Free Radical Research, October 2008; 42(10): 849–862

informa
healthcare

The involvement of nitric oxide in maneb- and paraquat-induced oxidative stress in rat polymorphonuclear leukocytes

ISRAR AHMAD[1], ASHUTOSH KUMAR[1], SMRITI SHUKLA[1], HAUSHILA PRASAD PANDEY[2], & CHETNA SINGH[1]

[1]*Indian Institute of Toxicology Research (IITR), Lucknow 226 001, India, and* [2]*Department of Biochemistry, Banaras Hindu University (BHU), Varanasi, India*

Accepted by Professor M. Smith

(Received 10 June 2008; revised 2 September 2008; accepted 23 September 2008)

Abstract

Oxidative stress plays a crucial role in the manifestations of maneb (MB) and paraquat (PQ)-induced toxicity including MB + PQ-induced Parkinson's disease (PD). Polymorphonuclear leukocytes (PMNs) actively participate in the oxidative stress-mediated inflammation and organ toxicity. The present study was undertaken to investigate the MB- and/or PQ-induced alterations in the indices of oxidative stress in rat PMNs. Animals were treated with or without MB and/or PQ in an exposure time dependent manner. In some sets of experiments, the animals were pre-treated with NOS inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AG) along with respective controls. A significant increase in myeloperoxidase (MPO), superoxide dismutase (SOD), nitric oxide, iNOS expression and lipid peroxidation (LPO) was observed in PMNs of MB- and/or PQ-treated animals, while catalase and glutathione S-transferase (GST) activities were attenuated. L-NAME and AG significantly reduced the augmented nitrite content, iNOS expression and MPO activity to control level in MB and PQ exposed animals. Although the augmented LPO was also reduced significantly in L-NAME and AG treated rat PMNs, the level was still higher as compared with controls. Alterations induced in SOD and GST activities were not affected by NOS inhibitors. The results thus suggest that MB and/or PQ induce iNOS-mediated nitric oxide production, which in turn increases MPO activity and lipid peroxidation, thereby oxidative stress.

Keywords: *Maneb, paraquat, polymorphonuclear leukocytes, oxidative stress, nitric oxide*

Introduction

Pesticide exposure in humans and experimental animals has been associated with the development of various types of cancers and organ toxicity including cardio-toxicity, immunological suppression and neurodegenerative diseases. The involvement of oxidative stress in the pathogenesis of pesticides-induced diseases has been extensively studied [1–7]. The prevention or reduction in the pesticides-induced cell injury by treatment with antioxidants, such as α-tocopherol and β-carotene, has demonstrated the role of oxidative stress in pesticides-induced toxicity [8].

Maneb (manganese ethylene bis-dithiocarbamate), a commonly used fungicide and paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride), a widely used herbicide, exposure is implicated in the development of various organ toxicities viz. lung, liver, thoracic cancers, cardio-toxicity, neural tube defects, etc., and associated with increased risk of Parkinson's disease (PD) development [7,9,10]. Animal experimentation using mouse model showed that co-exposure of maneb and paraquat selectively and

Correspondence: Dr Chetna Singh, Indian Institute of Toxicology Research (IITR), Council of Scientific and Industrial Research, Mahatma Gandhi Marg, Post Box 80, Lucknow 226 001, UP, India. Tel: +91-522-2613618 Ext. 342. Fax: +91-522-2628227. Email: singhchetnaitrc@rediffmail.com

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760802513733

synergistically target the nigrostriatal system leading to significant reduction in motor activity and other symptoms that mimic PD [11–14]. These investigations led to the development of a mouse model of MB- and PQ-induced PD phenotype for understanding the molecular mechanism of MB- and PQ-induced PD [11–14]. Farmers quite often use both the pesticides in the same field either simultaneously or consecutively, which greatly increases their chances of getting co-exposed to the pesticides.

The animal experimentation has clearly shown a crucial role of oxidative stress in the onset and progression of pesticides-induced PD [7,15,16]. PQ and MB facilitate generation of ROS via inhibition of complex I and complex III, respectively [10,17–20]. PQ^{2+} is converted to free radical by accepting an electron ($PQ^{\cdot +}$) that reacts with oxygen to generate superoxide free radicals, thereby starting a cascade of free radical generating reactions [21,22]. PQ has also been shown to produce superoxide radicals via activation of NADPH oxidase in microglial cells, which play an important role in the development of PD [23]. MB, besides inhibition of complex III, facilitates auto-oxidation of dopamine, which result in excess free radical production leading to oxidative stress [24].

Polymorphonuclear leukocytes (PMNs) actively participate in the defense system against pathogens and xenobiotics and are the first to reach the site of inflammation through infiltration. The mode of action of PMNs is mainly attributed to production of oxidative stress via the involvement of free radical generating enzymes, i.e. NADPH oxidase, myeloperoxidase (MPO) and nitric oxide synthase (NOS). Nitric oxide (NO) is known to exhibit both beneficial and deleterious effects depending upon its concentration. If present up to a threshold level, NO plays a vital role in signal transduction and contributes to the endothelial muscle relaxation process but, if exceeding beyond the normal limit, it changes its role and facilitates cell injury through the formation of peroxynitrite anion ($ONOO^{-\cdot}$), which is highly toxic and results in cell death by causing nitration of essential macromolecules (proteins, DNA, etc.) and rendering them inactive [25].

The role of NO in the case of pesticides-induced toxicity has been investigated in several studies and is reported to be controversial. Various studies have demonstrated involvement of different isoforms of nitric oxide synthase (NOS) in the manifestations of toxicity in different models. PQ-induced toxicity in isolated guinea pig lungs showed participation of constitutive NOS (cNOS) [26], while neuronal NOS (nNOS) is reported to be involved in rotenone-induced neurodegeneration [27]. Non-specific NOS inhibitors- N(G)-nitro monomethyl L-arginine hydrochloride (L-NMMA) and L-NAME showed a protective role against deltamethrin-induced apoptosis and PQ-induced neurotoxicity in rats, respectively, implicating the involvement of NO in deltamethrin and PQ-induced toxicity [28,29]. On the other hand, inducible NOS (iNOS) mediated PQ-induced cytotoxicity in primary cortical glial cultures and diquat-induced gastrointestinal inflammation [30,31].

Several studies have reported an increased level of oxidative stress and NO in circulating neutrophils in neurodegenerative diseases like Alzheimer's disease (AD) and sporadic form of PD along with increased expression of neuronal NOS in brain of PD patients, indicating the role of PMNs and NO in the pathogenesis of these diseases [32,33]. Circulating PMNs with higher levels of oxidative stress and NOS activity may act as a rich source of ROS and RNS, thereby aggravating the toxicity induced by various toxins and affecting non-target tissues as well [32]. The role of PMNs in the neurotoxicity through generation of soluble factors like ROS, cytokines and matrix metalloproteinases (MMPs) has been suggested recently [34]. Over-expression of NOS in neutrophils and brain of PD patients is reported, suggesting that PMNs may contribute in the pathogenesis of sporadic PD [32,33]. Oxidative stress has been implicated in neurodegeneration induced by PQ either alone or in combination with MB. The present study was, therefore, undertaken to investigate the MB- and PQ-induced oxidative stress in PMNs of exposed rats and to evaluate the role of NO. Additionally, the mechanism of MB- and PQ-induced oxidative stress has also been looked into.

Materials and methods

Materials

Acetic acid, disodium hydrogen phosphate, glutathione reduced (GSH), nicotinamide adenine dinucleotide reduced form (NADH), potassium chloride and sodium dihydrogen phosphate were procured from Sisco Research Laboratories (SRL; India). Hydrogen peroxide (H_2O_2), nitroblue tetrazolium (NBT), orthophosphoric acid, phenazine methosulphate (PMS) and potassium dichromate were purchased from Merck (Germany). Aminoguanidine (AG), Bradford's reagent, 1-chloro 2,4-dinitrobenzene (CDNB), dextran, histopaque 1119/1077, maneb (MB), N(1-naphthyl) ethylene diamine dihydrochloride, N^G-nitro-L-arginine methyl ester (L-NAME), o-dianisidine dihydrochloride, paraquat dihydrochloride (PQ), sodium azide, sodium dodecyl sulphate (SDS), sodium nitrite, sodium pyrophosphate, sulphanilamide, thiobarbituric acid (TBA), Tri reagent and trisodium citrate were obtained from Sigma-Aldrich (USA). cDNA synthesis kits were procured from MBI Fermentas (USA). dNTPs, $MgCl_2$, Taq buffer and Taq DNA polymerase were purchased from Bangalore Genei India Pvt. Ltd.

Gene specific primers were synthesized from Metabion GmbH (Germany).

