Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1399–1410
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats

Antonio Ramón Martín, Isabel Villegas, Carmen La Casa, Catalina Alarcón de la Lastra*

Department of Pharmacology, Faculty of Pharmacy, University of Seville, Profesor García González Street 2, 41012 Sevilla, Spain

Received 4 September 2003; accepted 15 December 2003

Abstract

Oxidative stress, neutrophil infiltration, proinflammatory cytokines and eicosanoid generation are clearly involved in the pathogenesis of intestinal bowel disease. Resveratrol, a polyphenolic compound found in grapes and wine, has been shown to have anti-inflammatory, antioxidant, antitumour and immunomodulatory activities, however, its effects on experimental colitis remain unknown. We have investigated the effects of resveratrol on the colon injury caused by intracolonic instillation of trinitrobenzenesulphonic acid (TNBS) in rats. We determined the production of prostaglandin (PG)E_2 and PGD_2 in colon mucosa and the expression of cyclo-oxygenases (COX)-1 and -2 immunohistochemically. The inflammatory response was assessed by histology and myeloperoxidase activity, as an index of neutrophil infiltration. Interleukin-1β production, histological and histochemical analysis of the lesions were also carried out. Finally, since resveratrol has been found to modulate apoptosis we intended to elucidate its effects on colonic mucosa under early acute inflammatory conditions. Resveratrol (5–10 mg/kg/day) significantly reduced the degree of colonic injury, the index of neutrophil infiltration and the levels of the cytokine. Resveratrol did not revert the increased PGE_2 levels but produced a significant fall in the PGD_2 concentration. Compared with inflamed colon, no changes in staining for COX-1 were observed in colon of resveratrol and TNBS-treated rats. In contrast, COX-2 expression was decreased. Furthermore, resveratrol enhanced apoptosis compared with already high level induced by TNBS. In conclusion, resveratrol reduces the damage in experimentally induced colitis, alleviates the oxidative events and stimulates apoptosis.

Keywords: Resveratrol; TNBS (trinitrobenzenesulphonic acid); Neutrophils; Interleukin (IL)-1β; Prostaglandin (PG); Apoptosis

1. Introduction

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a naturally occurring stilbene found in grape skins and the red wines. The compound exerts striking inhibitory effects on diverse cellular events associated with tumour initiation, promotion, and progression [1]. Resveratrol is also reported to be a neuroprotective [2] and cardioprotective agent [3]. The compound has been shown to exert a strong inhibitory effect on lipid peroxides production and also to modulate lipoprotein metabolism [4]. Several studies within the last few years have shown that resveratrol exhibits potent antioxidative and anti-inflammatory effects [5,6]. How exactly resveratrol exerts its anti-inflammatory effects is not understood but they have been ascribed to its ability to disrupt arachidonic acid metabolism by inhibiting COX-1 and hydroxyperoxidase of COX-1 with an ED_{50} of 3.7 μm [1]. This compound was also able to reduce COX-2 levels induced by lipopolysaccharide and phorbol 12-myristate and impaired the overexpression of COX-2 [7–9]. Besides inhibiting COX-2, resveratrol has been shown to suppress iNOS expression and subsequent NO production in culture cells [7,10,11].

Ulcerative colitis (UC) is a nonspecific inflammatory disorder involving primarily the mucosa and submucosa of the colon. Activated immune cells, primarily represented by neutrophils, macrophages, and cytotoxic T cells play the role of aggressors that attack and destroy the intestinal barrier either directly through physical contact or indirectly through the release of reactive oxygen and nitrogen metabolites, cytotoxic proteins, lytic enzymes, or cytokines

* Corresponding author. Tel.: +34-95-455-1666; fax: +34-95-423-3765.
E-mail address: calarcon@us.es (C.A. de la Lastra).
Abbreviations: TNBS, trinitrobenzenesulphonic acid; PG, prostaglandin; COX, cyclo-oxygenase; MPO, myeloperoxidase; IL, interleukin; UC, ulcerative colitis.

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.12.024

such as tumour necrosis factor (TNF-α) and interleukin (IL)-1β [12–14]. In particular, IL-1β appears to be a primary stimulator of diarrhoea, the major symptom of intestinal inflammation and is cleaved and activated by the IL-1β converting enzyme [15].

The role of prostanoids in the intestinal inflammatory process is not completely understood. Colonic prostanoid generation is increased in UC and correlates well with disease activity [16]. In contrast, previous reports suggest that decrease of local prostaglandin PGE_2 is correlated with colonic mucosal inflammation. In fact, experimental colitis can be attenuated by pre-treatment with exogenous PG [17]. PGE_2 is known to have immunoregulatory effects inhibiting production of various cytokines such as TNF-α, IL-1β and IL-12 by macrophages [18]. Indeed, PGD_2 is the major PG produced by mucosal mast cells and has been suggested to exert anti-inflammatory effects playing an important role in down-regulating colon inflammation in rats [19].

The anti-inflammatory and antioxidant activity of resveratrol has been well documented in many studies. However, there is no report related to its activity on experimental UC, thus here we investigated the effects of resveratrol on the colon injury caused by intracolonic administration of TNBS in rats. In order to gain a better insight into the mechanisms of action, we determined the production of PGE_2 and PGD_2 in colon mucosa and furthermore the expression of COX-1 and -2 by immunohistochemistry. The inflammation response was assessed by histology and myeloperoxidase (MPO) activity, as an index of quantitative inflammation and neutrophil infiltration in the mucosa. IL-1β production, histological and histochemical analysis of the lesions was also carried out. Finally, since resveratrol has been found to modulate apoptosis [20] we aimed to study its effects in colonic mucosa under early acute inflammatory conditions.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats supplied by Animal Services, Faculty of Medicine, University of Seville, Spain, and weighing 180–220 g, were placed singled in cages with wire-net floors in a controlled room (temperature 24–25°, humidity 70–75%, lighting regimen of 12-hr light:12-hr dark) and were fed a normal laboratory diet (Panlab). Rats were deprived of food for 24 hr prior to the induction of colitis, but were allowed free access to tap water throughout. They were randomly assigned to groups of 8–15 animals. Experiments followed a protocol approved by the local animal Ethics Committee and the Local Government. All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EC).

2.2. Induction of colitis

Colitis was induced according to the procedure described by Morris *et al.* [21]. Briefly, rats were slightly anaesthetised with ether following a 24 hr fast, and then a medical-grade polyurethane canal for enteral feeding (external diameter 2 mm) was inserted into the anus and the tip was advanced to 8 cm proximal to the anus verge. TNBS (Sigma-Aldrich Company Ltd.) dissolved in 50% ethanol were instilled into the colon through the cannula (10 mg in a volume of 0.25 mL to induce acute colitis). Following the instillation of the hapten, the animals were maintained in a head-down position for a few minutes to prevent leakage of the intracolonic instillate. Different control groups were created for comparison with TNBS/ethanol instillation: rats in the sham group received an enema of physiological saline instead of the TNBS solution, and ethanol group received 0.25 mL of 50% ethanol. Resveratrol (5–10 mg/kg; Sigma-Aldrich Company Ltd.) was suspended in 0.9% saline solution and administered by gavage 48, 24 and 1 hr prior to the induction of colitis and 24 hr later. Control groups received vehicle in a comparable volume (10 mL/animal). The animals were sacrificed, using an overdose of anaesthetic, 48 hr after induction of colitis. The rats were checked daily for behaviour, body weight, and stool consistency.

