

*Full Length Research Paper*

## The protective role of 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile against lung and colon injury induced by nitrosomorpholine in adult male albino rat

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Nitrites and morpholine are ubiquitous environmental contaminants found in drinking water and food. NMOR can be formed endogenously from nitrite and morpholine. Increased levels of reactive oxygen species/ reactive nitrite species (ROS/RNS) are involved in the mechanism of NMOR toxicity. Certain antimicrobial, antifungal and antioxidant potential were observed in heterocyclic benzimidazole derivatives and dimethyl sulfoxide (DMSO). This study was designed to evaluate the biological potential of 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile in the protection of lung and colon tissues against the increased levels of ROS/RNS that are induced by administration of nitrite and morpholine in drinking water for 15 weeks. Forty adult male rats were categorized into 4 groups, 10 rats each. The results showed a significant increase in NO, lipid peroxidation (LPO), total peroxides (TPO), superoxide anion (O<sub>2</sub><sup>-</sup>) and DNA fragmentation in lung and colon tissues of rats treated with nitrite and morpholine compared to the control group. Moreover, histological observation of the lung and colon tissues showed cell necrosis, increase in the leukocyte infiltration and blood vessel congestion. Immunostaining for inducible nitric oxide synthase (iNOS) showed positive reaction for lung and colon tissues. After the co-treatment of rats with DEMSO and 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile, all the previous biochemical changes were reduced in addition to the relative improvement in the morphological changes of both lung and colon. In conclusion, the injury in lung and colon tissues induced by nitrite and morpholine may return to the increased production of ROS and to the alterations in the levels of antioxidants. Co-treatment of rats with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile and DMSO may protect them against nitrite and morpholine toxicity.

**Key words:** Nitrite, morpholine, nitrosomorpholine (NMOR), inducible nitric oxide synthase (iNOS), benzimidazole derivatives, dimethyl sulfoxide (DMSO), colon, lung, rat.

### INTRODUCTION

The importance of considering chemical mixture exposure in dealing with environmental issues has recently become better recognized. Humans and domestic animals

are clearly exposed concomitantly to several environmental carcinogens at low doses under normal conditions (Mumtazz et al., 1993). Occupational and environmental

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exposure to N-nitroso compounds (NNC) represents a potential health hazard to humans (Kitano et al., 1997). Nitrites and secondary amines which represent precursors of NNC are contained in many kinds of common food and can react under acidic conditions in the stomach (Erkekoglu and Baydar, 2010). The secondary amine morpholine can be easily converted to nitrosomorpholine (NMOR) in the presence of nitrite both *in vivo* and *in vitro* (Mirvish, 1995). NMOR and their precursors in the environment in certain occupational location as well as their endogenous formation in the human body from dietary components are associated with an increased risk of gastrointestinal cancer (Hord et al., 2009).

Furthermore, NMOR causes the generation of reactive oxygen species/ reactive nitrite species (ROS/RNS), resulting in oxidative stress and cellular injury (Bartsch et al., 1989; Spiteller, 1996). However, organs that possess antioxidant defense system can react with ROS/RNS and neutralize them before they cause injury on vital cell components (Bansal et al., 2005). Nitric oxide (NO) that is produced from L-arginine by nitric oxide synthase (NOS) isoforms (neuronal, endothelial and inducible) may act as a protectant against cytotoxic stress or may enhance cytotoxicity when produced at elevated concentration (Wu and Cederbaum, 2006). Moreover, NO can react with superoxide anion to form peroxynitrite, which causes oxidative damage to DNA (Michel and Feron, 1997).

Thiazolo[3-2a]benzimidazole derivatives have been indicated as being associated with a wide range of biological properties, including antibacterial activity, hypoglycemic, antiviral and antitumor (Hjorth et al., 1994; Sekhar, 2009). DMSO as organic solvent can penetrate membranes without damaging them and could carry other compounds into a biological system; hence it is used as a drug delivery solvent (Geiss, 2001). The present study was designed to evaluate the biological activity of the 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile and its solvent DMSO, in the protection of lung and colon tissues against oxidative stress induced by nitrite and morpholine as precursor of NMOR and to evaluate the role of inducible nitric oxide synthase (iNOS) in NMOR toxicity.

## MATERIALS AND METHODS

### Experimental animals

Forty male Sprague-Dawley rats of 140 to 160 g were obtained from the Animal House of the Faculty of Medicine, Assiut University, Assiut, Egypt. Rats were housed in cages and were kept in a room temperature about 30°C with normal light/dark cycle. They were allowed to acclimatize for one week before the experiment and they had *ad libitum* access to pelleted diet and water throughout the study. All experimental protocols held on animals were done according to regulations set by the Institutional Animal Care and approved by Assiut University.

### Chemicals

3-Aminothiazolo[3-2a]benzimidazole-2-carbonitrile was synthesized

by Sarhan et al. (2008). DMSO, Folin-Ciocalteu, phenol, diphenylamine, tetramethylpyrazine (TMP), xlenol orange, 1-chloro-2,4-dinitrobenzol (CDNB), 5, 5'-dithiobis-(2-nitrobenzoic) acid (DTNB), idonitrotetrazolium,  $\alpha,\alpha$ -dipyridyl, vitamin C, sodium dodecyl sulphate, thiobarbituric acid, epinephrine and diaminobenzidine tetrahydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies were purchased from BD Biosciences, San Diego, CA, USA. Avidin-biotinylated peroxidase complex reagent was purchased from Vector Laboratories, Burlingame, CA, USA, while isotype-matched mouse IgG2a was obtained from BD Biosciences, U.K. All other chemicals were of the highest purity commercially available

### Experimental design

The rats were randomly divided into four groups, 10 rats each as follows: Group 1, control animals; Group 2, treated daily with sodium nitrite (1 g/L) and morpholine (2 ml/L) in drinking water for 15 weeks according to Mirvish et al. (1976); Group 3: treated daily with sodium nitrite (1 g/L) and morpholine (2 ml/L) and day after day with DMSO (0.2 ml/kg) as solvent for the 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile; Group 4, treated daily with sodium nitrite (1 g/L) and morpholine (2 ml/L) and day after day with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile (2 mg/kg) according to Spasov et al. (1999). All animals were exposed to similar volume of administration.