Animal treatment

Male Wistar rats (180–200 g) were obtained from the animal colony of Indian Institute of Toxicology Research (IITR; Lucknow). Animals were maintained under the standard conditions in the animal house. The animal ethics committee of IITR for the use of laboratory animals approved the study (No. 54/CPCSEA/1999). The animals were maintained under standard conditions of temperature and humidity with 12 h light/dark cycle and fed *ad libitum*. Animals were divided into controls, maneb (MB), paraquat (PQ) and MB + PQ treated groups. MB (30 mg/kg body wt) and PQ (10 mg/kg body wt) were administered intraperitoneally either alone or in combination, twice a week, for 1, 2 and 3 weeks. Control animals received normal saline. In some sets of experiments of 2 weeks exposure, animals were treated with either non-specific NOS inhibitor-L-NAME (20 mg/kg body wt, i.p.) or specific iNOS inhibitor-AG (20 mg/kg body wt, i.p.) 1 h prior to MB and/or PQ treatment. Doses for MB, PQ and NOS inhibitors (L-NAME and AG) were selected from previous studies [13,35,36].

Isolation of PMNs

Blood was collected from the animals through cardiac puncture under ether anaesthesia using sodium citrate (0.129 mol/L, pH 6.5, 9:1 v/v) as an anticoagulant. PMNs were isolated from buffy coat by dextran sedimentation and further purified by Histopaque density gradient centrifugation at $700 \times g$ for 30 min at 20°C [37,38]. The PMNs rich layer obtained at the interface of Histopaque 1119/1077 was washed with Hank's balanced salt solution (HBSS—sodium chloride 138 mmol/L, potassium chloride 2.7 mmol/L, potassium dihydrogen phosphate 1.5 mmol/L; pH 7.4) containing magnesium chloride (0.6 mmol/L), calcium chloride (1.0 mmol/L) and glucose (10 mmol/L). Viability of the cells was tested by trypan blue exclusion test and was never less than 95%.

Biochemical estimations

Superoxide dismutase (SOD). SOD was measured as described by Nishikimi et al. [39] with slight modifications. Briefly, assay mixture (3.0 ml) containing sodium pyrophosphate buffer (pH 8.3, 0.052 M), phenazine methosulphate (186 μM), nitroblue tetrazolium (300 μM), nicotinamide adenine dinucleotide reduced form disodium salt (NADH; 780 μM) and cell lysate was incubated at 30°C for 90 s followed by addition of glacial acetic acid and n-butanol. The absorbance of n-butanol layer was read at 560 nm. The results are expressed as units/ml/min.

Catalase. Catalase was estimated according to method described by Sinha [40]. In brief, assay mixture containing phosphate buffer (0.01 M; pH 7.0), hydrogen peroxide (0.2 M) and diluted cell lysate (1:100) was incubated at 37°C for 1 min. Reaction was stopped by the addition of potassium dichromate and glacial acetic acid. Samples were incubated in a boiling water bath for 15 min, cooled and the absorbance was read at 570 nm. The enzymatic activity was calculated in μmoles/min/mg protein.

Glutathione S-transferase (GST). Glutathione S-transferase activity was measured using spectrophotometric method [41]. In brief, cell lysate (75 μl) was mixed with 2.9 ml of 0.2 M phosphate buffer (pH 6.5). The reaction was initiated by addition of 20 μl of 3% 1-chloro-2, 4-dinitrobenzene and absorbance was read at 340 nm for 3 min at intervals of 30 s. Enzyme activity was calculated in nm/min/mg protein.

Myeloperoxidase (MPO) activity. Myeloperoxidase was estimated by the method of Bradley et al. [42]. Briefly, assay mixture (3.0 ml) comprised of cell lysate (0.1 ml) prepared in 0.5% hexadecyltrimethylammonium bromide (HTAB) containing phosphate buffer and reaction buffer (50 mM phosphate (pH 6.0) buffer containing 0.167 mg/ml *o*-dianisidine hydrochloride and 0.0005% hydrogen peroxide). The change in absorbance was recorded after 1 min at 460 nm and enzyme activity is expressed as μmoles/min/mg protein.

Lipid peroxidation (LPO). Lipid peroxidation was measured using the method described by Ohkawa et al. [43]. In brief, cell lysate ($\sim 1 \times 10^7$ cells) was mixed with 10% sodium dodecyl sulphate (SDS) solution and incubated for 5 min at room temperature. Glacial acetic acid (20%) was added to the reaction mixture and incubated for 2–5 min at room temperature. Finally, 0.8% thiobarbituric acid (TBA) was added followed by incubation for 1 h in a boiling water bath. The reaction mixture was cooled, centrifuged and the absorbance of the supernatant was read at 532 nm against control without lysate. LPO levels are expressed as nmoles MDA/1×10^7cells.

Protein estimation. Protein content was estimated in control and treated groups using Bradford's [44] method. Protein concentration (in mg/ml) was calculated against the standard curve of Bovine serum albumin (BSA).

Nitrite content. Nitrite content was estimated in controls and treated animals using Griess reagent [45]. Cell lysate ($\sim 1 \times 10^7$ cells), prepared in ammonium chloride (0.7 M), was mixed with an equal amount of Griess reagent (0.1% N (1-naphthyl) ethylene diamine dihydrochloride and 1% sulphanilamide in 2.5% orthophosphoric acid) and incubated for 30 min at 37°C. Absorbance was read at 548 nm and nitrite content was calculated using standard curve of sodium nitrite. The results are expressed in $\mu M/1 \times 10^7$ cells. Nitrite content was also measured in MB and/or PQ exposed animals pre-treated with L-NAME and AG.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The gene expression of iNOS was evaluated by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) as previously described [46]. Total RNA was extracted from PMNs using Tri reagent and cDNA was synthesized using oligo dT and Revert Aid™ H minus Mul V reverse transcriptase kit as per manufacturers' instructions. The primers synthesized (Metabion GmbH, Germany) were as follows—sense: 5′-ACCACCTCTATCAGGAA-3′, antisense: 5′CCTCATGATAACGTTTCTGGC-3′ (GeneBank Accession No. X76881) for iNOS and sense: 5′-TCTATGCCAACACAGT-3′, antisense: 5′-AGCCACCAATCCACACAG-3′ (GeneBank Accession No. V01217) for β-actin. The PCR reaction mixture consisted of 2 µl cDNA, Tris HCl (10 mM), KCl (50 mM), $MgCl_2$ (2.0 mM), dNTPs (1.0 mM), forward and reverse primers (0.4 µM each) and 2.5 U Taq DNA polymerase in total volume of 25 µl. Amplification was initiated by 3 min of pre-denaturation at 95°C followed by 30 cycles (1 min at 95°C; 1 min at 60°C and 1.5 min at 72°C) and final extension at 72°C for 7 min. PCR products were visualized using agarose gel electrophoresis. The band density was analysed using a computerized densitometry system (Alpha Imager System, Alpha Innotech Corporation, South Africa). The values of gene expression were normalized by β-actin as the reference.

Statistical analysis

Results are expressed as mean ± standard error of means (SEM) for separate groups. Two-way analysis of variance (ANOVA) was used for statistical analysis. Bonferroni post-test was used for multiple comparisons. The differences were considered statistically significant when p-value was less than 0.05.

Results

Superoxide dismutase

A significant increase in SOD activity following MB and/or PQ treatment was observed. MB + PQ co-treated animals exhibited more pronounced increase, resulting in a synergistic effect. The augmentation in SOD activity was exposure time-dependent (Figure 1A).

Catalase

MB and PQ significantly reduced catalase activity in PMNs of exposed animals. The reduction in the catalase activity was more pronounced in the co-treated group than MB and PQ treatment alone (Figure 1B).

Glutathione S-transferase

MB and PQ treatment inhibited GST activity in the PMNs in an exposure-time dependent manner. The MB + PQ treated animals' PMNs showed greater decrease in GST activity although it was not more statistically significant than PQ-treated animals (Figure 1C).

Myeloperoxidase activity

A time of exposure dependent elevation was observed in MPO activity following MB and/or PQ treatment. The elevation of MPO activity was more significant in the PMNs of animals co-treated with MB + PQ rather than alone, i.e. MB and PQ exhibited synergistic effect (Figure 2A).

Lipid peroxidation

A significant augmentation in the lipid peroxidation in PMNs of the MB and PQ treated group was observed. The augmentation was more pronounced in the co-treated group as compared with individual treatment, i.e. synergistic effect of MB and PQ was observed in combination (Figure 2B).

