Molecular Pharmacology Fast Forward. Published on September 13, 2016 as DOI: 10.1124/mol.116.105668
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Adelmidrol, a palmitoylethanolamide analogue, as a new pharmacological treatment for the management of inflammatory bowel disease

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Molecular Pharmacology Fast Forward. Published on September 13, 2016 as DOI: 10.1124/mol.116.105668
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Running title: Adelmidrol reduces colitis.

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Text pages: 33 pag.

Number of tables: 0

Figures: 10

References: 46

Number of words in the abstract: 240

Number of words in the introduction: 716

Number of words in the discussion: 1016

List of non-standard abbreviation: intercellular adhesion molecule 1 (ICAM-1); peroxisome proliferator-activated receptors (PPARs); Ulcerative colitis (UC); Crohn's disease (CD); inflammatory bowel disease (IBD); N-acylethanolamines (NAEs); N-palmitoylethanolamine (PEA); dinitrobenzene sulfonic acid (DNBS); 1,1,3,3-tetramethoxypropan/99% malondialdehyde bis (dymethylacetal)/99% (MDA);

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ABSTRACT

Leukocyte infiltration, improved levels of intercellular adhesion molecule 1 (ICAM-1) and oxidative stress in the colon are the most principal factors in inflammatory bowel disease. The goal of the current study was to explore the effects of adelmidrol, an analogue of the anti-inflammatory fatty acid amide signaling molecule palmitoylethanolamide, in mice subjected to experimental colitis. Additionally, in order to clarify if the protective action of adelmidrol is dependent on activation of peroxisome proliferator-activated receptors (PPARs), we investigated the effects of a PPARγ antagonist, GW9662, on adelmidrol action. Adelmidrol (10 mg/kg daily o.s.) was tested in a murine experimental model of colitis induced by intracolonic administration of dinitrobenzene sulfonic acid. Nuclear factor-κB translocation, cyclooxygenase-2 and phospho-extracellular signal-regulated kinase as well as tumor necrosis factor-α and interleukin-1β were significantly increased in colon tissues after dinitrobenzene sulfonic acid administration. Immunohistochemical staining for ICAM-1, P-selectin, nitrotyrosine and poly(ADP)ribose showed a positive staining in the inflamed colon. Treatment with adelmidrol decreased diarrhea, body weight loss and myeloperoxidase activity. Adelmidrol treatment, moreover, reduced nuclear factor-κB translocation, cyclooxygenase-2 and phospho-extracellular signal-regulated kinase expression, pro-inflammatory cytokine release, the incidence of nitrotyrosine and poly(ADP)ribose in the colon and decreased the up-regulation of ICAM-1 and P-selectin. Adelmidrol treatment produced a reduction of Bax and an intensification of Bcl-2 expression. This study clearly demonstrates that adelmidrol exerts important anti-inflammatory effects that are partly dependent on PPARγ, suggesting that this molecule may represent a new pharmacological approach for inflammatory bowel disease treatment.

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1. INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the foremost categories of inflammatory bowel disease (IBD), a class of autoimmune diseases, in which the body's own immune system assaults elements of the digestive apparatus(Reddy et al., 1991). In UC, the inflammatory process implies the mucosa and extends by continuity, beginning from the rectum. Two of the key characteristic symptoms of UC are bloody diarrhea and abdominal discomfort associated with fever(Ordas et al., 2012). In CD, the inflammatory event involves the entire wall of the gastrointestinal area and is propagated segmentally, not by continuity, from the oral cavity to the rectum(Baumgart and Sandborn, 2012). The most representative symptoms of CD are abdominal pain, loss of body mass, anemization and diarrhea associated with fever(Riordan et al., 1998). Chronic intestinal inflammation is linked with nitrosative and oxidative stress, which have been linked in numerous human diseases, including IBD(Wendland et al., 2001). Substantial evidence proposes that IBD is accompanied by an imbalance among reactive oxygen species (ROS) and antioxidant activity which generates oxidative stress as the result of ROS overproduction such as decreased antioxidant activity(Halliwell, 1997).

Macrophages play a focal role in the improvement of noncaseous epithelioid granuloma in the intestinal mucosa, which is typical of Crohn's disease; they are involved also in the mucosal immune response. Activated macrophages produce cytokines for example tumor necrosis factor (TNF)-α, interleukin (IL)-1β, among others. Additionally, recruitment of inflammatory cells from circulation is a main process in amplifying the inflammatory response(Sandborn and Hanauer, 1999). TNF-α and IL-1β increase the levels of adhesion molecules in the vascular endothelium, leading to the penetration of inflammatory cells into the mucosal layer. Critical performers in this process are the selectins, vascular cell adhesion molecule-1, and intercellular adhesion molecule 1 (ICAM-1), which are expressed on the vascular endothelium surface(Koizumi et al., 1992).

Several animal models of IBD have been established which show similarities to human CD, although most do not predict what happens in human immunology. Amidst these models, colonic

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inflammation provoked by the intrarectal administration of dinitrobenzene sulfonic acid (DNBS) in mice is considered a well-documented model for identification of new pharmacological targets to treat human CD(Impellizzeri et al., 2015a). Such approaches include anti-TNF-antibodies, whose main aim is to induce clinical remission on a long-term basis and thereby realize an improved patient quality of life.

Different studies have determined that N-acylethanolamines (NAEs), which are widely expressed in mammals, are involved in a range of physiological functions, including inflammation, neurotransmission, analgesia, reproduction, appetite and cytoprotection(Schmid and Berdyshev, 2002). Like other NAEs, N-palmitoylethanolamine (PEA) has been extensively studied for its anti-inflammatory and neuroprotective effects(Hansen et al., 2002; Scuderi et al., 2012). PEA inhibits mast cell activation, stabilizes mitochondrial function and inhibits degradation of the endocannabinoid anandamide, which also has neuroprotective effects. Further, PEA may activate peroxisome proliferator-activated receptor α (PPARα), and/or potentiate microglial cell motility in a cannabinoid receptor-independent manner (Franklin et al., 2003; Impellizzeri et al., 2015b; Nagayama et al., 1999; Skaper et al., 1996). Interestingly, as previously demonstrated, in addition to the PPARα receptor activation, also the PPARδ and PPARγ receptors contribute to the anti-inflammatory activity of PEA (D'Agostino et al., 2007; Paterniti et al., 2013).

Moreover, PEA reportedly improved outcome in a murine model of experimental colitis, the effect being mediated by cannabinoid CB2 receptors, GPR55 and PPARα, and modulated by transient receptor potential cation channel subfamily V member 1 - all of which are involve ed in the control of intestinal inflammation(Borrelli et al., 2015). The anti-inflammatory ethanolamide derivative adelmidrol (International Nonproprietary Name), is a naturally-occurring saturated dicarboxylic acid found in trace amounts in the human body and in some whole grains. Adelmidrol (*N,N'*-bis(2-hydroxyethyl)nonanediamide) possesses both hydrophilic and hydrophobic properties that favor its solubility both in aqueous and organic media(De Filippis et al., 2009). Topical treatment with adelmidrol is reported to increase mast cell granular density, thereby suggesting a

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decrease in their degranulation(De Filippis et al., 2009). In addition, adelmidrol displayed some benefit in a pilot study on mild atopic dermatitis(Pulvirenti et al., 2007).

Our study was designed to evaluate the effects of adelmidrol in an animal model of IBD. In order to investigate whether the mechanism of action of adelmidrol treatment was similar to one previously studied for PEA, we have investigated the role of the PPARα and PPARγ and CB2 receptors on the adelmidrol anti-inflammatory property.

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2. MATERIALS AND METHODS

2.1 Animals

Male adult CD1 mice (25-30 g, Envigo, Italy) and male mice (20-27 g, Envigo, Italy) with a targeted disruption of the PPARα gene (PPARα KO) and littermate wildtype controls (PPARα WT) were placed in a controlled environment and maintained on a 12-h light/dark cycle with food and water available *ad libitum*. The study was permitted by the University of Messina Review Board for the care of animals. (D.M.116192 and O.J. of E.C. L 358/1 12/18/1986).

2.2 Induction of experimental colitis

Colitis was made as previously described(Impellizzeri et al., 2015a). Following colitis animals were observed for four days. After this period, the animals were weighed and anaesthetized with chloral hydrate and the abdomen opened by a midline cut. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border, washed, weighed and processed for histological and biochemical studies.

2.3 Experimental groups

Mice were casually divided into the following groups (10 for each group):

1. Sham + vehicle group: vehicle solution (saline) was given by oral somministration (o.s.) for 4 days.
2. Sham + adelmidrol (10 mg/kg): administered by o.s. for 4 days.
3. DNBS + vehicle: Mice were injected by DNBS as above, and vehicle (saline) was given by o.s. daily, for 4 days, starting 60 minutes following the injection of DNBS.
4. DNBS + adelmidrol (10 mg/kg): Mice were injected by DNBS as above, and adelmidrol (10 mg/kg) was given by o.s. daily, for 4 days starting 60 minutes following administration of DNBS.

In order to better investigate whether the mechanism of action of adelmidrol is related to the

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activation of PPARα and PPARγ or CB2 receptors, we performed the following experimental groups:

1. Sham + vehicle group: vehicle solution (saline) was given by o.s. for 4 days.
2. Sham + adelmidrol (10 mg/kg): administered by o.s. for 4 days.
3. Sham + SR144528 (1mg/kg): administered by o.s. for 4 days.
4. Sham PPARα WT + vehicle group: vehicle solution (saline) was given by o.s. for 4 days.
5. Sham PPARα KO mice + vehicle group: vehicle solution (saline) was given by o.s. for 4 days.
6. Sham PPARα WT + adelmidrol (10 mg/kg): administered by o.s. for 4 days.
7. Sham PPARα KO mice + adelmidrol (10 mg/kg): administered by o.s. for 4 days.
8. DNBS + vehicle: Mice were injected by DNBS as above, and vehicle (saline) was given by o.s. daily, for 4 days, starting 60 minutes following the injection of DNBS.
9. DNBS+ vehicle: PPARα WT mice were injected by DNBS as above;
10. DNBS+ vehicle: PPARα KO mice were injected by DNBS as above;
11. DNBS+adelmidrol (10 mg/kg): PPARα WT mice were injected by DNBS as above, and adelmidrol (10 mg/kg) was given by o.s. daily, for 4 days starting 60 minutes following administration of DNBS
12. DNBS+adelmidrol (10 mg/kg): PPARα KO mice were injected by DNBS as above, and adelmidrol (10 mg/kg) was given by o.s. daily, for 4 days starting 60 minutes following administration of DNBS
13. DNBS + GW9662 (1mg/kg): Mice were injected by DNBS as above, but GW9662 (1mg/kg), was given by o.s. daily, for 4 days, starting 60 minutes following the injection of DNBS;

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14. DNBS + GW9662 (1mg/kg) + adelmidrol (10 mg/kg): Mice were injected by DNBS as above, but GW9662 (1mg/kg), was given orally 30 min before adelmidrol administration;

15. DNBS + SR144528 (1mg/kg): Mice were injected by DNBS as above, but SR144528 (1mg/kg), was given by o.s. daily, for 4 days, starting 60 minutes following the injection of DNBS

16. DNBS + SR144528 (1mg/kg) + adelmidrol (10 mg/kg): Mice were injected by DNBS as above, but SR144528 (1mg/kg), was given orally 30 min before adelmidrol administration;

2.4 Evaluation of colon damage

After removal, the entire colon was gently rinsed with saline solution, opened by a longitudinal cut and immediately observed under a microscope. Colon injury (macroscopic damage score) was evaluated and scored by two independent observers as previously described: 0, no damage; 1, restricted hyperemia without ulcers; 2, linear ulcers with not important inflammation; 3, linear ulcers with inflammation at one site; 4, two or more major sites of inflammation and ulceration extending >1 cm along the length of the colon; and 5–8, one point is added for each centimeter of ulceration beyond an initial 2 cm (Cuzzocrea et al., 2003; Zingarelli et al., 1993).

