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Benefit of nicorandil using an immunologic murine model of experimental colitis

Research Article

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Abstract: Inflammatory bowel disease (IBD) is a chronic inflammatory condition with an unknown etiology. Nicorandil, a potassium channel opener, has been used for many years for the treatment of angina. Recently, it has been shown that nicorandil possesses some novel traits such as anti-apoptotic, gastroprotective, free radical scavenging, and anti-inflammatory properties. Therefore, we set out to examine the possible beneficial effect of nicorandil in a rat model of IBD. Colitis was induced by rectal administration of 2,4,6-trintrobenzene sulphonic acid (TNBS) into rats. Groups of animals used in this study were sham, control, and exposure to dexamethasone, nicorandil, glibenclamid (a pure adenosine triphosphate sensitive potassium channel (KATP) blocker), or nicorandil plus glibenclamid. Drugs were administered by gavage and animals were sacrificed after 7 days. Biochemical markers, including TNF-α and IL-1β, ferric reducing/antioxidant power (FRAP), myeloperoxidase (MPO) activity and thiobarbituric acid-reactive substance (TBARS), were measured in the homogenate of colonic tissue. Results indicate that nicorandil significantly reduces macroscopic and histological damage induced by TNBS. Nicorandil diminishes MPO activity and levels of TBARS, TNF-α, and IL-1β in damaged colonic tissue with a concomitant increase in FRAP value ($P<0.01$). These effects were not reversed by coadministration of glibenclamide. In conclusion, nicorandil is able to ameliorate experimental IBD with a dose in which it does not show any anti-hypertensive effect, and the mechanism of which is partially or totally independent from KATP channels. It is hypothesized that nitric oxide donation and free-radical scavenging properties of nicorandil upregulate endothelial nitric oxide synthase may be responsible for this phenomenon. These findings suggest that nicorandil can be useful in treatment of IBD, although further investigations are needed to elucidate the mechanisms involved.

Keywords: *Nicorandil • Inflammatory bowel disease • Immunologic • Myeloperoxidase • Inflammatory cytokines • 2,4,6-trinitrobenzene sulphonic acid (TNBS)*

1. Introduction

Inflammatory bowel disease (IBD) which develops in the gastrointestinal (GI) tract is composed of a variety of chronic idiopathic inflammatory conditions. Traditionally, IBD is comprised of two types of diseases, namely ulcerative colitis and Crohn's disease.

The definite etiology of IBD is unknown, though it seems that the most important basis of this condition is a dysregulation of the immune system and imbalance between proinflammatory and anti-inflammatory mediators [1]. Reactive oxygen species (ROS), which are believed to be one of the effective immunoregulatory factors, are atypically produced in tremendous levels in IBD. It is thought that other factors such as environmental and genetic factors are involved in the initiation and perpetuation of this condition. A recent research has demonstrated that two phenomena, oxidative stress and nitrosative stress (toxic stress), have important

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etiologic roles in IBD [2]. ROS are by-products of the normal metabolism of oxygen and reactive nitrogen species (RNS) are by-products of the reaction of nitric oxide (NO) with O_2 or O_2^- [3,4].

The treatment of IBD remains challenging, although many medications are being used, such as salicylates, glucocorticoids, and immunosuppressives like azathiopurine, 6-mercaptopurine and cyclosporine [5]. Currently, the use of complementary and alternative therapies is also common in 50% of patients suffering from IBD [6]. Moreover, various herbal extracts [7-10], N-acetylcysteine (NAC) [11] and probiotics [12-15] have been suggested for the treatment of IBD, and the effectiveness of antibiotic therapy in IBD has been investigated [16,17]. Unfortunately, it is very difficult to reach maximum efficacy and quality of life because of the side effects [18] associated with the treatments described above. Therefore it is essential to develop other medicines with novel mechanisms, high efficacy, and fewer side effects.

Recent investigations in the area of GI protection have drawn attention to ion channel modulators, especially potassium channel openers (PCOs). Nicorandil, one such PCO and a nitrate donor, is enormously useful in the treatment of angina [19]. In addition, the known anti-ulcer activity of nicorandil is based on K channel opening that prevents a potentially dangerous increase in NO. Moreover, it has been shown that nicorandil exerts anti-free-radical and neutrophil modulating properties that are independent from its effect on KATP channels. One mechanism that has been suggested is that the nicotinamide moiety in its structure is a hydroxyl radical scavenger. Another study shows that nicorandil is able to reduce ROS generation in hypoxia-reoxygenation treated endothelial cells, thereby sustaining endothelial NO and suggesting a role for KATP channels.