Nitrite content and expression of iNOS in absence/presence of NOS inhibitors following MB and/or PQ exposure

The nitrite content was increased in the PMNs of MB and PQ treated animals. The MB + PQ co-treated animals exhibited more pronounced increase in nitrite content than that of either pesticide alone. The increase in the nitrite content was time of exposure dependent and the maximum increase in nitrite content was observed in the PMNs of the animals treated with MB + PQ for 3 weeks (Figure 3A). Similar results were obtained with expression of

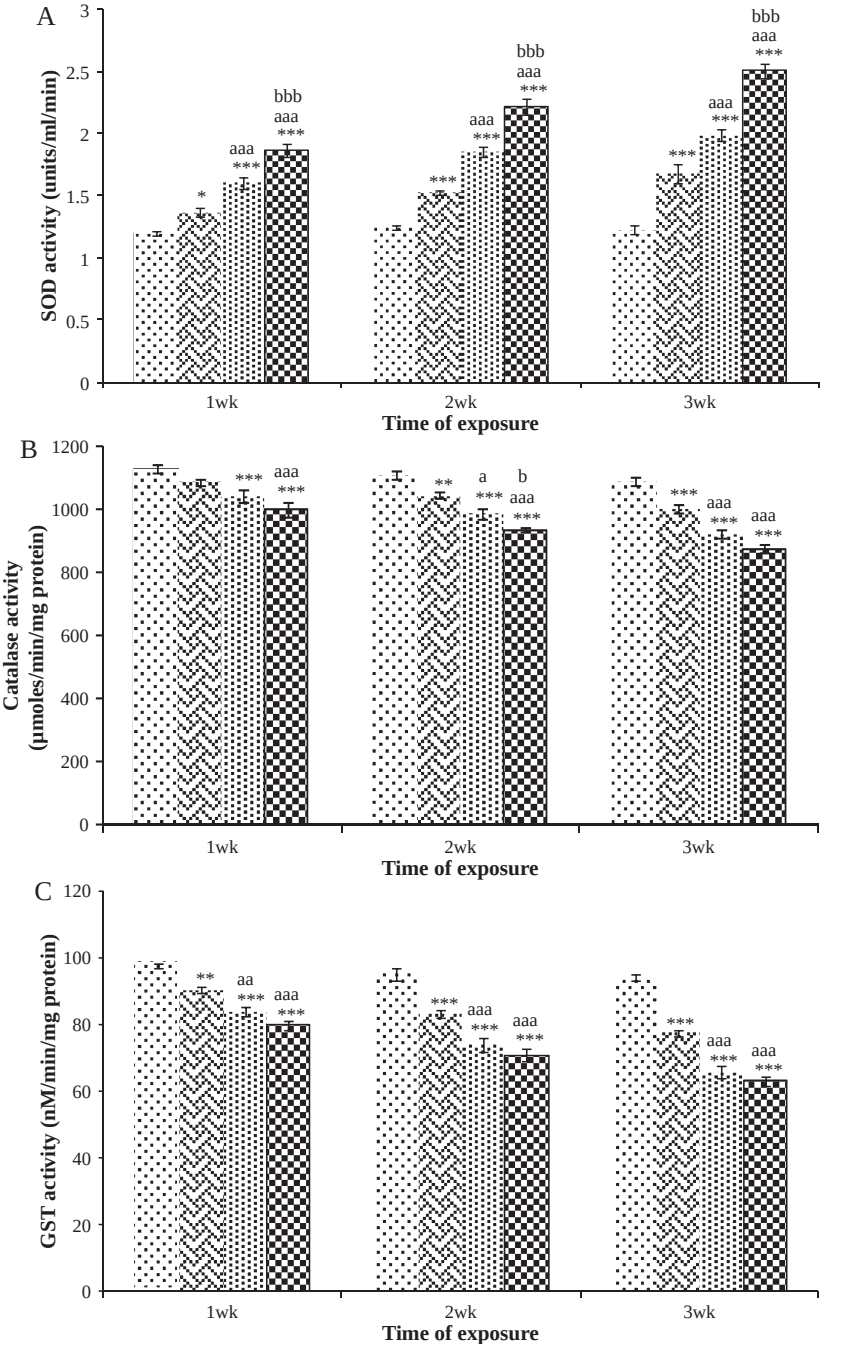

Figure 1. (A) Effect of MB and/or PQ on SOD activity of PMNs isolated from rats exposed for 1, 2 and 3 weeks (***$p < 0.001$ and *$p < 0.05$ as compared with control; aaa = $p < 0.001$ as compared with MB treated group and bbb = $p < 0.001$ as compared with PQ treated group); (B) depicts alterations in catalase activity of PMNs of treated rats following treatment with MB and PQ either alone or in combination (***$p < 0.001$ and **$p < 0.01$ as compared with control; aaa = $p < 0.001$ and a = $p < 0.05$ as compared with MB treated group and b = $p < 0.05$ as compared with PQ treated group); (C) shows inhibition of GST activity in isolated rat neutrophils following of MB and PQ treatment (***$p < 0.001$ and **$p < 0.01$ as compared with control; aaa = $p < 0.001$ and aa = $p < 0.01$ as compared with MB treated group). First, second, third and fourth bar in each panel represents control, MB, PQ and MB + PQ-treated groups, respectively.

iNOS gene, i.e. MB and/or PQ significantly increased the iNOS expression in a time of exposure dependent manner and effect of MB + PQ was more pronounced than PQ followed by MB treated animals (Figure 3B). Nitrite content was decreased significantly following treatment with both L-NAME and AG in vehicle control as well as in MB and/or PQ treated animals. L-NAME significantly decreased nitrite content as compared with saline control, MB and/or PQ treated groups. AG also significantly decreased nitrite content as compared with MB and/or PQ treated groups (Figure 4A) but did not show

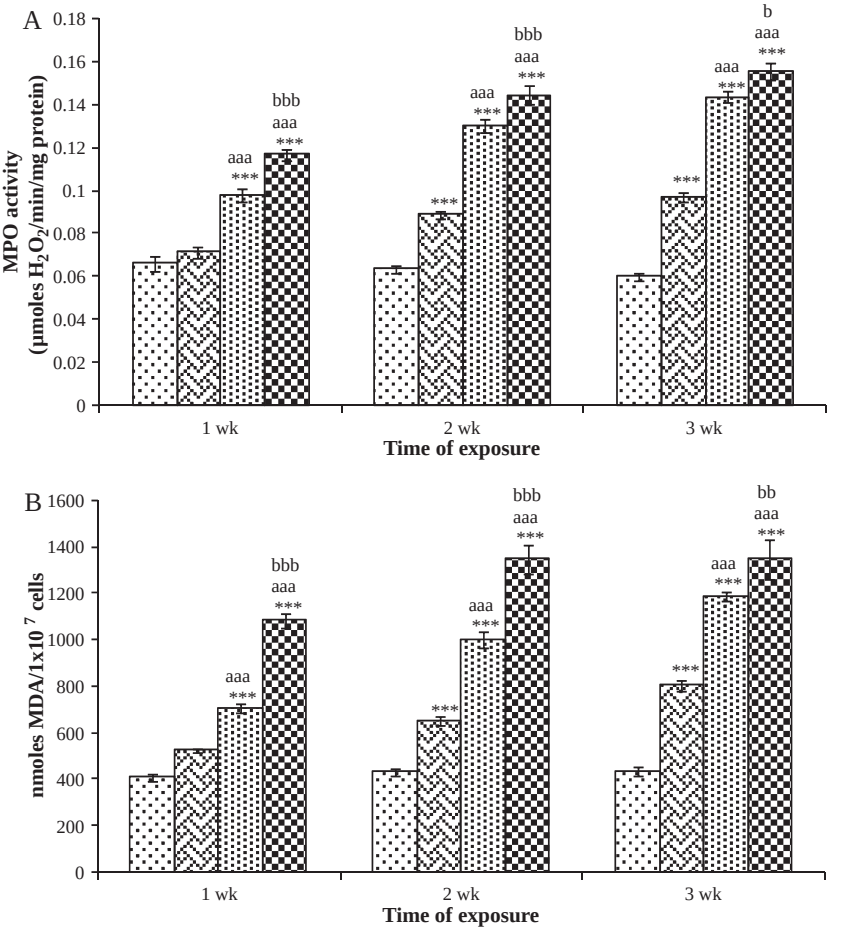

Figure 2. (A) Effect of MB and/or PQ exposure on MPO activity of isolated rat PMNs. (***$p < 0.001$ as compared with control; aaa $= p < 0.001$ as compared with MB treated group and bbb $= p < 0.001$ and $b = p < 0.05$ as compared with PQ treated group); (B) shows alterations in the status of lipid peroxidation (LPO) following MB and PQ treatment either alone or in combination in PMNs of exposed rats (***$p < 0.001$ as compared with control; aaa $= p < 0.001$ as compared with MB treated group; bbb $= p < 0.001$ and bb $= p < 0.01$ as compared with PQ treated group). First, second, third and fourth bar in each panel represents control, MB, PQ and MB + PQ treated groups, respectively.

significant alterations from the control group. Results obtained with iNOS gene expression studies were in concurrence with that of nitrite content, i.e. both L-NAME and AG did not alter mRNA expression *per se* but reduced augmented iNOS expression in MB and/or PQ exposed animals to control levels (Figure 4B).