2.3. Assessment of colitis

The severity of colitis was evaluated by an independent observer who was blinded to the treatment. For each animal, the distal 10 cm portion of the colon was removed and cut longitudinally, slightly cleaned in physiological saline to remove faecal residues and weighed. Macroscopic inflammation scores were assigned based on clinical features of the colon (score 0–10: 0 (no damage), 1 (focal hyperaemia), 2 (ulceration without hyperaemia or bowel wall thickening), 3 (ulceration with inflammation at 1 site), 4 (≥2 sites of ulceration and inflammation), 5 (major sites of inflammation >1 cm along the organ), 6–10 (major sites of inflammation >2 cm along the organ)) [22]. The presence of adhesions (score 0–2), and/or stool consistency (score 0–1) were evaluated according to the criteria of Bobin-Dubigeon *et al.* [22]. Pieces of inflamed colon were collected and frozen in liquid nitrogen for measurement of biochemical parameters.

2.4. Histological studies

For examination with the light microscope we used tissue samples from the distal colon of each animal fixed in 4% buffered paraformaldehyde, dehydrated in grade ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 μm on a rotary microtome (Leica Ultracut), mounted on clean glass slides and dried overnight at 37°. Sections were cleared, hydrated, and stained

with haematoxylin and eosin, Giemsa, and Alcian blue for histological evaluation of colonic damage, cell infiltration and mucus content, respectively, according to standard protocols, and the slides were coded to prevent observer bias during evaluation. All tissue sections were examined in an Olympus BH-2 microscope for characterization of histopathological changes.

Photographs taken from colon samples were digitised using Kodak D290 Zoom camera Eastman Kodak Co, USA and Motic® Images 2000 release 1.1 (MicroOptic Industrial Group Co, Ltd.; B1 Series System Microscopes). Analysis of the figures were carried out by Adobe® Photoshop® Version 5.0 (Adobe Systems) image analysis program.

2.5. Immunohistochemical study

Colonic tissues were fixed in 4% buffered paraformaldehyde, dehydrated through graded concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5 μm thick) were mounted on slides, cleared, and hydrated. All of them were treated with a buffered blocking solution (3% BSA) for 15 min. Then, sections were co-incubated with primary antibodies for COX-1 and COX -2 (goat polyclonal, M-19 and M-20 of Santa Cruz Biotechnologies) at a dilution of 1:400 at room temperature for 1 and 24 hr, respectively. Sections were washed with PBS and co-incubated with secondary antibody (anti-sheep IgG, peroxidase conjugated, Sigma-Aldrich Company Ltd.) (1:500 in PBS, v/v), at room temperature for 1 hr. Thereafter, sections were washed as before and with Tris–HCl 0.05 M, pH 7.66, and then co-incubated with a 3,3′-diaminobenzidine solution in the dark, at room temperature for 10 min. Sections were washed with Tris–HCl, stained with haematoxylin according to standard protocols and observed under an Olympus BH-2 microscope.

2.6. Assessment of leukocyte involvement

MPO activity was assessed as a marker of neutrophil infiltration according to the methods of Grisham *et al.* [23]. In all animals one sample from the distal colon was obtained. Samples were excised from each animal and rapidly rinsed with ice-cold saline, blotted dry, and frozen at −70°. The tissue was thawed, weighed and homogenized in 10 vol. 50 mM PBS, pH 7.4. The homogenate was centrifuged at 20,000 *g*, 20 min, 4°. The pellet was again homogenized in 10 vol. 50 mM PBS, pH 6.0, containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM EDTA. This homogenate was subjected to one cycle of freezing/thawing and a brief period of sonication. A sample of homogenate (0.5 μL) was added to a 0.5 mL reaction volume containing 80 mM PBS, pH 5.4, 0.5% HETAB and 1.6 mM 3,3′,5,5′-tetramethylbenzidine. The mixture was incubated at 37° for 5 min and the reaction started by the addition of 0.3 mM H_2O_2.

Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37°. The reaction was terminated by the sequential addition of catalase (20 μg/mL) and 2 mL 0.2 M sodium acetate, pH 3.0. The changes in absorbance at 655 nm were measured with a spectrophotometer. One unit of MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 U/min at 37° in the final reaction volume containing the acetate. Results were quantified as U/mg protein.

2.7. Production of PGE_2

Colonic mucosa was excised and rapidly rinsed with ice-cold saline. The tissue was weighed and homogenized in 6 mL triethylammonium phosphate (TEAP) buffer (pH 3.24), which contained a COX inhibitor, lysine acetyl salicylate (Sigma-Aldrich Company Ltd.). The homogenate was centrifuged (1500 *g*, 10 min, 4°) and the supernatant was removed and passed through a reverse-phase octadecylsilica C18 Sep Pak cartridge which was washed with 10 mL distilled water, 10 mL 15% ethanol, 10 mL hexane and 10 mL ethylacetate, and the eluate collected. Each fraction was evaporated with ethylacetate, and the dry residue redissolved in ethanol. PGE_2 was determined by a competitive ELISA kit (Assay Designs, Inc.). PGE_2 levels were quantified as PGE_2/mg tissue.

2.8. Production of PGD_2

PGD_2 also was determined in colon tissue samples obtained from each group. Briefly, colonic mucosa was excised and rapidly rinsed with ice-cold saline. The tissue was weighed and homogenized in TEAP buffer (pH 3.24) which contained a COX inhibitor, lysine acetyl salicylate (Sigma-Aldrich Company Ltd.). The homogenate was centrifuged (1500 *g*, 10 min, 4°) and an aliquot of supernatant must be methoximated due to its chemical instability and rapid degradation. Later the supernatant was removed and passed through a reverse-phase octadecylsilica C18 Sep Pak cartridge which was washed with 10 mL distilled water, 10 mL 15% ethanol, 10 mL hexane and 10 mL ethylacetate, and the eluate collected. Each ethylacetate fraction was evaporated, and the dry residue redissolved in buffer. PGD_2 was determined by an ELISA kit (Cayman Chemical). Results were expressed as PGD_2/mg tissue.

2.9. Measurement of IL-1β production

Mucosal level of this cytokine was assayed by using a commercially available IL-1β enzyme-immunometric assay (EIA) kit (Titerzyme® EIA rat IL-1β, Assay Designs, Inc.). Briefly, colonic mucosal samples kept at −70° were weighed and homogenized, after thawing, in 10 vol. of assay buffer. They were centrifuged at 2405 *g* for

20 min, at 4°. One hundred microliters of the supernatants and 100 μL of standards and assay buffer were added to the wells of a microtiter plate with provided with an immobilized polyclonal antibody to rat IL-1β. After incubation at 37° for 1 hr, the excess sample or standard was washed out and a monoclonal antibody to rat IL-1β with the enzyme horseradish peroxidase was added. This labelled antibody binds to the rat IL-1β captured on the plate. After a short incubation, the excess labelled antibody was washed out and substrate was added. The substrate reacts with the labelled antibody bound to the rat IL-1β captured on the plate. The colour generated with the substrate was read at 450 nm in a microplate reader (Labysistem Multiskan EX), and was directly proportional to the concentration of rat IL-1β in either standards or samples. The IL-1β content were expressed as IL-1β/mg protein.

2.10. Apoptosis

Cytoplasmic DNA fragments, which are indicators of apoptosis, were measured with a DNA cell death detection ELISA PLUS KIT (Roche Diagnostics) according to the manufacturer's instructions. Results were expressed as absorbance $\times 10^3$/mg protein.

2.11. Statistical analysis

All values in the figures and text are expressed as arithmetic means ± SEM. Data were evaluated with Graph Pad Prism® Version 2.01 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way ANOVA, using Tukey-Kramer multiple comparisons test as *post hoc* test. P values of <0.05 were considered statistically significant. In the experiment involving histology or immunohistochemistry, the figures shown are representative of at least six experiments performed on different days.