### Tissue preparation

At the end of the treatment period, blood was collected under ether anesthesia and lung and colon were removed and washed in 0.1 M phosphate buffer (pH 7.4), then divided into two parts, a small part was used for histopathological and immunohistochemical studies and a larger part was immersed in liquid nitrogen and stored at -20°C for biochemical analysis. In brief, 10% w/v homogenate of lung and colon tissue was prepared in 0.1 M phosphate buffer (pH 7.4) using IKA Yellow line DI homogenizer (18 Disperser, Germany). The homogenates were centrifuged at 10,000 g for 1 h at 4°C and the supernatant cytosols were used for subsequent biochemical assays.

### Biochemical measurements

The total protein was measured by the method of Lowry et al. (1951). Lipid peroxides as thiobarbituric acid reactive substances (TBARS) was determined according to the method of Ohkawa et al. (1979). Glutathione was determined using the method of Beutler et al. (1963). Measurement of DNA fragmentation was based on the hydrolysis of DNA leading to release of free deoxyribose that was colorimetrically measured at 575 nm after reaction with the diphenylamine reagent according to Kurita-Ochiai et al. (1999). Superoxide anion was measured according the procedure of Prodczasy and Wei (1988), which is based on the reduction of idonitrotetrazolium. Total peroxides was measured according to Harma and Erel (2005), based on the oxidation of zyleneol orange into purple colored chromogen that is directly in proportion with the peroxide content in the presence of ferrous sulfate as catalyzer. Superoxide dismutase (SOD) activity was determined according to its ability to inhibit the autoxidation of epinephrine at alkaline medium according to Misra and Fridovich (1972). Catalase (CAT) activity was estimated by the procedure of Luck (1963), depending on its ability to decompose hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Glutathione S-transferase (GST) activity was assayed using 1-chloro-2,4-dinitrobenzene as substrate according to the method of Superoxide dismutase (SOD) activity was determined according to its ability to

**Table 1.** Mean values  $\pm$  S.E.M. of lipid peroxidation (LPO), nitric oxide (NO), total peroxides (TPO), superoxide anion ( $O_2^-$ ) levels and DNA fragmentation % and Inhibition (I) and or stimulation (S) % in the lung and colon of control and different treated rats.

Group	Measurement (nmol /mg protein)									
	LPO		NO		TPO		$O_2^-$		DNA Fragmentation (%)	
	Lung	Colon	Lung	Colon	Lung	Colon	Lung	Colon	Lung	Colon
G1	0.29 $\pm$ 0.02	0.19 $\pm$ 0.01	0.28 $\pm$ 0.02	0.90 $\pm$ 0.18	27.66 $\pm$ 1.67	14.41 $\pm$ 1.28	14.38 $\pm$ 0.89	20.69 $\pm$ 1.22	27.72 $\pm$ 0.32	24.19 $\pm$ 0.77
G2	0.77 $\pm$ 0.04 <sup>a***</sup>	0.79 $\pm$ 0.04 <sup>a***</sup>	0.57 $\pm$ 0.04 <sup>a***</sup>	1.0 $\pm$ 0.11	79.92 $\pm$ 3.95 <sup>a***</sup>	77.99 $\pm$ 2.92 <sup>a***</sup>	18.67 $\pm$ 0.84 <sup>a*</sup>	24.81 $\pm$ 1.56 <sup>a*</sup>	62.01 $\pm$ 1.74 <sup>a***</sup>	77.88 $\pm$ 2.18 <sup>a***</sup>
G3	0.81 $\pm$ 0.04	0.51 $\pm$ 0.03 <sup>b***</sup>	0.57 $\pm$ 0.02	1.05 $\pm$ 0.19	53.58 $\pm$ 2.5 <sup>b***</sup>	41.45 $\pm$ 2.0 <sup>b***</sup>	16.05 $\pm$ 0.84	19.68 $\pm$ 0.99 <sup>b**</sup>	42.28 $\pm$ 0.23 <sup>b***</sup>	45.74 $\pm$ 0.57 <sup>b***</sup>
G4	0.88 $\pm$ 0.04	0.16 $\pm$ 0.01 <sup>b**</sup>	0.59 $\pm$ 0.04	0.65 $\pm$ 0.06 <sup>b***</sup>	34.35 $\pm$ 1.34 <sup>b***</sup>	19.52 $\pm$ 0.76 <sup>b***</sup>	19.73 $\pm$ 1.50	15.25 $\pm$ 0.98 <sup>b***</sup>	42.83 $\pm$ 0.30 <sup>b***</sup>	49.90 $\pm$ 0.75 <sup>b***</sup>

A = significance difference between normal control group (G1) and sodium nitrite + morpholine group (G2). b = significance difference between sodium nitrite + morpholine group (G2) and DMSO (G3) and 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile (G4) treated groups. \* = P < 0.05, \*\* = P < 0.01 and \*\*\* = P < 0.001.

inhibit the autoxidation of epinephrine at alkaline medium according to Misra and Fridovich (1972). Catalase (CAT) activity was estimated by the procedure of Luck (1963), depending on its ability to decompose hydrogen peroxide ( $H_2O_2$ ). Glutathione S-transferase (GST) activity was assayed using 1-chloro-2,4-dinitrobenzene as substrate according to the method of Habig et al. (1974). Vit E was measured using Emmerie-Engel reaction based on the reduction of ferric to ferrous ions, forming a red complex with  $\alpha,\alpha$ -dipyridyl according to the method of Roe (1961). Ceruloplasmin (CP) content was determined colorimetrically with 0.1% para-phenylenediamine reagent according the method of Houchin (1958).