2.5 Tissue processing and histology

Concisely, paraffin tissue slices (thickness, 7 μm) were deparaffinized with xylene, stained with hematoxylin and eosin and observed by light microscopy (AxioVision, Zeiss, Milan, Italy) by a qualified histopathologist. The degree of inflammation on microscopic cross-sections of the colon was graded semi-quantitatively from 0 to 4 as previously described by Impellizzeri et al(Impellizzeri et al., 2015a) in particular the following morphological criteria were considered: score 0, no damage; score 1 (mild), focal epithelial edema and necrosis; score 2 (moderate), diffuse swelling and necrosis of the villi; score 3 (severe), necrosis with presence of neutrophil infiltrate in the submucosa; score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and

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hemorrhage (Cuzzocrea et al., 2004).The scores from all sections of each colon were averaged to give a final score for each mouse. All histological analyses were performed in a blinded fashion.

2.6 Myeloperoxidase (MPO) assay

Neutrophil infiltration in the colon was examined by determining tissue MPO activity using a spectrophotometric assay with tetramethylbenzidine as substrate, according to a previously published method(Mullane et al., 1985). Following DNBS injection, colon tissues were collected and weighed. Every piece of tissue was homogenized in a mixture containing 0.5% hexadecyltrimethyl ammonium bromide dissolved in 10 mM potassium phosphate buffer pH 7 and centrifuged for 30 min at 20,000 x g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2. The degree of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was described as the quantity of enzyme degrading 1 mmol of peroxide per min at 37°C and was expressed in U/g wet tissue.

2.7 Thiobarbituric acid- reactant substances measurement

Thiobarbituric acid-reactant substances measurement was determined in colon tissue 4 days after DNBS administration, as a marker of lipid peroxidation,. Thiobarbituric acid-reactant substances were calculated by comparison the OD_{532} to standard mixture of 1,1,3,3-tetramethoxypropan/99% malondialdehyde bis (dymethylacetal)/99% (MDA) (Sigma, Milan). The absorbance of the supernatant was measured spectrophotometrically at 532 nm.

2.8 Immunohistochemical localization of TNF-α, IL-1β, ICAM-1, P-selectin, poly(ADP-ribose) (PAR) and nitrotyrosine

Seven days after DNBS administration colon tissues were collected and fixed for 24 h in paraformaldehyde (4% in 0.1M PBS) at room temperature, dehydrated through a graded series of ethanol and xylene and embedded in BioPlast Plus (Bio Optica, Milan, Italy). Thereafter, 7 μm

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sections were cut from the paraffin-embedded tissue. Following deparaffinization with xylene and graded ethanol as above, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Slices were permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was diminished by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin and avidin binding sites were blocked by progressive incubation for 15 min with biotin and avidin (Vector Laboratories, Burlingame, CA, USA), respectively. Subsequently, slices were incubated overnight with anti-TNF-α murine polyclonal antibody (1/100 in PBS, v/v, Santa Cruz Biotechnology), or anti- IL-1β murine polyclonal antibody (1/100 in PBS, v/v, Santa Cruz Biotechnology), anti-ICAM-1 murine polyclonal antibody (1/100 in PBS, v/v, Santa Cruz Biotechnology), anti-P-selectin murine polyclonal antibody (1/100 in PBS, v/v, Santa Cruz Biotechnology), anti-PAR murine polyclonal antibody (1/100 in PBS, v/v, Santa Cruz Biotechnology) and anti-nitrotyrosine rabbit polyclonal antibody (1:200 in PBS, v/v, Millipore). Sections were rinsed with PBS and incubated with peroxidase-conjugated bovine anti-mouse immunoglobulin G (IgG) secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2,000 Jackson Immuno Research, West Grove, PA, USA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG or biotin-conjugated goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA). To verify the binding specificity for TNF-α, IL-1β, ICAM-1, P-selectin, PAR and nitrotyrosine, control slices were incubated with only primary antibody or secondary antibody. In these controls no positive staining was detected. Immunohistochemical images were collected using a Zeiss microscope and Axio Vision software. For graphic display of densitometric analyses, the intensity of positive staining (brown staining) was measured by computer-assisted color image analysis (Leica QWin V3, UK). The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as percent of total tissue area (red staining). Replicates for every experimental condition and histochemical staining were acquired from each mouse in all experimental group. In sham-operated mice, the central areas of equivalent tissue

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sections were taken as reference points and a comparable number of optical fields counted(Shea, 1994). All histological analyses were carried out by an observer without knowledge of the treatments.

2.9 Western blot analysis for Ikb-α, nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2), phospho-extracellular signal-regulated kinase (p-ERK), Bcl-2, Bax, lamin a/c and β-actin

The levels of Ikb-α, NF-κB, COX-2, p-ERK, Bcl-2, Bax, lamin a/c and β-actin were calculated, as previously described, in cytosolic and nuclear fractions from colon tissue collected at the end of experiment with minor modifications(Bethea et al., 1998). Colon tissue from each mouse were suspended in extraction Buffer A containing 0.2mM phenylmethylsulfonyl fluoride, 0.15 mM pepstatin A, 20 mM leupeptin, 1 mM sodium orthovanadate, homogenized at the maximum setting for 2 min, and centrifuged at 12000 x rpm for 4 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in Buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM leupeptin, 0.2 mM sodium orthovanadate. After centrifugation 10 min at 12000 rpm at 4°C, the supernatants containing the nuclear protein were stored at -80°C for further analysis. The filters were blocked with 1x PBS, 5% (w/v) non-fat dried milk (PM) for 40 min at room temperature and successively probed with anti-Ikb-α (1/500 in PBS, v/v, Santa Cruz Biotechnology), anti-NF-κB (1/500 in PBS, v/v, Santa Cruz Biotechnology), anti-COX-2 (1/500 in PBS, v/v, Cayman), anti-p-ERK (1/500 in PBS, v/v, Santa Cruz Biotechnology), anti-Bax (SantaCruz Biotechnology 1/500 in PBS, v/v), anti-Bcl-2 (1/500 in PBS, v/v, Santa Cruz Biotechnology) and anti-lamin a/c (1/500 in PBS, v/v, Santa Cruz Biotechnology) in 1x PBS, 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT) at 4 ºC overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson Immuno Research, West Grove, PA) for 1 h at room temperature. To establish that blots were loaded with equivalent amounts of protein lysates, they were similarly

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incubated with antibody against β-actin (1/1000 in PBS, v/, Santa Cruz Biotechnology v). Relative expression of the protein bands for Ikb-α (37 kDa), NF-κB (65 kDa), COX-2 (72 kDa), p-ERK (46 kDa), Bcl-2 (29 kDa), Bax (23 kDa), lamin a/c (65 kDa) and and β-actin (42 kDa) were detected with the enhanced chemiluminescence detection system according the to manufacturer's instructions (SuperSignal West Pico Chemiluminescent Substrate, Pierce). Expression of protein bands was calculated by densitometry with Bio-Rad ChemiDoc™ XRS□+□software and standardized to β-actin levels. Images of blot signals (8-bit/600-dpi resolution) were imported to an analysis program (Image Quant TL, v2003). Commercially available molecular weight markers (10 to 250 kDa) were used to establish molecular weight positions.

2.10 Materials

Adelmidrol was obtained from Epitech Group Spa (Saccolongo, Italy). All compounds were purchased from Sigma Aldrich (Milan, Italy). All chemicals were of the maximum commercial grade available. All stock mixes were made in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

2.11 Statistical evaluation

All values in the images and text are expressed as mean ± standard error of the mean (SEM) of N observations. For *in vivo* studies N represents the number of animals. In experiments involving histology and immunoistochemistry, the pictures shown are demonstrative of at least three independent experiments. Moreover, western blot images are representative of three different gels obtained by dividing the number of samples from 10 animals for each experimental group on different days.A *p*-value of less than 0.05 was appraise significant. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons.

3. RESULTS

3.1 Effects of adelmidrol treatment on the degree of colitis in DNBS-challenged mice

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Histological analysis of colonic mucosa of sham-treated mice showed an intact epithelium (Fig. 1A, see macroscopic score 1D; Fig. 2A,A'; view histological score 2D). Four days after intra-colonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. Macroscopic examination of cecum, colon and rectum presented with mucosal congestion, erosion and hemorrhagic ulcerations (Fig. 1B; see macroscopic score 1D). The histopathological features included transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon sections from DNBS-injected mice (Fig. 2B,B'; view histological score 2D). Adelmidrol (10 mg/kg o.s.) reduced significantly the degree and severity of the macroscopic (Fig. 1C, see macroscopic score 1D) and histological signs of colon injury (Fig. 2C,C'; view histological score 2D). Moreover, four days after colitis induced by DNBS treatment, all mice had diarrhea and a reduction in body weight (compared with the sham groups) (Fig. 1E). Adelmidrol (10 mg/kg o.s.) treatment reduced significantly the loss of body weight (Fig. 1E). The IBD induced by DNBS intrarectal administration was also characterized by an augmentation in MPO activity, an indicator of neutrophil accumulating in the colon (Fig. 2E). This was consistent with light microscopic observations showing the colon of vehicle-treated DNBS-mice to contain a large number of neutrophils. On the contrary, adelmidrol (10 mg/kg o.s.) significantly reduced the degree of polymorphonuclear cell infiltration (determined as reduction in MPO activity) in inflamed colon (Fig. 2E).

3.2 Effects of adelmidrol treatment on NF-κB pathway, COX-2 and p-ERK expression in DNBS-challenged mice

In order to explore the cellular mechanism(s) underlying adelmidrol mitigation of the development of DNBS-induced colitis, we used Western blot analysis to evaluate IκB-α degradation and nuclear NF-κB p65 translocation. NF-κB p65 levels in the colon nuclear fractions were substantially improved four days after DNBS injection compared to sham mice (Fig. 3B; see densitometric examination 3B'). Treatment with adelmidrol (10 mg/kg o.s.) reduced the colon levels of NF-kB

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p65 (Fig. 3B; see densitometric examination 3B'). Basal expression of IkB-α detected in colon samples from sham-treated animals were significantly reduced in colon tissues from vehicle-treated animals four days after DNBS injection (Fig. 3A; see densitometric examination 3A'). Adelmidrol treatment prevented DNBS-induced IκB-α degradation (Fig. 3A; see densitometric examination 3A'). In addition, Phosphorylation of ERK1/2 plays a role in the control of gene expression, for example, COX-2, mediating the inflammatory responses. Basal levels of COX-2 in homogenates from colon tissues of sham-operated animals (Fig. 3C; see densitometric examination 3C') were significantly increased in colon tissue of DNBS-injected mice. Treatment of mice with adelmidrol (10 mg/kg o.s.) reduced DNBS-mediated COX-2 expression (Fig. 3C; see densitometric examination 3C'). This increase in COX-2 expression appeared to correspond to an activation of signal transduction pathways involved in its regulation, as pERK1/2 levels were significantly increased in the colon tissues of DNBS-injected mice (Fig. 3D; see densitometric examination 3D'). Likewise, treatment with adelmidrol significantly reduced pERK1/2 activation (Fig. 3D; see densitometric examination 3D').