3. Results

Forty-eight hours after intracolonic administration of TNBS, rats showed postration, piloerection and hypomotility. Macroscopic inspection of the cecum, colon and rectum showed evidence of severe colonic mucosal damage, with oedema, deep ulcerations and haemorrhage. Lesions in the distal colon were quantified using a macroscopic damage score (mean: 7.2 ± 1.1) (Fig. 1). In this experimental group, body weight loss was elevated compared with the sham animals. A significant increase of

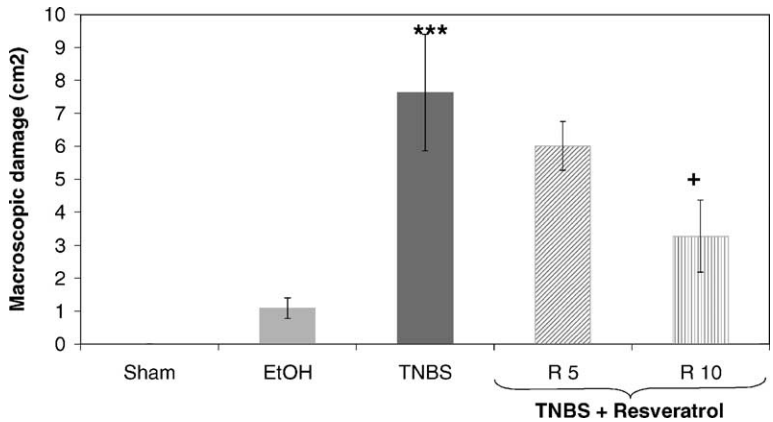

Fig. 1. Effects of acute administration of resveratrol on the colonic damage score. Colonic macroscopic damage resulting from TNBS (10 mg/animal) instilled into rat colon was scored as indicated in Section 2. Scores were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham, TNBS and ethanol groups), or in the presence of resveratrol (R: 5 and 10 mg/kg/day). Data are expressed as the mean ± SEM. $^{***}P < 0.001$ vs. sham. $^{+}P < 0.05$ vs. TNBS.

Table 1
Parameters quantified after administration of resveratrol (R: 5 and 10 mg/kg) in rats with acute colitis induced by TNBS intracolonic instillation (10 mg/animal)

Group	N	Body weight changes (g)	Food consumption (g/rat per day)	Adhesions (score 0–2)	Diarrhoea (score 0–1)	Colon weight/colon length (mg/cm)
Sham	11	16.0 ± 4.3	25.7	0	0	133.42 ± 6.06
EtOH	15	1.4 ± 2.9	27.5	0	0.2 ± 0.2	200.73 ± 24.16
TNBS	14	−8.3 ± 12.3	18.5	1.5 ± 0.3*,**	0.7 ± 0.2	261.80 ± 34.52*
R 5	12	10.3 ± 16.4	21.35	0.5 ± 0.2	0.5 ± 0.2	231.75 ± 22.39
R 10	12	12.5 ± 9.7	20.5	0.6 ± 0.3	0.5 ± 0.2	232.38 ± 21.60

Colonic parameters were quantified in the sham group (N = 11), which received saline instillation. TNBS group (N = 14) received TNBS intracolonically in a vehicle of 50% ethanol; ethanol group (N = 15) received 50% ethanol by intracolonic injection. Data are expressed as mean ± SEM.
$^{*}P < 0.05$ significantly different from sham.
$^{**}P < 0.05$ significantly different from EtOH.

weight/length of the rat colon, an indicator of inflammation, and presence of adhesions to adjacent organs were frequently observed in TNBS-treated rats (Table 1).

The histopathological features included transmural necrosis, oedema and diffuse inflammatory cells (polymorphonuclear leukocytes, lymphocytes, and eosinophils) infiltration in the mucosa. We assessed focal ulceration of the colonic mucosa extending through the muscularis mucosae as well as desquamated areas or loss of the epithelium. The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts, especially in basal areas (Figs. 2C, 3C and D). Some areas showed accumulation of mucus and cell remnants. However, Alcian blue positive cells were less numerous. In addition, the mucin layer of the epithelium was missing (Fig. 2D).

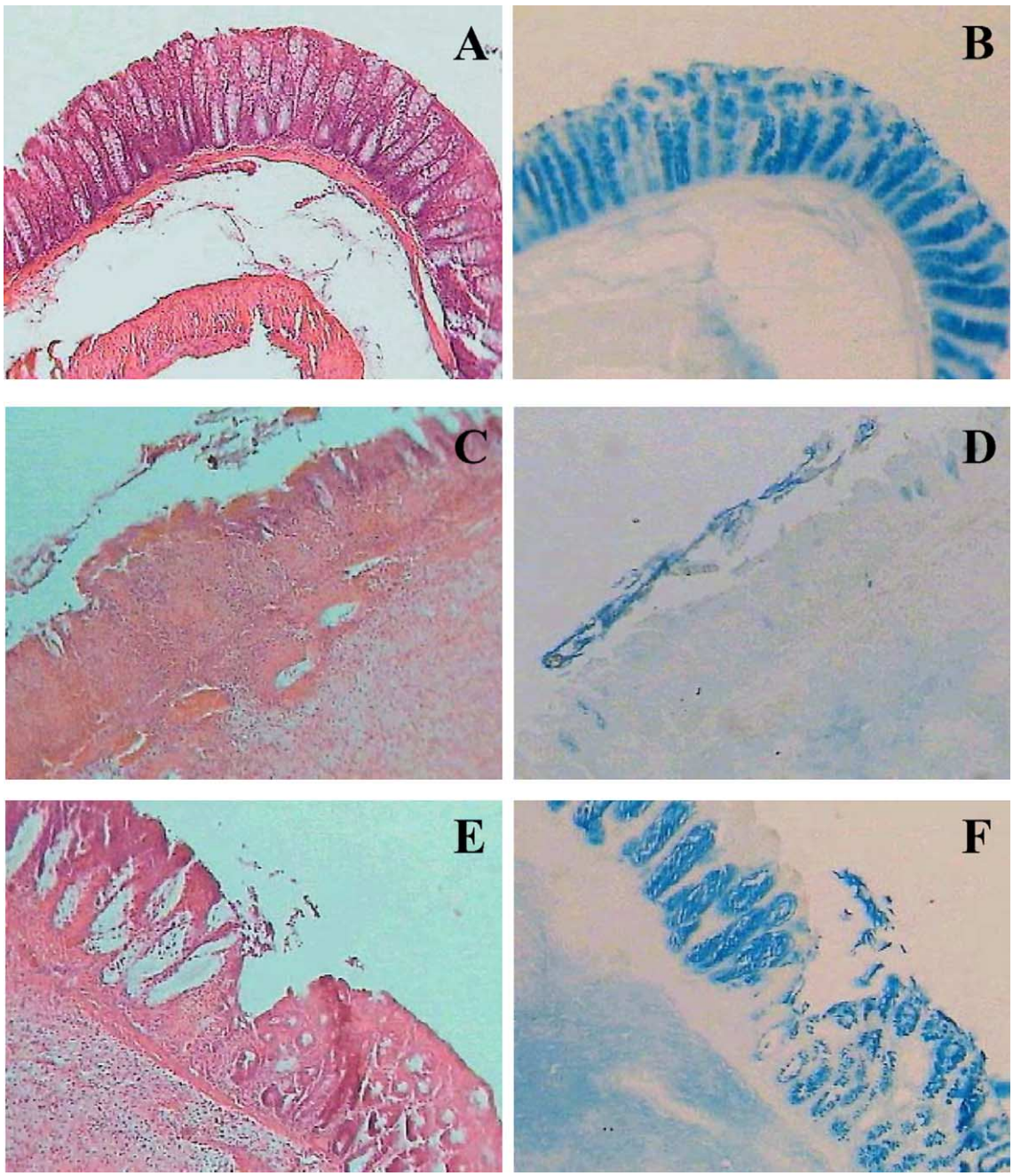

Fig. 2. Acute colitis model induced by TNBS: effect of resveratrol on colon injury. Histological appearance of rat colonic mucosa after haematoxylin and eosin stain (H–E) or Alcian blue stain (AB): sham (A and B), and treated with TNBS 10 mg/animal (C and D), and resveratrol 10 mg/kg (E and F). Histopathological features of the colon in association with colitis. (A and B) No histological modification was present in the sham animals. (C and D) Mucosal injury was produced after TNBS administration, characterized by necrosis of epithelium, focal ulceration of the mucosa and diffuse infiltration of inflammatory cells in the mucosa and submucosa. (E) Treatment with resveratrol (10 mg/kg) reduced the morphological alteration associated with TNBS administration protecting the mucosal architecture. (F) Some areas showed accumulation of mucus and cell remnants, however, Alcian blue positive cells were less numerous, and the mucin layer of the epithelium was missing. Original magnification 10×.

