Original article:

NEUROPROTECTIVE EFFECT OF NITRIC OXIDE DONOR ISOSORBIDE-DINITRATE AGAINST OXIDATIVE STRESS INDUCED BY ETHIDIUM BROMIDE IN RAT BRAIN

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ABSTRACT

This study investigated the effect of systemic administration of isosorbide-dinitrate (ISDN) on oxidative stress and brain monoamines in a toxic model of brain demyelination evoked by intracerebral injection (i.c.i) of ethidium bromide (10 µl of 0.1 %). Rats received saline (control) or ISDN at 5 or 10 mg/kg for 10 days prior to injection of ethidium bromide. Rats were euthanized one day later, and then the levels of reduced glutathione (GSH), lipid peroxidation (malondialdehyde; MDA), nitric oxide (nitrite/nitrate), acetylcholinesterase (AChE) activity, paraoxonase activity as well as monoamine levels (serotonin, dopamine and noradrenaline) were assessed in the brain cortex in different treatment groups. The i.c.i of ethidium bromide resulted in increased oxidative stress in the cortex one day after its injection; (i) MDA increased by 36.9 %; (ii) GSH decreased by 20.8 %, while (iii) nitric oxide increased by 60.3 %; (iv) AChE and paraoxonase activities in cortex decreased by 35.9 % and 29.4 %, respectively; (v) serotonin was significantly increased. In ethidium bromide-treated rats, pretreatment with ISDN at 10 mg/kg decreased cortical MDA by 23.9 %. Reduced glutathione was increased by 25.1 % ISDN at 10 mg/kg, while nitric oxide showed a 32.8 and 41.7 % decrease after 5 and 10 mg/kg of ISDN, respectively. Acetylcholinesterase activity increased by 24.3 % by 10 mg/kg of ISDN. Paraoxonase activity showed further decrease by 72.2 and 83.8 % after treatment with 5 and 10 mg/kg of ISDN, respectively. The administration of ISDN decreased the level of serotonin and noradrenaline compared with the ethidium bromide only treated group. Overall, the present findings suggest neuroprotective effect of ISDN against oxidative stress in this model of chemical demyelination.

Keywords: toxic demyelination, ethidium bromide, isosorbide dinitrate, rat brain

INTRODUCTION

Demyelinating diseases of the central nervous system are a heterogeneous group of chronic inflammatory disorders, the hallmark of which is loss of myelin sheath and nerve conduction deficits leading to motor and/or sensory dysfunction and are the leading cause of nontraumatic neurological disability in young adults (Hu and Lucchinetti, 2009). The spectrum of demyelinating disorders includes 'autoimmune' inflammatory demyelinating diseases, the inflammatory demyelinating diseases of infectious aetiology, and the demyelinating or dysmyelinating diseases of

genetic/hereditary background. In addition, primary demyelination is present in other conditions, such as brain ischaemia and intoxication (Lassmann, 2001). Multiple sclerosis is by far the most common inflammatory demyelinating disease leading to focal plaques of primary demyelination with a variable degree of axonal and neuronal degeneration (Love, 2006; Lassmann et al., 2007).

Oxidative stress has been implicated in both normal aging and in various neurodegenerative disorders. In the brain, the high content of polyunsaturated fatty acids, the high utilization of oxygen account for the susceptibility to free radical damage. The mechanisms of tissue injury in demyelinating diseases of the central nervous system are poorly understood but increasing evidence support a role for oxidative stress due to an imbalance between free radicals generation and endogenous antioxidant mechanisms. Reactive oxygen species, nitric oxide, and proinflammatory cytokines released by monocyte-derived macrophages contribute to neuroinflammation, demyelination and axonal damage and disease progression in multiple sclerosis (Mirshafiey and Mohsenzadegan, 2009; Smith, 2011; de Vries et al., 2011). Multiple sclerosis patients showed increased generation of superoxide free radicals in blood (Glabinski et al., 1993), elevated levels of thiobarbituric acid reactive substances and reduced protein sulfhydryl groups in cerebrospinal fluid and serum (Mitosek-Szewczyk et al., 2010), suggesting increased free radical production and lipid peroxidation. Oxidized lipids and DNA were highly enriched in active multiple sclerosis plaques (Haider et al., 2011). Evidence also implicates increased nitric oxide generated by the inducible form of nitric oxide synthase (iNOS) in the inflammation and demyelination in multiple sclerosis. Increased iNOS activity has been demonstrated in monocytes/macrophages and/or astrocytes in demyelinating lesions of postmortem tissues in multiple sclerosis (Bagasra et al., 1995; Oleszak et al., 1998; Liu et

al., 2001). Nitric oxide is increased in serum of patients with multiple sclerosis (Ibragic et al., 2012). Nitric oxide is likely to be involved in axonal and neuronal injury in demyelinating conditions (Kapoor et al., 2000; Garthwaite et al., 2002).