Effect of NOS inhibitors on MPO, LPO, catalase, SOD and GST

MPO activity was reduced back to control levels in the presence of both L-NAME and AG and the extent of reduction was similar with L-NAME and AG, indicating that iNOS activity is mainly responsible for increased activity in MB and PQ treated rat PMNs (Figure 5A). L-NAME *per se* showed significant reduction of basal MPO activity as compared with control. AG *per se* did not show significant reduction in MPO activity as compared to control animals.

NOS inhibitors significantly reduced the augmented levels of LPO in pesticides exposed animals but LPO levels were still significantly higher than that of the control group (Figure 5B). L-NAME and AG *per se* did not show any change in LPO levels as compared with the control/saline-treated group. Catalase activity was significantly augmented in the PQ and MB + PQ treated group co-treated with L-NAME and AG but no significant alteration was observed in the group treated with MB alone. NOS inhibitors did not alter catalase activity in control animals (Figure 6A). Augmentation in SOD activity induced by MB and/or PQ treatment was not altered by L-NAME and AG and these did not exhibit any alterations in the control activity *per se* also (Figure 6B).

No alterations were observed in attenuated GST activity following MB and/or PQ exposure as well as control GST levels in presence of NOS inhibitors (Con = 98.17 + 1.27; Con + L-NAME = 95.14 ± 1.53; Con + AG = 96.45 + 0.59; MB-treated = 82.79 + 1.17; MB + L-NAME = 84.37 + 1.45; MB + AG = 83.99 + 1.12; PQ-treated = 77.02 + 0.58; PQ + L-NAME = 78.86 + 1.32; PQ + AG = 80.72 + 0.54; MB + PQ-treated = 72.25 + 0.56; MB + PQ + L-NAME = 76.25 + 2.25;

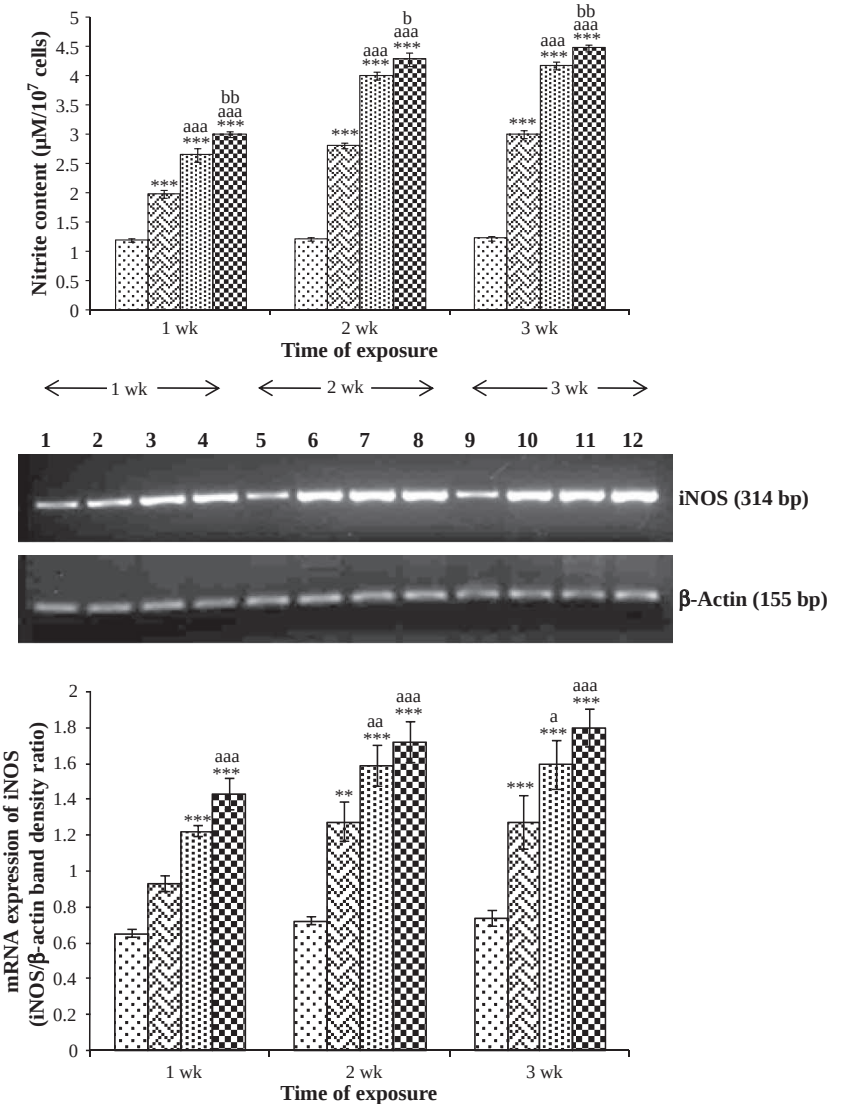

Figure 3. (A) Nitrite content of the PMNs isolated from control, MB and/or PQ exposed rats (***$p<0.001$ as compared with control; aaa $=p<0.001$ and aa $=p<0.01$ as compared with MB treated group and bb $=p<0.01$ and b $=p<0.05$ as compared with PQ treated group); (B) Upper panel shows representative gel picture depicting effect of MB and/or PQ treatment following exposure of 1, 2 and 3 weeks on iNOS expression along with expression of β-actin gene as reference. Lower panel shows densitometric analysis of the same ($n=4$) (***$p<0.001$, **$p<0.01$ and *$p<0.05$ as compared with control; aaa $=p<0.001$, aa $=p<0.01$ and a $=p<0.05$ as compared with MB treated group). First, second, third and fourth bar in each panel represents control, MB, PQ and MB+PQ treated groups, respectively.

MB+PQ+AG = 76.64+2.69; values are expressed in nM/min/mg protein).

Discussion

The present study investigated the alterations induced by MB and/or PQ in indices of oxidative stress in PMNs of exposed rats. Additionally, the role of NO was also investigated in the MB and/or PQ-induced oxidative stress in rat PMNs. Oxidative stress is implicated in pesticides-induced diseases including neurological disorders like PD [1,2,16,47,48]. PMNs play a critical role in inflammation and organ toxicity through generation of oxidative stress. The increased oxidative stress and over-expression of nNOS are reported in circulating neutrophils of PD patients [32,33]. A recent report has suggested the role of PMNs in neurotoxicity via generation of soluble factors like ROS, matrix metalloproteinases (MMPs) and cytokines [34].

Increased levels of SOD following MB and/or PQ treatment suggested its protective or scavenging action against enhanced production of superoxide radicals by these pesticides and combination exhibited significantly greater augmentation in SOD levels, which is in concurrence with other reports showing a synergistic effect of these pesticides in oxidative stress generation [8,16]. This is also supported by earlier studies where PQ is shown to cause augmentation in the mitochondrial SOD levels in rat liver, although cytosolic SOD was not affected [49] and *in vitro* study with human peripheral blood leukocytes resulting in