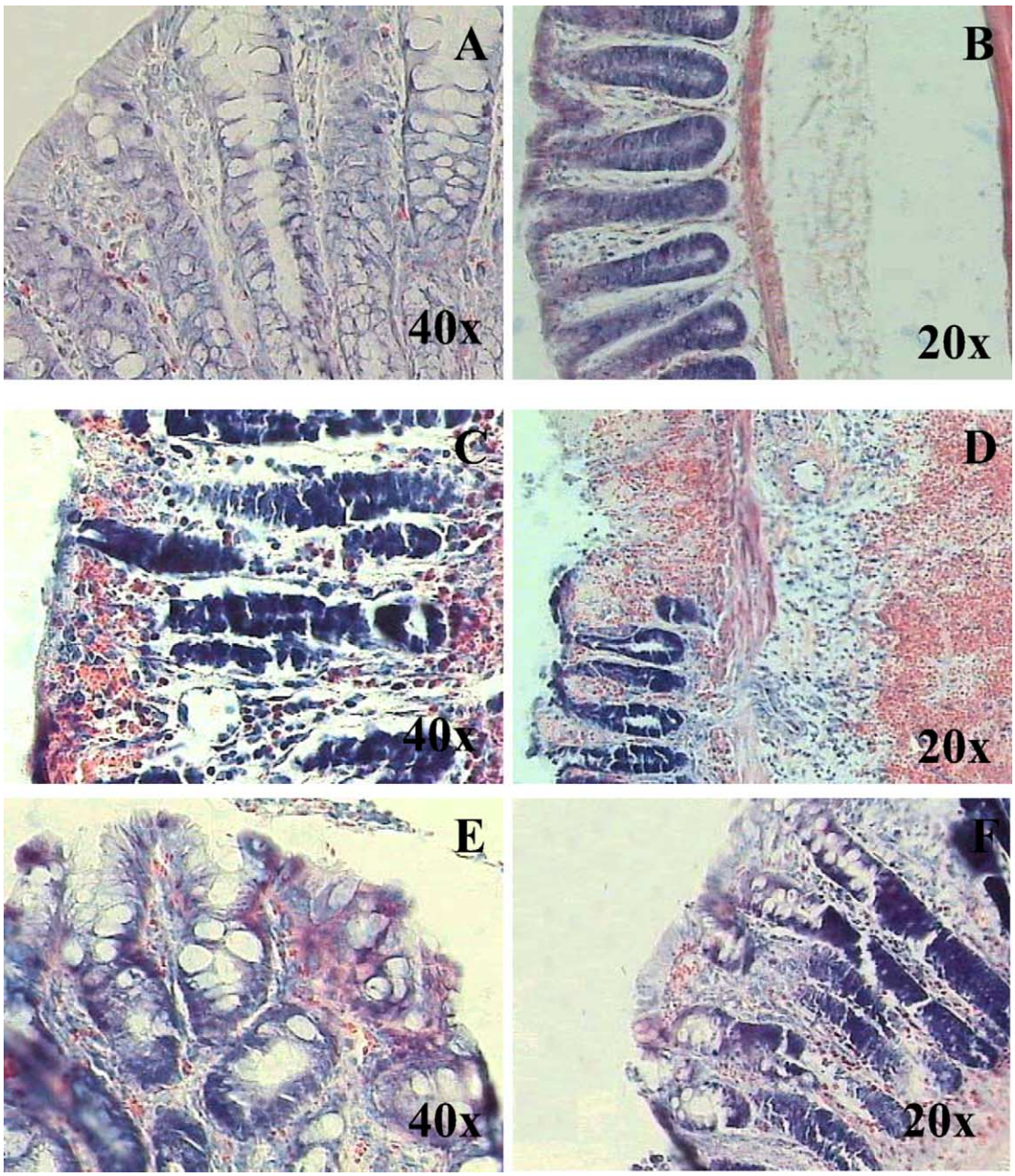

Fig. 3. Rat colon segments stained with Giemsa: sham (A and B), and treated with TNBS 10 mg/animal (C and D), and resveratrol 10 mg/kg (E and F). Infiltration of inflammatory cells was highly observed in the colonic mucosa of TNBS-treated animals. Resveratrol prevented development of inflammatory changes. Original magnifications 20 and 40×.

Treatment of TNBS-rats with resveratrol reduced the loss in body weight and the presence of adhesions to adjacent organs. There was also no significant increase in the weight/length of the rat colon, an indicator of inflammation in TNBS rats, which had been treated with resveratrol (Table 1). The polyphenolic compound attenuated the extent and severity of the colonic injury (Fig. 1). In fact, resveratrol was able to reduce the macroscopic damage score down to 3.28 ± 0.6 ($P < 0.05$) with the highest dose. On histological examination there was an attenuation of morphological signs of cell damage and inflammatory cells were not found in lamina propria. In some areas, the epithelium remained intact and the mucin layer was clearly visible with Alcian blue positive cells, however, in ulcerative areas, exfoliation of epithelial cells, dilated crypts, inflammatory cells and vascular congestion were observed (Figs. 2E, F and 3E, F).

As shown in Table 2, a marked increase in MPO activity, an indicator of the infiltration of the colon with polymorphonuclear leukocytes also characterized the colitis caused by TNBS. This result was consistent with the histological findings. Treatment of TNBS-treated rats with resveratrol significantly ($P < 0.05$) reduced the degree of polymorphonuclear neutrophil infiltration. Colonic injury by TNBS

Table 2
Myeloperoxidase activity (MPO, U/mg protein) and interleukin 1-beta levels (IL-1β, pg/mg protein) after resveratrol (R: 5 and 10 mg/kg, respectively) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/animal)

Group	N	MPO (U/mg protein)	N	IL-1β (pg/mg protein)
Sham	11	5.66 ± 0.63	8	0.25 ± 0.06
EtOH	15	7.12 ± 0.66	9	4.69 ± 1.71
TNBS	14	9.80 ± 0.83[a,b]	9	33.78 ± 7.86[c,d]
R 5	8	7.73 ± 0.84	11	18.06 ± 0.71[e]
R 10	11	7.20 ± 1.02[f]	12	14.06 ± 3.31[g]

Colonic mucosal MPO activity and IL-1β levels were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham, TNBS and ethanol groups), or in the presence of resveratrol (5 and 10 mg/kg/day). Data are expressed as the mean ± SEM.
[a] $P < 0.05$ significantly different from sham.
[b] $P < 0.05$ significantly different from EtOH.
[c] $P < 0.001$ significantly different from sham.
[d] $P < 0.001$ significantly different from EtOH.
[e] $P < 0.01$ significantly different from TNBS.
[f] $P < 0.05$ significantly different from TNBS.
[g] $P < 0.001$ significantly different from TNBS.

administration was also characterized by an increase of the proinflammatory cytokine IL-1β. In contrast, the levels of this cytokine were significantly lower ($P < 0.01$) in rats treated with resveratrol.

Our data showed that PGE_2 content increased significantly ($P < 0.01$) in colonic mucosa of TNBS group compared with sham animals. In addition, under our experimental conditions, the tested doses of resveratrol did not suppress the increased PGE_2 levels. The PGD_2 production in colon of sham-treated rats averaged 2414.34 ± 249.06 pg/mg tissue nearly five times higher than the PGE_2 values reached in basal conditions. As shown in Fig. 4 after TNBS administration, there was a decrease in the mucosal generation of PGD_2 and treatment with resveratrol produced a significant ($P < 0.05$ vs. TNBS) fall in the PGD_2 concentration.