Changes in neurotransmitter concentrations in multiple sclerosis and the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis are recognized to underlie many neurological symptoms associated with the disease, and there is accumulating evidence demonstrating that immune function is directly regulated by the activity of certain neurotransmitters (Bhat et al., 2010; Lee et al., 2011; Vollmar et al., 2009). It has been recently observed that a mouse model of EAE is associated with chronic deficits in spinal cord concentrations of noradrenaline (NE), 5-hydroxytryptamine (5-HT/ serotonin) and γ-aminobutyric acid (GABA) (Musgrave et al., 2011). Furthermore, recent studies have shown that therapeutic agents that increase GABAergic and monoaminergic signaling can lessen the severity of EAE (Bhat et al., 2010; Simonini et al., 2010; Taler et al., 2010). Nitric oxide may play a role in physiological neuronal functions such as long-term potentiation as a retrograde messenger (Shuman and Madison, 1994; Medina and Izquierdo, 1995) and in the regulation of gene expression (Yun et al., 1997). Furthermore, it can act as a potent vasodilator and an inhibitor of platelet aggregation (Iadecola, 1997; Szabo, 1996) and, as has been reported, in the S-nitrosylation of proteins (Arnelle and Stamler, 1995; Rauhala et al., 1998). Earlier studies have shown that nitric oxide exerts a regulatory influence on behavioral and physiological parameters in normal and stressed rats (Gulati and Chakraborti, 2007; Masood et al, 2003). The results of Hummel et al. (2006) described an antioxidant effect for nitric oxide.

Isosorbide dinitrate (ISDN) (an orally active form of nitrates) is a drug widely used for the management of coronary ischaemia by

virtue of its vasodilatory properties. ISDN is capable of releasing nitric oxide in a concentration- and pH-dependent manner (Jiang et al., 2001). Thus, the present study was designed to investigate the effect of the nitric oxide donor ISDN on oxidative stress and brain monoamines in a model of toxic demyelination evoked by intracerebral injection of ethidium bromide in the rat. Ethidium bromide is a DNA chelating agent that is commonly used to evoke transient central nervous system demyelination in experimental animals, which can be used to study the pathogenetic mechanisms and the possible therapeutic interventions (Yajima and Suzuki, 1979; Jeffery and Blakemore, 1997; Mazzanti et al., 2006).

MATERIALS AND METHODS

Animals

Twenty five adult male Sprague Dawley rats weighing (130 ± 10 g) (age: 10–11 weeks) were used in this study. The animals were obtained from the Animal House Colony of the National Research Centre (Cairo, Egypt). They were housed in stainless steel wire meshed suspended rodent cages under environmentally controlled conditions. The ambient temperature was 25 ± 2 °C and the light/dark cycle was 12/12 hours. The animals had free access to water and standard rodent chow diet (NRC rodent chow). All animals received human care in compliance with guidelines of the Ethical Committee of National Research Centre, Egypt Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Equal groups of 5 rats each were used in all experiments.

Drugs and chemicals

Ethidium bromide (Sigma, St Louis, MO, USA) and isosorbide dinitrate (Amrya Pharm. Ind., Cairo, Egypt) was used and dissolved in isotonic (0.9 % NaCl) saline solution immediately before use. The doses of isosorbide dinitrate in the study were based upon the human dose after conversion to that of rat according to Paget and Barnes (1964) conversion tables.

Surgical procedures

Rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and after shaving the hair from the fronto-occipital area antisepsis was performed with 2 % iodine solution. A hole of 0.5 Cm was made using orthodontic roof motor and number 2 drill to the right of the bregma until the dura matter was exposed. With the use of a Hamilton syringe fitted with a 30-gauge needle the solution of ethidium bromide (10 µl of 0.1 %) was injected in the cisterna pontis (basal), an enlargement of the subarachnoid space on the ventral surface of the pons. A group of rats (n=5) was undergone to the same surgical procedure but injected with saline (0.9 %) and served as negative control. The dura matter left open and the skin together with remainder of the subcutaneous tissue was sutured with a nylon thread 4.0.