3.3 Adelmidrol treatment reduces cytokine production in DNBS-challenged mice

Immunohistological analysis of colon tissue was carried out to determine whether adelmidrol may modulate the secondary inflammatory reaction also by regulating secretion of cytokines, such as TNF-α and IL-1β. A substantial increase of TNF-α and IL-1β production was found in colon samples collected from DNBS-injected mice compared to sham-operated mice (Fig. 4B,E; see densitometric examination 4G'). Colon levels of TNF-α and IL-1β were significantly attenuated in mice treated with adelmidrol (10 mg/kg o.s.) (Fig. 4C,F; see densitometric examination 4G'). There was no staining for TNF-α and IL-1β in colon obtained from sham groups of mice (Fig. 4A,D; see densitometric examination 4G').

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3.4 Adelmidrol treatment reduces ICAM-1 and P-selectin expression in DNBS-challenged mice

The potential contribution of intestinal expression of ICAM-1 and P-selectin to cell recruitment throughout colon inflammation was next examined. Positive staining for ICAM-1 and P-selectin (Fig. 5B,E; see densitometric examination 5G) was significantly increased in vessels of the lamina propria and submucosa as well as in epithelial cells of injured colon and in infiltrating inflammatory cells in damaged tissues from DNBS-injected mice. Treatment with adelmidrol (10 mg/kg o.s.) reduced staining for ICAM-I and P-selectin (Fig. 5C,F; see densitometric examination 5G) in colon tissues of DNBS-injected mice. No positive staining for ICAM-I and P-selectin was observed in colon tissues of sham-treated mice (Fig. 5A,D; see densitometric examination 5G).

3.5 Effects of adelmidrol treatment on nitrotyrosine and PAR formation and lipid peroxidation in DNBS-challenged mice

Immunohistochemistry for PAR revealed positive staining localized in the nuclei of inflammatory cells in colon tissues from DNBS-injected mice (Fig. 6B; see densitometric examination 6G). Adelmidrol (10 mg/kg o.s.) significantly reduced the extent of PAR immunoreactivity in the colon (Fig. 6C; see densitometric examination 6G). Four days after DNBS administration, nitrotyrosine, a indicator of nitrosative stress, was assessed by immunohistochemical analysis in terminal colon sections to identify “peroxynitrite formation” and/or other nitrogen derivatives formed during experimental colitis. Colon sections from sham-treated mice did not stain for nitrotyrosine (Fig. 6D; see densitometric examination 6G), but sections from DNBS-injected mice displayed positive nitrotyrosine immunostaining mainly in inflammatory cells and around vessels (Fig. 6E; see densitometric examination 6G). Treatment with adelmidrol (10 mg/kg o.s.) reduced the degree of nitrotyrosine immunoreactivity in the colon (Fig. 6F; see densitometric examination 6G). In addition, plasma levels of thiobarbituric acid-reactant substances, as an indicator of lipid peroxidation, increased significantly in from mice subjected to DNBS when compared with sham-

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operated mice (Fig. 6H). Thiobarbituric acid-reactant substance levels were significantly attenuated in DNBS-treated with adelmidrol (10 mg/kg o.s.) (Fig. 6H).

3.6 Effects of adelmidrol treatment on the apoptotic pathway in DNBS-challenged mice

To test whether colon damage was connected with apoptosis, four days after DNBS, the presence of proteic effectors of canonical mitochondrial apoptosis, such as Bax and Bcl-2, was investigated by Western blot analysis. Bax levels was appreciably increased in the colon from mice subjected to DNBS (Fig. 7B; see densitometric examination 7B'). In contrast, adelmidrol treatment (10 mg/kg o.s.) prevented DNBS-induced Bax expression (Fig. 7B; see densitometric examination 7B'). On the other hand adelmidrol administration (10 mg/kg o.s.) increased Bcl-2 expression in DNBS-injected mice (Fig. 7A; see densitometric examination 7A'), essentially reaching the basal level of Bcl-2 (Fig. 7A; see densitometric examination 7A').

3.7 Role of PPARγ receptors in the systemic anti-inflammatory effects of adelmidrol in DNBS-induced colitis

In order to investigate the mechanism of adelmidrol action, we used the potent PPARγ antagonist, GW9662, in the model of DNBS-induced colitis. Histological evaluation by hematoxylin and eosin staining revealed that mice that have received GW9662 after DNBS-injection showed an intensification of the damage as well as an increase in MPO levels similar to DNBS group (Fig. 8B,C; see macroscopic score 8F; Fig.9B,C; view histological score 9F). Moreover, DNBS-injected mice pretreated with GW9662 displayed limited protection by adelmidrol, suggesting a possible antagonist effect of GW9662 pretreatment on adelmidrol action (Fig. 8D,E; see macroscopic score 8F; Fig.9D,E; view histological score 9F). Additionally, four days after colitis induced by DNBS treatment, mice pretreated with GW9662 showed a major loss in body weight (Fig. 8G) and an intensification in myeloperoxidase activity (Fig. 9 G) compared with adelmidrol-treated mice.

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3.8 Role of PPARα and CB2 receptors in the systemic anti-inflammatory effects of adelmidrol in DNBS-induced colitis

Histological evaluation by hematoxylin and eosin staining showed a transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon sections from DNBS groups (data not shown). The histological alteration was also found in PPARα WT and KO mice and in DNBS injected mice that have received SR144528 (Fig. 10A,A'; 10C,C' and 10 E,E' view histological score 10G and 10H) compared to sham groups (data not shown). Moreover, adelmidrol treatment was able to decrease the histological alteration in all experimental groups suggesting that the absence of PPARα receptor or pretreatment with SR144528 did not modify the action of adelmidrol (Fig. 10B,B'; D,D' and 10F,F' view histological score 10G and 10H).

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4. DISCUSSION

Inflammatory bowel disease is a chronic, relapsing, inflammatory condition of the gastrointestinal tract and includes UC and CD, which show differences in pathology and clinical characteristics. Presently, the etiology and pathogenesis of IBD are poorly understood. It is widely accepted that the pathogenesis of IBD involves genetic and environmental factors(Pierik et al., 2005; Ye et al., 2015). Until recently the main goal of IBD therapy was to achieve and preserve clinical remission, whereas today's therapeutic aim is that of a prolonged remission characterized by sustained clinical remission, complete mucosal healing and normalization of serological markers of inflammation. The most frequently applied biological agents in CD and UC are TNF-α-targeted therapies, a class of medication that has modernized the treatment of these disorders and the quality of life for patients. However, they also pose risks of developing various side effects including infections, exacerbation of some neurological manifestations, cutaneous lesions or antibody production(Casili et al., 2015).

Adelmidrol, a diethanolamide derivative of azelaic acid, is effective in the treatment of human inflammatory disorders, and its mechanism of action has been extensively investigated(Mastrofrancesco et al., 2010). Adelmidrol belongs to the aliamide family with similar anti-inflammatory and anti-nociceptive proprieties as PEA and able to control mast cell hyper-reactivity in several pathophysiological conditions(Aloe et al., 1993; Costa et al., 2008; De Filippis et al., 2011; Esposito et al., 2011; Genovese et al., 2008).

Here, we investigated the beneficial effects of adelmidrol in an experimental model of IBD. In particular, adelmidrol attenuated DNBS-induced colitis in mice. At the macroscopic and histological level, epithelial disruption was significant reduced in mice treated with adelmidrol. Further, mice treated with adelmidrol were more resistant to DNBS-induced colitis, showing a marked resolution of the macroscopic and histological markers of the inflammatory process. This

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was also reflected in a decreased weight loss as well as a reduction of MPO activity in mice treated with adelmidrol compared to the DNBS-injected group.

NF-κB is an important mediator of inflammation. In response to a wide range of stimuli including oxidative stress, infection, hypoxia, extracellular signals and inflammation, IκBα is phosphorylated by IκB kinase(Bowie and O'Neill, 2000). This results is the release of the NF-κB dimer, which then translocates to the nucleus. DNBS induced a significant degradation of IκBα and a consequent increase in the nuclear translocation of the subunit p65, whereas adelmidrol treatment significantly reduced NF-κB translocation and inhibited IκBα degradation. Moreover, intestinal epithelial cells express COX-2 when stimulated by pro-inflammatory factors, such as lipopolysaccharide and oxidative stress(Longo et al., 1998). Adelmidrol reduced COX-2 and p-ERK DNBS-induced expression.

TNF-α and IL-1β are the most important cytokines present in colon tissues during the evolution of colitis(Kapsoritakis et al., 2001). The present study confirmed expression of TNF-α and IL-1β in colon tissues from DNBS-injected mice. Importantly, treatment with adelmidrol reduced these inflammatory cytokines levels. It has been suggested that during acute and chronic colitis the continuous production of pro-inflammatory cytokines plays a key role in the adherence and infiltration of leukocytes to endothelial cells, thus maintaining chronic inflammation within the cecal and colonic interstitium(Kawachi et al., 2000). Here, we confirmed that DNBS injection induced the manifestation of P-selectin on the endothelial vascular wall and up-regulated surface expression of ICAM-1 on endothelial cells. Treatment with adelmidrol halted expression of P-selectin and ICAM-1 up-regulation without effecting constitutive levels of ICAM-1 on endothelial cells. Activated neutrophils produce ROS and reactive nitrogen species within intestinal mucosa, which provoke oxidative stress, leading to DNA single-strand damage, PAR synthetase activation and cell death(Mandalari et al., 2011; Szabo and Dawson, 1998). In accord with this process, we detected an increased staining for nitrotyrosine and PAR in DNBS-injected mice, which was blunted by adelmidrol treatment. One form of epithelial cell damage in the inflamed colonic mucosa

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has been described to involve apoptosis(Araki et al., 2010). Interestingly, pro-apoptotic transcriptional changes, including up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2, were observed by Western blot. Treatment with adelmidrol lowered the signal for Bax when compared to DNBS injected mice while the signal for Bcl-2 in DNBS mice treated with adelmidrol was significantly greater than in the DNBS group.