#### Histological studies

Pieces of lung and colon were quickly removed and fixed in 10% neutral buffered formalin for histological and immunohistochemical investigations. Then sections (7  $\mu$ M) were mounted on slides and dried overnight at 37°C. The sections were dewaxed in xylene and hydrated in a graded series of alcohols and stained with Harris hematoxylin and eosin according to Drury and Wallington (1980).

#### Immunoperoxidase studies

Immunohistochemical staining for iNOS was performed using a standardized streptavidin-biotin-peroxidase method on formalin-fixed paraffin-embedded tissues according to

Zhou et al. (2002). After deparaffinization, sections were treated with 5% hydrogen peroxidase for 5 min to block endogenous peroxidase activity. Incubation with 1.5% normal horse serum for 35 min prevents nonspecific antigen binding. Sections were incubated with primary antibodies (BD Biosciences, San Diego, CA, USA) overnight at 4°C, followed by incubation for 45 min with the biotinylated secondary antibody, and subsequently incubated for 50 min in avidin-biotinylated peroxidase complex reagent (Vectastain Rabbit ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). Signals were developed by treatment with diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) for 5 min. The slides were counterstained with Mayer's hematoxylin dehydrated and mounted with D.P.X (BDH Ltd, Poole, UK). For negative controls, the primary antibody recognizing iNOS was omitted or replaced by the isotype-matched mouse IgG2a (BD Biosciences, UK) according to Zhang et al. (2008). The positive controls consisted of rat lung tissue.

#### Statistical analysis

The results were analyzed by one way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test as post-test by using Prism program for windows, version 3.0 (Graph pad software, Inc., San Diago CA, USA). The significant difference between groups was accepted at P < 0.05.

## RESULTS

The results of oxidative stress biomarkers in lung and colon tissues of control and different treated rats were presented in Tables 1 to 3. As shown in Table 1, administration of sodium nitrite plus morpholine significantly increased lipid peroxidation (LPO), NO, total peroxides (TPO) and superoxide anion ( $O_2^-$ ) levels and DNA fragmentation percent of the lung and colon versus those of control rats. When 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile or its solvent DMSO was given to sodium nitrite and morpholine administered rats, they significantly inhibited the increase of LPO, NO, TPO and  $O_2^-$  in colon and DNA fragmentation percent in lung and colon.

The activity of SOD was inhibited; however glucose-6-phosphate dehydrogenase (G6PD) was enhanced in the lung and colon of rats treated with nitrite and morpholine compared to the control. Moreover, CAT and GST activities were increased in the lung and decreased in the colon of rats treated with nitrite and morpholine versus those of controls. The administration of 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile or

**Table 2.** Mean Values  $\pm$  S.E.M of superoxide dismutase (SOD), catalase (CAT), glutathione S- transferase (GST) and glucose 6 phosphate dehydrogenase (G6PD) activities and inhibition (I) and or stimulation (S) % in lung and colon of control and different treated rats.

Group	SOD (U/min/mg protein)		CAT (U/min/mg protein)		GST (nmol/min/mg protein)		G6PD (nmol/min/mg protein)	
	Lung	Colon	Lung	Colon	Lung	Colon	Lung	Colon
G1	1.29 $\pm$ 0.07	2.77 $\pm$ 0.17	3.09 $\pm$ 0.21	3.13 $\pm$ 0.14	19.36 $\pm$ 0.88	35.37 $\pm$ 1.44	1.01 $\pm$ 0.07	1.07 $\pm$ 0.08
G2	0.92 $\pm$ 0.12 <sup>a*</sup>	0.82 $\pm$ 0.02 <sup>a***</sup>	11.5 $\pm$ 1.19 <sup>a***</sup>	2.25 $\pm$ 0.19	33.32 $\pm$ 1.3 <sup>a***</sup>	20.39 $\pm$ 2.17 <sup>a***</sup>	1.33 $\pm$ 0.080 <sup>a**</sup>	1.45 $\pm$ 0.09 <sup>a***</sup>
G3	1.45 $\pm$ 0.20 <sup>b**</sup>	3.17 $\pm$ 0.18 <sup>b***</sup>	7.2 $\pm$ 0.21 <sup>b*</sup>	2.91 $\pm$ 0.24	59.52 $\pm$ 2.87 <sup>b***</sup>	26.38 $\pm$ 1.09 <sup>b*</sup>	1.45 $\pm$ 0.13	1.34 $\pm$ 0.04
G4	0.54 $\pm$ 0.05 <sup>b**</sup>	1.15 $\pm$ 0.097 <sup>b***</sup>	16.4 $\pm$ 1.43 <sup>b**</sup>	6.20 $\pm$ 0.63 <sup>b***</sup>	28.41 $\pm$ 2.09	40.76 $\pm$ 1.59 <sup>b***</sup>	1.25 $\pm$ 0.10	1.63 $\pm$ 0.04

a= significance difference between normal control group (G1) and sodium nitrite + morpholine group (G2).b = significance difference between sodium nitrite + morpholine group (G2) and DMSO (G3) and 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile (G4) treated groups. \* = P < 0.05, \*\* =P < 0.01 and \*\*\* =P < 0.001.