Nicorandil can inhibit TNF-α release from immune cells in a dose-dependent manner. It subsequently inhibits local release of inflammatory mediators *via* both potassium channel opening and NO donating mechanism, although the second mechanism seems to be more involved [20]. Heywood and Thomas (2002) have shown that nicorandil prevents degranulation and TNF-α release of rat basophilic leukemia cells (RBL-2H3) *via* both mechanisms [21]. Nicorandil is also able to decrease production of TNF-α in macrophages which have already increased in response to ROS, and inhibits the release of free radicals from different cells [20].

Recent investigations demonstrate that activity of the mitochondrial KATP (mt-KATP) channel prevents the release of mitochondrial ROS; in fact, mt-KATP functions as a reactive oxygen sensor that diminishes mitochondrial free radical generation in response to increased local levels of oxidants. Consequently, opening of this channel prevents oxidative stress and cell death [22-24]. It has been shown that nicorandil is able to activate mt-KATP channels and, in so doing, prevent oxidative stress-induced apoptosis in neurons [25]. Nicorandil also shows anti-apoptosis property *via* a NO/cGMP-dependent mechanism and by activating mt-KATP channels [26,27].

The aim of the study reported here was to investigate the anti-inflammatory effect of nicorandil on experimental colitis in rats, to assess its influence on biochemical markers of colonic injury, and to compare the effects found with those of corticosteroids.

2. Experimental Procedures

2.1 Animals

Male Wistar-albino rats weighing between 220 and 230 g were used in this study. Animals were maintained under standard conditions of temperature (23±1ºC), relative humidity (55±10%), and 12/12 hours light/dark cycle, and fed with a standard pellet diet and water ad libitum. They were housed individually in standard polypropylene cages with a wire mesh top. All animals were treated ethically and humanely, and the experimental protocol was approved by the Ethics Committee of PSRC/TUMS.

2.2 Chemicals

2,4,6-trinitrobenzene sulphonic acid (TNBS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), n-butanol, hexadecyl trimethyl ammonium bromide (HETAB), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), hydrochloric acid (HCL), malondialdehyde (MDA), ethylene diamine tetra acetic acid (EDTA), O-dianisidine hydrochloride, hydrogen peroxide, acetic acid, sodium acetate, folin-ciocalteu reagent, bovine serum albumin (BSA), ferric chloride ($FeCl_3$-$6H_2O$), sodium sulphate (Na_2SO_4), sulphuric acid (H_2SO_4), phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen diphosphate (K_2HPO_4), peroxide hydrogen (H_2O_2), sodium carbonate (Na_2CO_3), Na K tartarate, cupric sulphate ($CuSO_4$-5H2O) were obtained from Merck (Tehran), nicorandil was obtained from Aventis (Tehran), glibenclamide was obtained from Apotex (Tehran), dexamethasone was obtained from DaruPakhsh (Tehran), and rat-specific TNF-α and IL-1β ELISA kits were obtained from BioSource (Belgium).

2.3 Experimental design

In this study, six groups of male rats consisting of 6 rats in each group, were used. Colitis was induced by application of TNBS in five groups, while the resting group was considered as sham group that received normal saline instead of TNBS. The five groups receiving TNBS were: 1- control group that received no treatment, 2- nicorandil-treated group (Nic) receiving nicorandil (6 mg/kg), a dose at which it does not show any antihypertensive effect [28], 3- glibenclamide-treated group (Glib) receiving glibenclamide (3 mg/kg) administered as a pure KATP channel blocker (PCB) in order to determine if nicorandil serves its possible effects through KATP channels, 4- nicorandil plus glibenclamide-treated group (Nic+Glib), in which nicorandil was administered 30 minutes after administration of glibenclamide [29], and 4- dexamethasone-treated group (Dexa) receiving dexamethasone (1 mg/kg) [30]. All the medications were prepared in a volume of 0.4 ml/ 200 g of body weight and administered by gavage for 7 days; the day of induction of colitis was considered as day 1.

