

Accepted Manuscript

Title: Beneficial effect of the non-psychoactive plant cannabinoid cannabigerol on experimental inflammatory bowel disease

Authors: Francesca Borrelli, Ines Fasolino, Barbara Romano, Raffaele Capasso, Francesco Maiello, Diana Coppola, Pierangelo Orlando, Giovanni Battista, Ester Pagano, Vincenzo Di Marzo, Angelo A. Izzo



PII: S0006-2952(13)00054-3
DOI: doi:10.1016/j.bcp.2013.01.017
Reference: BCP 11526

To appear in: *BCP*

Received date: 10-12-2012
Revised date: 22-1-2013
Accepted date: 22-1-2013

Please cite this article as: Borrelli F, Fasolino I, Romano B, Capasso R, Maiello F, Coppola D, Orlando P, Battista G, Pagano E, Di Marzo V, Izzo AA, Beneficial effect of the non-psychoactive plant cannabinoid cannabigerol on experimental inflammatory bowel disease, *Biochemical Pharmacology* (2013), doi:10.1016/j.bcp.2013.01.017

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

Francesca Borrelli^{a,1*}, Ines Fasolino^a, Barbara Romano^a, Raffaele Capasso^{a,1}, Francesco Maiello^b, Diana Coppola^b, Pierangelo Orlando^{c1}, Giovanni Battista^b, Ester Pagano^a, Vincenzo Di Marzo^{d1}, Angelo A. Izzo^{a1*}.

^aDepartment of Experimental Pharmacology, University of Naples Federico II, via D Montesano 49, 80131 Naples, Italy. franborr@unina.it; inesfasolino@gmail.com; barbara.romano@unina.it; rafcapas@unina.it, ester.pagano@unina.it; aaizzo@unina.it.

^bOspedale dei Pellegrini, Department of Diagnostic Services (Anatomy and Pathologic Histology Service), ASL 1, Naples, Italy. framai@iol.it; diana.coppola@virgilio.it; giovanni.battista.90@hotmail.it.

^cInstitute of Protein Biochemistry, National Research Council, Pozzuoli (NA), Italy. p.orlando@ibp.cnr.it.

^dInstitute of Biomolecular Chemistry, National Research Council, Pozzuoli (NA), Italy. vdimarzo@icmib.na.cnr.it.

¹Endocannabinoid Research Group.

*Corresponding author at: Department of Experimental Pharmacology, University of Naples Federico II, via D Montesano 49, 80131 Naples, Italy. Phone: +39.081.678665, fax: +39.081.678403, e-mail: franborr@unina.it (F. Borrelli), aaizzo@unina.it (AA. Izzo)

Abstract

Inflammatory bowel disease (IBD) is an incurable disease which affects millions of people in industrialised countries. Anecdotal and scientific evidence suggest that Cannabis use may have a positive impact in IBD patients. Here, we investigated the effect of cannabigerol (CBG), a non-psychoactive 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 β , interleukin-10 and interferon- γ 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 β , interleukin-10 and interferon- γ changes associated to DNBS administration. In macrophages, CBG reduced nitric oxide production and iNOS protein (but not mRNA) expression. Rimonabant (a CB₁ receptor antagonist) did not change the effect of CBG on nitric oxide production, while SR144528 (a CB₂ 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 CB₂ receptor) and reduced ROS formation in intestinal epithelial cells. CBG could be considered for clinical experimentation in IBD patients.

Keywords: Cannabigerol; Phytocannabinoids; Inflammatory bowel disease; Murine colitis; Macrophages; Dinitrobenzene sulphonic acid.

1. Introduction

Inflammatory bowel disease (IBD) comprises the chronic relapsing inflammatory disorders Crohn's disease (CD) and ulcerative colitis (UC) [1]. It is characterised 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 aminosalicylates, corticosteroids, thiopurines, methotrexate, and anti-tumor 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. Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the main Cannabis psychotropic ingredient which activates cannabinoid (CB₁ and CB₂) 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 Δ^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 antagonise 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_{1A} and α_2 adrenergic receptors [25].

In the present study, we investigated the effect of CBG in an experimental model of murine colitis. To further characterise 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-methylbenzyl)-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 (H₂O₂), FeCl₂·4H₂O, 2',7'-dichlorofluorescein-diacetate (H₂DCF-DA), lipopolysaccharide (LPS, from Escherichia coli serotype O111:B4), thioglycollate medium, cadmium, 2,3-diaminonaphthalene (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 hematoxylin 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 Microglass Heim 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 sulfoxide (DMSO). DNBS was dissolved in 50% ethanol (0.15 ml/mouse). The CBG vehicles (60 μ 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, $23\pm 2^{\circ}\text{C}$; 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_2 , 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- μ m sections were deparaffinized with xylene, stained with hematoxylin–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 hematoxylin. 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)-labeled-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 analyzed 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% HTAB in MOPS 10 Mm) in ratio 50 mg tissue /1 ml MOPS . The samples were then centrifuged for 20 minutes at 15,000 x 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, H₂O₂ (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 (15.000xg, 15 min, 4°C), 125 µl of the surnatant was incubated (for 20 min) with 613 µl of a reaction mixture containing 0.12 mM

xanthine, 48 mM Na₂CO₃, 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, CuCl₂ (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 MgCl₂, 10 mM ethylene glycol tetraacetic acid, and supplemented with 100 mM dithiothreitol and protease/phosphatase inhibitors (100 mM dimethylsulfonyl fluoride, 2 mg/ml aprotinin, 2 mM leupeptin, and 10 mM Na₃VO₄). 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 sulfate 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-1 β (*IL-1 β*), *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% fetal bovine serum (FBS), 80 U/ml penicillin and 80 μ g/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 μ g/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 ($\text{H}_2\text{O}_2/\text{Fe}^{2+}$ 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 μ M) for 24 h, macrophages (1×10^5 cells per well seeded in a 96-well plate) or *Ptk6* null colonic epithelial cells (3×10^4 cells per well seeded in a 96-well plate) were incubated with neutral red dye solution (50 μ g/ml) for 3 h, and then lysed by adding 1% acetic acid. The absorbance was read at 532 nm (iMarkTM Microplate Assorbance Reader, BioRad). Treatments were compared with a positive control, i.e., DMSO 20 % (v/v).

2.13 Nitrites measurement

Nitrites, stable metabolites of NO, were measured in macrophages medium as previously described [31]. Mouse peritoneal macrophages (5×10^5 cells per well seeded in a 24-well plate) were incubated with CBG (0.001–1 μ M) for 30 min and subsequently with LPS (1 μ g/ml) for 18 h. After reduction of nitrates to nitrites by cadmium, cell supernatants were incubated with DAN (50 μ 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 μ M, CB₁ receptor antagonist) and SR144528 (0.1 μ M, CB₂ receptor antagonist) were incubated 30 min before CBG (1 μ M).

2.14 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 lysis buffer (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 20mL 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].

2.45 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 3×10^4 cells per well. Ptk6 null colonic epithelial cells were incubated for 24 h at 37°C with CBG (0.1-10 μ M). After washing, cells were incubated for 1 h with 200 μ l of 100 μ 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).

2.16 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 ± 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. 2C).

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

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).

3.3 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).

3.4 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 neutrophil 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).

3.5 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 (Figure 4C).

3.6 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 (Figure 5A), but not COX-2 (Fig. 5B) over-expression induced by DNBS.

3.7 Interleukin-1 β (IL-1 β), interleukin-10 (IL-10) and interferon- γ (INF- γ) levels

The levels of IL-1 β and INF- γ were significantly increased by DNBS (Fig. 6A and 6B). By contrast, IL-10 production significantly decreased in the colon from DNBS-treated mice (Fig. 6 C).

Treatment with CBG (30 mg/kg, curative protocol) counteracted the changes in IL-1 β , IL-10 and INF- γ levels observed in the inflamed colons (Fig. 6A, 6B and 6C).