**Table 3.** Mean values  $\pm$  S.E.M. of vitamin E (Vit E), vitamin C (Vit C), glutathione (GSH) and ceruloplasmin (CP) in lung and colon of control and different treated rats.

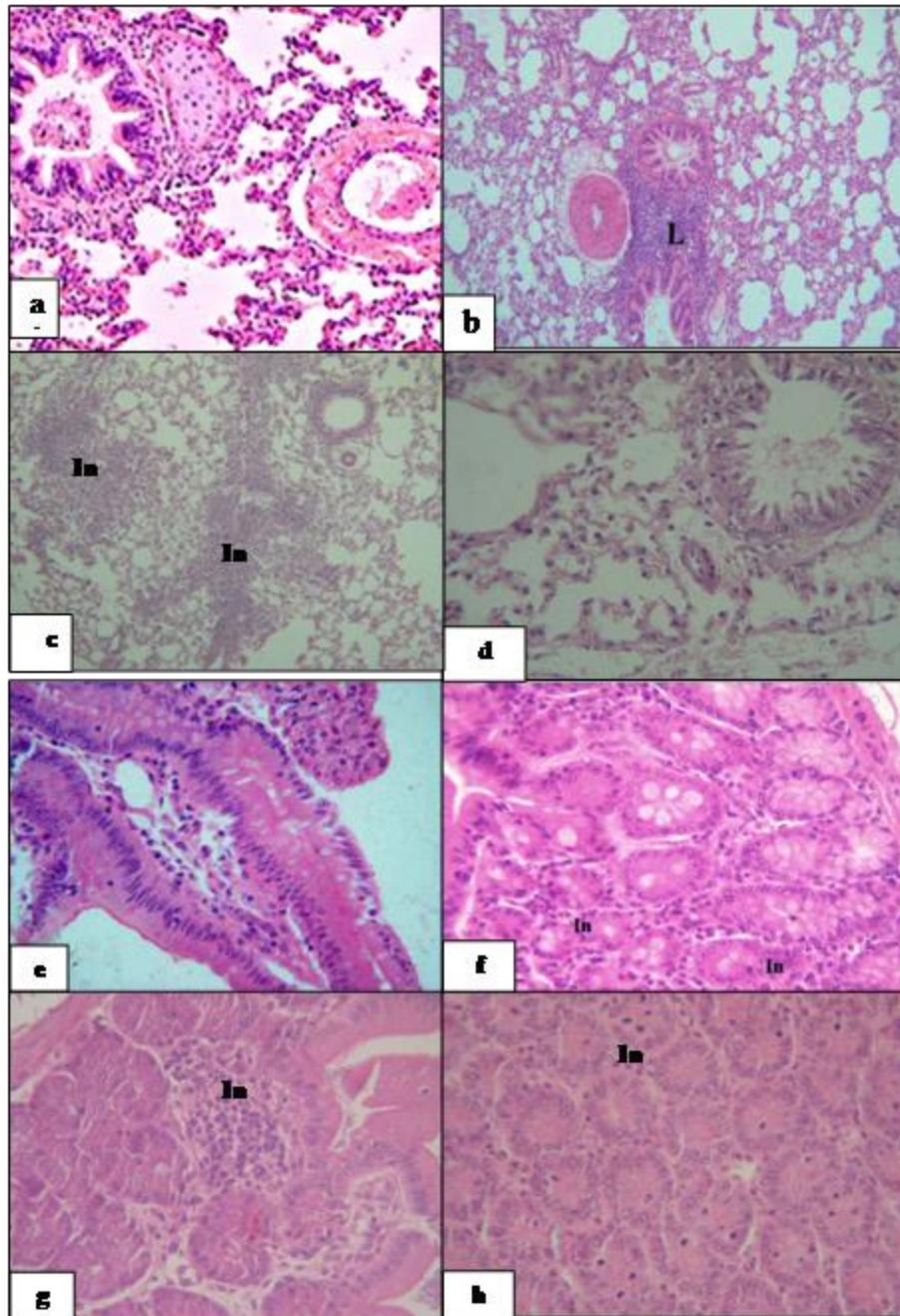
Groups	Vit E ( $\mu$ /mg protein)		Vit C ( $\mu$ /mg protein)		GSH ( $\mu$ /mg protein)		CP ( $\mu$ /mg protein)	
	Lung	Colon	Lung	Colon	Lung	Colon	Lung	Colon
G1	10.84 $\pm$ 0.35	8.87 $\pm$ 0.65	3.79 $\pm$ 0.27	4.83 $\pm$ 0.12	1.35 $\pm$ 0.040	1.54 $\pm$ 0.055	19.25 $\pm$ 0.74	8.82 $\pm$ 0.43
G2	8.75 $\pm$ 0.60 <sup>a*</sup>	13.11 $\pm$ 0.38 <sup>a***</sup>	3.84 $\pm$ 0.15	8.25 $\pm$ 0.27 <sup>a***</sup>	1.80 $\pm$ 0.060 <sup>a***</sup>	2.34 $\pm$ 0.065 <sup>a***</sup>	8.94 $\pm$ 0.57 <sup>a***</sup>	0.58 $\pm$ 0.04 <sup>a***</sup>
G3	10.30 $\pm$ 0.47 <sup>b*</sup>	11.16 $\pm$ 0.42 <sup>b**</sup>	2.81 $\pm$ 0.14 <sup>b**</sup>	3.79 $\pm$ 0.27 <sup>b***</sup>	1.24 $\pm$ 0.05 <sup>b***</sup>	1.29 $\pm$ 0.03 <sup>b***</sup>	5.63 $\pm$ 0.36 <sup>b***</sup>	1.06 $\pm$ 0.12
G4	5.20 $\pm$ 0.31 <sup>b***</sup>	8.58 $\pm$ 0.39 <sup>b***</sup>	2.97 $\pm$ 0.13 <sup>b**</sup>	4.87 $\pm$ 0.24 <sup>b***</sup>	1.33 $\pm$ 0.05 <sup>b***</sup>	1.41 $\pm$ 0.03 <sup>b***</sup>	6.32 $\pm$ 0.36 <sup>b***</sup>	2.03 $\pm$ 0.15 <sup>b***</sup>