As written above, adelmidrol shares similar pharmacological properties to PEA; this can be related to activation of nuclear receptor of PPAR family (Farquhar-Smith et al., 2002; Paterniti et al., 2013). Our previous studies in fact, have proven that the deletion of PPAR genes reduced protective effect of PEA and additionally we found that PPARα, PPARγ and PPARδ agonists exerted positive effects in several experimental models such as kidney ischemia/reperfusion and spinal cord trauma (Di Paola et al., 2012; Paterniti et al., 2013). Therefore, we have demonstrated that the anti-inflammatory effect of adelmidrol appeared to be related on PPARγ activation, because the PPARγ antagonist GW9662 efficiently neutralized the protective actions of adelmidrol against DNBS-induced colitis. On the contrary, the use of PPARα KO mice has clearly shown that the adelmidrol protective effect is not associated to the PPARα activation. Our results are also in agreement with a previous study in which the administration of GW6471, a PPARα antagonist, didn't reverse the anti-inflammatory effect of adelmidrol (De Filippis et al., 2009). Finally we have also showed by using the CB2 antagonist SR144528, that the adelmidrol protective effect is not related to the activation of the CB2 receptor pathways.

Our study clearly has established that the use of adelmidrol, compared to PEA, presents some important difference that may clearly justify the use in chronic inflammatory disease. In particular, it has been demonstrated that adelmidrol unlike than PEA, which is very lipophilic, presents both hydrophilic and lipophilic features, moreover adelmidrol increases PEA intracellular concentrations modulating the availability of endogenous anti-inflammatory lipids (Petrosino et al., 2016). In addiction our study clearly demonstrated in this experimental model that adelmidrol in contrast with PEA exerts its anti-inflammatory properties by action only to the PPARγ receptor but not PPARα or

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CB2 related pathways (Borrelli et al., 2015; Impellizzeri et al., 2015b). This evidence clearly represents a specific molecular mechanism that differentiate the adelmidrol treatment in comparison to PEA. In conclusion, adelmidrol could represent a novel therapeutic intervention in autoimmune/inflammatory disorders such as IBD.

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AUTHOR CONTRIBUTIONS

Participated in research design: Salvatore Cuzzocrea and Emanuela Esposito

Conducted experiments: Rosalba Siracusa, Daniela Impellizzeri and Enrico Gugliandolo

Performed data analysis: Rosalia Crupi

Wrote or contributed to the writing of the manuscript: Marika Cordaro

CONFLICT OF INTEREST STATEMENT

Salvatore Cuzzocrea is co-inventor on patent WO2013121449 A8 (Epitech Group SpA) which deals with compositions and methods for the modulation of amidases capable of hydrolysing N-acylethanolamines useable in the therapy of inflammatory diseases as well as is also a co-inventor on patent just submitted entitled "ADELMIDROL PER L'USO NELLE PATOLOGIE CARATTERIZZATE DA INSUFFICIENTE AGONISMO DEL RECETTORE PPARgamma" number 102015000067344 del 30/10/2015 (Italy) (Epitech Group SpA) .

Moreover, Dr Cuzzocrea is also a co-inventor with Epitech group on the following patent:

1. EP 2 821 083

2. MI2014 A001495

3. 102015000067344

All the other authors don't have any conflict of interest.

Molecular Pharmacology Fast Forward. Published on September 13, 2016 as DOI: 10.1124/mol.116.105668
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Molecular Pharmacology Fast Forward. Published on September 13, 2016 as DOI: 10.1124/mol.116.105668
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FOOTNOTES

FUNDING

This study was supported by PON01-02512 grant.

Molecular Pharmacology Fast Forward. Published on September 13, 2016 as DOI: 10.1124/mol.116.105668
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FIGURE LEGENDS

Figure 1. Effects of adelmidrol on macroscopic damage and body weight in DNBS-induced colitis

No macroscopic alteration was observed in colon tissue from sham-treated mice (1A; see macroscopic score 1D). Macroscopic inspection of cecum, colon and rectum evidenced mucosal congestion, erosion and hemorrhagic ulcerations (Fig. 1B, see macroscopic score 1D). Adelmidrol (10 mg/kg o.s.) reduced significantly the extent and severity of this macroscopic damage (Fig. 1C, see macroscopic score 1D). Moreover, Adelmidrol treatment reduced also the loss of body weight (Fig. 1E). ***P<0.001 vs sham group; ###P<0.001 vs DNBS group.

Figure 2. Effects of adelmidrol on histological damage and MPO activity in DNBS-induced colitis

Histological alteration was absent in colon tissue from sham-treated mice (2A,A'; view histological score 2D). Four days after intra-colonic administration of DNBS, the colon presented a transmural necrosis and edema with a diffuse leukocyte cellular infiltrate in the submucosa (Fig. 2B,B'; view histological score 2D). Adelmidrol (10 mg/kg o.s.) reduced significantly the histological signs of colon injury (Fig. 2C,C'; view histological score 2D). Myeloperoxidase (MPO) activity was increased in DNBS-injected mice in comparison to sham-treated mice (C), and treatment with adelmidrol (10 mg/kg o.s.) reduced the extent of PMN infiltration (determined as decrease in MPO activity) in inflamed colon (Fig. 2E). *P<0.05 vs sham group; #P< 0.05 vs DNBS group; ***P<0.001 vs sham group; ###P<0.001 vs DNBS group.

Figure 3. Effects of adelmidrol treatment on NF-κB pathway, COX-2 and p-ERK expression in DNBS-induced colitis

Basal expression of IkB-α was detected in colon samples from sham-treated animals were

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substantially reduced in colon tissues from vehicle-treated animals 4 days after DNBS injection (Fig. 3A; see densitometric examination 3A'). Adelmidrol treatment prevented DNBS-induced IκB-α degradation (Fig. 3A; see densitometric examination 3A'). NF-kB p65 levels in the colon nuclear fractions were substantially increased 4 days after DNBS injection compared to sham-treated mice (Fig. 3B; see densitometric examination 3B'). Treatment with adelmidrol (10 mg/kg o.s.) reduced the levels of NF-kB p65 (Fig. 3B; see densitometric examination 3B'). Moreover, COX-2 levels were significantly increased in the colon tissue of DNBS-injected mice compared to sham mice. Treatment with adelmidrol (10 mg/kg o.s.) significantly reduced DNBS-mediated COX-2 expression (Fig. 3C; see densitometric examination 3C'). In addition, pERK1/2 levels were significantly increased in the colon tissues of DNBS-injected mice (Fig. 3D; see densitometric examination 3D') compared to sham-treated mice. Adelmidrol significantly reduced the level of pERK1/2 activation (Fig. 3D; see densitometric examination 3D'). The densitometric expression of protein bands was normalized to β-actin and lamin a/c levels. *P<0.05 vs. sham; #P<0.05 vs. DNBS.

Figure 4. Adelmidrol treatment reduces cytokine production in DNBS-induced colitis

The substantial increase in TNF-α and IL-1β formation observed in colon samples collected from DNBS-injected mice compared to sham mice (Fig. 4B,E; see densitometric examination 4G') was significantly attenuated in mice treated with adelmidrol (10 mg/kg o.s.) (Fig. 4C,F; see densitometric examination 4G'). TNF-α and IL-1β immunostaining was not detected in colon from sham mice (Fig. 4A,D; see densitometric examination 4G'). ***P<0.001 vs sham group; ###P< 0.001 vs DNBS group:

Figure 5. Adelmidrol treatment reduces ICAM-1 and P-selectin expression in DNBS-induced

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ICAM-1 and P-selectin immunoreactivity (Fig. 5B,E; see densitometric examination 5G) was substantially increased in vessels of the lamina propria and submucosa as well as in epithelial cells of injured colon and in infiltrated inflammatory cells in tissues from DNBS-injected mice. Treatment with adelmidrol (10 mg/kg o.s.) reduced ICAM-I and P-selectin immunostaining (Fig. 5C,F; see densitometric examination 5G) in the colon tissues from DNBS-injected mice. No positive staining for ICAM-I and P-selectin was observed in colon tissues from sham-treated mice (Fig. 5A,D; see densitometric examination 5G). ***P<0.001 vs sham group; ###P<0.001 vs DNBS group.

Figure 6. Effects of adelmidrol treatment on nitrotyrosine and PAR formation and lipid peroxidation in DNBS-induced colitis

PAR immunoreactivity was visible in the nuclei of inflammatory cells in colon tissues from DNBS-injected mice compared to sham-treated animals (Fig. 6B; see densitometric examination 6G). Adelmidrol (10 mg/kg o.s.) significantly reduced the extent of immunostaining for PAR in the colon (Fig. 6C; see densitometric examination 6G). While colon sections from sham-treated mice did not stain for nitrotyrosine (Fig. 6D; see densitometric examination 6G), sections from DNBS-injected mice exhibited positive immunostaining for nitrotyrosine (Fig. 6E; see densitometric examination 6G). Treatment with adelmidrol (10 mg/kg o.s.) reduced the degree of positive immunostaining for nitrotyrosine (Fig. 6F; see densitometric examination 6G) in the colon. In addition,,plasma levels of thiobarbituric acid-reactant substances (an indicator of lipid peroxidation) were significantly increased (Figure 6H) in plasma collected from mice subjected to DNBS when compared with sham-treated animals. Treatment with adelmidrol (10 mg/kg o.s.) markedly attenuated the DNBS-induced rise in thiobarbituric acid-reactant substances (Figure 6H). ***P<0.001 vs sham group; ###P<0.001 vs DNBS group.

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Figure 7. Effects of adelmidrol treatment on apoptotic pathway in DNBS-induced colitis

Basal Bcl-2 levels in sham mice were markedly diminished following DNBS injection (Fig. 7A; see densitometric examination 7A'), an effect overcome with adelmidrol administration (10 mg/kg o.s.) (Fig. 7A; see densitometric examination 7A'). Bax levels appreciably increased in colon tissue from mice subjected to DNBS, and were prevented from doing so in adelmidrol-treated mice (10 mg/kg o.s.) (Fig. 7B; see densitometric examination 7B'). Densitometric analysis of protein bands was normalized to the level of β-actin. *P<0.05 vs. sham; #P<0.05 vs. DNBS; **P<0.01 vs. sham; ##P<0.01 vs. DNBS.

Figure 8. Role of PPARγ receptors in the systemic anti-inflammatory effects of adelmidrol in DNBS-induced colitis

Macroscopic inspection of cecum, colon and rectum evidenced mucosal congestion, erosion and hemorrhagic ulcerations (Fig. 8B, see macroscopic score 8F) compared to sham (Fig. 8A, see macroscopic score 8F). GW9662 administration did not show a significant difference compared to DNBS group (Fig. 8C, see macroscopic score 8F). Adelmidrol (10 mg/kg o.s.) significantly reduced the extent and severity of this macroscopic damage (Fig. 8D, see macroscopic score 8F). Moreover, Adelmidrol treatment reduced also the loss of body weight (Fig. 8G). DNBS-injected mice pretreated with GW9662 did not show a reduction of macroscopic damage (Fig. 8E; see macroscopic score 8F). Moreover, mice pretreated with GW9662 showed a major loss in body weight (Fig. 8G) compared with adelmidrol-treated mice. ***P<0.001 vs sham group; #P<0.05 vs DNBS group; ###P< 0.001 vs DNBS group.