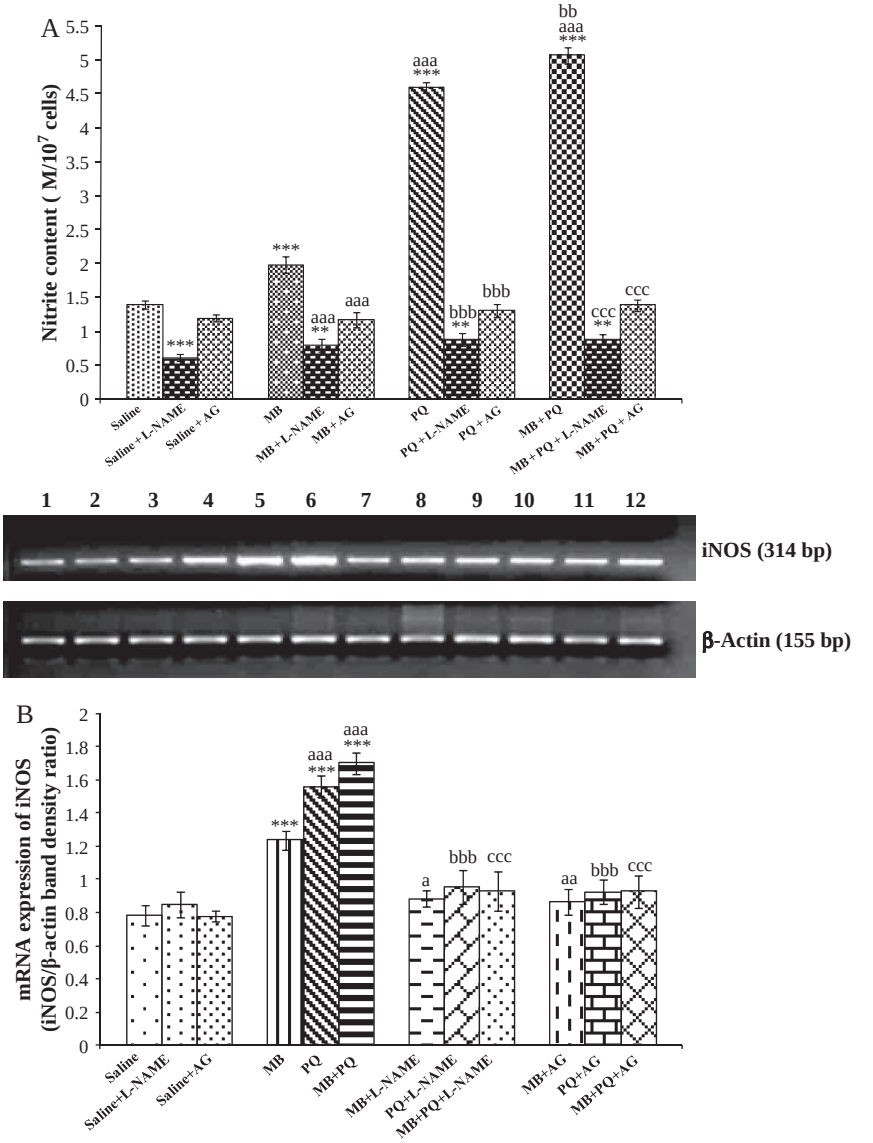

Figure 4. (A) Nitrite content in the PMNs of saline control, MB and/or PQ exposed group with or without L-NAME and AG pre-treatment (***$p < 0.001$ and **$p < 0.01$ as compared with control; aaa $= p < 0.001$ as compared with MB treated group; bbb $= p < 0.001$ and bb $= p < 0.01$ as compared with PQ treated group; ccc $= p < 0.001$ as compared with MB+PQ treated group); (B) Upper panel shows representative gel picture depicting effect of MB and/or PQ treatment in presence or absence of L-NAME and AG on iNOS expression along with β-actin expression following 2 weeks of exposure. Lower panel shows densitometric analysis of the same (***$p < 0.001$ and **$p < 0.01$ as compared with control; aa $= p < 0.01$ and a $= p < 0.05$ as compared with MB treated group; bb $= p < 0.01$ as compared with PQ treated group and ccc $= p < 0.001$ as compared with MB+PQ treated group).

significant increase in SOD levels following PQ exposure [50]. PQ exposure is also known to cause increases in Cu, Zn-SOD and Mn-SOD levels in isolated rat astrocytes [51]. Additionally, over-expression of SOD and administration of SOD mimetics provided protection against PQ-induced toxicity, reaffirming that an increase in SOD might be the defense mechanism to counteract the PQ-induced increased formation of superoxide radicals [52–54]. Increased H_2O_2 production is also reported in brain mitochondria following PQ exposure, which is indirect evidence for high SOD activity induced in the brain by PQ [22].

The decrease in catalase levels observed is in concurrence with an earlier report showing that paraquat inhibits catalase activity along with augmentation of Cu/Zn-SOD activity in rat alveolar type II cells *in vitro* [55]. The attenuation in catalase activity in this study may either be due to increased myeloperoxidase (MPO) (marker enzyme of PMNs) activity, which utilizes H_2O_2, the substrate of catalase, to generate hypochlorous acid (HOCl), another form of ROS involved in cytotoxicity leading to cell death or due to inhibition of catalase activity by increased NO, which is reported to reduce the activity of catalase *in vitro* [56,57] and *in vivo* [58–60].

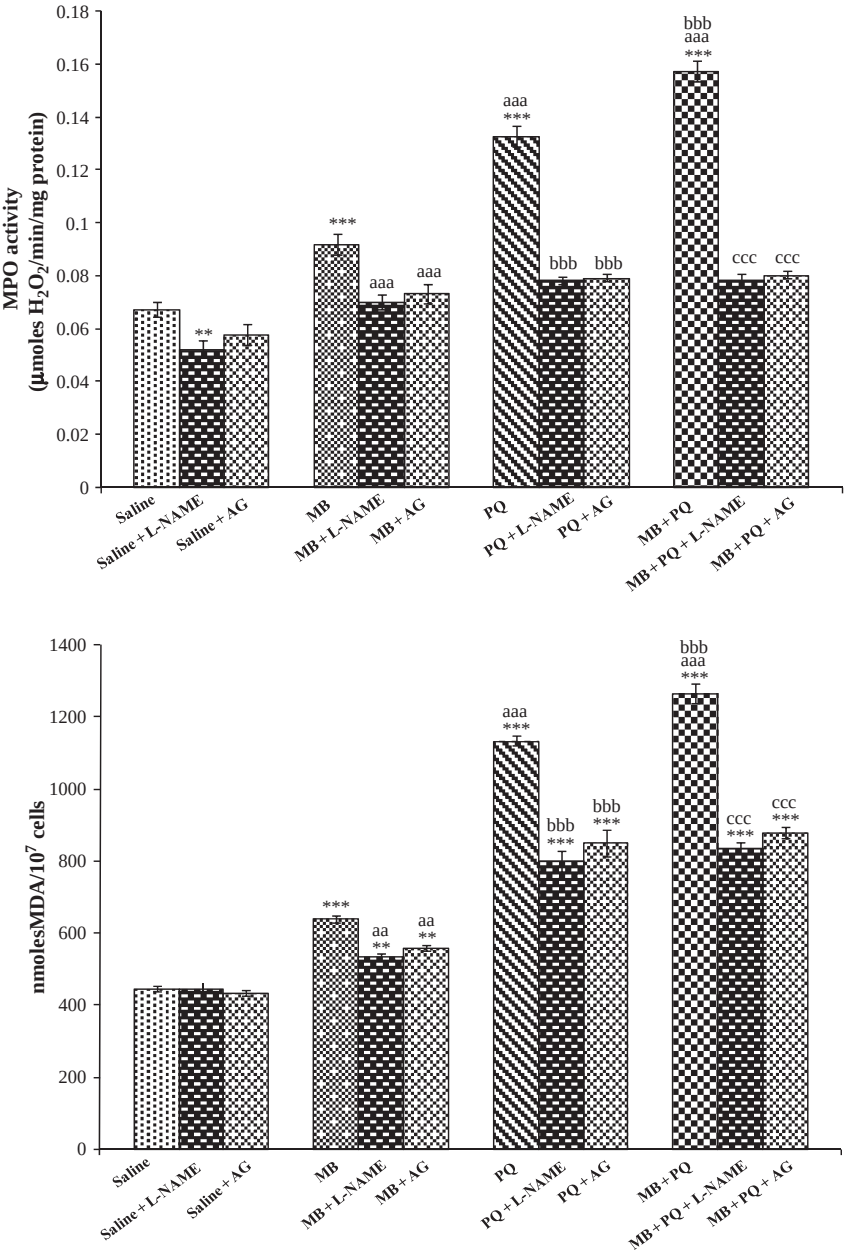

Figure 5. (A) Effect of L-NAME and AG on MPO activity in control, MB and/or PQ treated groups (***$p<0.001$, **$p<0.01$ and *$p<0.05$ as compared with control; aaa $=p<0.001$ as compared with MB treated group; bbb $=p<0.001$ as compared with PQ treated group and ccc $=p<0.001$ as compared with MB+PQ treated group); (B) effect of MB and/or PQ on LPO in rat PMNs following 2 weeks exposure with or without NOS inhibitors- L-NAME and AG (***$p<0.001$, **$p<0.01$ and *$p<0.05$ as compared with control; aaa $=p<0.001$ and aa $=p<0.01$ as compared with MB treated group; bbb $=p<0.001$ as compared with PQ treated group and ccc $=p<0.001$ as compared with MB+PQ treated group).

Attenuation observed in GST activity following treatment with the PQ either alone or in combination with MB is in accordance with earlier reports on effect of mancozeb and ziram on GST activity. Mancozeb showed dual effect by increasing GST activity *in vivo* and decreasing the enzyme activity *in vitro*, but ziram was shown to inhibit GST activity both *in vivo* and *in vitro* [61]. PQ treatment is also reported to decrease GST activity in the digestive gland of brown mussel [62]. On the contrary, Patel et al. [16] reported that MB and PQ co-treatment results in increased brain GST levels.

