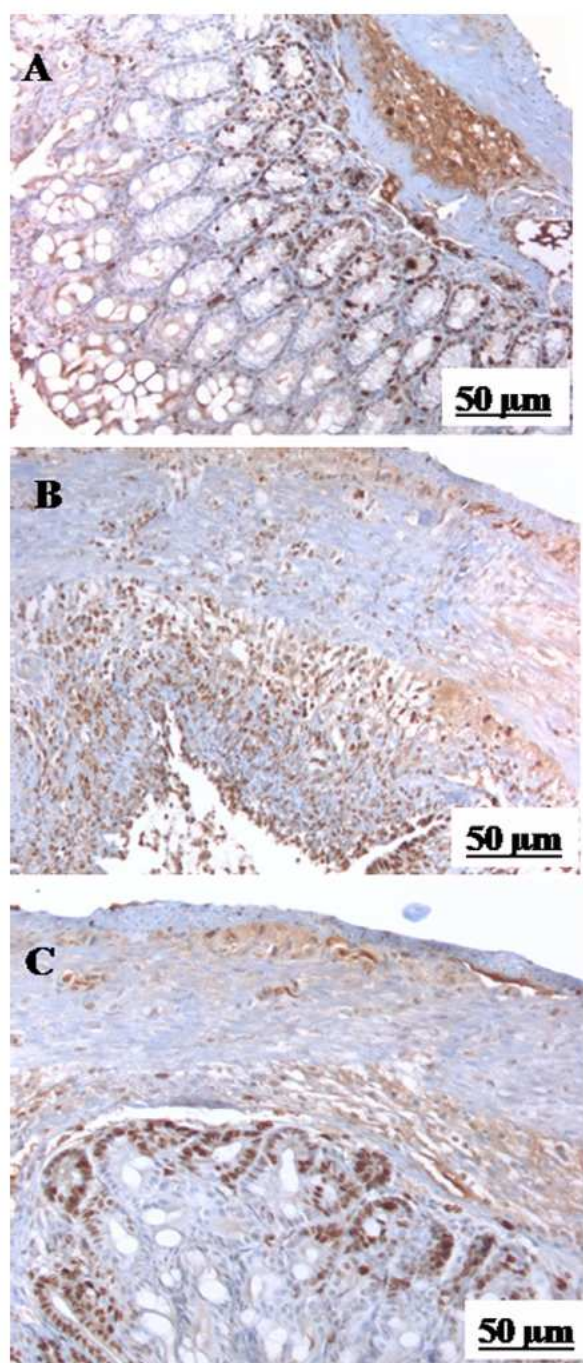


**Fig. 2.** Histological evaluations of inflamed and non-inflamed colons: effect of cannabigerol (CBG). No histological modification was observed in the mucosa and submucosa of control mice (A); mucosal injury induced by dinitrobenzene sulfonic acid administration (B); treatment with CBG reduced colon injury by stimulating regeneration of the glands (C). Histological analysis was performed 3 days after DNBS administration. CBG (30 mg/kg) was administered (i.p.) for two consecutive days starting 24-h after the inflammatory insult (curative protocol). Original magnification  $\times 200$ . The figure is representative of 4 experiments.

phil infiltration, as evaluated by MPO (Fig. 4B). CBG, given after the inflammatory insult at the dose of 30 mg/kg (curative protocol), counteracted DNBS-induced increase in MPO activity (Fig. 4B).

### 3.6. Superoxide dismutase activity

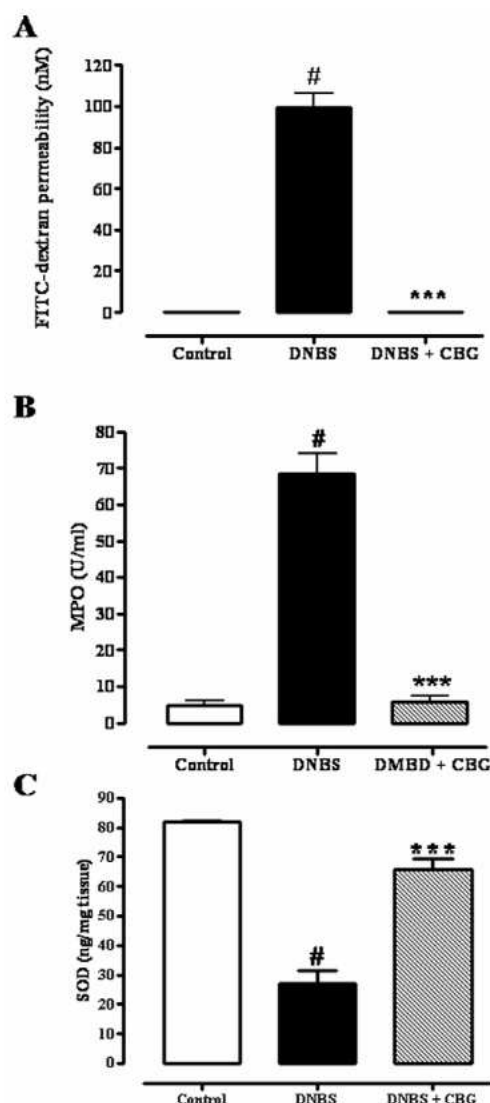
DNBS produced a significant decrease in SOD activity (Fig. 4C). CBG, at the dose of 30 mg/kg (curative protocol), counteracted DNBS-induced reduction in SOD activity (Fig. 4C).