a= Significance difference between normal control group (G1) and sodium nitrite + morpholine group (G2). b = Significance difference between sodium nitrite + morpholine group (G2) and DMSO (G3) and 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile (G4) treated groups. \* = P < 0.05, \*\* =P < 0.01 and \*\*\* =P < 0.001.

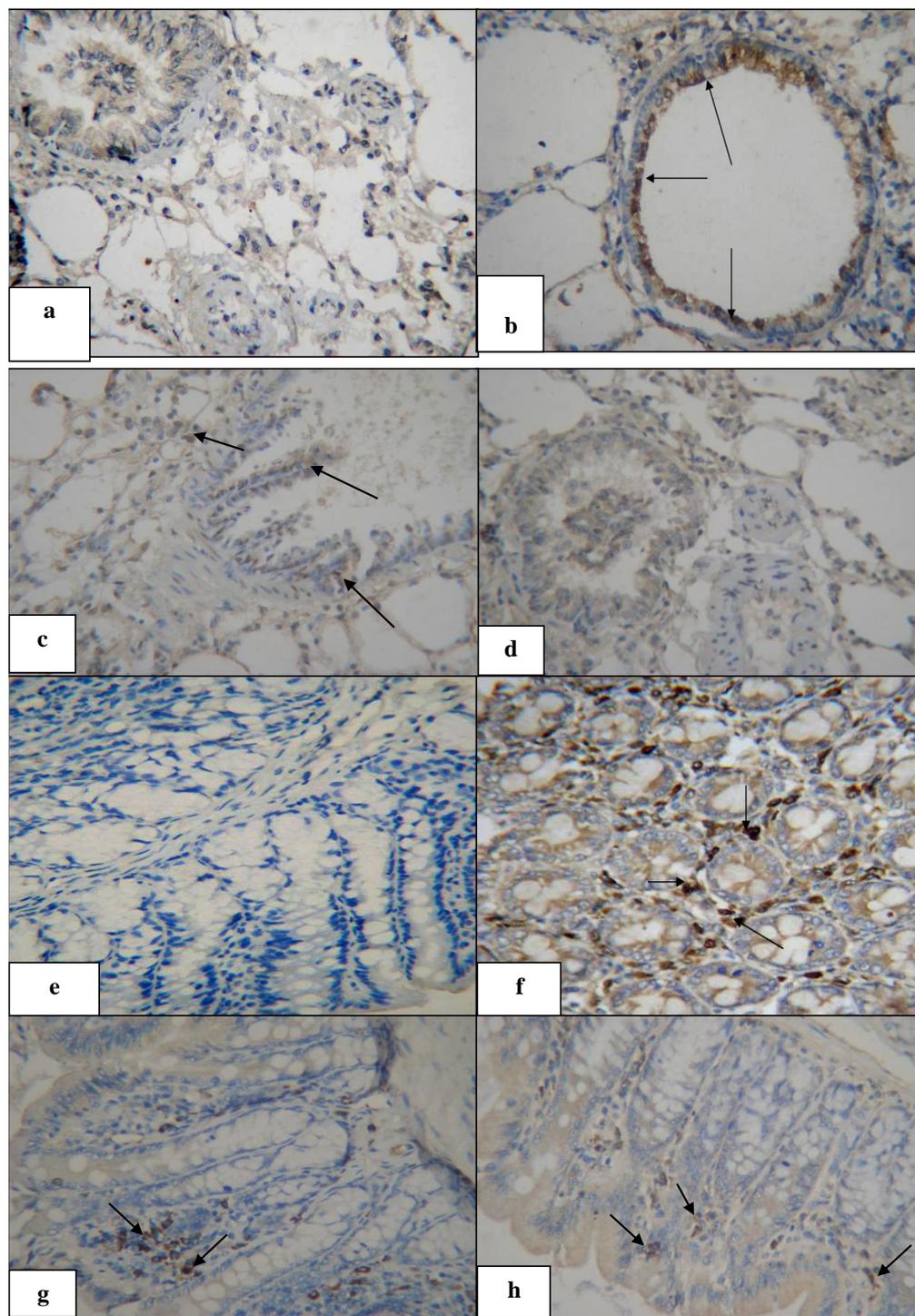
its solvent DMSO stimulated the SOD, CAT and GST activities, but it did not significantly change the G6PD activity in the lung and colon tissues (Table 2). Nitrite and morpholine administration to the rats also increased the vit E, vit C and GSH concentration, but decreased CP levels in the lung and colon tissues compared to those of controls. In addition, treatment of rats with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile or its solvent DMSO restored the changes that were caused by nitrite plus morpholine treatment (table 3). Histological examination of the lung sections of control rats after staining with H&E showed normal appearance of the bronchiole lined by pseudo stratified columnar epithelium with healthy