In normal colon, specific immunosignals for COX-1 were obtained in surface epithelium, and in the upper half of the crypts. Mononuclear cells of the lamina propria and the regional lymphatic nodules as well as cells of the muscularis mucosae showed COX-1 specific immunosignals (Fig. 5B). In the basal part of the crypts, COX-1 expression was restricted to individual cells, which according to morphological criteria are endocrine cells, a specialized epithelial cell type of the lower crypt (Fig. 5C). COX-2 specific immunolabelling was occasionally observed in colonocytes of the normal surface epithelium of matched control colon as shown in Fig. 6A.

Compared with normal colon, significant changes in the cellular distribution of COX-1 and COX-2 were observed in animals treated with TNBS in that colonocytes of the surface and the crypt epithelium were only weakly decorated by the COX-1 specific antiserum (Fig. 5G and F), whereas prominent COX-2 expression was found in cells of surface epithelium and in cells of the inflammatory infiltrate (Fig. 6B and D). Compared with inflamed colon, no significant changes in the cellular localization and the degree of positive staining for COX-1 were observed in the colon of resveratrol-treated rats (Fig. 5D and E) whereas COX-2 expression was decreased in apical epithelial cells of inflamed colon from resveratrol-treated rats (Fig. 6C, E and F).

Since COX inhibitors have been found to modulate apoptosis we wished to know what were their effects in colonic mucosa under early acute inflammatory conditions by an ELISA that specifically detected cytoplasmic histone-associated DNA fragments, mononucleosomes, and oligonucleosomes. As shown in Fig. 7 apoptosis was rarely observed in the colonic mucosa of sham animals. DNA fragmentation was dramatically increased in TNBS-treated

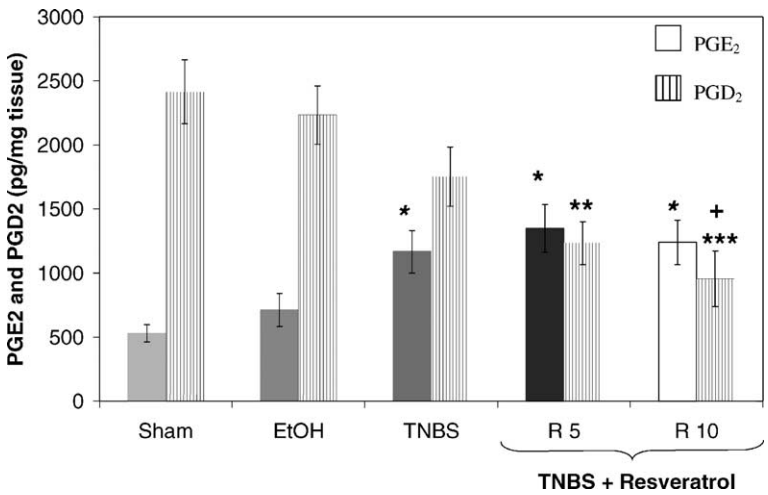

Fig. 4. Prostaglandin E_2 (pg/mg tissue) and D_2 (pg/mg tissue) after resveratrol administration (5 and 10 mg/kg) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/animal). Prostanoids synthesis in the colonic tissue was quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham, ethanol and TNBS groups), or in the presence of resveratrol (R: 5 and 10 mg/kg/day). Data are expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. sham. $^{+}P < 0.05$ vs. TNBS.

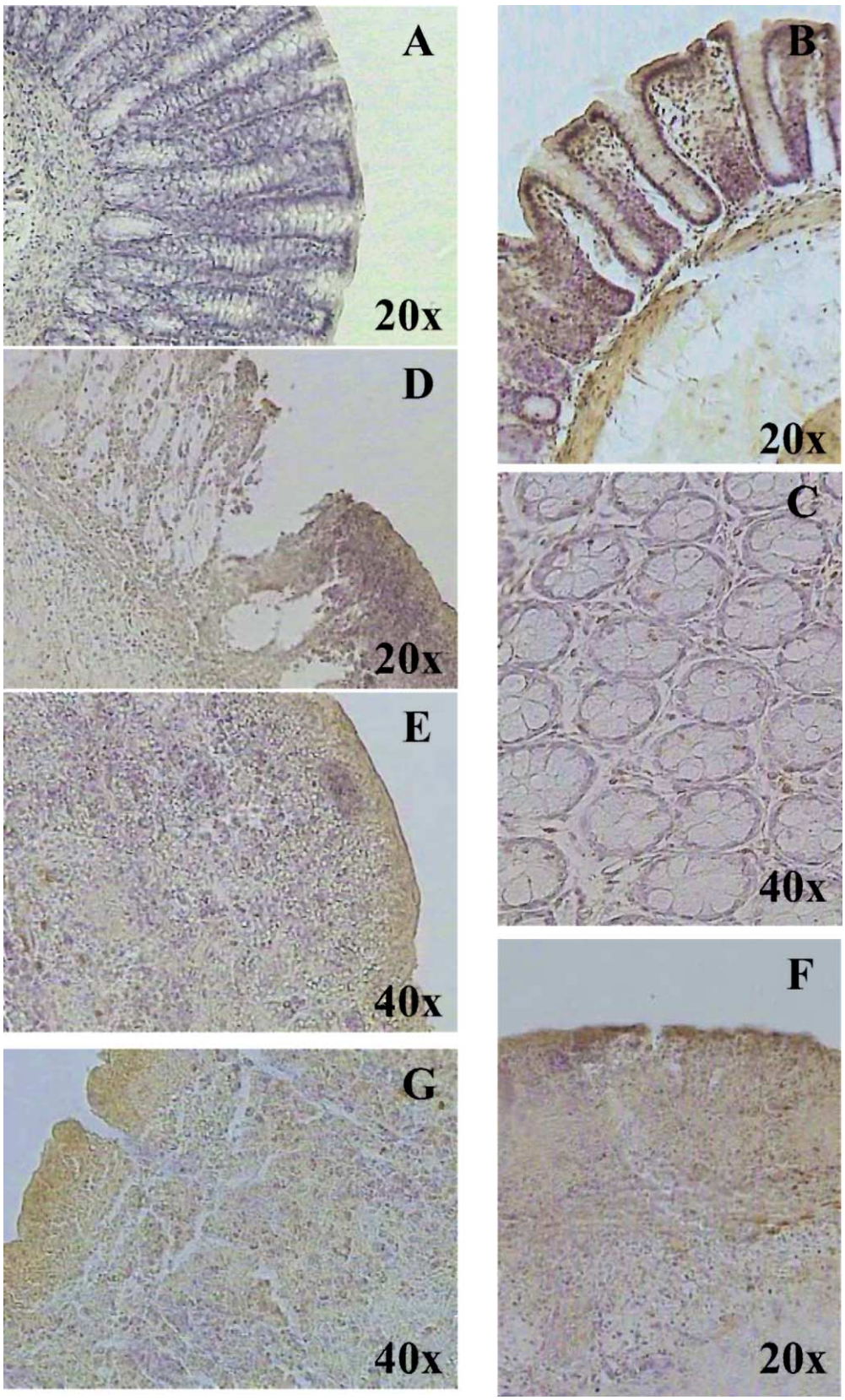

Fig. 5. Immunohistochemical localization of COX-1 isoenzyme in sections of colon. Negative control (A). In normal colon, colonocytes of the upper half of the crypts were found to be COX-1-positive (B). COX-1 expression in endocrine cells is particularly evident in this image of normal colonic mucosa (C). COX-1 expression in the colon of TNBS-control rats (F and G). COX-1 expression of inflamed colon treated with resveratrol 10 mg/kg (D and E). Original magnifications 20 and 40×.