2.4 Induction of colitis

All the rats were fasted for 36 h before the induction of colitis [30]. Rats were anesthetized by intraperitoanal (i.p.) administration of 45 mg/kg phenobarbital sodium [31]. First, colitis was induced by rectal administration of the mixture of 30 mg TNBS in 0.25 ml of ethanol 50% [32]. However, because of high rate of mortality, the method was modified, based on macroscopic- and microscopic evidence that the colitis was induced properly: the rats were positioned on their right side and 0.3 ml of a mixture containing 6 vol of 5% TNBS plus 4 vol of 99% ethanol was instilled intracolonically using a rubber cannula (8 cm long). After the instillation of TNBS, rats were maintained in a supine Trendelenburg position to prevent anal outflow of TNBS. Over the next seven days, medication was administered to the rats as described. On the eighth day, animals were anaesthetized using phenobarbital sodium, after which the abdomen was dissected open and the colon removed. All animals were sacrificed at the end of the procedure using an overdose of ether inhalation. The pieces of colons were cut open while on ice, cleansed gently using normal saline, and the macroscopic score of inflammation was determined for each one. Each sample was cut into two pieces, one piece for histopathology assessment (maintained in 5 ml formalin 10% as a fixative) [33] and one piece for measuring biomarkers. The pieces for analysis of biomarkers were weighed and maintained in -20º C for 24 h. Then, the colonic samples were homogenized in 10 vol ice cold 50 mM potassium phosphate buffer (pH 7.4), for which 100 µl of each sample was taken for FRAP assay and maintained at -80ºC until analysis. The remained homogenate for each sample was then sonicated and centrifuged for 30 min at 3500 g. The plates were maintained separately and the supernatants were transferred into several microtubes for separate biochemical assays and maintained at -80ºC until the analyses were performed.

2.5 Macroscopic assessment of colonic damage

The severity of colitis was assessed using the colon macroscopic scoring as described previously [34] (Table 1).

2.6 Microscopic assessment of colonic damage

The fixed segments in formalin 10% were embedded in paraffin for staining with hematoxylin and eosin. The scoring was performed by an observer blind to the identity of each treated group, as described previously [34] (Table 2).

2.7 Determination of tumor necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β)

An enzyme-linked immunosorbaent assay (ELISA) kit was used for quantitative detection of TNF-α and IL-1β levels in colon tissues (Bender Medsystem Rat TNF-α

Macroscopic features	Score
Normal appearance with no damage	0
Localized hyperemia without ulceration	1
Linear ulceration without significant inflammation	2
Linear ulceration with inflammation at one site	3
Two or more sites of ulceration extending more than 1 cm along the length of colon	4
Damage extending more than 2 cm along the length of colon (score is increased by 1 for each increased cm of involvement)	5-8

Table 1. Macroscopic scoring of colonic damage.

Microscopic criteria	Score
No damage	0
Focal epithelial edema and necrosis	1
Dispersed swelling and necrosis of the villi	2
Necrosis with neutrophil infiltration in submucosa	3
Wide spread necrosis with massive neutrophil infiltration and hemorrhage	4

Table 2. Microscopic scoring of colonic damage.

ELISA and Bender Medsystem Rat IL-1β ELISA). According to the procedure, a color product is formed in proportion to the amount of cytokine present in the sample. After adding stop solution to terminate the reaction, absorbance was measured at 450 nm as the primary wave length and 620 nm as the reference wave length. TNF-α and IL-1β levels were expressed as pg of cytokine per mg protein of tissue.

2.8 Ferric Reducing/Antioxidant Power (FRAP) assay

The efficiency of antioxidants is measured by their ability to reduce Fe^{3+} to Fe^{2+}. The interaction of TPTZ with Fe^{2+} leads to formation of a blue color, which has a maximum absorbance at 593 nm, as described previously [35]. The data was expressed as μM ferric ions reduced to ferrous form per g of tissue.

2.9 Myeloperoxidase (MPO) activity measurement

MPO activity in colon tissue was measured spectrophotometrically by measuring the absorbance for 3 min in 460 nm, as described previously [7]. MPO activity was reported as units per mg protein of tissue.

2.10 Thiobarbituric acid-reactive substance (TBARS) assay

MDA, which is the main by-product of polyunsaturated fatty acids oxidation, is a known biomarker of lipid peroxidation, and its concentration was assessed in colon tissue using the TBARS assay. In this method, MDA reacts with thiobarbituric acid to produce a pink color and its absorbance is measured spectrophotometrically at 532 nm, as described previously [36]. The data was reported as μM/g of tissue.