Experimental design

Rats randomly assigned into 4 groups (n=5 each) received saline (control) or ISDN at 5 or 10 mg/kg orally for 10 days weeks prior to injection of ethidium bromide. Next day after ethidium bromide injection, the animals were euthanized by decapitation in deep ether anesthesia. Brains were then removed, washed with ice-cold saline solution (0.9 % NaCl), and sectioned into cortex, weighed and stored at -80 °C for further determination of biochemical parameters. The brain was homogenized with 0.1 M phosphate buffer saline at pH 7.4, to give a final concentration of 10 % w/v for the biochemical assays. For the determination of monoamine neurotransmitters, frozen samples were homogenized in cold 0.1 N-perchloric acid.

Biochemical studies

Determination of brain lipid peroxidation

Lipid peroxidation was assayed by measuring the level of malondialdehyde (MDA) in the brain tissues. Malondialdehyde was determined by measuring thiobarbituric reactive species using the method of Ruiz-Larrea et al. (1994) in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex having peak absorbance at 532 nm.

Determination of brain reduced glutathione content

Reduced glutathione (GSH) was determined in brain tissue by Ellman's method (1959). The procedure is based on the reduction of Ellman´s reagent by –SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid, the nitromercaptobenzoic acid anion has an intense yellow color which can be determined spectrophotometrically. A mixture was directly prepared in a cuvette: 2.25 ml of 0.1 M K-phosphate buffer, pH 8.0; 0.2 ml of the sample; 25 µl of Ellman's reagent (10 mM 5,5′-dithio-bis-2-nitrobenzoic acid in methanol). After 1 min the assay absorbance was measured at 412 nm and the GSH concentration was calculated by comparison with a standard curve.

Determination of brain acetylcholinesterase activity

The procedure used for the determination of acetylcholinesterase activity in the cortex was a modification of the method of Ellman et al. (1961) as described by Gorun et al. (1978). The principle of the method is the measurement of the thiocholine produced as acetylthiocholine is hydrolyzed. The colour was read immediately at 412 nm.

Determination of brain nitric oxide

Nitric oxide measured as nitrite was determined by using Griess reagent, according to the method of Moshage et al. (1995). Where nitrite, stable end product of nitric oxide radical, is mostly used as indicator for the production of nitric oxide.

Determination of brain paraoxonase activity

Arylesterase activity of paraoxonase was measured spectrophotometrically in supernatants using phenylacetate as a substrate (Higashino et al. 1972; Watson et al., 1995).

Determination of brain monoamines

Determination of brain serotonin, noradrenaline and dopamine was carried out using high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series, equipped with a quaternary pump (Quat pump, G131A model). Separation was achieved on ODS reversed phase column (C18, 25 x 0.46 cm i.d. 5 µm). The mobile phase consisted of potassium phosphate buffer/methanol 97/3 (v/v) and was delivered at a flow rate of 1 ml/min. UV detection was performed at 270 nm and the injection volume was 20 µl. The concentration of both catecholamines and serotonin were determined by external standard method using peak areas. Serial dilutions of standards were injected and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the curve.

Statistical analysis

Data are expressed as mean ± SE. Data were analyzed by one-way analysis of variance, followed by Duncan's multiple range test for *post hoc* comparison of group means. Effects with a probability of $p < 0.05$ were considered to be significant.

RESULTS

Oxidative stress

In saline treated rats, i.c. ethidium bromide injection resulted in a significant increase in the level of MDA by 36.9 % (48.6 ± 4.1 *vs* 35.5 ± 3.0 nmol/g, $p < 0.05$) (Figure 1). Reduced glutathione decreased by 20.8 %