3.8 Nitrites measurement in murine macrophages

LPS (1 μ g/ml for 18 h) administration caused a significant increase in nitrite production (Fig. 7A). A pre-treatment with CBG (0.001-1 μ M, 30 min before LPS) caused a significant reduction in nitrite production. The inhibitory effect of CBG (1 μ M) on nitrite production in LPS-treated macrophages was accompanied by decrease of iNOS protein with no significant changes in its transcriptional levels (i.e. of iNOS mRNA) (Fig. 7B and 7C). CBG (up to 1 μ 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 CB₁ and CB₂ receptor antagonists. We found that rimonabant (0.1 μ M, CB₁ receptor antagonist) did not modify the inhibitory effect of CBG (1 μ M) (Fig. 8A). By contrast, SR144528 (0.1 μ M, CB₂ receptor antagonist) enhanced the inhibitory effect of CBG (1 μ 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 8B)

3.9 CB₁ and CB₂ mRNA expression in murine macrophages

A challenge with LPS (1 μ g/ml for 18 h) caused up-regulation of CB₁ receptors and down-regulation of CB₂ receptors (Fig. 9A and 9B). CBG (1 μ M) did not modify cannabinoid CB₁ and CB₂ receptors mRNA expression both in control and in LPS-treated macrophages (Fig. 9A and 9B).

3.10 Reactive oxygen species (ROS) production in intestinal epithelial cells

The exposure of Ptk6 null colonic epithelial cells to H₂O₂/Fe²⁺ (2 mM) produced a significant increase in ROS formation (Fig. 10). A pre-treatment for 24 h with CBG (0.1-10 μ M) reduced ROS formation as measured by the inhibition of DCF fluorescence intensity. The effect was significant starting at the concentration of 1 μ M (Fig. 10). CBG (up to 10 μ M) 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 Δ^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-psychoactive 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 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 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 β (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- γ , 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 β and interferon- γ 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 catalyzes 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 μ 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 μ 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 CB₁ and CB₂ 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 CB₁ and CB₂ 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 CB₁ receptor inverse agonist/antagonist rimonabant. By contrast, the CB₂ receptor inverse agonist/antagonist SR144528 further augmented the inhibitory effect of CBG on nitrite production, suggesting a modulatory role of CB₂ receptors. In other words, our results suggest that an endogenous cannabinoid tone may exist, via CB₂ 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 CB₂ [52], and thus possibly the most likely to synergize with a CB₂ inverse agonist/antagonist. Moreover, we found that CBG did not modify the effect of LPS on CB₁ and CB₂

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 IC₅₀ value calculated to block the TRPA1 (Izzo and Di Marzo, unpublished). 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_{1A} receptors have not been involved so far in colitis, whereas α_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 μ 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-psychotropic 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 β , IL-10 and interferon- γ) 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 CB₂ 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.

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'-dichlorfluorescein-diacetate; IBD, Inflammatory bowel disease; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; UC, ulcerative colitis.

Conflict of interest

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

References

- [1] Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011;474:307-317.
- [2] Glocker E, Grimbacher B. Inflammatory bowel disease: is it a primary immunodeficiency? *Cell Mol Life Sci* 2012;69:41-48.
- [3] Cosnes J, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011;140:1785-1794.
- [4] Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012;142:46-54.e42.
- [5] Burger D, Travis S. Conventional medical management of inflammatory bowel disease. *Gastroenterology* 2011;140:1827-1837.e2.
- [6] Blonski W, Buchner AM, Lichtenstein GR. Inflammatory bowel disease therapy: current state-of-the-art. *Curr Opin Gastroenterol* 2011;27:346-357.
- [7] Izzo AA, Camilleri M. Cannabinoids in intestinal inflammation and cancer. *Pharmacol Res* 2009;60:117-125.
- [8] Alhouayek M, Muccioli GG. The endocannabinoid system in inflammatory bowel diseases: from pathophysiology to therapeutic opportunity. *Trends Mol Med* 2012. [Epub ahead of print].
- [9] Naftali T, Lev LB, Yablecovitch D, Half E, Konikoff FM. Treatment of Crohn's disease with cannabis: an observational study. *Isr Med Assoc J* 2011;13: 455-458.
- [10] Lal S, Prasad N, Ryan M, Tangri S, Silverberg MS, Gordon A, Steinhart H. Cannabis use amongst patients with inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2011;23:891-896.
- [11] Lahat A, Lang A, Ben-Horin S. Impact of cannabis treatment on the quality of life, weight and clinical disease activity in inflammatory bowel disease patients: a pilot prospective study. *Digestion* 2012;85:1-8.

- [12] Borrelli F, Aviello G, Romano B, Orlando P, Capasso R, Maiello F, Guadagno F, Petrosino S, Capasso F, Di Marzo V, Izzo AA. Cannabidiol, a safe and non-psychoactive ingredient of the marijuana plant *Cannabis sativa*, is protective in a murine model of colitis. *J Mol Med* 2009; 87:1111-1121.
- [13] Jamontt JM, Molleman A, Pertwee RG, Parsons ME. The effects of Delta-tetrahydrocannabinol and cannabidiol alone and in combination on damage, inflammation and in vitro motility disturbances in rat colitis. *Br J Pharmacol* 2010; 60:712-723.
- [14] Schicho R, Storr M. Topical and Systemic Cannabidiol Improves Trinitrobenzene Sulfonic Acid Colitis in Mice. *Pharmacology* 2012; 89:149-155.
- [15] Turner CE, Elsohly MA, Boeren EG. Constituents of *Cannabis sativa* L. XVII. A review of the natural constituents. *J Nat Prod* 1980;43:169-234.
- [16] Mechoulam R, Shani A, Edery H, Grunfeld Y. Chemical basis of hashish activity. *Science* 1970;169:611-612.
- [17] Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. Non-psychoactive plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends Pharmacol Sci* 2009;30:515-527.
- [18] Hill AJ, Williams CM, Whalley BJ, Stephens GJ. Phytocannabinoids as novel therapeutic agents in CNS disorders. *Pharmacol Ther* 2012;133:79-97.
- [19] Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, Laezza C, Portella G, Bifulco M, Di Marzo V. Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *J Pharmacol Exp Ther* 2006;318:1375-1387.
- [20] Appendino G, Gibbons S, Giana A, Pagani A, Grassi G, Stavri M, Smith E, Rahman MM. Antibacterial cannabinoids from *Cannabis sativa*: a structure-activity study. *J Nat Prod* 2008;71:1427-1430.
- [21] Colasanti BK. A comparison of the ocular and central effects of delta 9-tetrahydrocannabinol and cannabigerol. *J Ocul Pharmacol* 1990;6:259-269.

- [22] Rock EM, Goodwin JM, Limebeer CL, Breuer A, Pertwee RG, Mechoulam R, Parker LA. Interaction between non-psychotropic cannabinoids in marijuana: effect of cannabigerol (CBG) on the anti-nausea or anti-emetic effects of cannabidiol (CBD) in rats and shrews. *Psychopharmacology* 2011;215:505-512.
- [23] De Petrocellis L, Ligresti A, Moriello AS, Allarà M, Bisogno T, Petrosino S, Stott CG, Di Marzo V. Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *Br J Pharmacol* 2011;163:1479-1494.
- [24] Ruhaak LR, Felth J, Karlsson PC, Rafter JJ, Verpoorte R, Bohlin L. Evaluation of the cyclooxygenase inhibiting effects of six major cannabinoids isolated from *Cannabis sativa*. *Biol Pharm Bull* 2011;34:774-778.
- [25] Cascio MG, Gauson LA, Stevenson LA, Ross RA, Pertwee RG. Evidence that the plant cannabinoid cannabigerol is a highly potent alpha2-adrenoceptor agonist and moderately potent 5HT1A receptor antagonist. *Br J Pharmacol* 2010;159:129-141.
- [26] Massa F, Marsicano G, Hermann H, Cannich A, Monory K, Cravatt BF, Ferri GL, Sibaev A, Storr M, Lutz B. The endogenous cannabinoid system protects against colonic inflammation. *J Clin Invest* 2004;113:1202-1209.
- [27] D'Argenio G, Valenti M, Scaglione G, Cosenza V, Sorrentini I, Di Marzo V. Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation. *FASEB J* 2006;20:568-570.
- [28] Osanai M, Nishikiori N, Murata M, Chiba H, Kojima T, Sawada N. Cellular retinoic acid bioavailability determines epithelial integrity: Role of retinoic acid receptor alpha agonists in colitis. *Mol Pharmacol* 2007;71:250-258.
- [29] Goldblum SE, Wu KM, Jay M. Lung myeloperoxidase as a measure of pulmonary leukostasis in rabbits. *J Appl Physiol* 1985;59:1471-1480.
- [30] Kuthan H, Haussmann HJ, Werringloer J. A spectrophotometric assay for superoxide dismutase activities in crude tissue fractions. *Biochem J* 1986;237:175-180.