The significant increase in the MPO and LPO indicated that MB and PQ induced oxidative stress in PMNs, which is in concurrence with studies showing induction of oxidative stress in pulmonary and neuronal cell lines and in brain of MB + PQ exposed PD phenotype model in mice [16,63,64]. Increased MPO in exposed rat PMNs is also in accordance with a study showing involvement of myeloperoxidase-mediated oxidative stress in benzo (a) pyrene-induced cytotoxicity in rat neutrophils [46]. Augmentation in MPO activity was more pronounced in the case of combined treatment, confirming a synergistic

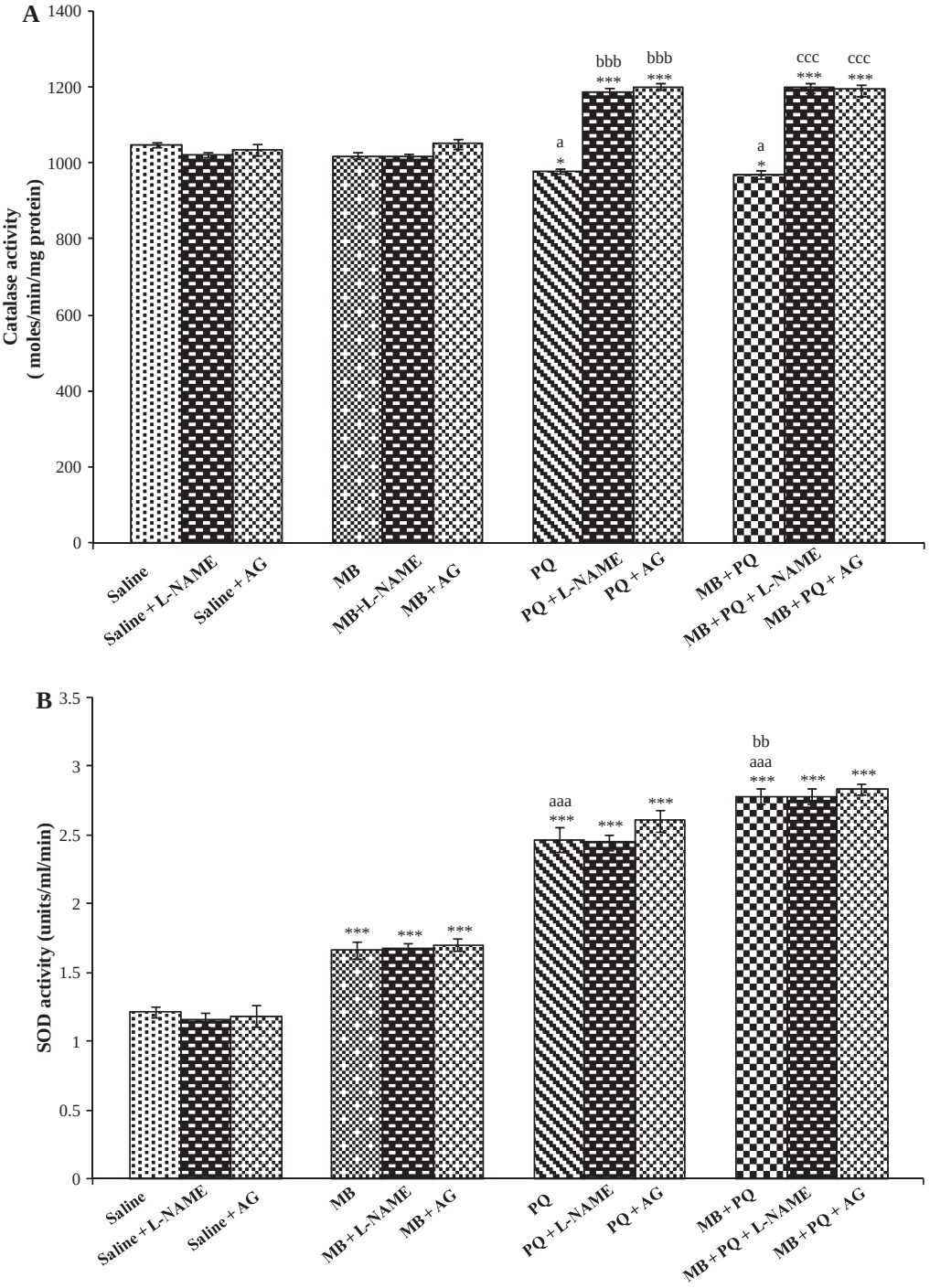

Figure 6. (A) Alterations in catalase activity in control, MB and/or PQ treated groups in presence and absence of NOS inhibitors (*$p <$ 0.05 as compared with control; a $= p < 0.05$ as compared with MB treated group; bbb $= p < 0.001$ as compared with PQ treated group and ccc $= p < 0.001$ as compared with MB+PQ treated group); (B) effect of L-NAME and AG on SOD activity in control, MB and/or PQ treated groups after 2 weeks exposure (***$p < 0.001$ as compared with control; aaa $= p < 0.001$ as compared with MB treated group and bb $= p < 0.01$ as compared with PQ treated group).

effect of MB and PQ, as reported earlier [16]. Augmented MPO levels are in line with increased SOD levels, which resulted in increased production of H_2O_2, thereby providing increased substrate for MPO leading to formation of HOCl.

NOS inhibitors and NOS knockout animals have shown a protective effect against neurodegeneration caused by 6-OHDA, MPTP and lipopolysaccharide (LPS, iNOS inducer) and PQ, indicating a deleterious role of nitric oxide in these models [29,65–71]. Involvement of both cNOS and iNOS is reported in PQ-induced toxicity in different models [26,31]. NOS enzyme is also reported to increase ROS generation by acting as PQ diaphorase [72].

Augmentation in nitrite content obtained in the present study following treatment with MB and/or PQ suggested the role of NO in MB and/or PQ-induced oxidative stress in rat PMNs and is in accordance with the earlier reports implicating NO in pesticides-induced toxicity [26–31]. The expression pattern of iNOS also showed similar results, i.e. increased iNOS expression was obtained with MB and/or PQ exposure and combined treatment showed greater increase. Pre-treatment with both L-NAME (non-specific NOS inhibitor) and AG (selective iNOS inhibitor) totally abolished the augmented nitrite content induced by MB and/or PQ, implying that the increase was due to iNOS induction in PMNs. Expression studies of the iNOS gene also yielded concurrent results, i.e. increased level of iNOS expression was reduced back to control levels by L-NAME and AG pre-treatment in MB and/or PQ exposed animals. These results are in accordance with earlier reports showing the involvement of NO in PQ-induced toxicity and show similarity with PQ-induced neurotoxicity, which also exhibited a deleterious effect of NO and protection with NOS inhibitor [26,29,31]. Reduction of iNOS mRNA obtained in our study is in accordance with earlier reports showing the effect of NOS inhibitors on NOS mRNA expression [46,73–76].

L-NAME and AG pre-treatment resulted in significant attenuation of MB and PQ-mediated increase in MPO activity, causing its reversal up to control levels. L-NAME significantly reduced MPO activity in the control group but AG did not alter MPO activity significantly as compared to control. L-NAME is a non-specific NOS inhibitor and inhibits both cNOS and iNOS, leading to a greater decrease in NO levels, and this could be the reason for inhibition of MPO activity by L-NAME in PMNs. AG, on the other hand, inhibits only iNOS, which contributes an almost negligible amount of NO under normal conditions; therefore, it did not produce a significant effect on control MPO activity. Since L-NAME and AG reduced MPO activity to the same extent in MB and/or PQ treated groups, it indicated that this increase was entirely due to an increase in iNOS and not due to the effect of L-NAME *per se*.

Increased LPO levels were also reduced significantly following pre-treatment with L-NAME and AG, but were still significantly greater than the control levels, suggesting that NO could not be the only factor responsible for MB and/or PQ-induced augmentation in LPO. Control LPO levels were not affected by L-NAME and AG treatment *per se*. NOS inhibitors did not affect augmented SOD levels activity induced by MB and/or PQ treatment, ruling out the direct involvement of SOD in the NO-mediated pathway leading to oxidative stress in PMNs. NOS inhibitors *per se* also did not affect SOD levels as compared with the control group.

Although with the current experimental design we do not have direct evidence indicating the exact reason for a significant increase in catalase activity observed in PQ and MB + PQ treated animals in the presence of NOS inhibitors, there could be two possibilities. The increase in catalase activity could be due to the defensive mechanism of PMNs to neutralize increased H_2O_2 production due to an increase in SOD. Normally, MB and/or PQ exposure could increase MPO activity, which may utilize increased H_2O_2. As NOS blockers inhibited increase in MPO, therefore, catalase activity could be increased to counteract H_2O_2 production. Another possibility could be that iNOS-induced NO production by MB and/or PQ could be inhibiting catalase activity. NOS inhibitors blocked iNOS-induced NO production, thereby abolishing the inhibitory effect of NO on catalase leading to an increase in the enzyme activity. This is in accordance to earlier reports showing reversal of catalase activity in the presence of NOS inhibitors [56–60]. NOS inhibitors did not alter catalase activity of control animals. NOS inhibitors neither affected GST status *per se* in control animals nor attenuated GST activity in MB and/or PQ exposed rats, ruling out the role of GST in the NO-mediated oxidative stress induced by MB and PQ in rat PMNs.