Figure 9. Role of PPARγ receptors on histological damage and MPO activity compared to adelmidrol

Four days after intra-colonic administration of DNBS, the colon presented a transmural necrosis and edema with a diffuse leukocyte cellular infiltrate in the submucosa (Fig. 9B,B'; view

Molecular Pharmacology Fast Forward. Published on September 13, 2016 as DOI: 10.1124/mol.116.105668
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histological score 9F) compared to sham (Fig. 9A,A'; view histological score 9F). No significant difference was appreciate between DNBS and DNBS+GW9662 group (Fig. 9C,C'; view histological score 9F). Adelmidrol (10 mg/kg o.s.) reduced significantly the histological signs of colon injury (Fig. 9D,D'; view histological score 9F). The protective effects of adelmidrol (10 mg/kg o.s.) were inhibited in mice injected with DNBS and pretreated with the PPARγ antagonist GW9662, in terms of both histological damage (Fig.9E,E' view histological score 9F) and myeloperoxidase (MPO) activity (Fig. 9G). *P<0.05 vs sham group; ***P<0.001 vs sham group; #P<0.05 vs DNBS group; ###P< 0.001 vs DNBS group.

Figure 10. Role of PPARα and CB2 receptors in the systemic anti-inflammatory effects of adelmidrol in DNBS-induced colitis

As showed in panel 10 a transmural necrosis and edema with a diffuse leukocyte cellular infiltrate in the submucosa was found in the colon sections from DNBS-injected mice (Fig. 10A,A'; 10C,C' and 10 E,E' view histological score 10G and 10H) compared to sham (data not shown). Adelmidrol (10 mg/kg o.s.) reduced significantly the histological signs of colon injury (Fig. 10B,B'; D,D' and 10F,F' view histological score 10G and 10H). *P<0.05 vs sham group; ***P<0.001 vs sham group; #P<0.05 vs DNBS group; ###P< 0.001 vs DNBS group.

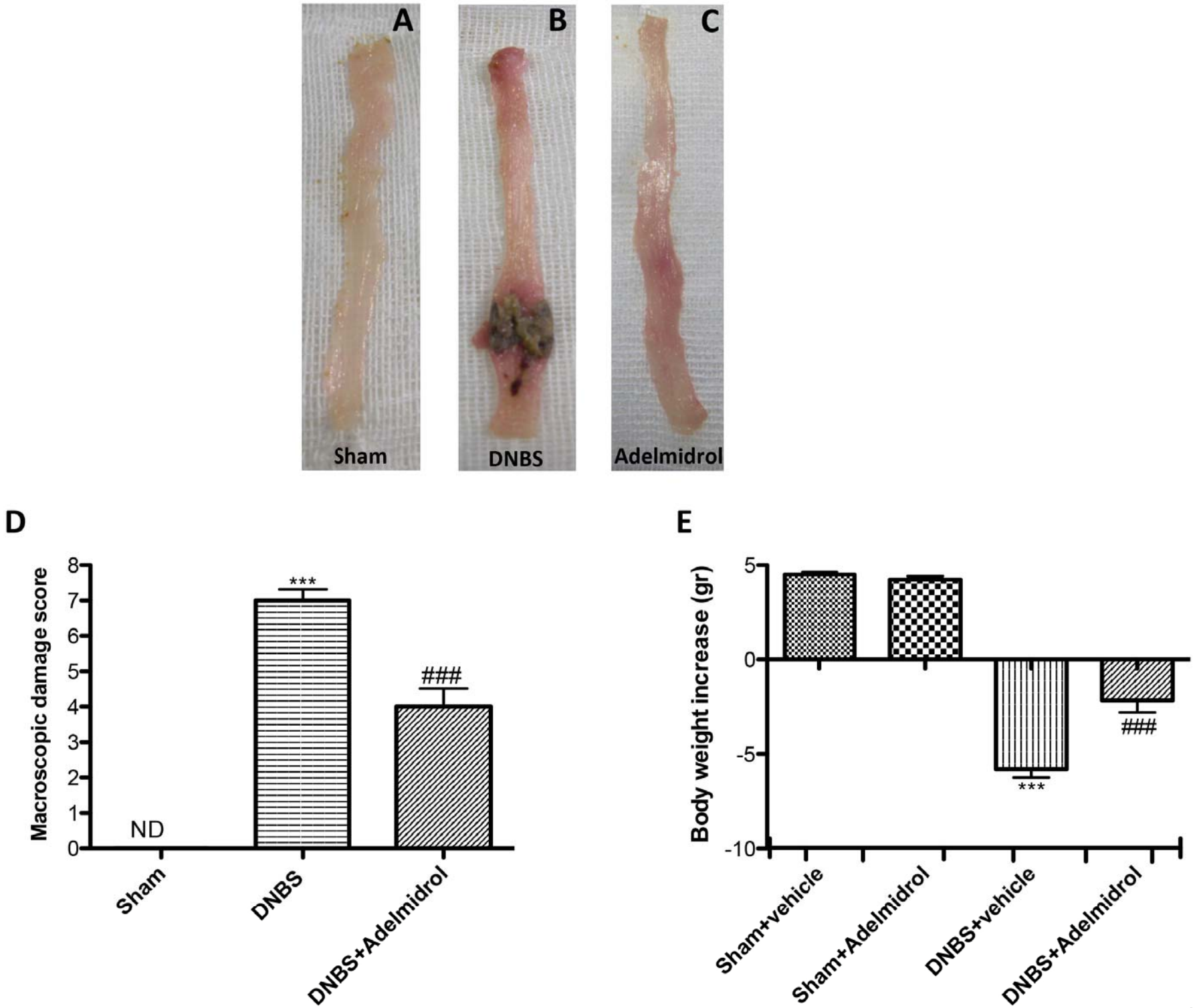


Figure 1

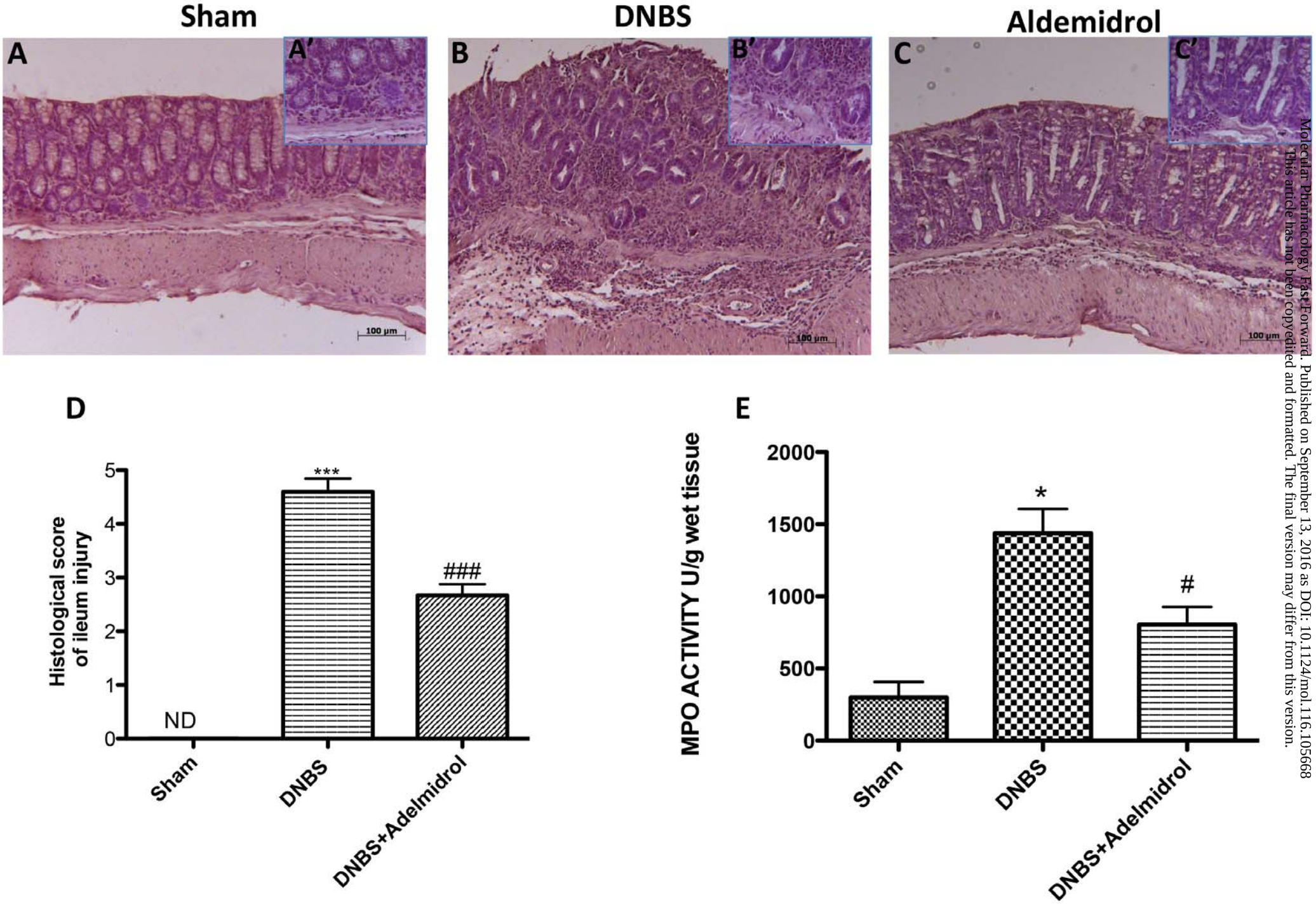
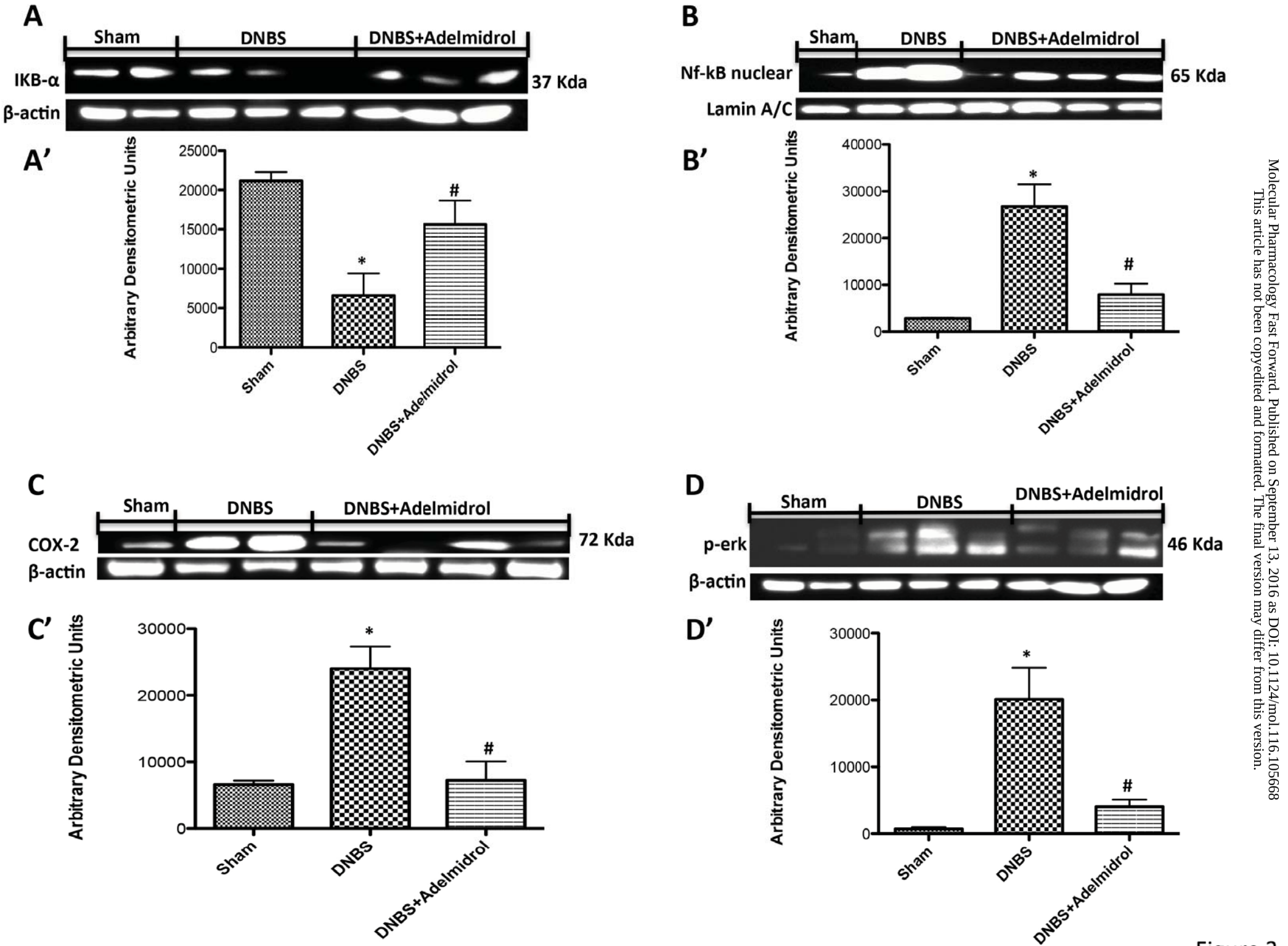