interstitial tissue (Figure 1a). Treatment of rats with nitrite plus morpholine showed large sized aggregated lymph follicle in a close relation to bronchioles (Figure 1b). Co-treatment of rats with DMSO revealed that aggregated groups of mononuclear cell are still found between bronchioles (Figure 1c). Concerning the co-treatment of rats with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile, the histological examination showed more or less normal appearance of the bronchioles with almost normal interstitial tissue (Figure 1d). Histological observation of the colon sections of control rats (Figure 1e) showed longitudinal section in the crypts that are lined by columnar cells with oval basal nuclei and separated

by goblet cells. Scattered mononuclear cells are also observed in the interstitial tissue. Treatment of rats with nitrite plus morpholine for 15 weeks showed disturbed mucosal architecture and pronounced infiltration of mononuclear cells (Figure 1f). Rats co-treated with DMSO showed localized large aggregation of mononuclear cells infiltration in lamina propria (Figure 1g), while colon of rats co-treated with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile showed few scattered mononuclear cells in lamina propria with more or less normal appearance of the crypts (Figure 1h). Immuno-staining lung for iNOS showed negative reaction either in the bronchiole or in the interstitial tissue (Figure 2a), After treatment



**Figure 1.** A photomicrograph for H&E staining in lung and colon X400 showing: (a) normal appearance of the bronchiole lined by pseudostratified columnar epithelium with healthy interstitial tissue; (b) large sized aggregated lymph follicle (L) in a close relation to bronchioles in rat treated by nitrite and morpholine; (c) aggregated groups of mononuclear cell infiltration (In) found between bronchioles in rat co-treated with DMSO; (d) more or less normal appearance of the bronchioles with almost normal interstitial tissue in lung of 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile co-treated rat; (e) longitudinal section in the crypts that are lined by columnar cells with oval basal nuclei and separated by goblet cells, few scattered mononuclear cells are observed in the interstitial tissue of normal colon; (f) disturbed mucosal architecture and pronounced infiltration of inflammatory cells (In) in colon of rats treated with nitrite and morpholine; (g) localized large aggregation of mononuclear cells infiltration (In) in lamina propria in colon of rats co-treated with DMSO; (h) few scattered mononuclear cells in lamina propria with more or less normal appearance of the crypts in colon of rats co-treated with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile.



**Figure 2.** A photomicrograph for iNOS staining in lung and colon X400 showing (a) nearly negative reaction either in the bronchiole or in the interstitial tissue; (b) positive reaction in the lining cells of the small bronchiole (arrows) in lung of nitrite and morpholine treated rats; (c) scattered positive cells in the bronchiole and in the interstitial tissue (arrows) in rat co-treated with DMSO; (d) nearly negative reaction either in the bronchiole or in the interstitial tissue in lung of 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile co-treated rat; (e) no reaction was observed in normal colon; (f) positive reaction localized primarily in the mononuclear cells of the lamina propria (arrows) in colon of rats treated with nitrite and morpholine; (g) scattered positive mononuclear cells in lamina propria (arrow) in colon of rats co-treated with DMSO; (h) areas with less intense reaction localized in the apical cytoplasm (arrows) in colon of rats co-treated with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile.

treatment with nitrite plus morpholine for 15 weeks, positive reaction in the lining cells of the small bronchiole was detected (Figure 2b). Lung of rats co-treated with DMSO showed scattered positive cells in the bronchiole and in the interstitial tissue (arrows) (Figure 2c). However, lung of rats co-treated with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile showed nearly negative reaction either in the bronchiole or in the interstitial tissue (Figure 2d). Immuno-staining colon for iNOS revealed negative reaction (Figure 2e), after treatment with nitrite plus morpholine for 15 weeks, colon showed intense positive reaction in mononuclear cells of the lamina propria (Figure 2f). Furthermore, the colon of rats co-treated with DMSO showed scattered positive mononuclear cells in lamina propria (arrow) (Figure 2g), however, colon of rats co-treated with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile showed areas with less intense reaction; this is primarily localized in the apical cytoplasm (arrows) (Figure 2h).