It can thus be concluded that although MB and PQ are both capable of generating oxidative stress in PMNs of exposed animals, MB + PQ in combination possess significantly higher potential to induce oxidative stress. These results are in concurrence with the combined effect of MB + PQ-induced oxidative stress in PD phenotype in mouse [16]. The results from the present study suggest that MB and PQ-induced oxidative stress is contributed partly by increased iNOS induction, which in turn increases MPO activity of PMNs and lipid peroxidation leading to oxidative stress. The results also suggest the presence of some NO-independent mechanism that appears to be acting in parallel to the NO-mediated pathway to increase lipid peroxidation, yielding a higher degree of oxidative stress by MB and PQ in rat PMNs than contributed via iNOS-mediated increased MPO activity alone, which needs further investigation. Several signalling pathways have been suggested for NOS induction in many cell types, indicating the role of cytokines and cytochrome P450s (CYPs). Induction of iNOS by CYP1A1 through TNFα, IL-β and NF-κB-mediated pathway is reported in benzo(a)pyrene treated rat PMNs [77], while TNFα, IL-β and IFN-γ are reported to cause NOS induction in insulin producing cells [78]. The role of TNFα-induced NOS is shown in MPTP-induced toxicity and PQ-induced increased production of TNFα is also reported [79]; however, the exact mechanism of iNOS-mediated MPO and LPO increase resulting

in oxidative stress in PMNs following MB and PQ exposure needs to be elucidated.

Acknowledgements

The authors sincerely thank the Council of Scientific and Industrial Research (CSIR), New Delhi, India, for providing a research fellowship to Israr Ahmad and University Grants Commission (UGC), New Delhi, India for providing a research fellowship to Ashutosh Kumar. The IITR communication number of this article is 2668.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Koner BC, Bannerjee BD, Ray A. Organochlorine pesticide-induced oxidative stress and immune suppression in rats. Ind J Exp Biol 1998;36:395–398.

[2] Yavuz T, Altuntas I, Delibas N, Yildirim B, Candir O, Cora A, Karahan N, Ibrisim E, Kutsal A. Cardiotoxicity in rats by methidathion and maeliorating effect of vitamins E and C. Hum Exp Toxicol 2004;23:323–329.

[3] Parvez S, Raisuddin S. Copper modulates non-enzymatic antioxidants in the freshwater fish Channa punctata (Bloch) exposed to deltamethrin. Chemosphere 2005;62:1324–1332.

[4] Terzi A, Iraz M, Sahin, S, Ilhan A, Idiz N, Fadillioglu F. Protective effect of erdosteine on rotenone-induced oxidant injury in liver tissue. Toxicol Ind Health 2004;20:141–147.

[5] Kale M, Rathore N, John S, Bhatnagar D. Lipid peroxidative damage on pyrethroid exposure and alterations in antioxidant status in rat erythrocytes: a possible involvement of reactive oxygen species. Toxicol Lett 1999;105:197–205.

[6] Testa CM, Sherer TB, Greenamyre JT. Rotenone induces stress and dopaminergic neuron damage in organotypic substantia nigra cultures. Brain Res Mol Brain Res 2005;134:109–118.

[7] McCormack AL, Atienza JG, Johnston LC, Anderson JK, Vu S, Di Monte DA. Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. J Neurochem 2005;93:1030–1037.

[8] Sohn HY, Kwon CS, Kwon GS, Lee JB, Kim E. Induction of oxidative stress by endosulfan and protective effect of lipid soluble antioxidants against endosulfan-induced oxidative damage. Toxicol Lett 2004;151:357–365.

[9] Priydarshi A, Khuder SA, Schaub EA, Priyadarshi SS. Environmental risk factors and Parkinson's disease: a metaanalysis. Environ Res 2001;86:122–127.

[10] Zhang J, Fitsanakis V, Gu G, Jing D, Ao M, Amarnath V, Montine T. Manganese ehtylene-bis-dithiocarbamate and selective dopaminergic neurodegenration in rat: a link through mitochondrial disruption. J Neurochem 2003;84:1–11.

[11] Thiruchelvum M, Richfield EK, Baggs RB, Tank AW, Cory-Slechta DA. The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and maneb: implications for Parkinson's disease. J Neurosci 2000; 20:9207–9214.

[12] Thiruchelvam M, McCormack A, Richfield EK, Baggs RB, Tank AW, Di Monte DA, Cory-Slechta DA. Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. Eur J Neurosci 2003;18:589–600.

[13] Cicchetti F, Lapointe N, Roberge-Tremblay A, Saint-Pierre M, Jimenez L, Ficke BW, Gross RE. Systemic exposure to paraquat and maneb models early Parkinson's disease in young adult rats. Neurobiol Dis 2005;20:360–371.

[14] Cory-Slechta DA, Thiruchelvam M, Barlow BK, Richfield EK. Developmental pesticide models of the Parkinson disease phenotype. Environ Health Perspect 2005;113:1263–1270.

[15] Groc L, Bezin L, Foster JA, Jiang H, Jackson TS, Weissmann D, Levine RA. Lipid peroxidation mediated oxidative stress and dopamine neuronal apoptosis in the substantia nigra during development. Neurochem Int 2001;39:127–133.

[16] Patel S, Singh V, Kumar A, Gupta YK, Singh MP. Status of antioxidant defense system and expression of toxicant responsive genes in striatum of maneb- and paraquat-induced Parkinson's disease phenotype in mouse: mechanism of neurodegeneration. Brain Res 2006;1081:9–18.

[17] Blaszczynski M, Litwinska J, Zaborowska D, Bilinski T. The role of respiratory chain in paraquat toxicity in yeast. Acta Microbiol Pol 1985;34:243–254.

[18] Hirai K, Witschi H, Cote MG. Mitochondrial injury of pulmonary alveolar epithelial cells in acute paraquat intoxication. Exp Mol Pathol 1985;43:242–252.

[19] Thakar JH, Hassan MN. Effects of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), cyperquat (MPP+) and paraquat on isolated mitochondria from rat striatum, cortex and liver. Life Sci 1988;43:143–149.

[20] Tomita M. Comparison of one-electron reduction activity against the bipyridylium herbicides, paraquat and diquat, in microsomal and mitochondrial fractions of liver, lung and kidney (*in vitro*). Biochem Pharmacol 1991;42:303–309.

[21] Bus JS, Aust SD, Gibson JE. Paraquat toxicity: proposed mechanism of action involving lipid peroxidation. Environ Health Perspect 1976;16:139–146.

[22] Castello PR, Drechsel DA, Patel M. Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. J Biol Chem 2007;282:14186–14193.

[23] Miller RL, Sun GY, Sun AY. Cytotoxicity of paraquat in microglial cells: involvement of PKC delta- and ERK1/2-dependent NADPH oxidase. Brain Res 2007;1167:129–139.

[24] Fitsanakis VA, Amarnath V, Moore JT, Montine KS, Zhang J, Montine TJ. Catalysis of catechol oxidation by metal-dithiocarbamate complexes in pesticides. Free Radic Biol Med 2002;33:1714–1723.

[25] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA1990;87:1620–1624.

[26] Berisha HI, Pakbaz H, Absood A, Said SI. Nitric oxide as a mediator of oxidant lung injury due to paraquat. Proct Natl Acad Sci USA 1994;91:7445–7449.

[27] He Y, Imam SZ, Dong Z, Jankovic J, Ali SF, Appel SH, Le W. Role of nitric oxide in rotenone-induced nigro-striatal injury. J Neurochem 2003;86:1338–1345.

[28] El-Gohary M, Awara WM, Nassar S, Hawas S. Deltamethrin-induced testicular apoptosis in rats: the protective effect of nitric oxide synthase inhibitor. Toxicology 1999;132:1–8.

[29] Djukic M, Jovanovic MC, Ninkovic M, Vasiljevic I, Jovanovic M. The role of nitric oxide in paraquat-induced oxidative stress in rat striatum. Ann Agric Environ Med 2007;14:247–252.

[30] Noack H, Possel H, Rethfeldt C, Keilhoff G, Wolf G. Peroxynitrite mediated damage and lowered superoxide tolerance in primary cortical glial cultures after induction of the inducible isoform of NOS. Glia 1999;28:13–24.

[31] Anton PM, Theodorou V, Bertrand V, Eutamene H, Aussenac T, Feyt N, Fioramonti J, Bueno L. Chronic ingestion of a potential food contaminant induces gastrointestinal inflammation in rats: role of nitric oxide and mast cells. Dig Dis Sci 2000;45:1842–1849.

[32] Vitte J, Michel BF, Bongrand P, Gastaut J-E. Oxidative stress level in circulating neutrophils is linked to neurodegenerative diseases. J Clin Immunol 2004;24:683–692.

[33] Gatto EM, Riobo NA, Carreras MC, Chernavsky A, Rubio A, Satz ML, Poderoso JJ. Overexpression of neutrophil neuronal nitric oxide synthase in Parkinson's disease. Nitric Oxide Biol Chem 2000;4:534–539.