**Fig. 3.** Different patterns of Ki-67 immunoreactivity in the colonic mucosa of control mice (A), DNBS-treated mice (B) and mice treated with DNBS plus cannabigerol (C). (A) Ki-67 immunopositive cells were localized to the lower part of the crypts. (B) Ki-67 immunopositive cells were observed on inflammatory cells. (C) Ki-67 immunopositive cells were observed only in the expanded basal zone. CBG (30 mg/kg) was administered (i.p.) for two consecutive days starting 24-h after the inflammatory insult (curative protocol). The figure is representative of 4 experiments.

### 3.7. Inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) expression

Densitometric analysis indicated a significant increase in the expression of both iNOS and COX-2 in the inflamed colons (Fig. 5). CBG (30 mg/kg, curative protocol) reduced iNOS (Fig. 5A), but not COX-2 (Fig. 5B) over-expression induced by DNBS.



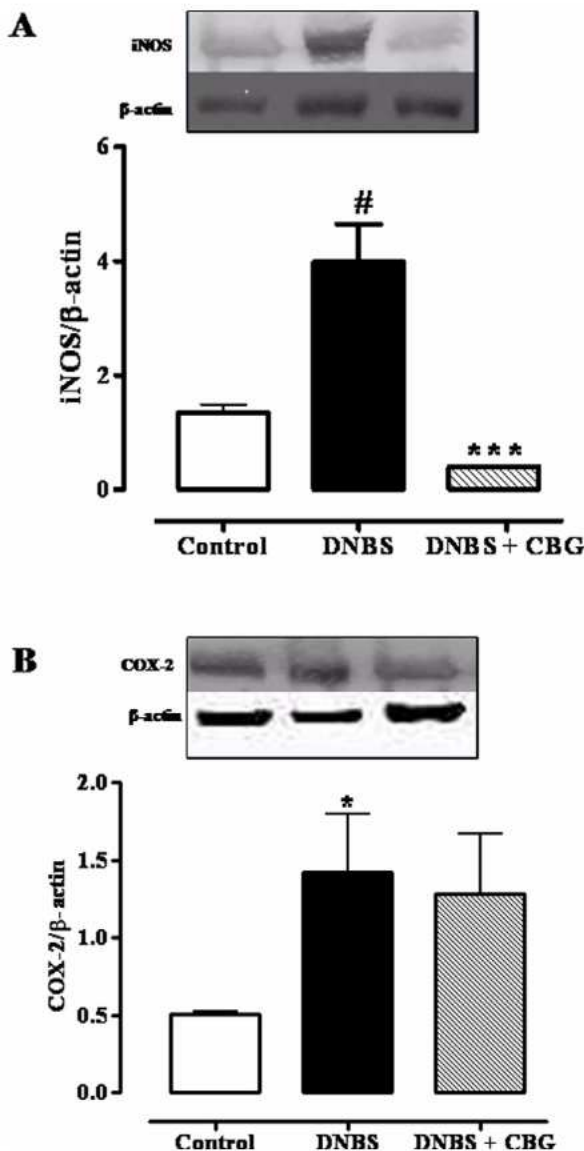
**Fig. 4.** Effect of cannabigerol (CBG) on intestinal permeability (evaluated as FITC-dextran permeability, see methods), myeloperoxidase (MPO, a marker of intestinal inflammation) activity (B) and superoxide dismutase (SOD) activity (C) in DNBS-induced colitis in mice. Colons (for MPO and SOD activities) and blood (for intestinal permeability) were analysed 3 days after vehicle or DNBS (150 mg/kg, intracolonic) administration. CBG (30 mg/kg) was administered (i.p.) for two consecutive days starting 24-h after the inflammatory insult (curative protocol). Bars are mean  $\pm$  SEM of 5 mice for each experimental group. <sup>#</sup> $p < 0.001$  vs control and <sup>\*\*\*</sup> $p < 0.001$  vs DNBS alone.