## DISCUSSION

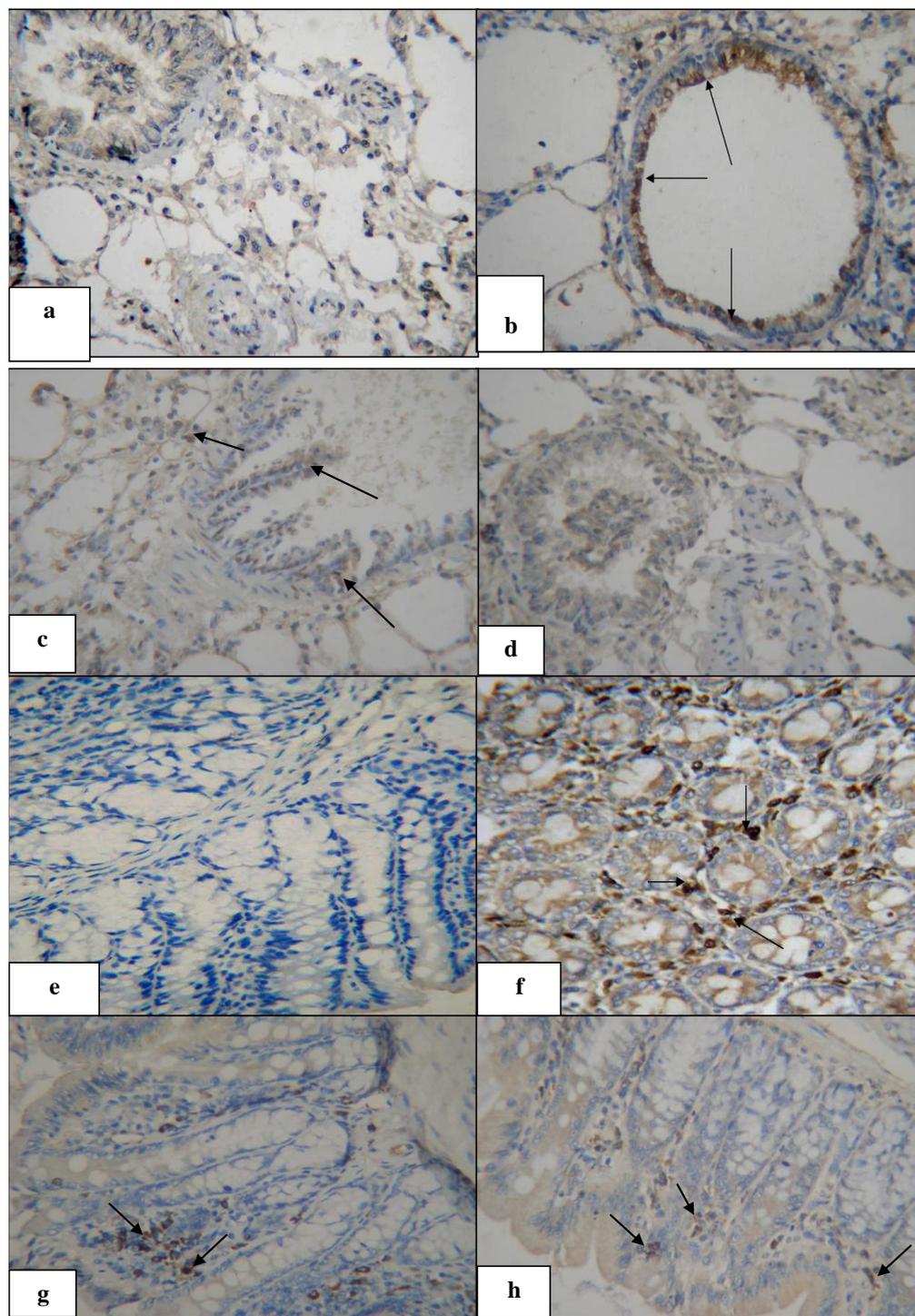
Many human cancers may be caused by the environmental factors such as traces of nitrosamines, so it was important to check up the effects of those chemicals at relatively low doses (Futakuchi et al., 1999). It is well known that in the presence of nitrite, MOR can be converted to NMOR both *in vitro* and *in vivo*. NMOR is widely used as hepatocarcinogen in experimental models and might induce DNA damage via formation of ROS/RNS (Robichová et al., 2004; Erkekoglu and Baydar, 2010). In the current study, TPO, LPO, NO and  $O_2^-$  were significantly increased in the lung and colon tissues of rats treated with nitrite combined with MOR for 15 weeks. In this aspect, elevation of ROS may be one of the contributing factors in the mechanisms of nitrate tolerance (Watanabe et al., 1998). Moreover, nitrosamines are metabolized by cytochrome P-450 dependent monooxidases system, generating a variety of ROS which are responsible for the onset of the toxic effects of nitrosamines (Mostafa and Shewita, 1992). Induction of carcinogen-metabolizing enzymes plays a critical step in initiation of carcinogenesis caused by nitrosamines (Shewita, 2000). Increased concentration of ROS is involved in pathological processes leading to the development of cancer and other diseases (Cerutti, 1994). Nitrite production by inflammatory cells, in combination with amine production by bacteria, results in formation of carcinogenic nitrosamines (Roediger et al., 1990).

The possible production of ROS is reflected in an increase in LPO, which is an important process in cellular damage. Consequently, in the present study, DNA fragmentation and LPO were significantly elevated in the lung and colon tissues of rats treated with nitrite plus MOR. The present data confirmed that ROS such as  $O_2^-$ ,

$OH^-$  and  $H_2O_2$  produced in the metabolism of NMOR was formed endogenously from the administration of nitrite plus MOR (Bansal et al., 2005). In this aspect, NMOR is widely used as a carcinogen in experimental animal models and its DNA-damaging activity was confirmed by Korr et al. (2001) and Robichová and Slameňová (2001). NMOR induce DNA damage via at least two different pathways: directly via formation of  $NO^-$  and indirectly through N-hydroxylamines generated by drug-metabolizing enzymes (Robichová et al., 2004). Moreover, ROS/RNS are potent intrinsic stimuli for apoptosis that modulate apoptosis when cell are under stress by mitochondrial damage (Reed, 1997). Also, increased ROS/RNS levels are extensively involved in the mechanisms of chronic lung inflammation contributing to the development of lung cancer (Azad et al., 2008).

Synthetic antioxidant compounds are required from external sources for direct interference in the carcinogenic process or scavenging and quenching carcinogens or preventing their synthesis (Frenech and Ferguson, 2001). In the present study, co-treatment of rats with either 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile or DMSO resulted in reduction of the TPO and LPO levels and DNA fragmentation % in the lung and colon tissues. In consistence, 5-nitro-2-(phenoxymethyl) benzimidazole significantly inhibited LPO, which was similar to that observed with butylated hydroxytoluene (BHT), a well-known antioxidant. This action was returned to the  $O_2^-$  scavenging activity of the synthesized compounds at different concentration (Olorunisola et al., 2011). In addition, synthesized benzimidazole depending on the tissue have variable effects on cytochrome P<sub>450</sub> system which is also known to be expressed and regulated differentially in various tissues by numerous xenobiotics (Iscan et al., 1989). DMSO, a known to be an effective hydroxyl radical scavenger (Del Maestro et al., 1980), may act to neutralize the cytotoxic effects of hydroxyl radicals in mitochondria themselves (Schlafer et al., 1982) and can lower oxygen utilization during cellular ischemia (Ghosh et al., 1976).