$(5.10 \pm 0.28$ *vs* 6.44 ± 0.33 µmol/g, $p < 0.05)$ (Figure 2), while nitric oxide increased by 60.3 % $(15.45 \pm 0.83$ *vs.* 9.64 ± 0.51 µmol/g, $p < 0.05)$ after ethidium bromide injection compared with the saline control group (Figure 3).
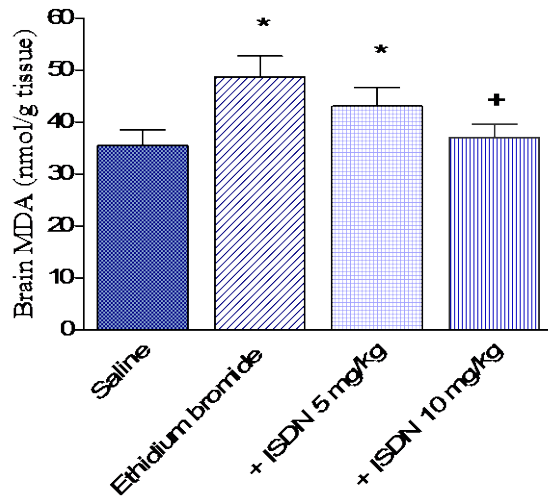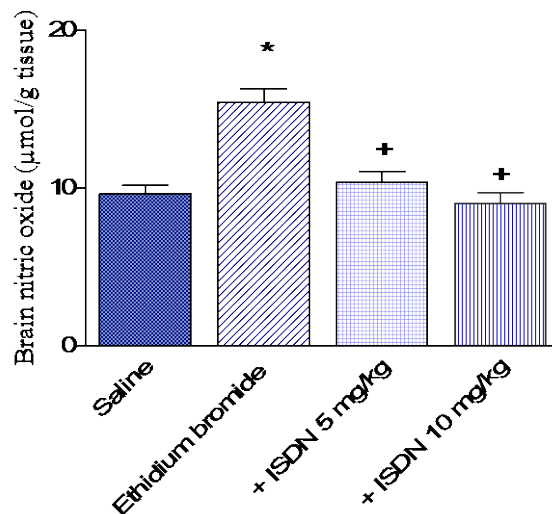

Figure 1: Effect of isosorbide dinitrate (ISDN) treatment on the concentration of malondialdehyde (MDA) in the cortex of rats subjected to intracerebral injection of ethidium bromide. Data are means ± SEM. *: $p < 0.05$ *vs* the saline control group. +: $p < 0.05$ *vs* the ethidium bromide control group.

Pretreatment with ISDN at 5 mg/kg for 10 days prior to ethidium bromide injection had no significant effect on cortical MDA $(43.0 \pm 3.6$ *vs* 48.6 ± 4.1 nmol/g, $p > 0.05)$. However, ISDN administered at 10 mg/kg resulted in a significant decrease in MDA in cortex by 23.9 % compared with the ethidium bromide control group $(37.0 \pm 2.6$ *vs* 48.6 ± 4.1 nmol/g, $p < 0.05)$ (Figure 1). Reduced glutathione was not significantly altered by ISDN treatment at 5 mg/kg, but increased by 25.1 % after treatment with the higher dose of ISDN $(6.38 \pm 0.41$ *vs* 5.1 ± 0.28 µmol/g) (Figure 2). Meanwhile, nitric oxide decreased by 32.8 and 41.7 % following ISDN administration at 5 and 10 mg/kg, respectively, compared with the ethidium bromide control group $(10.38 \pm 0.64$ and 9.0 ± 0.71 *vs* 15.45 ± 0.83 µmol/g, $p < 0.05$ (Figure 3).
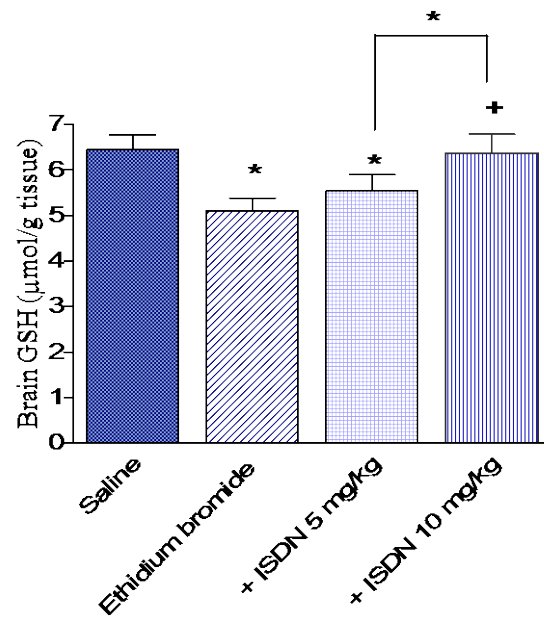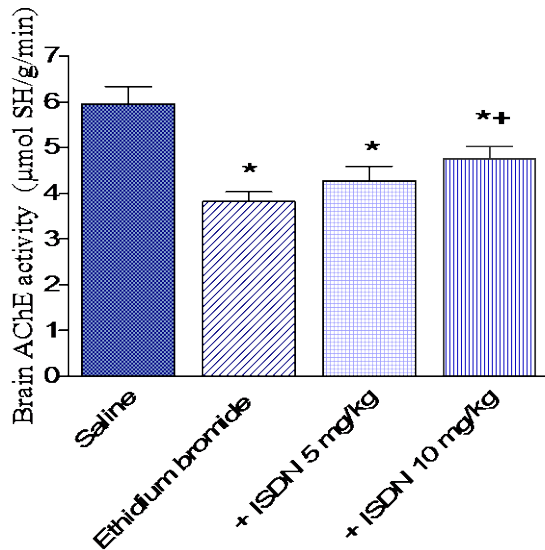