2.11 Total protein (TP) of colon homogenate

According to Lowry method, BSA was used at different concentrations as a protein standard against which to measure TP (mg/ml) in colon homogenate [37].

2.12 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The data was analyzed by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons to ensure the variances of data are distributed properly. A P-value of less than 0.05 was considered significant.

3. Results

3.1 Macroscopic and microscopic evaluation of the colonic damage

Data are shown in Table 3. Intracolonic administration of TNBS/ethanol caused severe ulcerations, adhesions, wall thickening and inflammation in control group; and colons of sham group had normal appearance (P<0.01). In contrast, treatment with nicorandil significantly decreased the inflammation and ulcer (P<0.01) and, in some samples, local hyperemia was observed. In the glibenclamide group, some hyperemia and inflammation, but no mucosal inflammation, were observed and there was a significant difference compared to colitis group (P<0.01). Also, treatment of rats by nicorandil and glibenclamide improved ulcers significantly (P<0.01); less adhesion and wall thickening were observed though some inflamed areas were evident. Finally, scores of the colitis group were reversed noticeably by dexamethasone.

Microscopic evaluation of the control group showed severe edema, hemorrhage, and mucosal and submucosal polymorphonuclear leucocyte (PMN) infiltration with crypt abscess, whereas in the sham group the features of the colons were within normal limits (P<0.01). Histological examination of the Nic group showed diffuse edema and mild focal mucosal and submucosal PMN infiltration without crypt abscess and necrosis. In addition, in the Glib- and Nic+Glib groups, focal non-hemorrhagic edema, focal submucosal PMN infiltration with crypt abscess, and goblet cell depletion were observed (P<0.01). Finally, no significant difference was observed in histological evaluation of colons between sham and Dexa groups (Figure 1).

Groups	Macroscopic Score (Mean + SEM) Median (Min-Max)	Microscopic Score (Mean + SEM) Median (Min-Max)
Sham	(0.0 ± 0.0) 0 (0.0-0.0)	(0.0 ± 0.0) 0 (0.0-0.0)
Control	(5.75 ± 0.47)[a] 5.5 (5.0-7.0)	(4.0 ± 0.0)[a] 4 (4.0-4.0)
Dexa	(1.0 ± 0.36)[b] 0.5 (0.0-2.0)	(0.8 ± 0.2)[b] 1 (0.0-1.0)
Nic	(1 ± 0.31)[b] 1 (0.0-2.0)	(1.0 ± 0.4)[b] 0.5 (0.0-2.0)
Glib	(1.0 ± 0.25)[b] 1 (0.0-2.0)	(1.00 ± 0.31)[b] 1 (0.0-2.0)
Nic+Glib	(1.2 ± 0.37)[b] 1 (0.0-2.0)	(0.8 ± 0.2)[b] 1 (0.0-1.0)

Table 3. Extent of colonic damage according to macroscopic and microscopic scores.

[a] Significantly different from Sham group at P<0.01. [b] Significantly different from control group at P<0.01.