Figure 2



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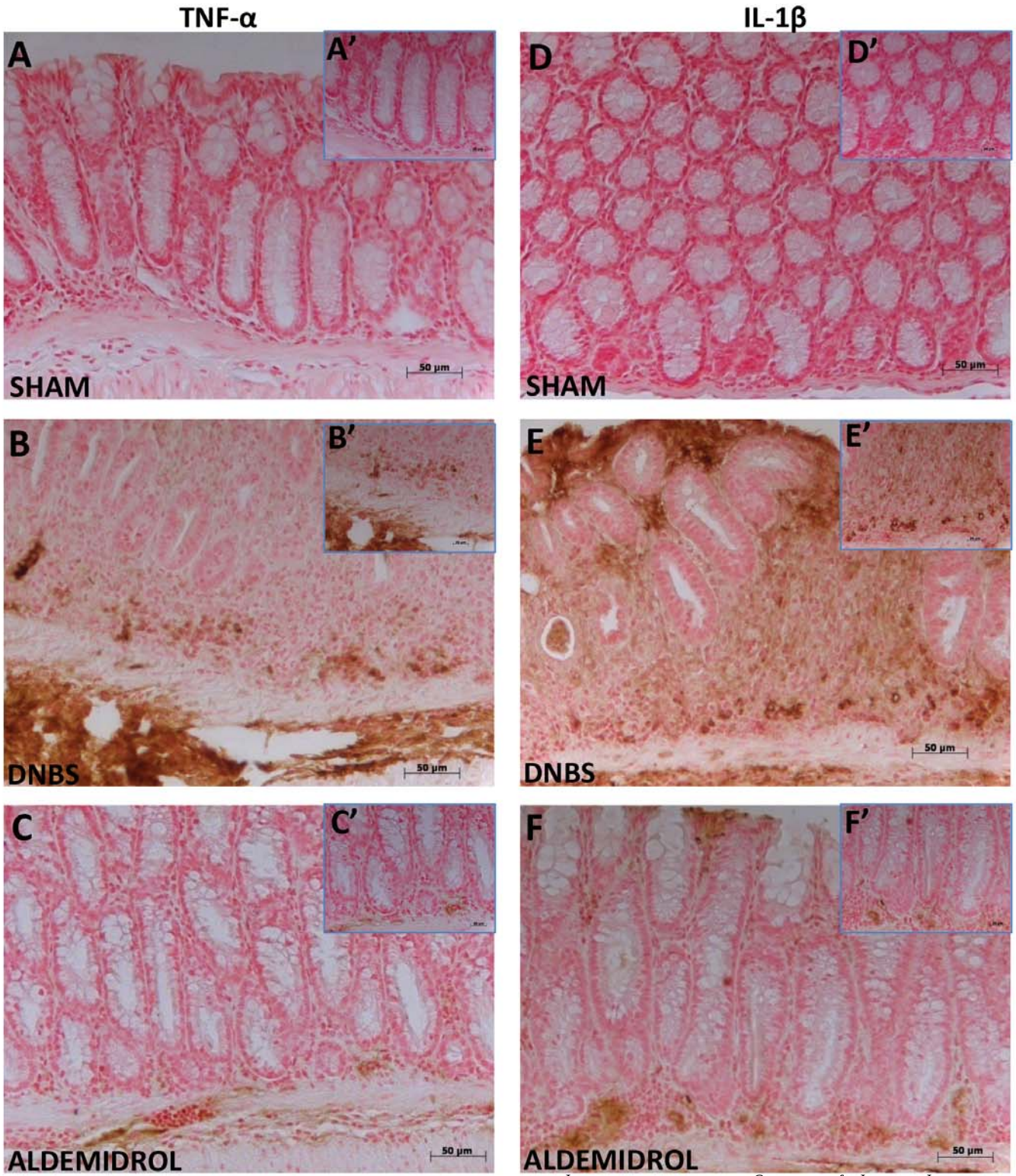

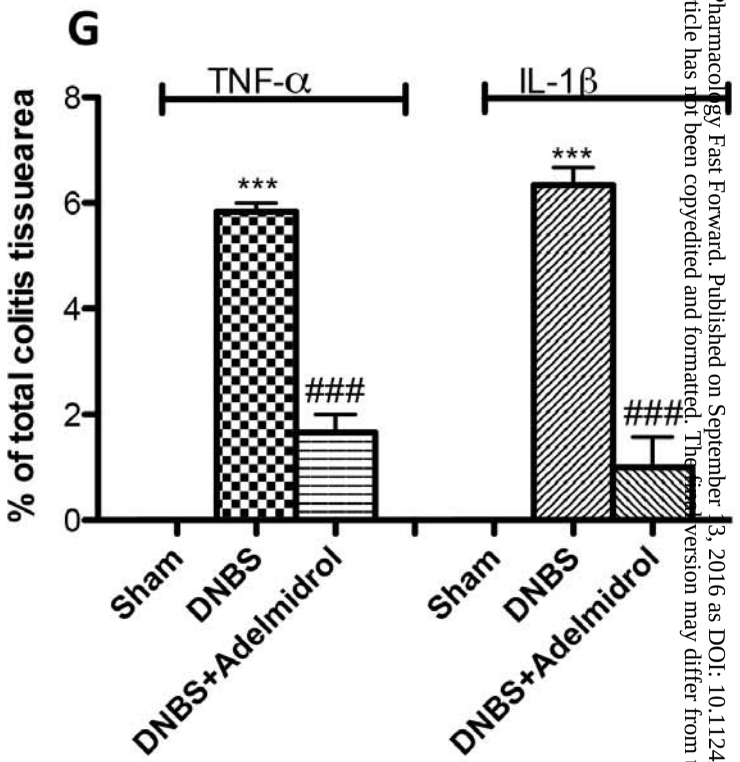

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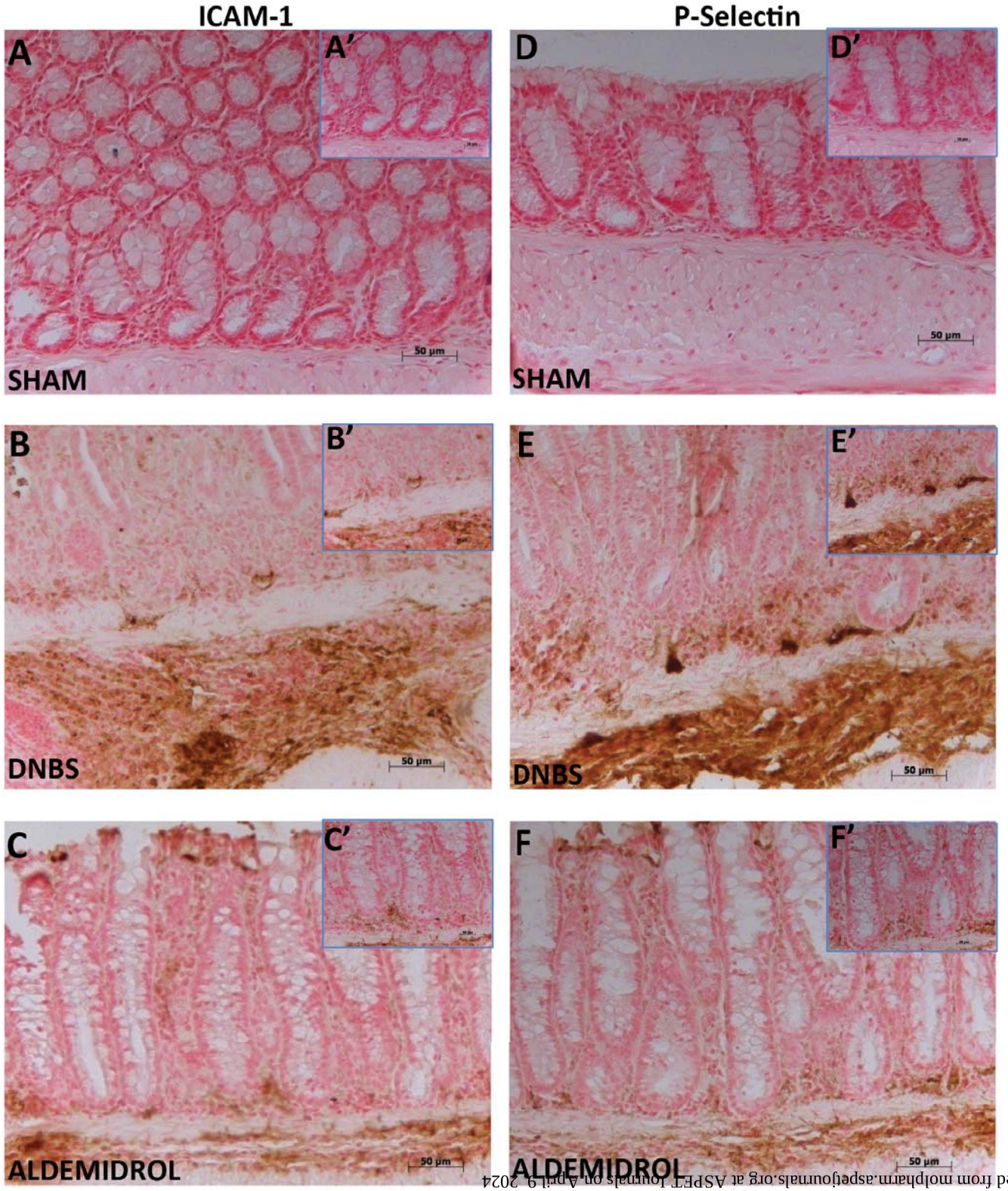