Figure 2: Effect of isosorbide dinitrate (ISDN) treatment on reduced glutathione (GSH) in the rat cortex after intracerebral administration of the demyelinating agent ethidium bromide. Data are means ± SEM. *: $p < 0.05$ vs the saline control group and between different groups as indicated. +: $p < 0.05$ vs the ethidium bromide control group.

Figure 3: Effect of isosorbide dinitrate (ISDN) treatment on nitric oxide concentration in the rat cortex after intracerebral administration of the demyelinating agent ethidium bromide. Data are means ± SEM. *: $p < 0.05$ *vs* the saline control group. +: $p < 0.05$ *vs* the ethidium bromide control group.

Acetylcholinesterase activity

In saline treated rats, AChE activity decreased by 35.9 % after i.c. ethidium bromide injection (3.82 ± 0.21 *vs* 5.96 ± 0.38 µmol SH/g/min). AChE activity was unaltered in rats treated with ISDN at 5 mg/kg. The higher dose of ISDN, however, increased AChE activity by 24.3 % compared with the ethidium bromide control group (4.75 ± 0.28 *vs* 3.82 ± 0.21 µmol SH/g/min, $p < 0.05$ (Figure 4).
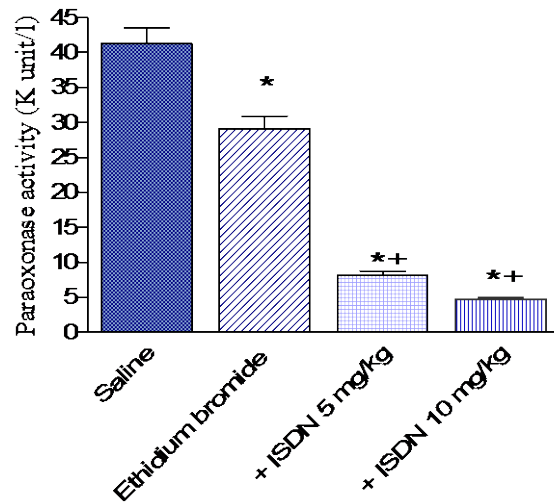

Figure 4: Effect of isosorbide dinitrate (ISDN) treatment on nitric oxide concentration in the rat cortex after intracerebral injection of the demyelinating agent ethidium bromide. Data are means ± SEM. *: p < 0.05 *vs* the saline control group. +: p < 0.05 *vs* the ethidium bromide control group.

Paraoxonase activity

In saline treated rats, paraoxonase activity decreased by 29.4 % after i.c. ethidium bromide injection (29.1 ± 1.8 *vs* 41.22 ± 2.3 kU/l). Paraoxonase activity showed a further decrease by 72.2 and 83.8 % after treatment

with ISDN at 5 or 10 mg/kg, respectively (8.1 ± 0.62 and 4.7 ± 0.38 *vs* 29.1 ± 1.8 kU/l, $p < 0.05$ (Figure 5).

Figure 5: Effect of isosorbide dinitrate (ISDN) treatment on paraoxonase activity in the rat cortex after intracerebral injection of the demyelinating agent ethidium bromide. Data are means ± SEM. *: p < 0.05 *vs* the saline control group. +: p < 0.05 *vs* the ethidium bromide control group.