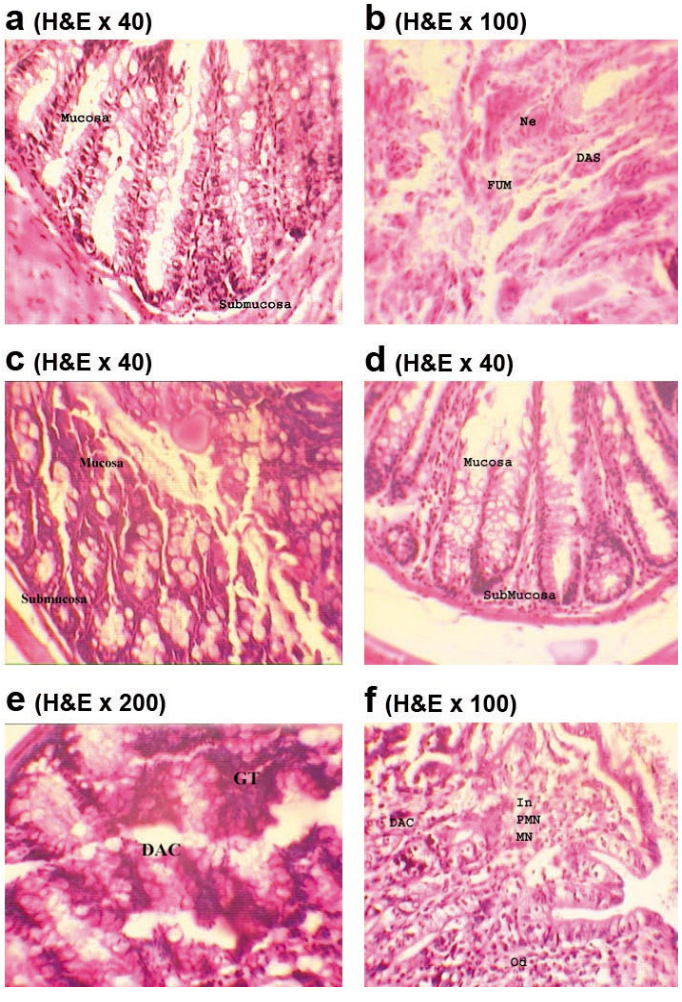

Figure 1. Histological images of colonic samples.
a) Sham group, b) Control group, c) Dexa group, d) Nic group, e) Glib group, f) Nic+Glib group. H&E, hematoxyllin and eosin; NE, Necrosis; OD, Oedema; IN, inflammation; GT, Granulated Tissue; PMN, Polymorphonuclear leukocytes; MN, Mononuclear; FUM, Focal Ulceration of Mucosa; DAC, Disrupted Architecture of Crypt.

3.2 Colonic TNF-α levels

TNF-α level in the control group was elevated significantly when compared to that of the sham group (P<0.01). Nicorandil, glibenclamide, and co-administration of these compounds significantly lowered the level of TNF-α (P<0.01). The TNF-α level in the Nic group (P=0.07) and the Nic+Glib group (P=0.27) approached the levels observed by administration of dexamethasone, although its level in the Glib group was significantly higher than in the Dexa (P=0.01) group. Administration of nicorandil resulted in TNF-α levels which were not significantly different from those in the Glib (P=0.95) and Nic+Glib (P=0.96) groups. Finally, the TNF-α level in the Glib-treated animals was not significantly different from Nic+Glib group (P=0.57) (Figure 2).

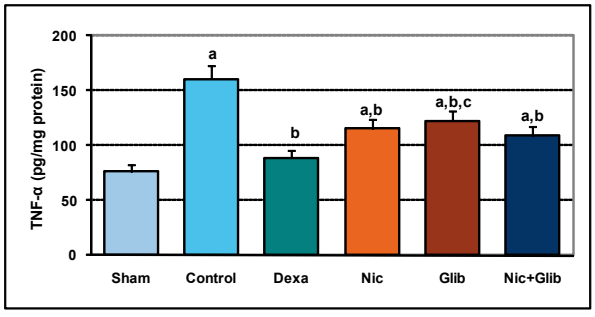

Figure 2. Tumor necrosis factor-alpha (TNF-α) levels in colon. Values are mean±SEM. [a] Significantly different from sham group at P<0.01. [b] Significantly different from control group at P<0.01. [c] Significantly different from dexamethason group at P<0.01.

3.3 Colonic IL-1β levels

As shown in Figure 3, there was a significant increase (P<0.01) in the level of IL-1β in TNBS-induced colitis rat when compared with the sham group. Administration of nicorandil, glibenclamide, and a combination of the two improved colitis in terms of IL-1β level significantly (P<0.01). There was no significant difference in the Nic group (P=0.18) and the Glib group (P=0.99) when compared to the Dexa group, although its level was significantly higher in the Nic+Glib group (P<0.01). There was no significant difference in IL-1β levels in the Nic group when compared to the Glib group (P=0.42) and the Nic+Glib group (P=0.45). The Nic group value was near the normal range. Additionally, the level of IL-1β was significantly lower in the Nic+Glib group when compared with the Glib group (P=0.01).