- [31] Aviello G, Borrelli F, Guida F, Romano B, Lewellyn K, De Chiaro M, Luongo L, Zjawiony JK, Maione S, Izzo AA, Capasso R. Ultrapotent effects of salvinorin A, a hallucinogenic compound from *Salvia divinorum*, on LPS-stimulated murine macrophages and its anti-inflammatory action in vivo. *J Mol Med* 2011;89:891-902.
- [32] Whitehead RH, Robinson PS, Williams JA, Bie W, Tyner AL, Franklin JL. Conditionally immortalized colonic epithelial cell line from a Ptk6 null mouse that polarizes and differentiates in vitro. *J Gastroenterol Hepatol* 2008;23:1119-1124.
- [33] De Petrocellis L, Orlando P, Moriello AS, Aviello G, Stott C, Izzo AA, Di Marzo V. Cannabinoid actions at TRPV channels: effects on TRPV3 and TRPV4 and their potential relevance to gastrointestinal inflammation. *Acta Physiol* 2012;204:255-266.
- [34] Gálvez J, Garrido M, Merlos M, Torres MI, Zarzuelo A. Intestinal anti-inflammatory activity of UR-12746, a novel 5-ASA conjugate, on acute and chronic experimental colitis in the rat. *Br J Pharmacol* 2000;130:1949-1959.
- [35] Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology* 1984;87:1344-1350.
- [36] Alhamoruni A, Wright KL, Larvin M, O'Sullivan SE. Cannabinoids mediate opposing effects on inflammation-induced intestinal permeability. *Br J Pharmacol* 2012;165:2598-2610.
- [37] Capasso R, Borrelli F, Aviello G, Romano B, Scalisi C, Capasso F, Izzo AA. Cannabidiol, extracted from *Cannabis sativa*, selectively inhibits inflammatory hypermotility in mice. *Br J Pharmacol* 2008;154:1001-1008.
- [38] De Filippis D, Esposito G, Cirillo C, Cipriano M, De Winter BY, Scuderi C, Sarnelli G, Cuomo R, Steardo L, De Man JG, Iuvone T. Cannabidiol reduces intestinal inflammation through the control of neuroimmune axis. *PLoS One* 2011;6:e28159.
- [39] Izzo AA, Capasso R, Aviello G, Borrelli F, Romano B, Piscitelli F, Gallo L, Capasso F, Orlando P, Di Marzo V. Inhibitory effect of cannabichromene, a major non-psychotropic

cannabinoid extracted from *Cannabis sativa*, on inflammation-induced hypermotility in mice. *Br J Pharmacol* 2012;166:1444-1460.

- [40] Romano B, Fasolino I, Borrelli F, Capasso R, Piscitelli F, Orlando P, Di Marzo V, Izzo AA. The non-psychotropic cannabinoid cannabichromene inhibits nitric oxide production in macrophages and ameliorates experimental inflammatory bowel disease. First joint Spanish-Italian meeting on cannabinoid research/13^a reunion anual de la sociedad espanola de investigacion sobre cannabinoids. Madrid 29th November-1th December, 2012.
- [41] Andersen SN, Rognum TO, Bakka A, Clausen OP. Ki-67: a useful marker for the evaluation of dysplasia in ulcerative colitis. *Mol Pathol* 1998;51: 327-332.
- [42] Madsen K. Combining T cells and IL-10: a new therapy for Crohn's disease? *Gastroenterology* 2002;123:2140-2144.
- [43] Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011;140:1756-1767.
- [44] Barbara G, Xing Z, Hogaboam CM, Gaudie J, Collins SM. Interleukin 10 gene transfer prevents experimental colitis in rats. *Gut* 2000;46:344-349.
- [45] Ito R, Shin-Ya M, Kishida T, Urano A, Takada R, Sakagami J, Imanishi J, Kita M, Ueda Y, Iwakura Y, Kataoka K, Okanoue T, Mazda O. Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clin Exp Immunol* 2006;146:330-338.
- [46] Lamine F, Eutamène H, Fioramonti J, Buéno L, Théodorou V. Colonic responses to *Lactobacillus farciminis* treatment in trinitrobenzene sulphonic acid-induced colitis in rats. *Scandinavian Journal of Gastroenterology* 2004;39:1250-1258.
- [47] Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* 2004;113:427-437.
- [48] Wallace JL, Devchand PR. Emerging roles for cyclooxygenase-2 in gastrointestinal mucosal defense. *Br J Pharmacol* 2005;145:275-282.

- [49] Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1988;333:664-666.
- [50] Moncada S, Higgs A, Furchgott R. International Union of Pharmacology Nomenclature in Nitric Oxide Research. *Pharmacol Rev* 1997;49:137-142.
- [51] Deiana S, Watanabe A, Yamasaki Y, Amada N, Arthur M, Fleming S, Woodcock H, Dorward P, Pigliacampo B, Close S, Platt B, Riedel G. Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarin (CBDV), Δ -tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive-compulsive behaviour. *Psychopharmacology* 2012;219:859-873.
- [52] Pollastro F, Tagliatalata-Scafati O, Allarà M, Muñoz E, Di Marzo V, De Petrocellis L, Appendino G. Bioactive prenylogous cannabinoid from fiber hemp (*Cannabis sativa*). *J Nat Prod* 2011;74: 019-2022.
- [53] Engel MA, Leffler A, Niedermirtl F, Babes A, Zimmermann K, Filipović MR, Izydorczyk I, Eberhardt M, Kichko TI, Mueller-Tribbensee SM, Khalil M, Siklosi N, Nau C, Ivanović-Burmazović I, Neuhuber WL, Becker C, Neurath MF, Reeh PW. TRPA1 and substance P mediate colitis in mice. *Gastroenterology* 2011;141:1346-1358.
- [54] Bai A, Lu N, Guo Y, Chen J, Liu Z. Modulation of inflammatory response via alpha2-adrenoceptor blockade in acute murine colitis. *Clin Exp Immunol* 2009;156:353-362.
- [55] D'Aldebert E, Cenac N, Rousset P, Martin L, Rolland C, Chapman K, Selves J, Alric L, Vinel JP, Vergnolle N. Transient receptor potential vanilloid 4 activated inflammatory signals by intestinal epithelial cells and colitis in mice. *Gastroenterology* 2011;140: 275-285.
- [56] Watterlot L, Rochat T, Sokol H, C. Cherbuy, I. Bouloufa, F. Lefevre, J.J. Gratadoux, E. Honvo-Hueto, S. Chilmonczyk, S. Blugeon, G. Corthier, P. Langella, L.G. Bermudez-Humaran. 2010 Intra-gastric administration of a superoxide dismutase-producing recombinant *Lactobacillus casei* BL23 strain attenuates DSS colitis in mice. *Int. J. Food Microbiol.* 144: 35-41.

[57] Kruidenier, L., and HW. Verspaget. 2002. Review article: oxidative stress as a pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Aliment Pharmacol Ther.* 16: 1997-2015.

Accepted Manuscript

FIGURE LEGENDS

FIGURE 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 analyzed 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.

FIGURE 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 x 200. The figure is representative of 4 experiments.

FIGURE 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.

FIGURE 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. # $p < 0.001$ vs control and *** $p < 0.001$ vs DNBS alone.