Brain monoamines

The levels of dopamine and noradrenaline were not significantly altered by ethidium bromide injection, whereas serotonin was increased compared with the saline control group. ISDN given at 5 or 10 mg/kg resulted in 41.6, 70.6 % decrease in serotonin and 28.6, 31.9 % decrease in noradrenaline, respectively when compared with the ethidium bromide control group (Table 1).

Table 1: Effect of ISDN on serotonin, dopamine and noradrenaline in mice cortex after ethidium bromide injection

	Serotonin (µg/g tissue)	Dopamine (µg/g tissue)	Noradrenaline (µg/g tissue)
Saline	2.96 ± 0.18	3.20 ± 0.21	2.22 ± 0.12
Ethidium	$5.10 \pm 0.32^{*}$	3.39 ± 0.12	2.13 ± 0.18
ISDN 5 mg/kg	$2.98 \pm 0.12^{+}$	3.21 ± 0.18	$1.52 \pm 0.07^{*+}$
ISDN 10 mg/kg	$1.50 \pm 0.08^{*+}$	3.26 ± 0.11	$1.45 \pm 0.11^{*+}$

Results are mean ± S.E. Six mice were used per each group. Data were analyzed by one way ANOVA and means of different groups were compared by Duncan's multiple range test. $P < 0.05$ was considered statistically significant. *: $p < 0.05$ *vs* saline control group. +: $p < 0.05$ *vs* the ethidium control group.

DISCUSSION

The present study provides evidence that the administration of the vasodilator and nitric oxide releasing agent ISDN in a model of toxic demyelination resulted in amelioration of oxidative stress markers. The intracerebral or intraspinal administration of the DNA chelating agent ethidium bromide has been widely utilized to evoke toxic demyelination in rodents, which can be used to study the pathogenetic mechanisms involved in the destruction of myelin as well as to evaluate possible therapeutic interventions (Yajima and Suzuki, 1979; Honmou et al., 1996; Jeffery and Blakemore, 1997; Graça et al., 2001; Mazzanti et al., 2006). In the present study, the local injection of ethidium bromide into the rat brain resulted in elevated MDA, an index of lipid peroxidation (Gutteridge, 1995), which indicates increased free radical production in cerebral cortex. There was also a significant decrease in the level of GSH, the major thiol present in brain tissue, and the most important redox buffer in cells, which has an important role in the protection against oxidative injury due to reactive oxygen species (Wang and Ballatori, 1998). This suggests consumption of GSH by the increased free radical production following ethidium bromide injection. Nitric oxide was markedly increased after ethidium bromide. These findings suggest increased oxidative stress by ethidium bromide in the cerebral cortex and are in line with other studies indicating increased oxidative stress in different brain areas by the toxin (Abdel-Salam el al., 2011). The increase in oxidative stress following ethidium bromide injection was decreased by prior treatment with the nitric oxide donor ISDN, which decreased MDA and increased GSH in the cortex. These findings have important clinical implications in view of the evidence that oxidative stress is involved in demyelination disorders. Multiple sclerosis patients were found to have elevated lipid peroxidation and decreased levels endogenous antioxidants, suggesting consumption of the scavenger molecules by free radical excess (Karg et al., 1999; Mitosek-Szewczyk et al., 2010). In addition, GSH levels measured in the brain with magnetic resonance spectroscopy were lower in patients with multiple sclerosis compared with control (Srinivsan et al., 2010; Choi et al., 2011). Oxidized lipids and DNA were highly enriched in active multiple sclerosis plaques and oxidative injury of oligodendrocytes and neurons were associated with demyelination and axonal or neuronal injury (Haider et al., 2011). Studies have also indicated increased oxidative and nitrosative stress in experimental models eg., the ethidium bromide-induced damage (Abdel-Salam et al., 2011) and in autoimmune encephalomyelitis (Ljubisavljevic et al., 2011; Vana et al., 2011).

Nitric oxide is an important molecule involved in synaptic transmission and regulation of vascular tone. Nitric oxide is produced within the central nervous system from L-arginine by a constitutive (neuronal) form of nitric oxide synthase (nNOS), an endothelial form in vascular endothelium (eNOS) or an inducible form (iNOS) localized to glia, and requires activation by endotoxin and cytokines (Moncada and Bolaños, 2006). The production of nitric oxide is increased in brain and serum of multiple sclerosis patients (De Groot et al., 1997; Liu et al., 2001; Koch et al., 2008). Nitric oxide is a free radical and can react with many other free radicals e.g., superoxide radical generating peroxynitrite radical, capable of causing oxidative changes to macromolecules e.g., proteins, lipids and DNA (Moncada and Bolaños, 2006).