3.4 Colonic FRAP

Inflammatory conditions caused a significant decrease in FRAP value in the control group when compared to normal rats (P<0.01). Nicorandil, glibenclamide, and

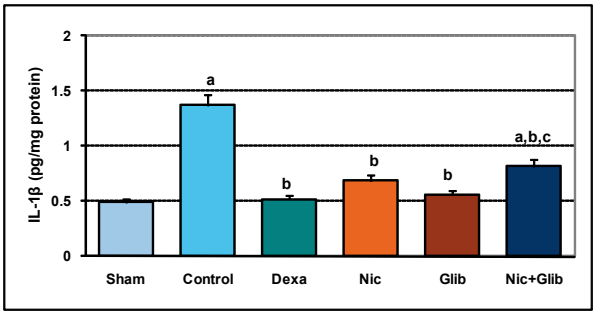

Figure 3. Interleukin-1β (IL-1β) levels in colon. Values are mean±SEM. [a] Significantly different from sham group at P<0.01. [b] Significantly different from control group at P<0.01. [c] Significantly different from dexamethason group at P<0.01.

coadministration of these compounds increased its value significantly (P<0.01). Although nicorandil significantly augmented the FRAP value (P<0.01) when compared to dexamethasone, the FRAP value approached that observed in the Dexa group, in the Glib (P=0.203) and Nic+Glib (0.96) groups. Nicorandil caused a significant elevation in FRAP by comparison with glibenclamide (P<0.01) and the combination of nicorandil and glibenclamide (P<0.01). In Glib group, however, there was no significant difference in terms of FRAP value with the Nic+Glib group (P=0.59) (Figure 4).

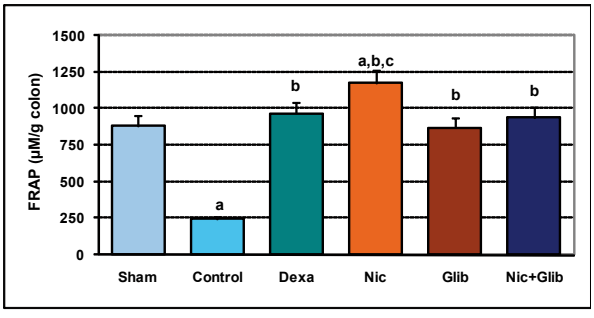

Figure 4. Ferric Reducing Antioxidant Power (FRAP) of colon. Values are mean±SEM. [a] Significantly different from sham group at P<0.01. [b] Significantly different from control group at P<0.01. [c] Significantly different from dexamethason group at P<0.01.

3.5 Colonic MPO activity

Colonic MPO activity in the non-treated colitis group was noticeably higher than that in the sham group (P<0.01). Treatment with nicorandil, glibenclamide and coadministration of these compounds decreased MPO activity significantly, in comparison with the control group (P<0.01). MPO activity in the Nic group approached the values observed by dexamethasone administration (P=0.08), but its value was significantly higher in the Glib- (P<0.01) and Nic+Glib groups (P=0.01), when compared

to the Dexa group. MPO activity decreased significantly in the Nic group as compared to the Glib group (P<0.01) but there was no noticeable difference when compared to the Nic+Glib group (P=0.94). Furthermore, MPO activity in the Glib group was significantly higher than that observed in the Nic+Glib group (P<0.01) (Figure 5).

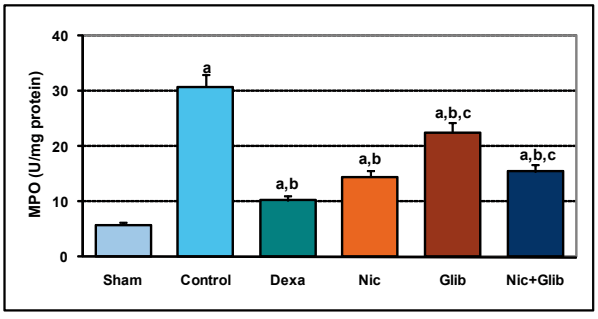

Figure 5. Myeloperoxidase (MPO) activity in colon. Values are mean±SEM. [a] Significantly different from sham group at P<0.01. [b] Significantly different from control group at P<0.01. [c] Significantly different from dexamethason group at P<0.01.

3.6 Colonic lipid peroxidation level as TBARS

Induction of colitis in control animals significantly increased the TBARS value (P<0.01) as compared to the sham group. Nicorandil, glibenclamide and coadministration of these compounds significantly (P<0.05) decreased the TBARS value in the colon of IBD rat. The TBARS value was significantly higher in the Nic (P<0.01), Glib (P<0.01), and Nic+Glib (P<0.01) groups, compared to those in the Dexa group. The TBARS value was significantly reduced in the Nic group when compared to the Glib (P<0.01) and Nic+Glib (P<0.01) groups. The TBARS value in the Glib group was not significantly different from that observed in the Nic+Glib group (P=0.99) (Figure 6).