[34] Nguyen HX, O'Barr TH, Anderson AJ. Polymorphonuclear leukocytes promote neurotoxicity through release of matrix metalloproteinases, reactive oxygen species, and TNF-α. J Neurochem 2007;Postprint;10.1111/j.1471-4159.2007.04643.x.

[35] Zhang J, Benveniste H, Klitzman B, Piantadosi CA. Nitric oxide synthase inhibition and extracellular glutamate concentration after ischemia/reperfusion. Stroke 1995;26:298–304.

[36] Scott JA, McCormack DG. Selective in vivo inhibition of inducible nitric oxide synthase in a rat model of sepsis. J Appl Physiol 1999;86:1739–1744.

[37] Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Lab Invest 1968;21(Suppl 97):77–89.

[38] Sethi S, Singh MP, Dikshit M. Nitric oxide-mediated augmentation of polymorphonuclear free radical generation after hypoxia-reoxygenation. Blood 1999;93:333–340.

[39] Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 1972;46:849–855.

[40] Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972;47:389–394.

[41] Pabst MJ, Habig WH, Jakoby WB. Glutathione S-transferase assay. J Biol Chem 1974;249:7140–7150.

[42] Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol 1982;78:206–209.

[43] Ohkawa H, Oshishi N, Yagi K. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–358.

[44] Bradford MA. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.

[45] Granger DL, Taintor RR, Read R, Boockvars KS, Hibb JB. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reagent. Methods Enzymol 1996;268:142–145.

[46] Kumar A, Patel S, Gupta YK, Singh MP. Involvement of endogenous nitric oxide in myeloperoxidase mediated benzo(a)pyrene induced polymorphonuclear leukocytes injury. Mol Cell Biochem 2006;286:43–51.

[47] Kanthaswamy AG, Wagner JR. Environmental factors-induced oxidative stress in dopaminergic degeneration: relevance to the etiopathogenesis of Parkinson's disease. Prominent Press; 2000.

[48] Klaunig JE, Xu Y, Isenberg JS, et al. The role of oxidative stress in chemical carcinogenesis. Environ Health Perspect 1998;106(Suppl 1):289–295.

[49] Konstantinova SG, Russanov EM. Studies on paraquat-induced oxidative stress in rat liver. Acta Physiol Pharmacol Bulg 1999;24:107–111.

[50] Niwa Y, Ishimoto K, Kanoh T. Induction of superoxide dismutase in leukocytes by paraquat: correlation with age and possible predictor of longevity. Blood 1990;76:835–841.

[51] Schmuck G, Rohrdanz E, Tran-Thi QH, Kahl R, Schluter G. Oxidative stress in rat cortical neurons and astrocytes induced by paraquat *in vitro*. Neurotox Res 2002;4:1–13.

[52] Day BJ, Shawen S, Liochev SI, Crapo JD. A metalloporphyrin superoxide dismutase mimetic protects against paraquat-induced endothelial cell injury, *in vitro*. J Pharmacol Exp Ther 1995;275:1227–1232.

[53] Mollace V, Iannone M, Muscoli C, Palma E, Granato T, Rispoli V, Nistico R, Rotiroti D, Salvemini D. The role of oxidative stress in paraquat induced neurotoxicocity in rats: protection by non-peptidyl superoxide dismutase mimetic. Neurosci Lett 2003;335:163–166.

[54] Thiruchelvam M, Prokopenko O, Cory-Slechta DA, Richfield EK, Buckley B, Mirochnitchenko O. Overexpression of superoxide dismutase or glutathione peroxidase protects against the paraquat+maneb-induced Parkinson disease phenotype. J Biol Chem 2005;280:22530–22539.

[55] Weidaur E, Lehmann T, Ramissch A, Rohrdanz E, Foth H. Response of alveolar type II cells and human lung tumor cells towards oxidative stress induced by hydrogen peroxide and paraquat. Toxicol Lett 2004;151:69–78.

[56] Brown GC. Reversible binding and inhibition of catalase by nitric oxide. Eur J Biochem 1995;232:188–191.

[57] Brunelli L, Yermilov V, Beckman JS. Modulation of catalase peroxidatic and catalatic activity by nitric oxide. Free Radic Biol Med 2001;30:709–714.

[58] Farias-Eisner R, Chaudhuri G, Aeberhard E, Fukuto JM. The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. J Biol Chem 1996;271:6144–6151.

[59] Dobashi K, Pahan K, Chahal A, Singh I. Modulation of endogenous antioxidant enzymes by nitric oxide in rat C6 glial cells. J Neurochem 1997;68:1896–1903.

[60] Hashida K, Sasaki K, Makino N. Interactions of nitric oxide and oxygen in cytotoxicity: proliferation and antioxidant enzyme activities of endothelial cells in culture. Free Radic Res 2000;33:147–156.

[61] Siddiqui A, Ali B, Srivastava S. Effect of mancozeb on hepatic glutathione S-transferase in rat. Toxicol Lett 1993;68:301–305.

[62] Dafre AL, Medeiros ID, Muller IC, Ventura EC, Bainy ACD. Antioxidant enzymes and thiol/disulfide status in the digestive gland of the brown mussel Perna perna exposed to lead and paraquat. Chem Biol Interact 2004;149:97–105.

[63] Barlow BK, Lee DW, Cory-Slechta DA, Opanashuk LA. Modulation of antioxidant defense systems by the environmental pesticide maneb in dopaminergic cells. Neurotoxicol 2005;26:63–75.

[64] Olesen BT, Clausen J, Vang O. Characterization of the transcriptional profile in primary astrocytes after oxidative stress induced by paraquat. Neurotoxicol 2008;29:13–21.

[65] Arimoto T, Bing G. Up-regulation of inducible nitric oxide synthase in the substantia nigra by lipopolysaccharide causes microglial activation and neurodegeneration. Neurobiol Dis 2003;12:35–45.

[66] Barthwal MK, Srivastava N, Dikshit M. Role of nitric oxide in a progressive neurodegenration model of Parkinson's disease. Redox Rep 2001;6:297–302.

[67] Castano A, Herrera AJ, Cano J, Machado A. Lipopolysaccharide (LPS) intranigral injection induced inflammatory reaction and damage in nigrostriatal dopaminergic system. J Neurochem 1998;70:1584–1592.

[68] Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, Dawson VL, Dawson TM, Przedborski S. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model Parkinson's disease. Nat Med 1999;5:1403–1409.

[69] Kavya R, Saluja R, Singh S, Dikshit M. Nitric oxide synthase regulation and diversity: implications in Parkinson's disease. Nitric Oxide 2006;15:280–294.
[70] Ebadi M, Sharma SK. Peroxynitrite and mitochondrial dysfunction in the pathogenesis of Parkinson's disease. Antioxd Redox Signal 2003;5:319–335.
[71] Iravani MM, Leung CCM, Sadeghian M, Haddon CO, Rose S, Jenner P. The acute and long term effects of nigral lipopolysaccharide administration on dopaminergic dysfunction and glial cell activation. Eur J Neurosci 2005;22:317–330.
[72] Day BJ, Patel M, Calavetta L, Chang L-Y, Stamler JS. A mechanism of paraquat toxicity involving nitric oxide synthase. Proc Nat Acad Sci USA 1999;96:12760–12765.
[73] Islas-Carbajal MC, Covarrubias A, Grijalva G, Alvarez-Rodriguez A, Armendariz-Borunda J, Rinco n-Sanchez AR. Nitric oxide synthases inhibition results in renal failure improvement in cirrhotic rats. Liver Int 2005;25:131–140.
[74] Wei C-L, Hon W-M, Lee K-H, Khoo H-E. Chronic administration of aminoguanidine reduces vascular nitric oxide production and attenuates liver damage in bile duct-ligated rats. Liver Int 2005;25:647–656.
[75] Eum H-A, Park S-W, Lee S-M. Role of nitric oxide in the expression of hepatic vascular stress genes in response to sepsis. Nitric Oxide 2007;17:126–133.
[76] Lukivskaya O, Patsenker E, Lis R, Buko VU. Inhibition of inducible nitric oxide synthase activity prevents recovery in rat thioacetamide-induced fibrosis reversal. Eur J Clin Invest 2008;38:317–325.
[77] Kumar A, Upadhyay G, Modi DR, Singh MP. The involvement of secondary signaling molecules in cytochrome P-450 1A1-mediated inducible nitric oxide synthase expression in benzo(a)pyrene-treated rat polymorphonuclear leukocytes. Life Sci 2007;81:1575–1584.
[78] Sigfrid LA, Cunningham JM, Beeharry N, Lortz S, Tiedge M, Lenzen S, Carlsson C, Green IC. Cytokines and nitric oxide inhibit the enzyme activity of catalase but not its protein or mRNA expression in insulin-producing cells. J Mol Endocrinol 2003;31:509–518.
[79] Mitra S, Abraham E. Participation of superoxide in neutrophil activation and cytokine production. Biochim Biophys Acta 2006;1762:732–741.