Elevated nitric oxide concentrations which occur in neuroinflammatory states can thus result in neurodegeneration. Increased levels of nitric oxide causes axonal degeneration (Kapoor et al., 2000; Garthwaite et al., 2002) and activation of nNOS in oligodendrocytes leads to oligodendrocyte injury resulting in demyelination (Yao et al., 2010). Evidence also implicates iNOS in the inflammation and demyelination of optic neuritis, where localized loss of myelin proteins, myelin breakdown, and the presence of iNOS and nitrotyrosine were associated with inflammatory infiltrates on the edges of the nerve and reactive astrocytes (Tsoi et al., 2006). In experimental allergic encephalomyelitis (EAE), nitrotyrosine, an indicator of peroxynitrite formation is increased in the spinal cord white matter, which correlated with loss of mature oligodendrocytes (Li et al., 2011).

Given that nitric oxide is likely to be involved in axonal and neuronal injury in demyelinating conditions (Kapoor et al., 2000; Garthwaite et al., 2002), in the present study, the nitric oxide donor ISDN was used to evaluate a possible modulating effect. ISDN is capable of releasing nitric oxide in a concentration- and pH-dependent manner. ISDN increased nNOS and eNOS activities in the presence oxyhemoglobin under hypoxia due to the increase in molecular oxygen concentration (Jiang et al., 2001). Interestingly, pretreatment with ISDN decreased MDA, whilst elevating the level of reduced glutathione in cerebral cortex. Moreover, ISDN resulted in marked decrease in the level of nitric oxide in cortex. These results suggest that nitric oxide donors are likely to exert beneficial effects on the demyelination process. The results are also unexpected in view of the evidence that implicates nitric oxide in demyelinating diseases of the central nervous system. Studies thus have shown that the production of nitric oxide is increased in brain and serum of multiple sclerosis patients (De Groot et al., 1997; Liu et al., 2001; Koch et al., 2008). Increased

levels of nitric oxide causes axonal degeneration (Kapoor et al., 2000; Garthwaite et al., 2002) and activation of nNOS in oligodendrocytes which leads to oligodendrocyte injury resulting in demyelination (Yao et al., 2010). Evidence also implicates iNOS in the inflammation and demyelination of optic neuritis, where localized loss of myelin proteins, myelin breakdown, and the presence of iNOS and nitrotyrosine were associated with inflammatory infiltrates on the edges of the nerve and reactive astrocytes (Tsoi et al. 2006). In experimental allergic encephalomyelitis, nitrotyrosine, an indicator of peroxynitrite formation is increased in the spinal cord white matter, which correlated with loss of mature oligodendrocytes (Li et al., 2011). Nitric oxide is a free radical and can react with many other free radicals e.g., superoxide radical generating peroxynitrite radical, capable of causing oxidative changes to macromolecules e.g., proteins, lipids and DNA. Increased nitric oxide production by microglia which occurs in neuroinflammatory states can thus result in neurodegeneration. While nitric oxide normally functions as a physiological neuronal mediator, excess production of nitric oxide mediates cellular toxicity by damaging critical metabolic enzymes and by reacting with superoxide to form an even more potent oxidant, peroxynitrite (Bredt, 1999).

On the other hand, the effect of nitric oxide donors in neurodegenerative and demyelinating conditions is not clear. Nitric oxide donors exerted cytotoxic effects on dopaminergic neurons (Nunes et al., 2008; Di Matteo et al., 2009; Kurauchi et al., 2009) via mechanisms that include mitochondrial dysfunction (Nunes et al., 2008). Nitric oxide donors cause reversible conduction block in both normal and demyelinated axons of the central and peripheral nervous systems. Notably, conduction in demyelinated and early remyelinated axons is particularly sensitive to block by nitric oxide (Redford et al., 1997). In cultured hippocampal neurons, ISDN as well as another newly developed nitric oxide-