### 3.8. Interleukin-1 $\beta$ (IL-1 $\beta$ ), interleukin-10 (IL-10) and interferon- $\gamma$ (INF- $\gamma$ ) levels

The levels of IL-1 $\beta$  and INF- $\gamma$  were significantly increased by DNBS (Fig. 6A and 6B). By contrast, IL-10 production significantly decreased in the colon from DNBS-treated mice (Fig. 6C). Treatment with CBG (30 mg/kg, curative protocol) counteracted the changes in IL-1 $\beta$ , IL-10 and INF- $\gamma$  levels observed in the inflamed colons (Fig. 6A–C).

### 3.9. Nitrites measurement in murine macrophages

LPS (1  $\mu$ g/ml for 18 h) administration caused a significant increase in nitrite production (Fig. 7A). A pre-treatment with CBG (0.001–1  $\mu$ M, 30 min before LPS) caused a significant reduction in nitrite production. The inhibitory effect of CBG (1  $\mu$ M) on nitrite production in LPS-treated macrophages was accompanied by decrease of iNOS protein with no significant changes in its



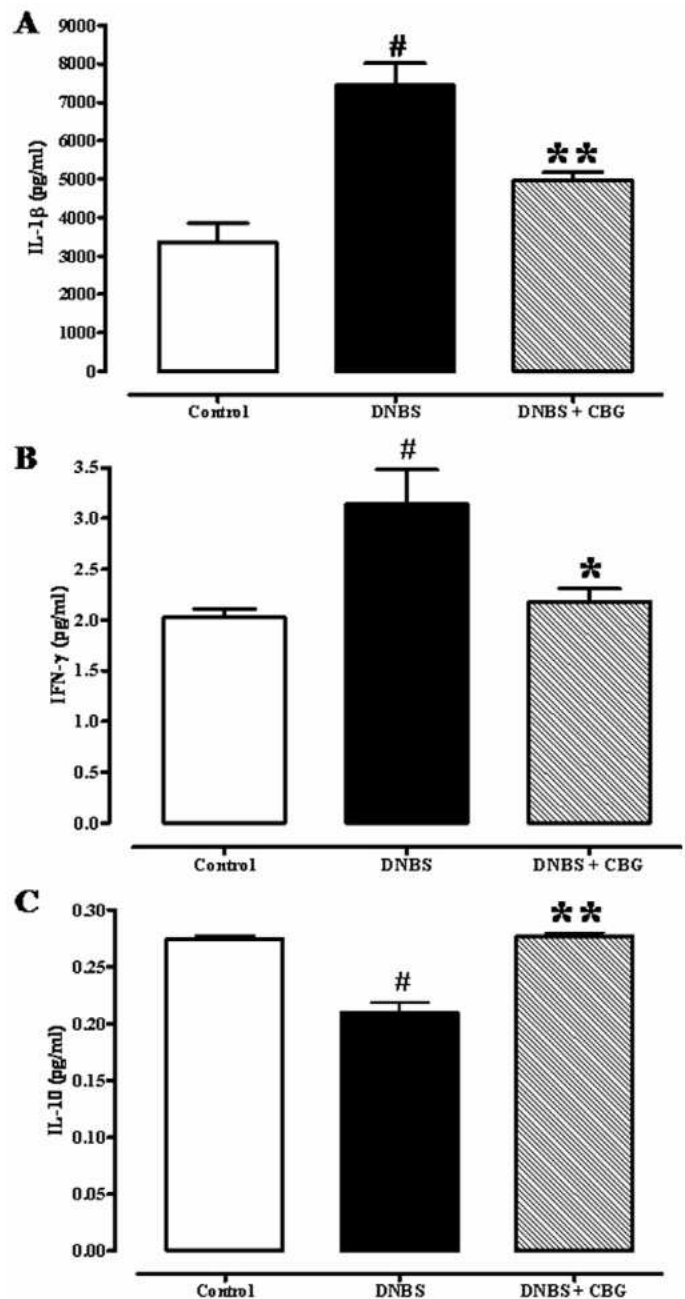
**Fig. 5.** Inducible nitric oxide synthase (iNOS) (A) and cyclooxygenase-2 (COX-2) (B) expression in colonic tissues of animals treated or not with dinitrobenzene sulfonic acid (DNBS): effect of cannabigerol (CBG). Measurements were performed 3 days after DNBS (150 mg/kg, intracolonic) administration. CBG (30 mg/kg) was administered (i.p.) for two consecutive days starting 24-h after the inflammatory insult (curative protocol). Results are mean  $\pm$  SEM of 3–4 experiments. <sup>\*</sup> $p < 0.05$  and <sup>\*\*</sup> $p < 0.001$  vs control; <sup>\*\*\*</sup> $p < 0.001$  vs DNBS alone.

transcriptional levels (i.e. of iNOS mRNA) (Fig. 7B and C). CBG (up to 1  $\mu$ M) had no significant cytotoxic effect on peritoneal macrophages after a 24-h exposure (data not shown).