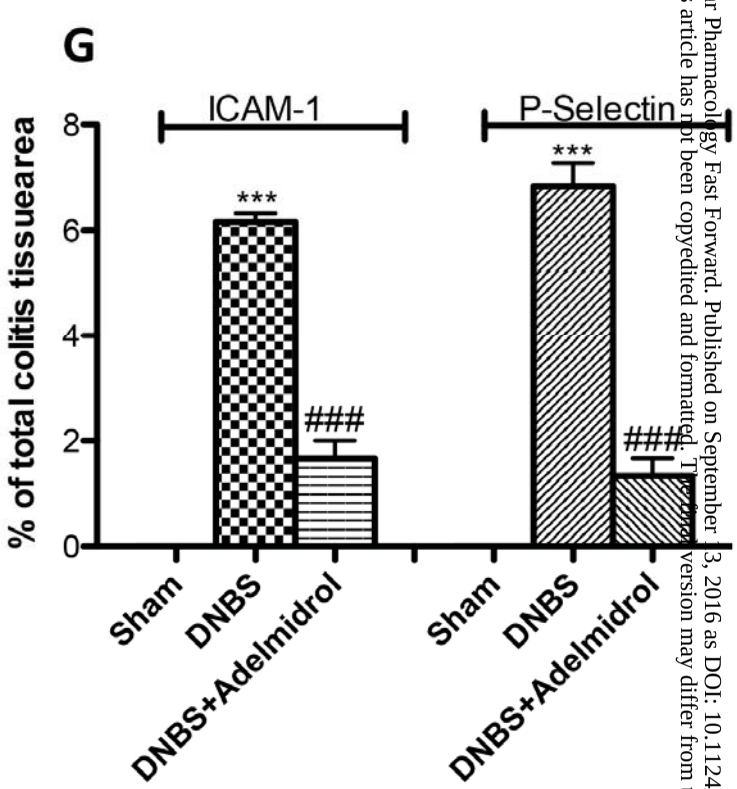

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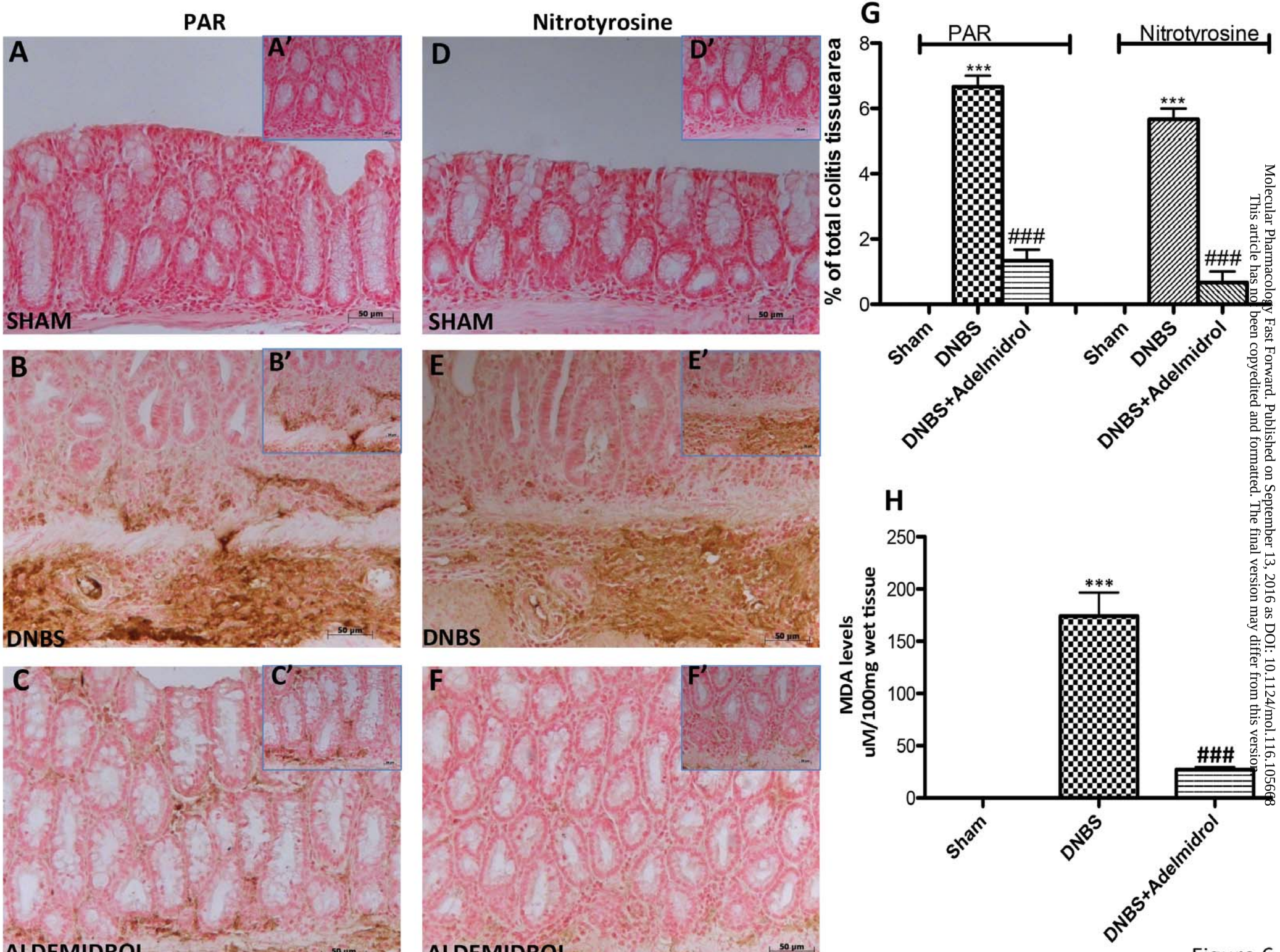

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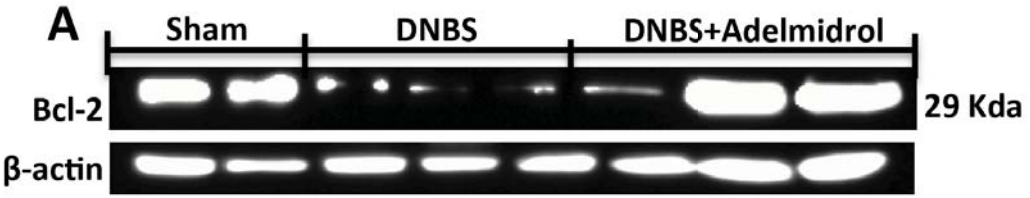