FIGURE 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 means \pm SEM of 3-4 experiments. * $p < 0.05$ and # $p < 0.001$ vs control; *** $p < 0.001$ vs DNBS alone.

FIGURE 6. Effect of cannabigerol (CBG) on interleukin-1 β (IL-1 β) (A), interferon γ (IFN- γ) (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. # $p < 0.01-0.001$ vs control, * $p < 0.05$ and ** $p < 0.01$ vs DNBS alone.

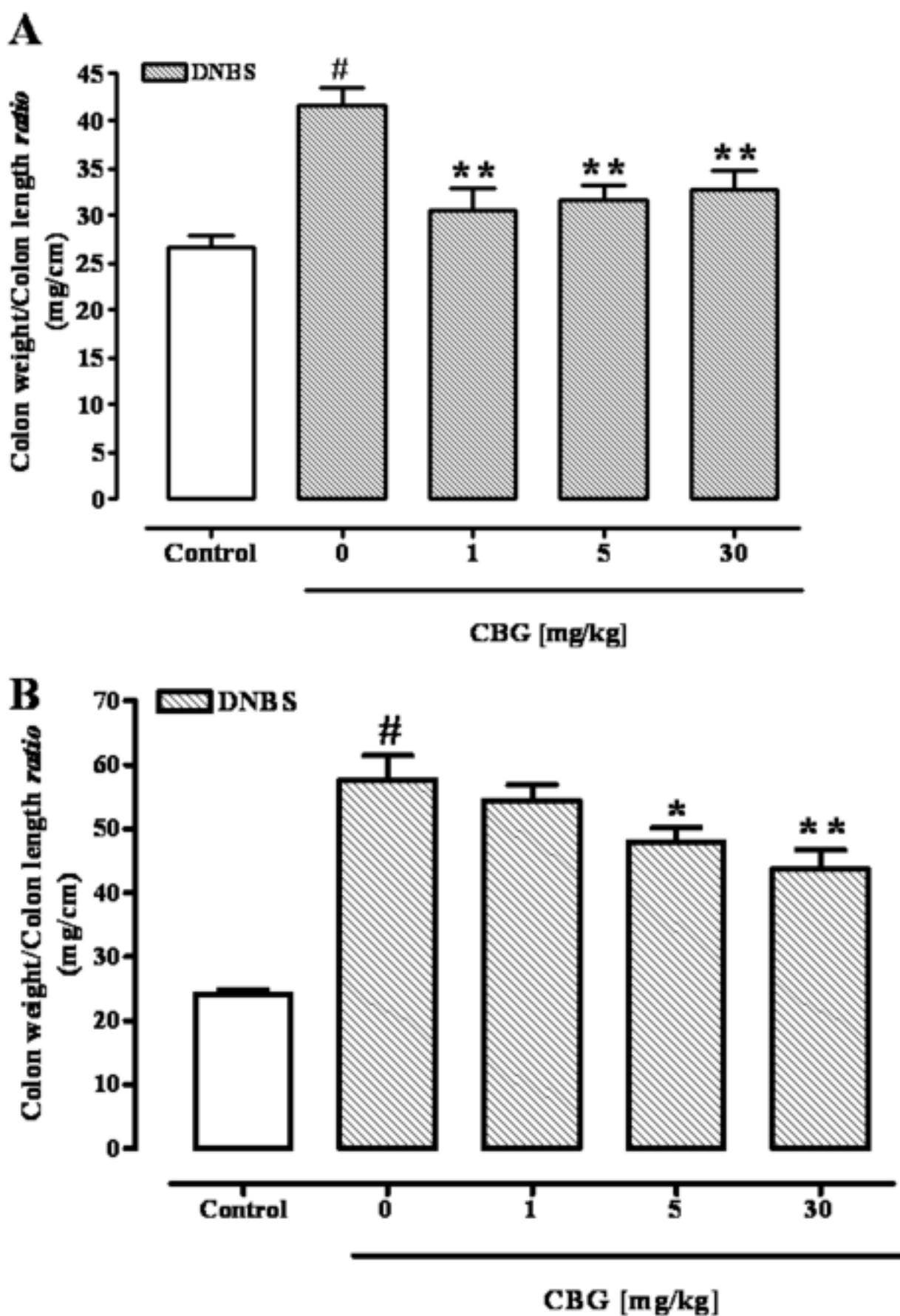
FIGURE 7. Effect of cannabigerol (CBG) on nitrite levels (A) in the cell medium of murine peritoneal macrophages incubated with lipopolysaccharide (LPS, 1 μ g/ml) for 18 h. CBG (0.001–1 μ M) was added to the cell media 30 min before LPS challenge. Results, expressed as nitrite concentration (nM), are mean \pm SEM of four experiments (in triplicates). Figures 7B and 7C show

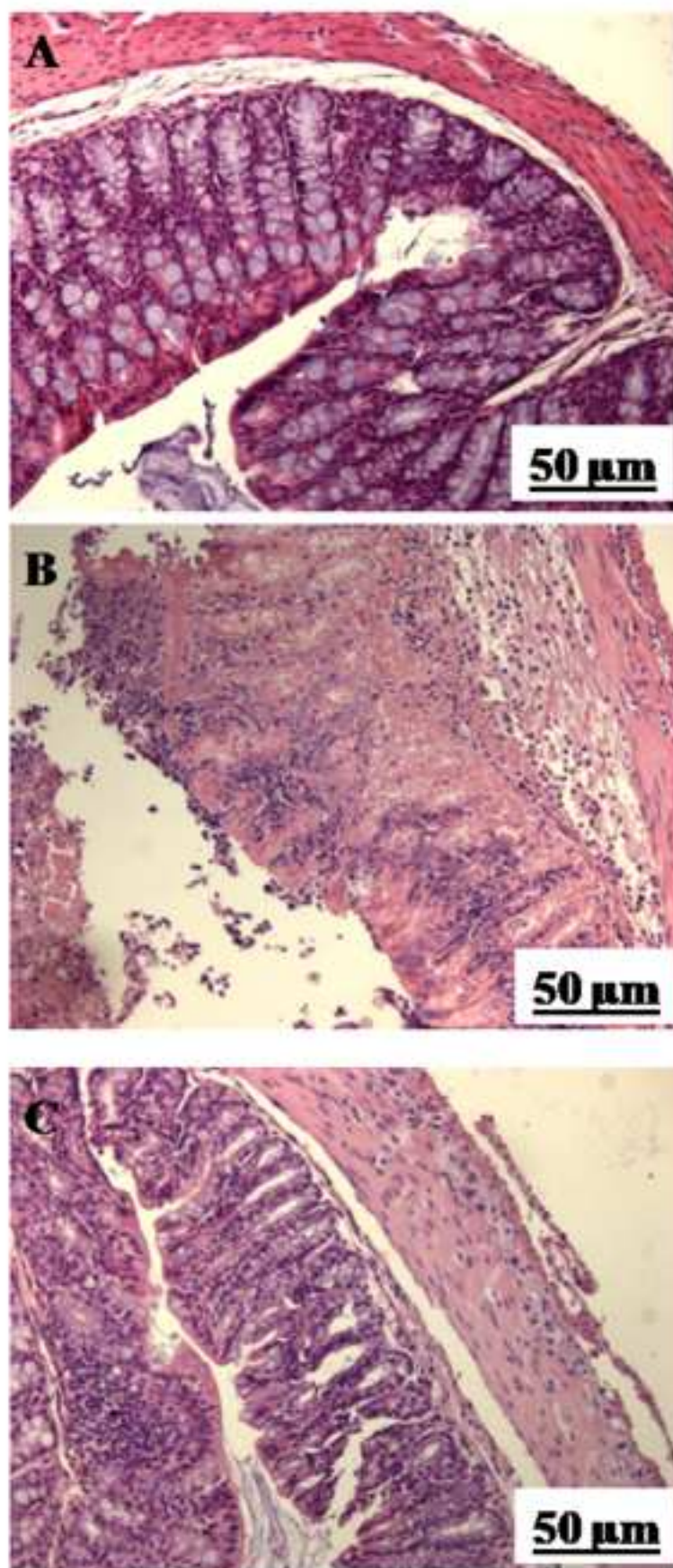
the effect of CBG (1 μ M) on inducible nitric oxide synthase (iNOS) expression in cell lysates, evaluated by western blot analysis (B, n=5) or RT-PCR (C, n=4), respectively. # p <0.001 vs control; * p <0.05, ** p <0.01 and *** p <0.001 vs LPS alone.