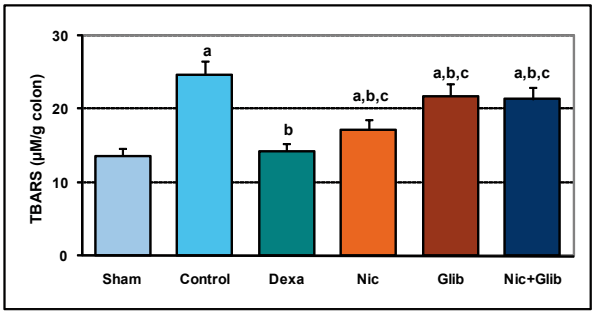

Figure 6. Lipid peroxidation as TBARS in colon. Values are mean±SEM. [a] Significantly different from sham group at P<0.01. [b] Significantly different from control group at P<0.05. [c] Significantly different from dexamethasone group at P<0.01.

4. Discussion

The present study demonstrates for the first time the beneficial effects of nicorandil in an animal model of TNBS-induced colitis. Nicorandil significantly improved TNBS-induced macroscopic and histological damages and diminished neutrophil infiltration and markers of inflammation and toxic stress.

In order to assess the effects of different medications in the treatment of IBD, an appropriate animal model is needed which covers all the characteristics of a typical human IBD including acute and chronic features, and biochemical and pathological changes. TNBS-induced colitis used in this project is a well-defined animal model of colitis meeting all of the above-mentioned criteria and high reproducibility [38,39]. In this model, after devastation of the mucosal barrier by ethanol, TNBS acts as a hapten and induces a delayed-type hypersensitivity that leads to chronic colitis. According to previous studies the main symptom of IBD, diarrhea, is evident in this model as a result of poor absorption of water from the inflamed area and closely mimics the symptoms of human IBD [1].

Overexpression of pro-inflammatory cytokines such as TNF-α and IL-1β is marked during inflammation especially colitis. It has been shown that the levels of these cytokines increase in both experimental model and human IBD [11,40-42]. These major inflammatory mediators in the progression of IBD can induce production of other inflammatory mediators, which can amplify tissue damage [43]. TNF-α is widely secreted during inflammation from monocytes and macrophages, T cells, B cells, NK cells, and mast cells [40]. This cytokine is able to trigger a cascade of pro-inflammatory cytokine production [10]. Attempts to block TNF-α or reduce its levels have been successful in the treatment of IBD. Currently, Infliximab, a monoclonal antibody against TNF-α, is in widespread use [41,42]. Further, IL-1β is responsible for diarrhea, the major complication of IBD and associated tissue damage [43]. Our results show that nicorandil is able to diminish TNF-α and IL-1β levels in damaged colon tissue most likely through its ability to reduce TNF-α production and release from lymphocytes and also lessen neutrophil recruitment, thereby reducing production of inflammatory cytokines such as IL-1β.

As mentioned before, ROS and RNS are extremely reactive molecules that cause detrimental damages to macromolecules. As a consequence of a marked imbalance between the production of oxidants and the neutralization role of antioxidants, oxidative stress plays an important role in the initiation of IBD. As described

previously [2,44], free radicals are not only mediators involved in the progression of IBD but also they can contribute to the regulation of genes that control other aspects of inflammation, both the immune and acute phase response. One suggested mechanism for the antioxidant activity characteristics of nicorandil is the role of mt-KATP channels. Although some investigations suggest that mt-KATP channels have a role in ischemic preconditioning, especially in intestinal tissue [24], there are others who question this property [45,46]. For instance, one suggested mechanism to explain ischemic preconditioning in cardiac tissue is involvement of sarcolemmal KATP channels rather than mt-KATP channels. After a report of mt-ATP channel activity by Inoue *et al.* (1991) [47], there have been no further investigations focusing on the structure of the channels; more research is needed to evaluate their role and effectiveness under the conditions described in this study.

In order to evaluate the antioxidant potential in colon samples following induction of colitis and treatment, we used the FRAP method and, as expected, nicorandil was able to increase its the antioxidant properties and confirming its role as a free radical scavenger.