Because CBG can inhibit endocannabinoid metabolism and hence indirectly activate cannabinoid receptors [23], in another set of experiments we verified if CBG effect on nitrite production was sensitive to selective CB<sub>1</sub> and CB<sub>2</sub> receptor antagonists. We found that rimonabant (0.1  $\mu$ M, CB<sub>1</sub> receptor antagonist) did not modify the inhibitory effect of CBG (1  $\mu$ M) (Fig. 8A). By contrast, SR144528 (0.1  $\mu$ M, CB<sub>2</sub> receptor antagonist) enhanced the inhibitory effect of CBG (1  $\mu$ M) on nitrite production (Fig. 8B). Rimonabant, and SR144528, at the concentrations used, did not modify *per se* nitrite levels induced by LPS stimulation (Fig. 8A and B)

### 3.10. CB<sub>1</sub> and CB<sub>2</sub> mRNA expression in murine macrophages

A challenge with LPS (1  $\mu$ g/ml for 18 h) caused up-regulation of CB<sub>1</sub> receptors and down-regulation of CB<sub>2</sub> receptors (Fig. 9A and B).

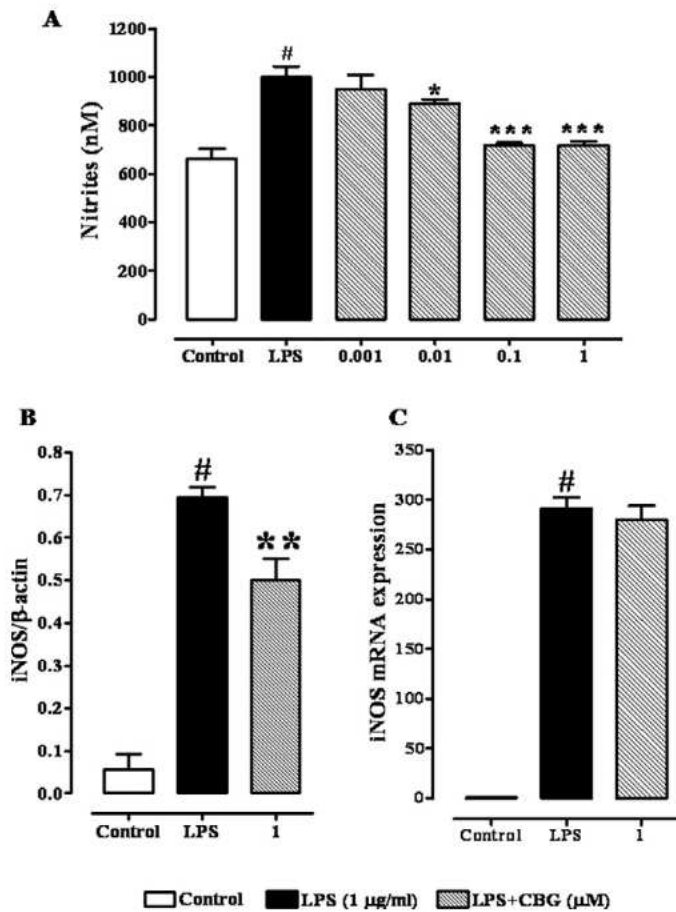


**Fig. 6.** Effect of cannabigerol (CBG) on interleukin-1 $\beta$  (IL-1 $\beta$ ) (A), interferon  $\gamma$  (IFN- $\gamma$ ) (B) and interleukin-10 (IL-10) (C) levels in mouse colons treated with dinitrobenzene sulfonic acid (DNBS). Measurements were performed 3 days after DNBS (150 mg/kg, intracolonic) administration. CBG (30 mg/kg) was administered (i.p.) for two consecutive days starting 24-h after the inflammatory insult (curative protocol). Results (expressed as picograms per ml of proteic extract) are mean  $\pm$  SEM of 3–4 experiments. <sup>#</sup> $p < 0.01$ – $0.001$  vs control, <sup>\*</sup> $p < 0.05$  and <sup>\*\*</sup> $p < 0.01$  vs DNBS alone.

CBG (1  $\mu$ M) did not modify cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors mRNA expression both in control and in LPS-treated macrophages (Fig. 9A and B).