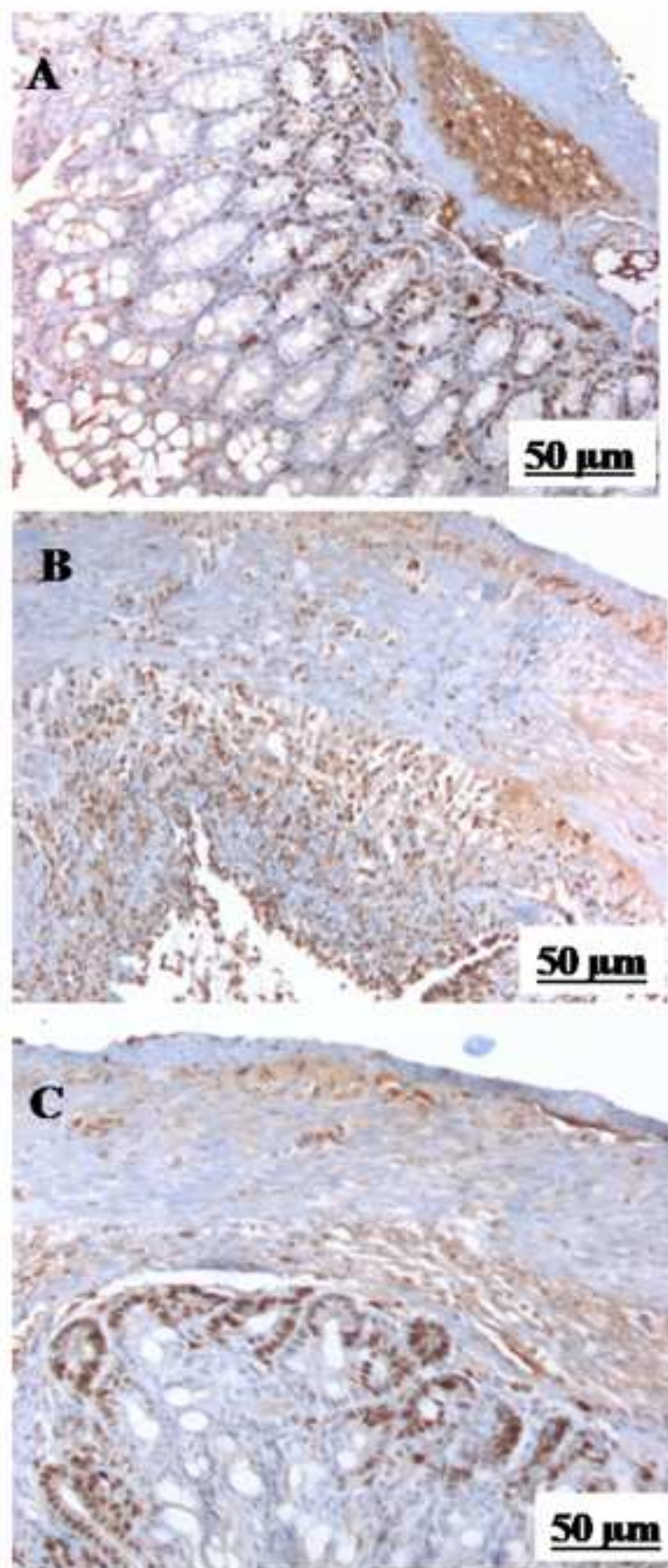
FIGURE 8. Effect of cannabigerol (CBG, 1 μ M) alone or in presence of the cannabinoid CB₁ receptor antagonist rimonabant (Rim, 0.1 μ M) (A) or the cannabinoid CB₂ receptor antagonist SR144528 (0.1 μ M) (B) on nitrite levels in the cell medium of murine peritoneal macrophages incubated with lipopolysaccharide (LPS, 1 μ g/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 \pm SEM of three experiments (in triplicates). # p <0.001 vs control; * p <0.05 and *** p <0.001 vs LPS alone; ° p <0.001 vs LPS+CBG.

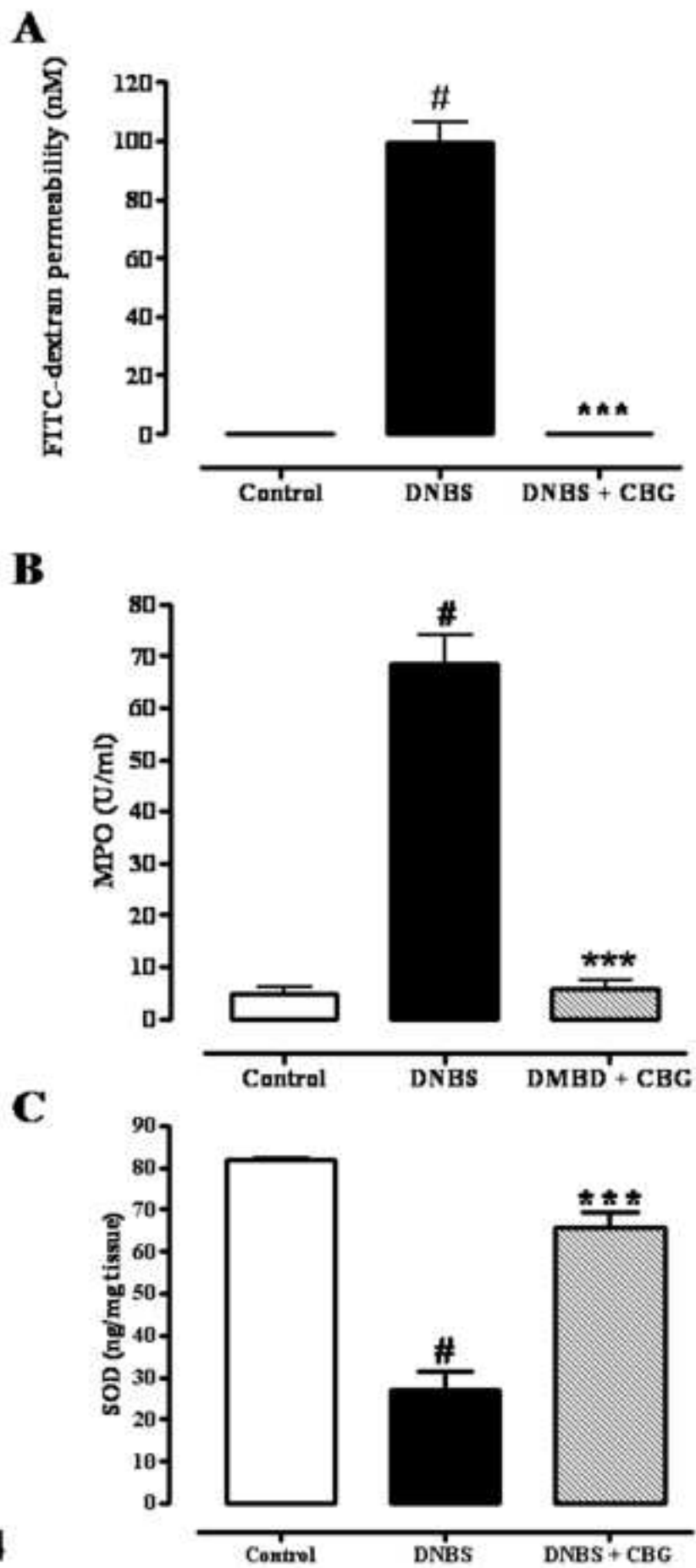
FIGURE 9. Relative mRNA expression of cannabinoid CB₁ receptor (A), cannabinoid CB₂ receptor (B) in cell lysates from macrophages incubated or not with lipopolysaccharide (LPS, 1 μ g/ml) for 18 h. Cannabigerol (CBG, 1 μ M) was added alone to the cell media or 30 min before LPS challenge. Data were analyzed by GENEX software for group wise comparisons and statistical analysis. Results are means \pm SEM of four experiments. # p <0.001 vs control.