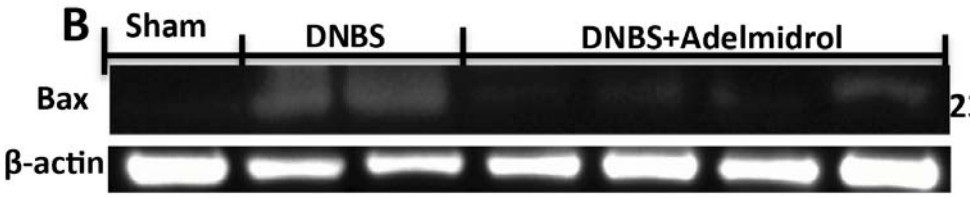

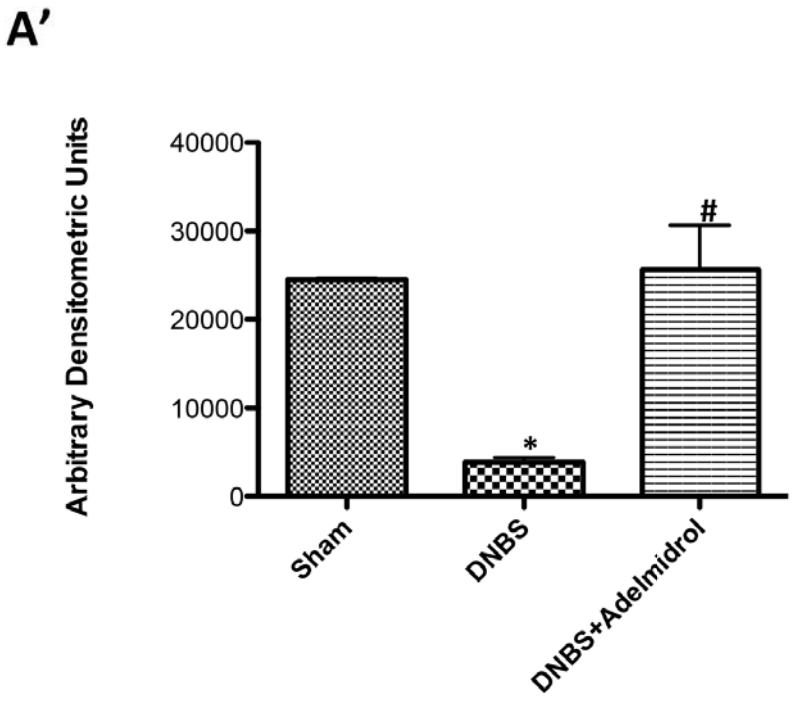

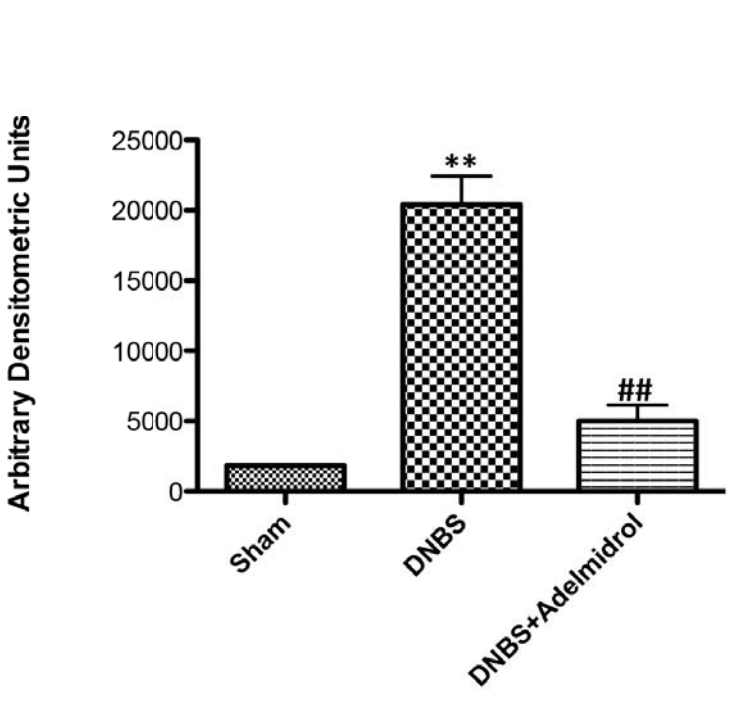

Figure 7

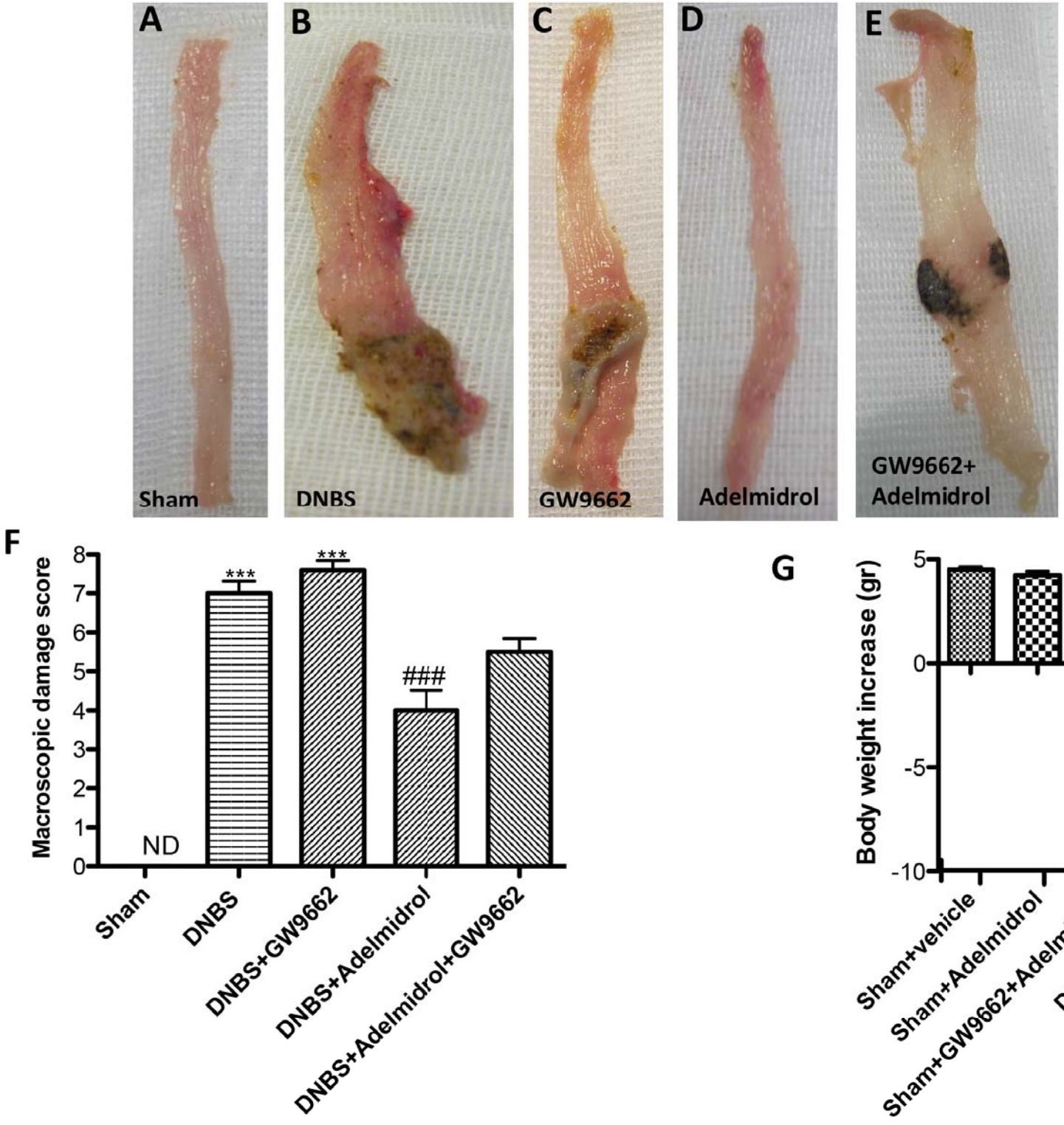

Figure 8

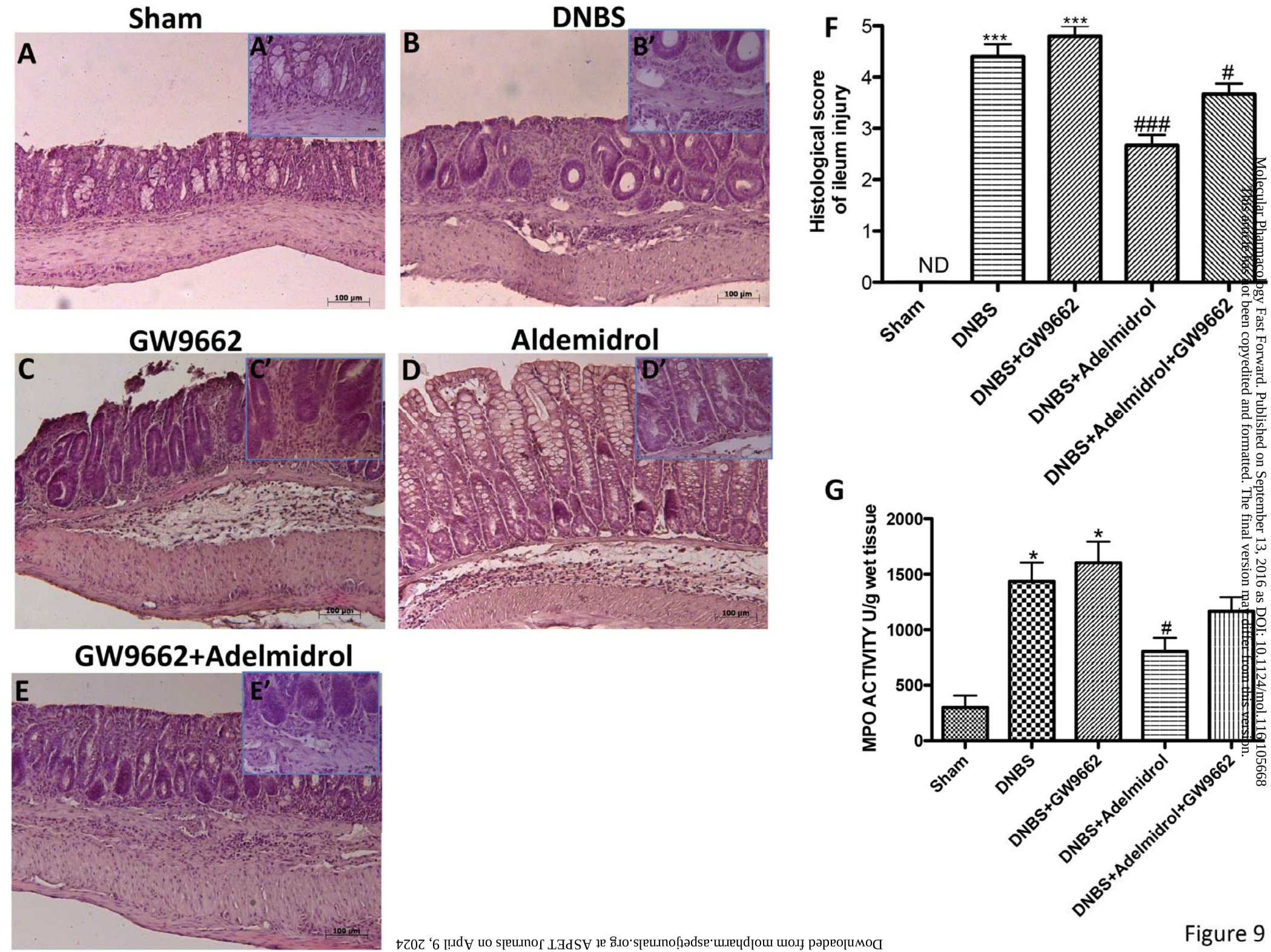

Figure 9

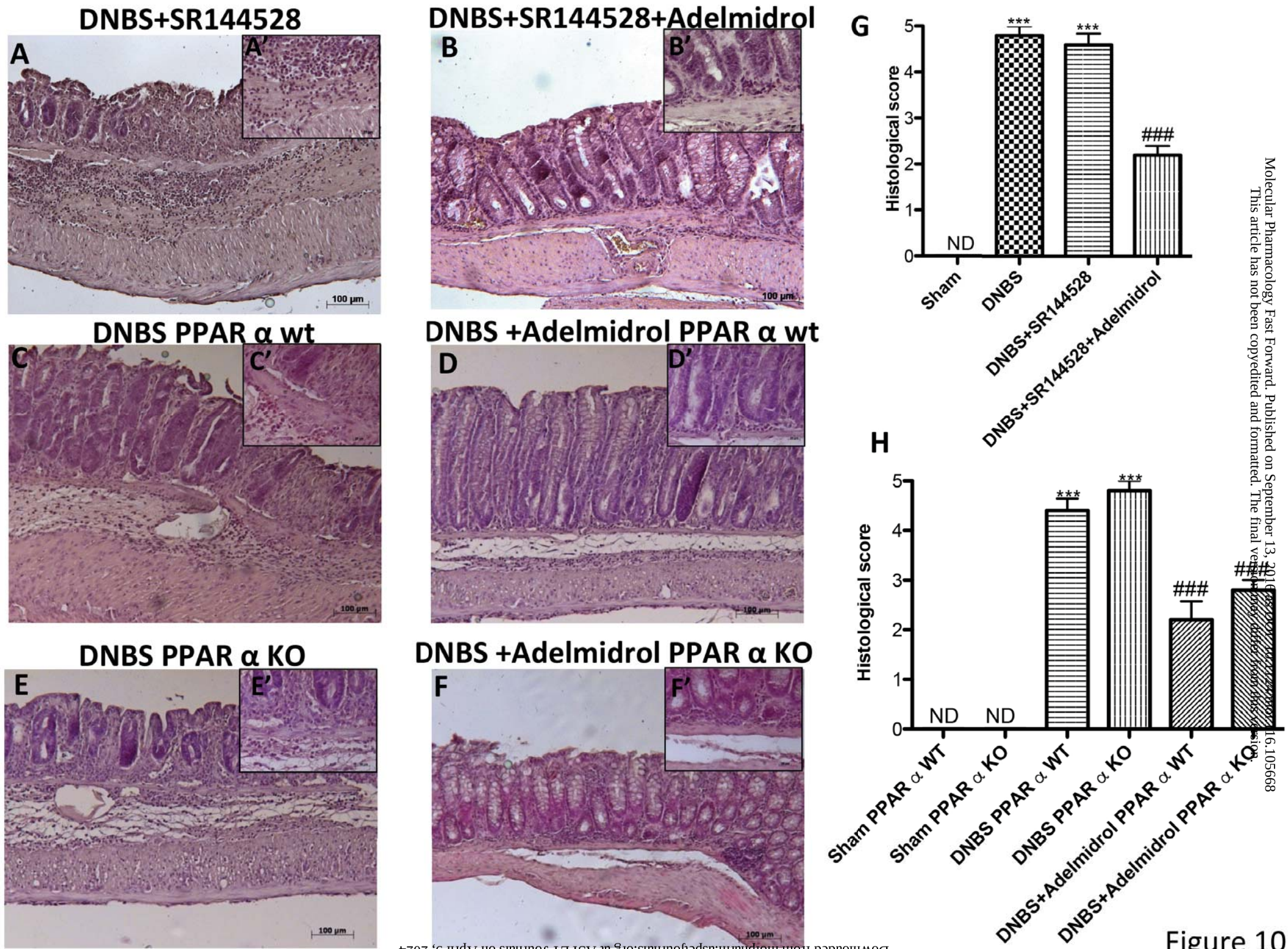

Figure 10