Dietary nitrate increased gastric NO levels and potently protected against the macroscopic injury caused by nonsteroidal anti-inflammatory drugs (NSAID) exposure (Lundberg et al., 2008). Additionally, nitrate pretreatment decreased mucosal myeloperoxidase activity and the expression of iNOS, which is indicative of reduced tissue inflammation. The protection afforded by nitrate probably is related to increase gastric mucosal blood flow (Peterson et al., 2007). GSH-GSSG antioxidant system protects macrophages from large amounts of NO produced by iNOS as in the present study (Coleman, 2001). Oxygen free radical enzymatic scavengers like SOD, CAT, glutathione peroxidase (GPx), GST, glutathione reductase (GR), and G6PDH may protect the system from deleterious effect (Banerjee et al., 1999). In the present study, the alterations in the activities of SOD, CAT, GST and G6PD in lung and colon tissues of all the



**Figure 2.** A photomicrograph for iNOS staining in lung and colon X400 showing (a) nearly negative reaction either in the bronchiole or in the interstitial tissue; (b) positive reaction in the lining cells of the small bronchiole (arrows) in lung of nitrite and morpholine treated rats; (c) scattered positive cells in the bronchiole and in the interstitial tissue (arrows) in rat co-treated with DMSO; (d) nearly negative reaction either in the bronchiole or in the interstitial tissue in lung of 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile co-treated rat; (e) no reaction was observed in normal colon; (f) positive reaction localized primarily in the mononuclear cells of the lamina propria (arrows) in colon of rats treated with nitrite and morpholine; (g) scattered positive mononuclear cells in lamina propria (arrow) in colon of rats co-treated with DMSO; (h) areas with less intense reaction localized in the apical cytoplasm (arrows) in colon of rats co-treated with 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile.

treated rats might result from increased ROS production. In consistence, increased oxidative stress due to increased production of ROS may alter the antioxidant system (Dröge, 2002). SOD is responsible for the dismutation of  $O_2^-$  to  $H_2O_2$ , which is neutralized by the combined action of CAT and GPx (Mates, 2000). The decreased level of SOD activity, suggests an increased  $O_2^-$  production. In the present study, reduced CAT activity in colon tissue of nitrite and morpholine-treated rats may be due to the fact that colon cells have reduced amount of peroxisomes. GST provides protection to the tissues by catalyzing the conjugation of a variety of electrophilic xenobiotics to GSH (Chasseaud, 1979). G6PDH enzymes play an important role in maintaining GSH redox state. The stimulated activity of G6PDH in the present study may be accrued to the increased supply of NADPH for catalytic activity of GR (Markey, 1998). GSH quenches toxic hydroperoxides catalyzed by GPx and conjugation reactions catalyzed by GST (Chasseaud, 1979). In the present study, decreased level of GSH by GMSO and 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile could seriously impair the optimal functioning catalytic activities of various GSH dependent enzymes (Mates, 2000).

Vitamin E is the most effective chain-breaking, lipid-soluble antioxidant weapon that inhibits LPO induced by ROS. The altered level of Vit E in the present study indicates the subnormal scavenging of LPO. Vit C scavenges many different types of free radicals and is also capable of regenerating Vit E from the tocopheroxy radicals that are generated during the  $\alpha$ -tocopherol mediated inhibition of LPO (Dieber-Rotheneder et al., 1991). The reduction of Vit C in lung and colon tissues of GMSO and 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile treated rats may be due to the decrease in the recycling of Vit C (Rumpsey and Levine, 1998). The capability of DMSO as antioxidant to scavenge free radicals that gather at the site of injury has been observed in experiments with laboratory animals and in ulcerative colitis patients (Itoh and Guth, 1985; Salim, 1992). Moreover, DMSO was found to prolong the survival rates in rats and protect non cancer cells while potentiating the chemotherapeutic drug (Salim, 1992). Also, promising group of antitumor compounds is represented by benzimidazole [1,2-c] quinazolines and thiazolo [3,4-a] benzimidazole (Spasov et al., 1999).

In the present study, ceruloplasmin levels were significantly increased in lung and colon of rats treated with nitrite and morpholine. Inoue et al. (1999) reported that ceruloplasmin favors the formation of S-nitrosoglutathione when added to cultured monocytes expressing iNOS. Whereas, nitrothiol adducts of NO function as NO transporters and are involved in modulation of intracellular and intercellular signal transduction (Akailke, 2000). Moreover, different mechanism highlight the enhancement of LPO by catalytic amounts of ceruloplasmin in the presence of iron plus GSH-derived thiols or ascorbate, a system which is

also able to promote mutagenesis (Glass and Stark, 1997). In contrast, Beieli and Calabrese (2002) claimed that ceruloplasmin could benefit the organism under some pathophysiological conditions, such as neo-vascularization of injured tissues.

## Conclusions

Nitrite plus morpholine administration to rats results in oxidative injury in pulmonary and colon tissues as evidenced by biochemical changes and morphological changes. This injury may cause the increased production of ROS/RNS and changes in the levels of antioxidants which were ameliorated by 3-aminothiazolo[3-2a]benzimidazole-2-carbonitrile and DMSO.

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