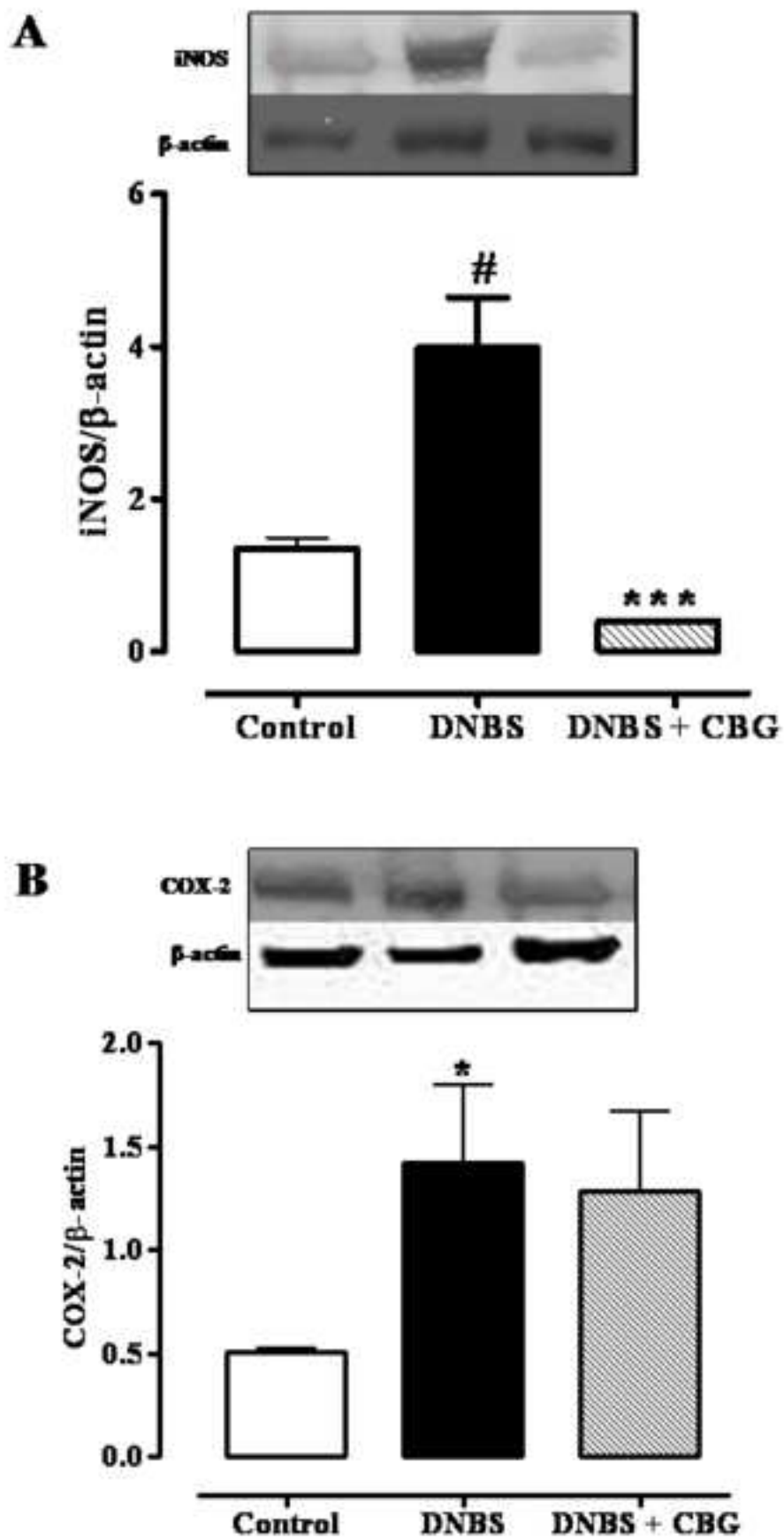
FIGURE 10. Reactive oxygen species (ROS) production produced by Fenton's reagent (2 mM H₂O₂/Fe²⁺) in Ptk6 null colonic epithelial cells after 24-h exposure to cannabigerol (0.1-10 μ M). Results are mean \pm SEM of five experiments. # p <0.001 vs control, * p <0.05 and ** p <0.01 vs H₂O₂/Fe²⁺ alone.