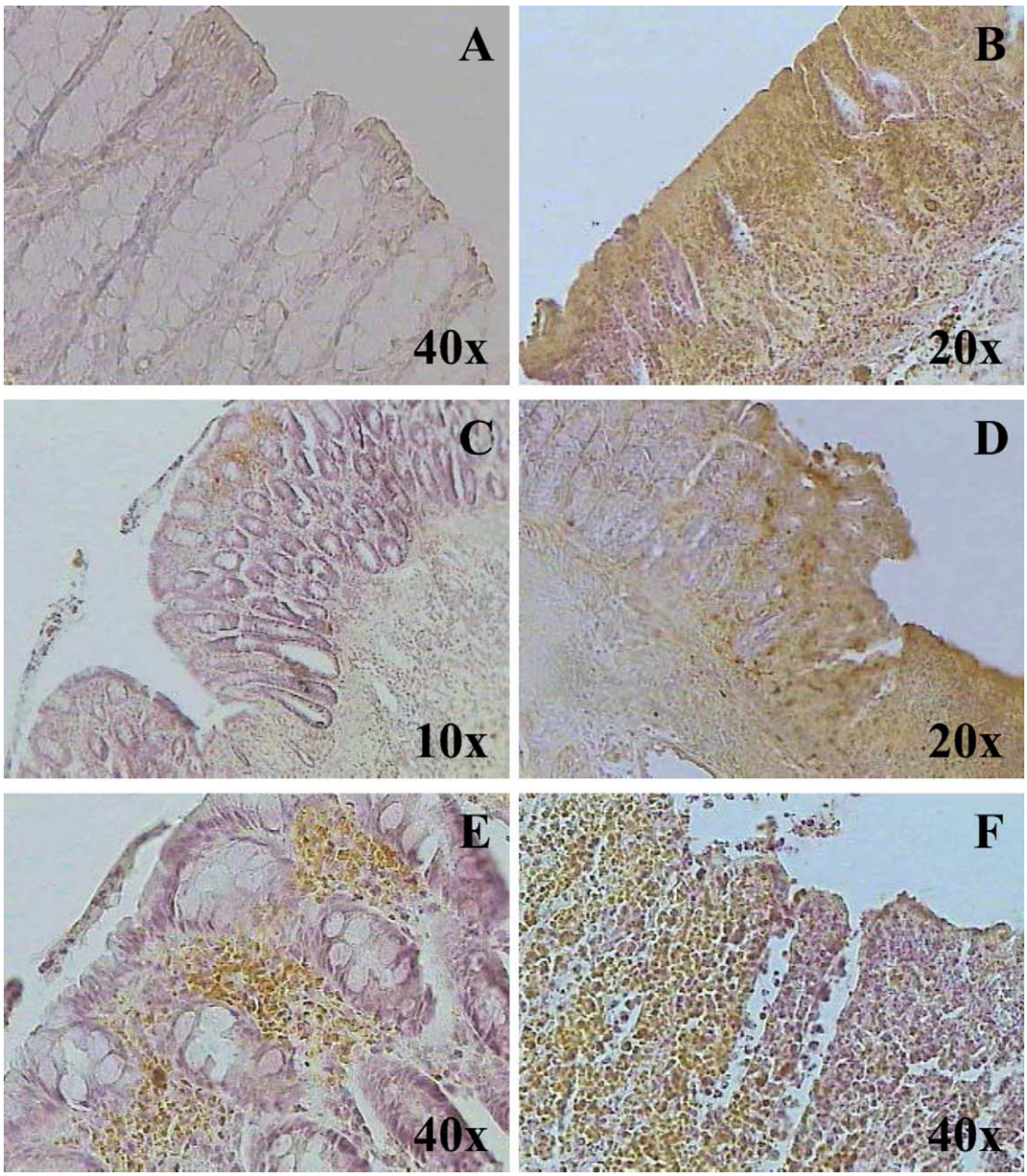

Fig. 6. Immunohistochemical localization of COX-2 isoenzyme in sections of colon. COX-2 expression in normal colonic mucosa (A). COX-2 is strongly expressed in the colon of TNBS-control rats (B and D). COX-2 expression was decreased in apical epithelial cells of inflamed colon treated with resveratrol 10 mg/kg (C, E and F). Original magnifications 20 and 40×.

rats. Furthermore, treatment of rats with resveratrol caused a significant ($P < 0.05$) increase of TNBS-induced apoptosis.

4. Discussion

Intestinal bowel disease (IBD), which include UC and Crohn's disease are chronic nonspecific inflammatory disorders with unknown aetiology. Various mucosal inflammatory mediators (cytokines, eicosanoids and reactive oxygen metabolites (ROM)) and immunoreactive cells (neutrophils, lymphocytes and macrophages) have been investigated concerning their contribution to the pathogenesis of this disease. Activated neutrophils pass out of the circulation and enter the inflamed mucosa and submucosa of the large intestine during acute inflammation, leading to overproduction of reactive oxygen and nitrogen species. Neutrophils can also release proteases, lactoferrin and lipid mediators that can contribute to intestinal injury [12,22]. Quantitatively, the principal free radical in tissues is superoxide anion (O_2^-) which is converted to the secondary oxidant H_2O_2 by superoxide dismutase. O_2^- can be produced by both endothelial cells through xanthine oxidase

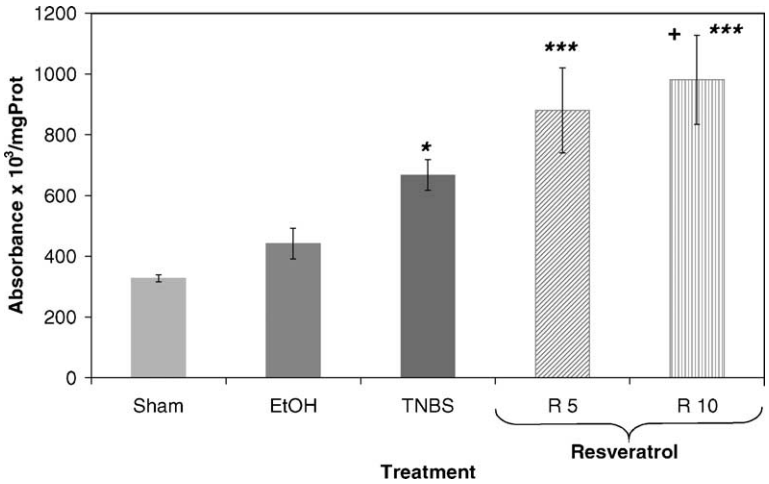

Fig. 7. Apoptosis observed in the colonic mucosa after acute colitis induced by TNBS (10 mg/animal). Apoptosis were quantified in the absence of treatment, but with daily administration of the vehicle saline solution (sham group, trinitrobenzene sulphonic acid group and ethanol group), or in the presence of resveratrol (R: 5 and 10 mg/kg/day). Data are expressed as the mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. sham. $^{+}P < 0.05$ vs. TNBS.

and activated neutrophils through NADPH oxidase, which reduces molecular oxygen to the O_2^- radical, and through the enzyme MPO. This enzyme catalyzes the formation of such potent cytotoxic oxidants as hypochlorous acid from H_2O_2 and chloride ions and *N*-chloramines.