Tissue MPO activity, a marker of neutrophil sequestration and infiltration into GI tract, has been used extensively as a biomarker of inflammation [7-9]. This marker is stored in some immune cells, such as neutrophils, and is responsible for mediating microbial killing as well as initiation and progression of both acute and chronic inflammatory reactions [48]. Infiltrated and activated neutrophils produce a large amount of cytotoxic components, especially ROS and RNS, leading to progression of inflammation. It has been shown that experimental and human IBD is associated with a significant increase in mucosal MPO activity and also in the number of MPO positive neutrophils [33,49]. With respect to our results, nicorandil is able to significantly reduce MPO activity in colonic tissue by decreasing granulocytes at the inflammation site, thereby reducing their harmful effects.

Lipid peroxidation was assessed using the TBARS assay. MDA, the lipid peroxidation by-product, has been reported to be increased in animal models of colitis, suggesting another role of ROS in progression of inflammation and damage. Superoxide and H_2O_2 are able to increase mucosal penetration of neutrophils and activation of others [2], resulting in the release of pro-inflammatory cytokines and exacerbating the injury. Moreover, there are indications that TBARS increases significantly in the inflamed mucosa of patients suffering from IBD [50]. Results of the TBARS assay in colon tissue show that nicorandil is capable of reducing lipid peroxidation, supporting its role as an antioxidant. However, there are other studies that suggest a minor anti-free-radical activity for nicorandil in necrotic tissue during ischemia, and this finding confirms the existence of more effective mechanisms than KATP channel opening. To determine whether nicorandil exerts its protective effects in IBD through KATP channel or by another mechanism, glibenclamide was used as a PCB. According to our results, glibenclamide did not reverse the above-mentioned effects of nicorandil. The first thing comes to mind is that nicorandil does not exert its remissive properties through KATP channels totally, suggesting possible involvement of mechanisms other than KATP channel opening.

One promising mechanism might be the involvement of NO, an endogenous mediator that not only has indispensable physiological roles but also shows various toxic effects [51-53]. Several investigations have shown that in acute colitis constitutive production of NO from endothelial NO synthase (eNOS) is advantageous, but chronic overproduction of NO through activation of inducible NOS (iNOS) is associated with progression of inflammation and tissue injury [53,54]. Recent clinical studies indicated changes of NO levels in saliva of IBD patients that is in correlation with the disease activity index [55]. As mentioned previously, nicorandil contains a nitrate moiety in its chemical structure, allowing it to function as a NO donor. On the other hand, other investigations [56,57] showed that nicorandil is able to upregulate eNOS expression and increase NO release following ischemia. Thus, it seems that upregulation of eNOS, which leads to acute production of NO, in addition to the NO donor property of nicorandil and the nicotinamide moiety that makes it a potent hydroxyl radical scavenger, is partially or totally responsible for its beneficial effects in remission of IBD. However, our experimental design did not include a measurement of NO levels in colonic tissue to evaluate this hypothesis.

Interestingly, beneficial effects of glibenclamide in attenuation of experimental IBD were observed. There is some evidence that shows an anti-inflammatory property associated with glibenclamide.

Studies have shown that glibenclamide is able to reduce levels of TNF-α and IL-1β alongside oxidative stress biomarkers [58-61]. On the other hand, Cocks *et al.* (1990) [62] discovered a new property for glibenclamide which is a competitive antagonism of TXA2 receptors in coronary artery. It has been shown that thromboxanes (TX) may play a pathologic role in IBD [63]. Although several mechanisms can be proposed to explain the beneficial effects of glibenclamide in IBD, our data is not sufficient to draw such conclusions, not was it the

aim of this study. Further experimentation is needed to evaluate glibenclamide as a potential therapy for IBD and discern possible mechanisms for its effects.

In conclusion, the present study elucidated the beneficial effects of nicorandil treatment using a murine model of colitis. Nicorandil had a positive effect on colonic injury, lipid peroxidation, neutrophil infiltration, and associated inflammatory mediators. There are some reports of rare dose-related nicorandil-induced ulceration in some tissues that may appear months after starting nicorandil therapy [64-66]. These reports, however, are in contrast to the results presented here. The protective effect observed in this study confirms previous reports on the anti-inflammatory properties of nicorandil. Given the low incidence of side effects and long duration of action, nicorandil may find a place in the management of IBD, albeit extensive clinical investigations are needed to support these results and, in so doing, elucidate the mechanisms which are responsible for protective effects of nicorandil in this disease.

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