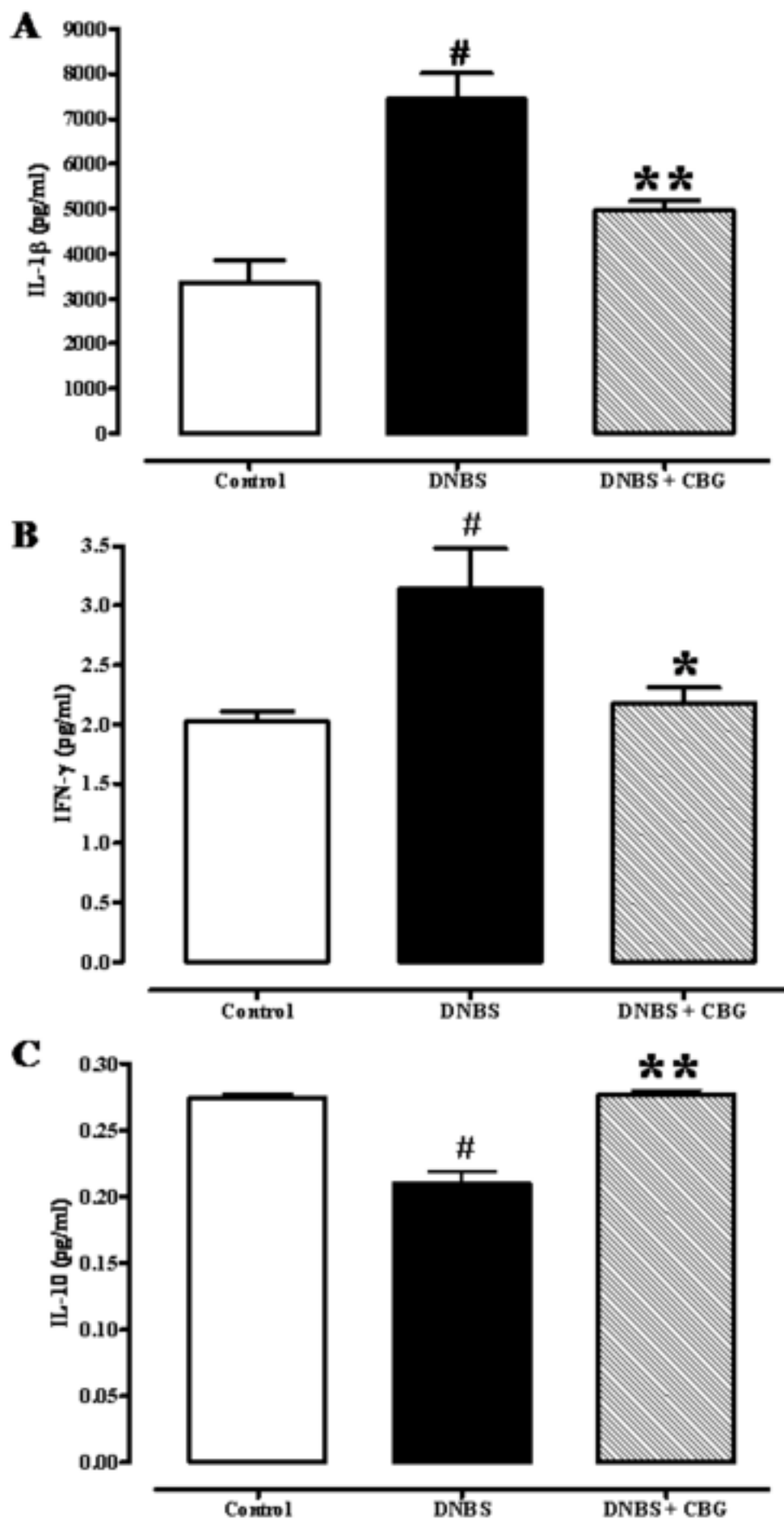
**Figure 1**

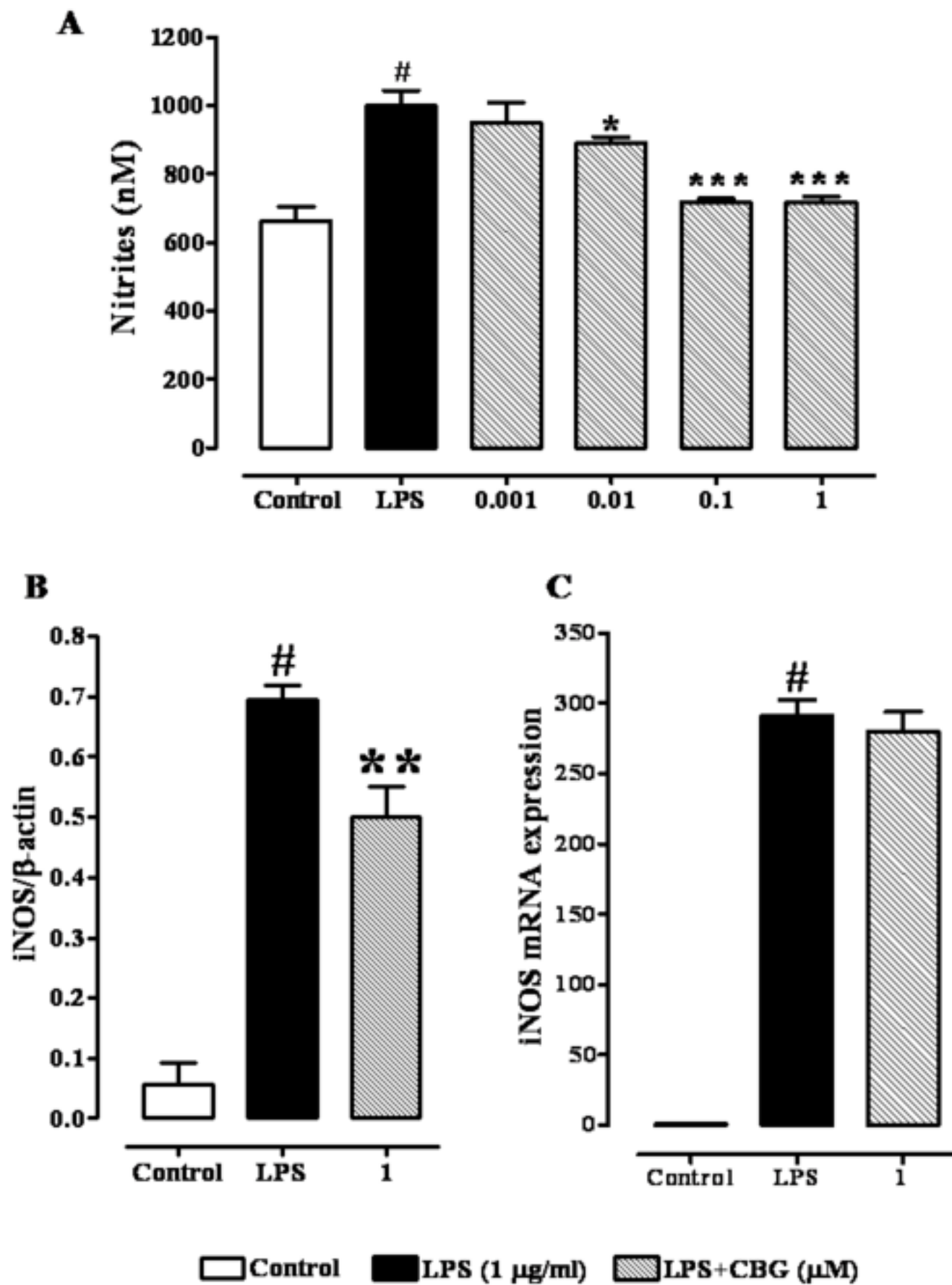
**Figure 2**

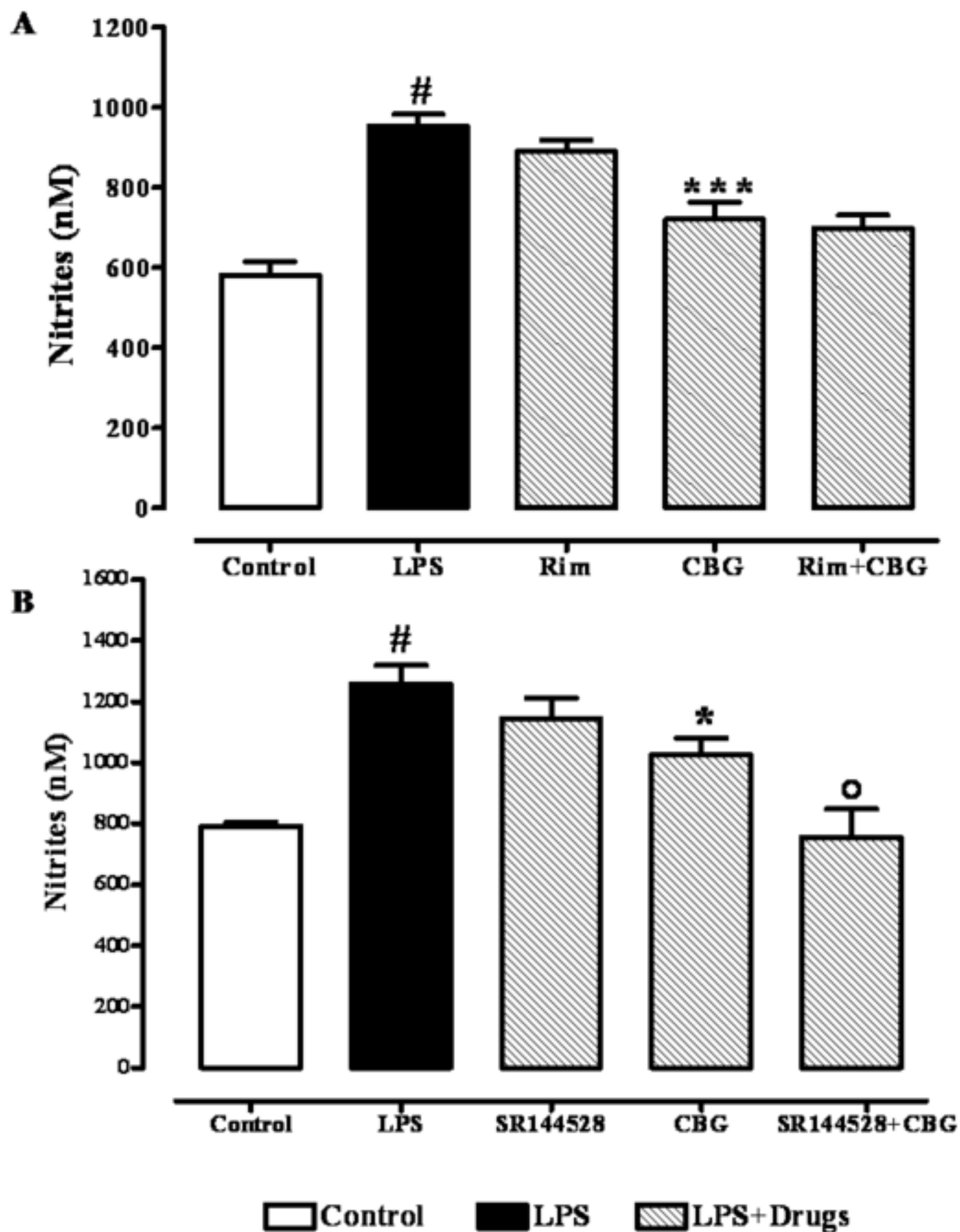