In our experiments, the cellular infiltrate and the inflammation of colon tissue caused by TNBS were substantially reduced in rats treated with the polyphenolic compound. Previous reports have shown the anti-inflammatory action and antioxidant effects of resveratrol [8,24]; this drug in particular has been effective in reducing carrageenan-induced inflammation in rats [1] and also in inhibiting MPO, oxidized glutathione reductase and superoxide dismutase activities [24]. Likewise, using unopsonized zymosan-stimulated cells and human monocytes and neutrophils, it was demonstrated that resveratrol inhibited ROM generation [25]. Moreover, resveratrol exerted a strong inhibitory effect on ROM production stimulated by lipopolysaccharide (LPS) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [8]. We suggest that the antioxidant properties of resveratrol might be exerted through a biochemical mechanism related to its anti-inflammatory and protective effects in UC.There is good evidence that the proinflammatory cytokine IL-1β helps to propagate the extension of a local or systemic inflammatory process. IL-1β is a key immunoregulatory cytokine that amplifies the inflammatory response by activating a cascade of immune cells and appears to be a primary stimulator of diarrhoea, the major symptom of intestinal inflammation [26]. IL-1β in high doses produces epithelial cell necrosis, oedema, neutrophil infiltration and, globet cell depletion. Blocking of the action of endogenous IL-1β attenuates acute and chronic experimental colitis and its systemic complications [27]. Our results showed a significant suppression of IL-1β in the resveratrol-treated rats. The ability of the polyphenolic stilbene to partially reduce the inflammatory cell infiltrate in the colon could in part explain the observed reduction in the levels of the cytokine. Similar reduction of cytokine production by resveratrol was observed in cultured rat hepatic Kupffer cells stimulated with LPS [28], in splenic lymphocytes, peritoneal macrophages [29] and leukaemia cell lines [30]. These effects were produced in part through the inhibition of the transcription factor NF-κB [29,31].

In additional studies, we determined the production of PGE_2 and PGD_2 in colon mucosa and the expression of COX-1 and -2 by immunohistochemistry. Our data showed that PGE_2 content increased significantly in colonic mucosa of TNBS group compared with that of sham animals. Resveratrol did not suppress the increased PGE_2 levels but produced a significant fall in the PGD_2 concentration. Previous studies found that the increased PGE_2 production during TNBS-induced acute colitis is due to enhanced activity of COX-2 pathway [32,33]. In accordance with reports in the literature, resveratrol inhibited COX-1 activity without affecting COX-2 activity when incubated with microsomes derived from sheep seminal vesicles as a crude source of COX-1 and the recombinant human COX-2 [1]. In *in vivo* studies resveratrol has been described as a selective COX-1 inhibitor in doses of 5–10 mg/kg [34,35]. These findings support our data and further suggest that resveratrol reduced colonic damage by a mechanism independent of PGE_2 synthesis by the COX-2 pathway. Topically applied reservation did not alter the expression of COX-1 and -2 induced by TPA in mouse skin [24], however, as judged by previous reports [8,36] and also shown in the current investigation, expression of COX-2 was enhanced by TNBS treatment, these increases could contribute to the inflammatory responses and tissue damage. Nevertheless, resveratrol although did not alter the expression of COX-1, down-regulated COX-2 expression. Our data are consistent with a previous study by Kankuri *et al.* [33] where the glucocorticoid dexamethasone that did not inhibit COX-2 activity, suppressed COX-

2 expression and, also reduced colonic damage and neutrophil infiltration [33].

PGD_2 is known to be an important mediator during acute and chronic inflammation. Production of this prostanoid was markedly elevated very early after induction of colitis (1–3 hr) but not thereafter. The initial increase in PGD_2 synthesis was paralleled by an increase in COX-2 expression, but it declined despite the continued elevation of COX-2 expression [19]. Accordingly, our results showed no significant changes of this prostanoid in TNBS-treated animals, which is in agreement with the worsening of colitis damage and with the augment in MPO activity. In contrast, a significant reduction of PGD_2 content in resveratrol-treated rats was produced. The possible explanation of this data, i.e. the factors responsible for the down regulation of PGD_2 synthase expression by resveratrol remains to be identified.

The protective effect of mucus as an active barrier may be attributed largely to its viscous and gel-forming properties that are derived from mucin glycoprotein constituents. Our results revealed that resveratrol increased the amount of mucus stained by Alcian blue (acid glucoproteins such as sialomucins) in colon mucosa. Alcian blue positive cells seem to be associated with regenerative processes of the mucosa [37]; by contrast, its reduction has been related to a decreased resistance of the mucosa and paralleled by alterations in the normal pattern of maturation of mucin in globet cells [38].

Apoptosis of individual cells may represent a protective mechanism against neoplasic development in the organism by eliminating genetically damaged cells or excess cells that have improperly been induced to divide by a mitotic stimulus. However, deregulated apoptosis seems to be a major cause of the impaired barrier function, leading to the invasion of pathogenic microorganisms, to an increase of leukocyte survival with exacerbation of the disease [39]. Previous studies have shown significant apoptosis in colonic epithelial cells during mild acute inflammation induced by dextran sulphate sodium [40] and TNBS-induced colitis [39]. These findings are in accordance with the present study, in which colonic cell death was associated with apoptosis in the colon lesion 48 hr after intracolonic administration of TNBS. In addition, treatment of rats with resveratrol caused a significant increase of TNBS-induced apoptosis.

An increase in apoptosis linked to resveratrol has already been documented in *in vitro* studies, for instance, various cell lines including colon cancer lines, JB6 epidermal cells, *human* leukaemia HL-60 cells, human oesophageal carcinoma cells, HCT116 colon carcinoma cells among others [20,41–46]. In these studies, it has been suggested that the p53 protein (wildtype or mutant) probably is required for resveratrol-induced apoptosis. The MAP kinases, ERKs, JNKs, or p38 kinases, might also be involved in resveratrol-induced activation of p53 and apoptosis [41]. However, in certain cancer lines, resveratrol also induced apoptosis independently of p53 [20,41].

Together, our data confirm that resveratrol exerts protective effects in acute experimental colitis. The anti-inflammatory effects seem to be related to impairment of neutrophil function, absence of up-regulation of IL-1β and increase of mucus production in colon mucosa. Resveratrol also reduced PGD_2 production and the over-expression of COX-2. We also found that resveratrol caused a significant increase of TNBS-induced apoptosis. In conclusion, taking into account the anti-inflammatory and powerful antioxidant properties of resveratrol our data suggest that the polyphenolic compound may have a chemopreventive role in UC.

References

[1] Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275:218–20.

[2] Virgili M, Contestabile A. Partial neuroprotection of in vivo excitotoxic brain damage by chronic administration of the red wine antioxidant agent, *trans*-resveratrol in rats. Neurosci Lett 2000;281:123–6.

[3] Hung LM, Chen JK, Huang SS, Lee RS, Su MJ. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. Cardiovasc Res 2000;47:549–55.

[4] Belguendouz L, Fremont L, Gozzelino MT. Interaction of *trans*-resveratrol with plasma lipoproteins. Biochem Pharmacol 1998;55:811–6.

[5] Manna SK, Mukhopadhyay A, Aggarwal BB. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. J Immunol 2000;164:6509–19.

[6] Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutat Res 2001;480:243–68.

[7] Chan MM, Mattiacci JA, Hwang HS, Shah A, Fong D. Synergy between ethanol and grape polyphenols, quercetin, and resveratrol, in the inhibition of the inducible nitric oxide synthase pathway. Biochem Pharmacol 2000;60:1539–48.

[8] Martinez J, Moreno JJ. Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. Biochem Pharmacol 2000;59:865–70.

[9] Moreno JJ. Resveratrol modulates arachidonic acid release, prostaglandin synthesis, and 3T6 fibroblast growth. J Pharmacol Exp Ther 2000;294:333–8.

[10] Tsai SH, Lin-Shiau SY, Lin JK. Suppression of nitric oxide synthase and the down-regulation of the activation of NFkappaB in macrophages by resveratrol. Br J Pharmacol 1999;126:673–80.

[11] Wadsworth TL, Koop DR. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. Biochem Pharmacol 1999;57:941–9.

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

[13] Nitta M, Hirata I, Toshina K, Murano M, Maemura K, Hamamoto N, Sasaki S, Yamauchi H, Katsu K. Expression of the EP4 prostaglandin E2 receptor subtype with rat dextran sodium sulphate colitis: colitis suppression by a selective agonist, ONO-AE1-329. Scand J Immunol 2002;56:66–75.