### 3.11. Reactive oxygen species (ROS) production in intestinal epithelial cells

The exposure of Ptk6 null colonic epithelial cells to H<sub>2</sub>O<sub>2</sub>/Fe<sup>2+</sup> (2 mM) produced a significant increase in ROS formation (Fig. 10). A pre-treatment for 24 h with CBG (0.1–10  $\mu$ M) reduced ROS formation as measured by the inhibition of DCF fluorescence



**Fig. 7.** Effect of cannabigerol (CBG) on nitrite levels (A) in the cell medium of murine peritoneal macrophages incubated with lipopolysaccharide (LPS, 1 μg/ml) for 18 h. CBG (0.001–1 μM) was added to the cell media 30 min before LPS challenge. Results, expressed as nitrite concentration (nM), are mean ± SEM of four experiments (in triplicates). Panels B and C show the effect of CBG (1 μM) on inducible nitric oxide synthase (iNOS) expression in cell lysates, evaluated by western blot analysis (B,  $n=5$ ) or RT-PCR (C,  $n=4$ ), respectively. <sup>#</sup> $p < 0.001$  vs control; <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$  and <sup>\*\*\*</sup> $p < 0.001$  vs LPS alone.

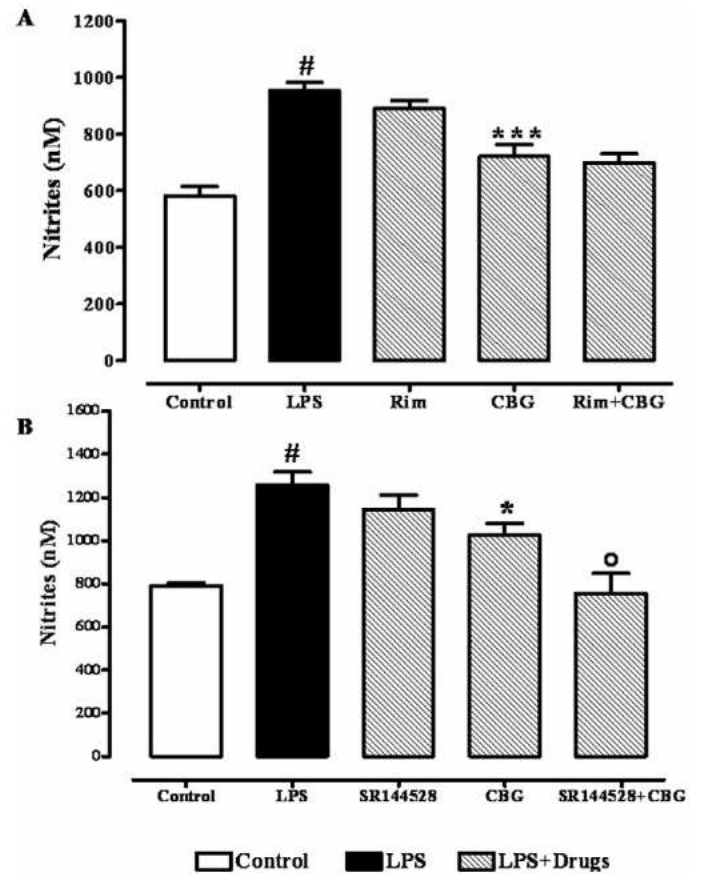
intensity. The effect was significant starting at the concentration of 1 μM (Fig. 10). CBG (up to 10 μM) had no significant cytotoxic effect on colonic epithelial cells after a 24-h exposure (data not shown).

#### 4. Discussion

Anecdotal and scientific evidence suggests that Cannabis use may have favourable effects in IBD patients [9–11]. The intestinal anti-inflammatory effects of  $\Delta^9$ -THC and CBD, *i.e.* the best studied among *Cannabis* ingredients, have been previously documented, both *in vitro* and *in vivo* [12–14,36–38]. In addition, CBC, another phytocannabinoid, has been recently shown to inhibit intestinal motility in an experimental model of intestinal inflammation [39] and to exert beneficial effects in experimental colitis [40]. In the present study we have shown that CBG, a non-psychotropic phytocannabinoid, exerts preventive and curative effects in the DNBS model of colitis and also attenuates both nitrite production in macrophages and ROS production in intestinal epithelial cells.