**Figure 3**

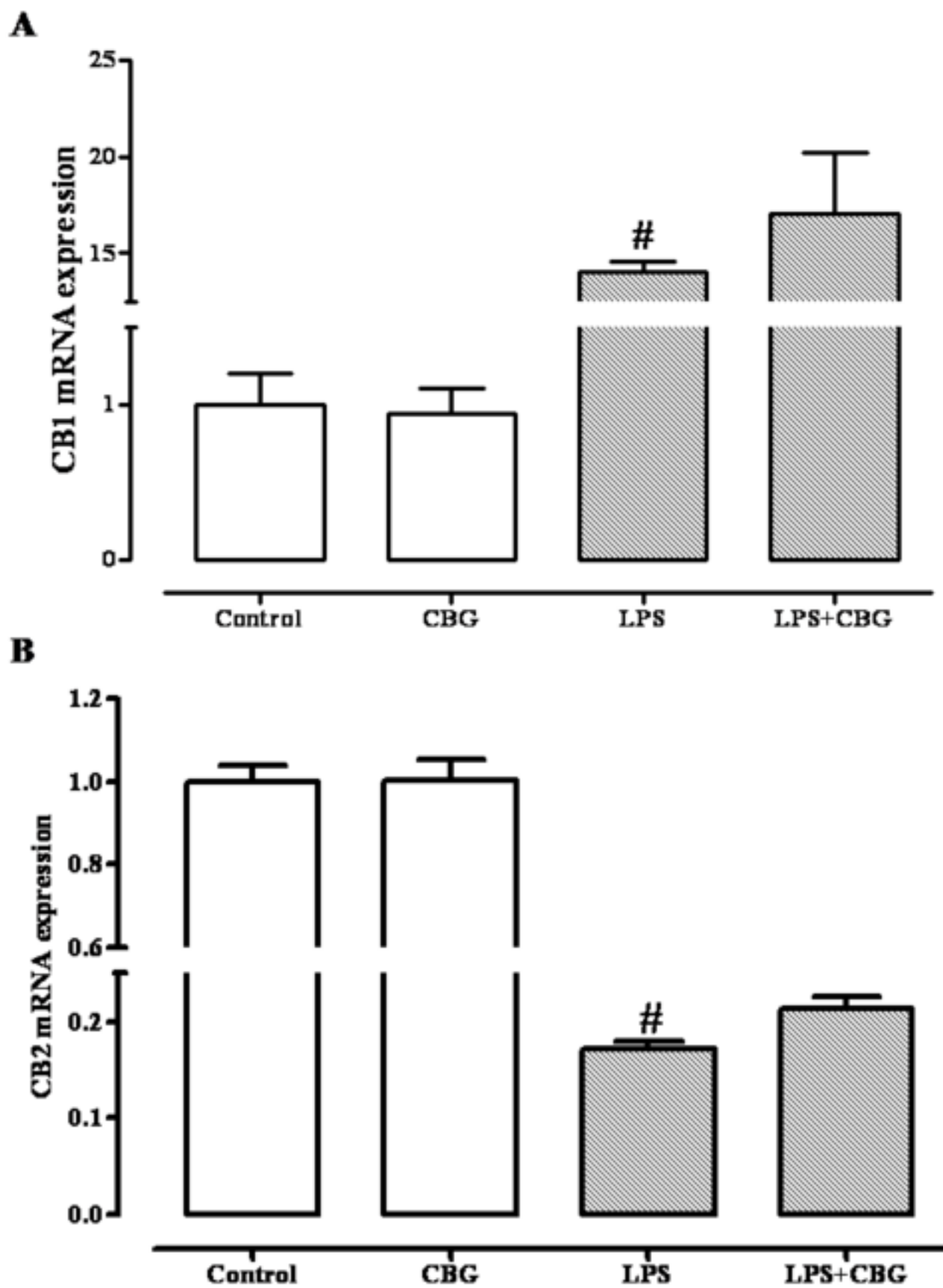
**Figure 4**

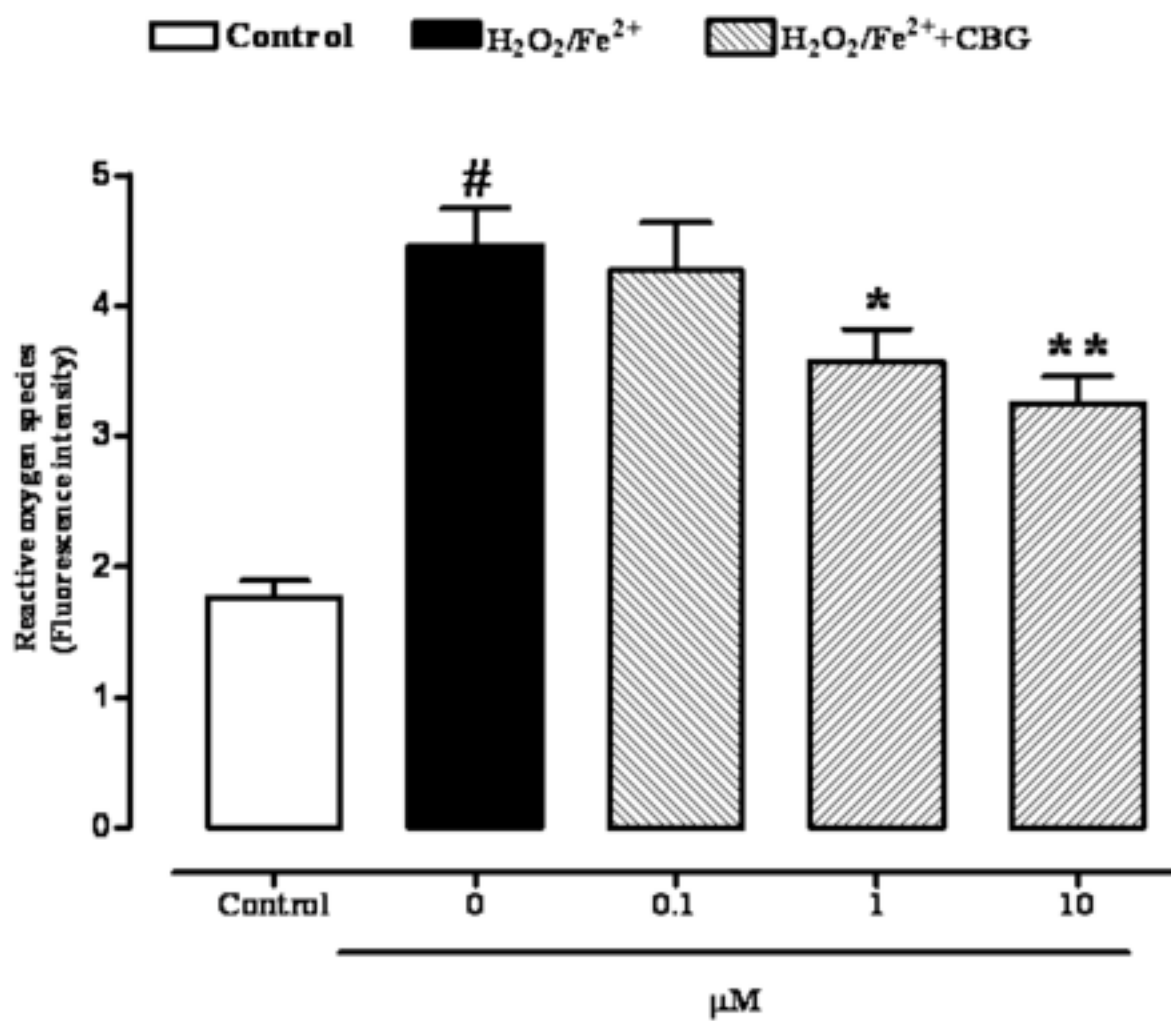
**Figure 5**

**Figure 6**

**Figure 7**

**Figure 8**

**Figure 9**

**Figure 10**