[14] Abreu MT. The pathogenesis of inflammatory bowel disease: translational implications for clinicians. Curr Gastroenterol Rep 2002;4:481–9.
[15] Siegmund B. Interleukin-1beta converting enzyme (caspase-1) in intestinal inflammation. Biochem Pharmacol 2002;64:1–8.
[16] Rachmilewitz D, Simon PL, Schwartz LW, Griswold DE, Fondacaro JD, Wasserman MA. Inflammatory mediators of experimental colitis in rats. Gastroenterology 1989;971:326–37.
[17] Fedorak RN, Empey LR, MacArthur C, Jewell LD. Misoprostol provides a colonic mucosal protective effect during acetic acid-induced colitis in rats. Gastroenterology 1990;98:615–25.
[18] Zhong WW, Burke PA, Drotar ME, Chavali SR, Forse RA. Effects of prostaglandin E2, cholera toxin and 8-bromo-cyclic AMP on lipopolysaccharide-induced gene expression of cytokines in human macrophages. Immunology 1995;84:446–52.
[19] Ajuebor MN, Singh A, Wallace JL. Cyclooxygenase-2-derived prostaglandin D(2) is an early anti-inflammatory signal in experimental colitis. Am J Physiol Gastrointest Liver Physiol 2000;279:G238–44.
[20] Mahyar-Roemer M, Katsen A, Mestres P, Roemer K. Resveratrol induces colon tumor cell apoptosis independently of p53 and precede by epithelial differentiation mitochondrial proliferation and membrane potential collapse. Int J Cancer 2001;94:615–22.
[21] Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 1989;96(3):795–803.
[22] Bobin-Dubigeon C, Collin X, Grimaud N, Robert JM, Le Baut G, Petit JY. Effects of tumor necrosis factor-α synthesis inhibitors on rat trinitrobenzene sulphonic acid-induced chronic colitis. Eur J Pharmacol 2001;42:103–10.
[23] Grisham MB, Beniot JN, Granger DN. Assessment of leukocyte involvement during ischemia and reperfusion on the intestine. In: Packer L, Glazer AE, editors. Methods in enzymology. Oxygen radicals in biological systems. San Diego: Academic Press; 1990. p. 729–41.
[24] Jang M, Pezzuto JM. Effects of resveratrol on 12-*O*-tetradecanoylphorbol-13-acetate-induced oxidative events and gene expression in mouse skin. Cancer Lett 1998;134:81–9.
[25] Jang DS, Kang BS, Ryu SY, Chang IM, Min KR, Kim Y. Inhibitory effects of resveratrol analogs on unopsonized zymosan-induced oxygen radical production. Biochem Pharmacol 1999;57:705–12.
[26] Sartor RB. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. Gastroenterology 1994;106:533–9.
[27] Rogler G, Andus T. Cytokines in inflammatory bowel disease. World J Surg 1998;22(4):382–9.
[28] Nawada N, Seki S, Inoue M, Kuroki T. Effect of antioxidants, resveratrol, quercetin, and *N*-acetylcysteine, on the functions of cultural rat hepatic stellate cells and Kupffer cells. Hepatology 1998;27:1265–74.
[29] Gao X, Xu YX, Janakiraman N, Chapman RA, Gautam SC. Immunomodulatory activity of resveratrol: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production. Biochem Pharmacol 2001;62:1299–308.
[30] Estrov Z, Shishodia S, Faderl S, Harris D, Van Q, Kantarjian HM, et al. Resveratrol blocks interleukin-1 β-induced activation of the nuclear transcription factor NF-κB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. Blood 2003;102:987–95.
[31] Manna SK, Mukhopadhyay A, Aggarwal BB. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. J Immunol 2000;164:6509–19.
[32] Guo X, Liu ES, Ko JK, Wong BC, Ye Y, Lam S, Cho C. Protective role of cyclooxygenase inhibitors in the adverse action of passive cigarette smoking on the initiation of experimental colitis in rats. Eur J Pharmacol 2001;41:193–203.
[33] Kankuri E, Vaali K, Korpela R, Paakkari I, Vapaatalo H, Moilanen E. Effects of a COX-2 preferential agent nimesulide on TNBS-induced acute inflammation in the gut. Inflammation 2001;25:301–9.
[34] Brzozowski T, Konturek PC, Konturek SJ, Drozdowicz D, Pajdo R, Pawlik M, Brzozowska I, Hahn EG. Expression of cyclooxygenase (COX)-1 and COX-2 in adaptive cytoprotection induced by mild stress. J Physiol (Paris) 2000;94:83–91.
[35] Korolkiewicz RP, Ujda M, Dabkowski J, Ruczynski J, Rekowski P, Petrusewicz J. Differential salutary effects of nonselective and selective COX-2 inhibitors in postoperative ileus in rats. J Surg Res 2003;109(2):161–9.
[36] Li ZG, Hong T, Shimada Y, Komoto I, Kawabe A, Ding Y, Kaganoi J, Hashimoto Y, Imamura M. Suppression of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. Carcinogenesis 2002;23:1531–6.
[37] Alarcón de la Lastra C, Martín MJ, Motilva V. Antiulcer and gastroprotective effect of quercetin: a gross and histologic study. Pharmacology 1994;48:56–62.
[38] Torres MI, García-Martín M, Fernández MI, Nieto N, Gil A, Ríos A. Experimental colitis induced by trinitrobenzenesulphonic acid. An ultrastructural and histochemical study. Dig Dis Sci 1999;44:2523–9.
[39] Yue G, Lai PS, Yin K, Sun FF, Nagele RG, Liu X, Linask KK, Wang C, Lin KT, Wong PY. Colon epithelial cell death in 2,4,6-trinitrobenzenesulfonic acid-induced colitis is associated with increased inducible nitric-oxide synthase expression and peroxynitrite production. J Pharmacol Exp Ther 2001;297:915–25;21:973–6.
[40] Tardieu D, Jaeg JP, Deloly A, Corpet DE, Cadet J, Petit CR. The COX-2 inhibitor nimesulide suppresses superoxide and 8-hydroxy-deoxyguanosine formation, and stimulates apoptosis in mucosa during early colonic inflammation in rats. Carcinogenesis 2000;21:973.
[41] Dong Z. Molecular mechanism of the chemopreventive effect of resveratrol. Mutat Res 2003;523/524:145–50.
[42] Hayashibara T, Yamada Y, Nakayama S, Harasawa H, Tsuruda K, Sugahara K, Miyanishi T, Kamihira S, Tomonaga M, Maita T. Resveratrol induces downregulation in survivin expression and apoptosis in HTLV-1-infected cell lines: a prospective agent for adult T cell leukemia chemotherapy. Nutr Cancer 2002;44:193–201.
[43] Zhou HB, Yan Y, Sun YN, Zhu JR. Resveratrol induces apoptosis in human esophageal carcinoma cells. World J Gastroenterol 2003;9:408–11.
[44] Boscolo P, del Signore A, Sabbioni E, Di Gioacchino M, Di Giampaolo L, Reale M, Conti P, Paganelli R, Giaccio M. Effects of resveratrol on lymphocyte proliferation and cytokine release. Ann Clin Lab Sci 2003;33:226–31.
[45] Cal C, Garban H, Jazirehi A, Yeh C, Mizutani Y, Bonavida B. Resveratrol and cancer: chemoprevention, apoptosis, and chemoimmunosensitizing activities. Curr Med Chem Anti-Cancer Agents 2003;3:77–93.
[46] Bernhard D, Schwaiger W, Crazzolara R, Tinhofer I, Kofler R, Csordas A. Enhanced MTT-reducing activity under growth inhibition by resveratrol in CEM-C7H2 lymphocytic leukemia cells. Cancer Lett 2003;195:193–9.