##### 4.1. CBG exerts protective and curative effects in the DNBS model of murine colitis

We have found that CBG reduced colon weight/colon length ratio of the inflamed colonic tissue, which is considered a reliable

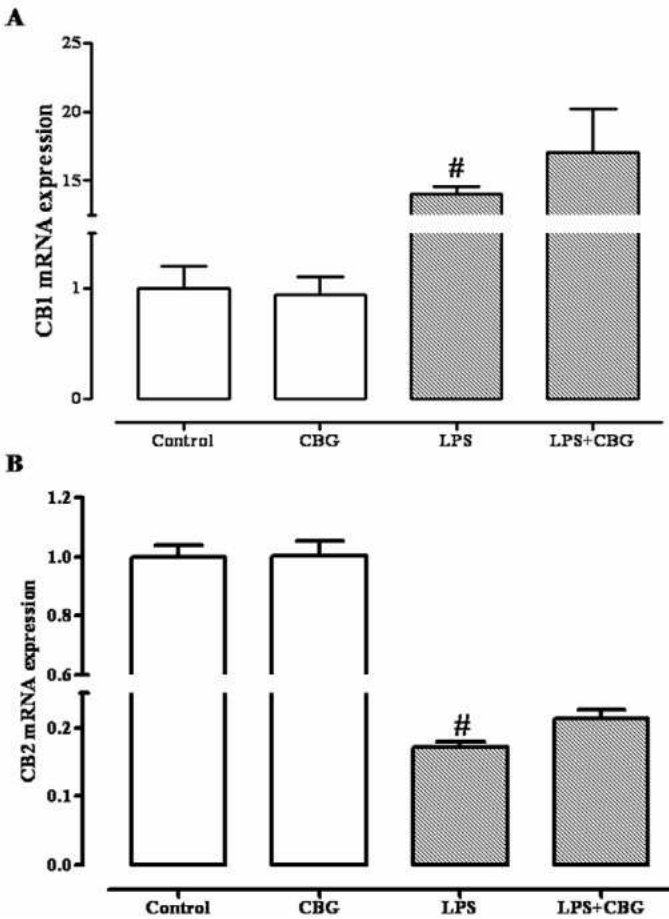


**Fig. 8.** Effect of cannabigerol (CBG, 1 μM) alone or in presence of the cannabinoid CB<sub>2</sub> receptor antagonist rimonabant (Rim, 0.1 μM) (A) or the cannabinoid CB<sub>2</sub> receptor antagonist SR144528 (0.1 μM) (B) on nitrite levels in the cell medium of murine peritoneal macrophages incubated with lipopolysaccharide (LPS, 1 μg/ml) for 18 h. The antagonists were added to the cell media 30 min before CBG exposure. LPS was incubated 30 min after CBG. Results are means ± SEM of three experiments (in triplicates). <sup>#</sup> $p < 0.001$  vs control; <sup>\*</sup> $p < 0.05$  and <sup>\*\*\*</sup> $p < 0.001$  vs LPS alone; <sup>o</sup> $p < 0.001$  vs LPS + CBG.

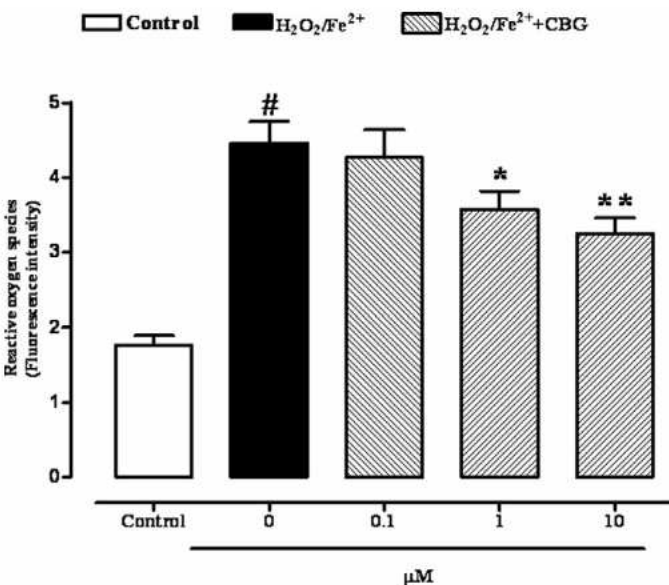
and sensitive indicator of the severity and extent of the inflammatory response [34]. CBG was effective when given both before and after the inflammatory insult, suggesting a preventive and a curative (therapeutic) beneficial effect. Significant protective effects were achieved starting from the 1 mg/kg dose (preventive protocol) and 5 mg/kg (curative protocol). Maximal efficacy was achieved with the 1 mg/kg dose and the 30 mg/kg dose in the preventive and in the curative protocol, respectively.

Because the main goal in IBD is to cure rather than to prevent, we performed further studies (histological analysis, immunohistochemistry, neutrophil infiltration, intestinal membrane integrity as well as cytokines and enzymes assay) by evaluating the effect of CBG given after the inflammatory insult and at the most effective dose of 30 mg/kg. Thus, histological examination showed that CBG 30 mg/kg reduced the signs of colon injury; specifically, in the colon of CBG-treated animals, the glands were regenerating, the oedema in submucosa was reduced and the infiltration of granulocytes into the mucosa and submucosa was decreased. The curative effect of CBG was further demonstrated by its capacity of abrogated the increase in intestinal permeability observed in mice with DNBS treatment (CBG restored the integrity of intestinal epithelium). Accordingly, neutrophil infiltration, revealed by measuring MPO activity [35], was likewise reduced. Furthermore immunohistochemical analyses demonstrated that CBG limited





**Fig. 9.** Relative mRNA expression of cannabinoid CB<sub>1</sub> receptor (A), cannabinoid CB<sub>2</sub> receptor (B) in cell lysates from macrophages incubated or not with lipopolysaccharide (LPS, 1  $\mu$ g/ml) for 18 h. Cannabigerol (CBG, 1  $\mu$ M) was added alone to the cell media or 30 min before LPS challenge. Data were analysed by GENEX software for group wise comparisons and statistical analysis. Results are means  $\pm$  SEM of four experiments. <sup>#</sup> $p < 0.001$  vs control.



**Fig. 10.** Reactive oxygen species (ROS) production produced by Fenton's reagent (2 mM H<sub>2</sub>O<sub>2</sub>/Fe<sup>2+</sup>) in Ptk6 null colonic epithelial cells after 24-h exposure to cannabigerol (0.1–10  $\mu$ M). Results are mean  $\pm$  SEM of five experiments. <sup>#</sup> $p < 0.001$  vs control, <sup>\*</sup> $p < 0.05$  and <sup>\*\*</sup> $p < 0.01$  vs H<sub>2</sub>O<sub>2</sub>/Fe<sup>2+</sup> alone.

the colonic diffusion of Ki-67, a useful marker for the evaluation of dysplasia in ulcerative colitis [41].

Thereafter we measured some cytokines which are known to be involved in IBD [42], namely IL-1 $\beta$  (a cytokine which plays an important pro-inflammatory role in the initiation and amplification of the intestinal inflammatory response) [43], IL-10 (a regulatory cytokine which inhibits pro-inflammatory cytokine release, resulting in anti-inflammatory effects within the gut) [44] and interferon- $\gamma$ , another pro-inflammatory cytokine that plays a crucial function in the initiation of experimental colitis [43,45]. Consistent with previous studies, we observed that intracolonic administration of DNBS caused an increase in colonic IL-1 $\beta$  and interferon- $\gamma$  as well as a decrease in IL-10 levels [12,46]. More importantly, we found that CBG counteracted the colonic variations of the three cytokines, thus suggesting the possible involvement of these cytokines in CBG-mediated anti-inflammatory effects. Finally, we measured iNOS and COX-2 expression, two key enzymes that mediate several of the most important components of intestinal mucosal defense and play a pivotal role in gut inflammation [47,48]. We demonstrated here that the expression of both iNOS and COX-2 was increased in the colon of DNBS-treated mice and that CBG reduced the expression of the iNOS, but not COX-2, protein. Others have recently reported that CBG inhibits COX-2 activity in intestinal cells, but in a higher concentration range, and decreases prostaglandin production in the human colon adenocarcinoma (HT29) cell line [24].

#### 4.2. CBG inhibits nitric oxide production in macrophages

In order to give some insights into the mode of CBG action, we investigated the effect of this phytocannabinoid on isolated cells. Because we have shown that CBG inhibits iNOS expression in the inflamed intestine (see above) and considering that activated macrophages, which play a key role in the pathogenesis of colitis, express iNOS [49], we evaluated the effect of CBG on nitric oxide production in macrophages activated with LPS.

Stimulation of murine macrophages by LPS results in the increased expression of iNOS, which catalyses the production of large amounts of NO from L-arginine and molecular oxygen [50]. We found here that CBG reduced the levels of nitrites, the stable metabolites of NO. Maximal inhibitory effects were achieved with the 0.1 and 1  $\mu$ M concentrations of CBG. These concentrations can be easily reached in the plasma after *in vivo* administration of the phytocannabinoid, since it has been recently demonstrated that *i.p.* administration of CBG (120 mg/kg) yields a peak plasma value of 373  $\mu$ M [51]. The inhibitory effect of CBG on LPS-induced nitrite levels was associated with down-regulation of iNOS, suggesting that inhibition of induction of such enzyme is one of the mechanisms underlying the inhibition of NO production by the phytocannabinoid. We have recently demonstrated that also cannabichromene (CBC), another non-psychoactive cannabinoid, reduces nitrites production in macrophages [40].

In order to explore the possible molecular target of CBG action, we considered the possible involvement of cannabinoid receptors since CBG was shown to behave as a partial agonist of CB<sub>1</sub> and CB<sub>2</sub> receptors [25], although exhibiting low affinity for these receptors [52], and to inhibit the reuptake of the endocannabinoid anandamide [23]. The possible involvement of cannabinoid receptors in CBG action was studied by evaluating: 1) the effect of selective CB<sub>1</sub> and CB<sub>2</sub> receptor antagonists on CBG-induced inhibition of nitrite production, and 2) possible alterations in cannabinoid receptor mRNA produced by CBG in LPS-challenged macrophages. We found that the inhibitory effect of CBG on nitrite production was not modified by the CB<sub>1</sub> receptor inverse agonist/antagonist rimonabant. By contrast, the CB<sub>2</sub> receptor inverse agonist/antagonist SR144528 further augmented the inhibitory

effect of CBG on nitrite production, suggesting a modulatory role of CB<sub>2</sub> receptors. In other words, our results suggest that an endogenous cannabinoid tone may exist, via CB<sub>2</sub> receptors, influencing negatively CBG signalling inhibition of nitrite production. Interestingly, among the non-THC plant cannabinoids, CBG is of the few with sub-micromolar affinity for CB<sub>2</sub> [52], and thus possibly the most likely to synergize with a CB<sub>2</sub> inverse agonist/antagonist. Moreover, we found that CBG did not modify the effect of LPS on CB<sub>1</sub> and CB<sub>2</sub> receptor mRNA expression. In a different study, we have shown that CBC altered the mRNA expression of cannabinoid receptors in the inflamed intestine [39].

CBG was also shown to potently activate and desensitize TRPA1 channels [23]. Therefore, at least the preventive effect of this compound, observed here, could have been due to desensitization of these channels to the action of DNBS, an analogue of which (*i.e.* TNBS) was recently shown to induce colitis by directly activating TRPA1 [53]. Unfortunately, we were not able to verify the possible involvement of TRPA1 in macrophages since two well-characterized TRPA1 antagonists, namely AP-18 and HC-030031, administered alone, completely blocked nitrite productions at concentrations below the IC<sub>50</sub> value calculated to block the TRPA1 [40]. Similarly, the TRPA1 antagonist HC-030031 attenuated experimental colitis in mice [53]. Furthermore, we recently showed that CBC, another phytocannabinoid, as potent at TRPA1 and more selective towards other TRP channels than CBG, did not act in the inflamed gut by activating TRPA1 [39]. Therefore, we did not investigate the involvement of this channel in the present effects of CBG. As to the other proposed targets for CBG, 5-HT<sub>1A</sub> receptors have not been involved so far in colitis, whereas  $\alpha_2$ -adrenergic receptor activation by the compound [25], if anything, should have worsened inflammation rather than reducing it [54]. Finally, CBG also potently activates and desensitizes TRPV2 channels and desensitizes TRPV4 channels *in vitro* in the low  $\mu$ M range [23,33]. Of these two TRP channels, only the latter has been implicated so far in the initiation of inflammatory cytokine release in experimental colitis [55]. Unfortunately, no TRPV4 antagonist is commercially available and we could not test whether CBG protective or curative effects against DNBS-induced colitis were mediated at least in part through inactivation of this channel.

#### 4.3. CBG exerts antioxidant effects

Finally, we explored the possibility that CBG could protect the intestinal mucosa by reducing oxidative stress. We measured SOD activity, an important antioxidant defence in the gut [56] and ROS production, a major tissue-destructive force which contributes significantly to the pathogenesis of IBD [57]. Previously, we showed that another phytocannabinoid, namely CBD, exerted antioxidant activity in human colorectal cancer cells [12]. We found here that CBG restored the decreased SOD activity induced by DNBS administration in colonic tissues as well as reduced ROS production induced by Fenton's reagent in mouse intestinal epithelial cells. These results suggest that the curative effect of CBG could be due, at least in part, to its antioxidant action.

In conclusion, our results show that the non-psychoactive plant cannabinoid CBG exerts protective effects in a murine experimental model of IBD. The effect of CBG was associated to modulation of cytokine (IL-1 $\beta$ , IL-10 and interferon- $\gamma$ ) levels and down-regulation of iNOS (but not COX-2) expression. Studies on peritoneal macrophages suggest that CBG inhibits iNOS-derived nitric oxide production and that this effect may be negatively modulated by cannabinoid CB<sub>2</sub> receptors. Also, CBG exerts antioxidant effects in the inflamed gut as well as in intestinal epithelial cells exposed to oxidative stress. On the whole, these results could provide a pharmacological basis to explain, at least in part, the beneficial

effects of *Cannabis* preparations observed in IBD patients using *Cannabis*. In a therapeutic prospective, our results suggest that CBG may represent a new therapeutic opportunity in IBD.

#### Conflict of interest

There is no other relationships/conditions/circumstances that present a potential conflict of interest.

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