releasing agent rapidly and significantly reduced axonal transport in anterograde and retrograde directions (Kiriyama et al., 2002). Nitric oxide donors exert metabolic effects e.g., nitroglycerin, ISDN, molsidomine, and sodium nitroprusside induced stimulation of glycolysis and shortened adenosine triphosphate (ATP)-turnover time in rat erythrocytes (Maletic et al., 2000). In rat reticulocytes, ISDN, stimulated glycolysis and decreased ATP production *via* oxidative phosphorylation (Maletic et al., 1999). The NO donor "spermine NONOate" decreased stimulated release of ATP from rabbit erythrocytes (Olearczyk et al., 2004). In hippocampal synaptosomes of rats, sodium nitroprusside (but not other nitric oxide donors such as S-nitroso-N-acetyl-penicillamine and ISDN) inhibited adenosine triphosphate diphosphohydrolase and 5'-nucleotidase involved in an enzymatic chain for the hydrolysis of ATP to adenosine in the synaptic cleft (Kirchner et al., 2001). Thus, whilst nitric oxide plays a physiological role in neuronal cell signaling, its over-production may cause neuronal energy compromise leading to neurodegeneration. Other researchers provided data suggesting that the administration of the exogenous nitric oxide donor molsidomine, a drug used for the treatment of coronary artery disease, limits the development of autoimmune encephalomyelitis and other T helper 1 (Th1) cell-mediated inflammatory diseases (Kwak et al., 2003). Studies also suggested that enhanced nitric oxide production by the nitric oxide donor SIN-1 (3-morpholinosydnonimine hydrochloride) during the priming phase of autoimmune encephalomyelitis promotes apoptosis, down-regulates disease-promoting immune reactivities, and ameliorates clinical EAE, without depending on NOS (Xu et al., 2001). Moreover, the increase in iNOS, nNOS and nitrotyrosine induced in the cerebral cortex of rats subjected to ischemia was prevented by the nitric oxide donor LA 419 (Serrano et al., 2007). It has been suggested that nitric oxide may be a double-edged sword, mediating tissue damage on the one hand and on the other hand modulating complex immunological functions which may be protective (Giovannoni et al., 1998).

Isosorbide dinitrate has been reported to preserve cell viability in the hippocampus after focal ischemia (Ramos-Zúñiga et al., 1998). In addition, intravenous administration of nitric oxide donors reduces the infarct size after transient focal cerebral ischemia in rats (Salom et al., 2000). Although there are several potential mechanisms for nitric oxide neuroprotective effects during brain ischemia (Verrecchia et al., 1995), a rationale for the use of nitric oxide promoting strategies lies on the ability of nitric oxide to increase brain perfusion in areas of compromised perfusion around the ischemic core. NO has many additional roles outside the cardiovascular system. It appears to promote or prevents cellular inflammation and death. Evidence shows that nitric oxide can be anti-inflammatory through several activities; inhibition of maturation of cytokines, such as IL-18 and IL 1β (Kim et al., 1997); blocking the effect of INF-γ (Murphy, 2000) and preventing the expression of cellular expression molecules *via* effects on NF-κB (Laroux et al, 2001; Brüne et al., 1998). Alternatively, nitric oxide can enhance neuronal survival by attenuation of Ca^{++} influx via antagonism of the NMDA glutamate receptors. The reasons for the dual effects of nitric oxide are unclear, although it may be that where there are high local concentrations of nitric oxide or where it is derived from a particular source (such as iNOS or nNOS) the toxic effects predominate (Willmot and Bath, 2003).

An important new observation in the present study was the decrease in paraoxonase activity in cortex by ethidium bromide. Paraoxonase is a calcium-dependent serum esterase that is synthesized by the liver and is released into the circulation, where it associates mainly with high density lipoproteins and protects LDL and cellular membranes against lipid peroxidation (La Du, 1992; Pri-

mo-Parmo et al., 1996). The paraoxonase gene family in humans includes three members: PON1, PON2 and PON3. PON1 possesses organophosphatase, arylesterase and lactonase activity and it hydrolyzes many different substrates (Rajkovic et al., 2011). Serum PON1 and PON3 are inactivated under oxidative stress (Marsillach et al., 2004). The enzyme PON1 is largely thought to have a role in protection against oxidative stress (Watson et al., 1995; Mackness et al., 2006; Amengual-Cladera et al., 2011). It has been proposed that this enzyme might have a function related to the inactivation of oxidative stress by-products (either at a cellular level or blood-vessel wall) and other environmental chemicals (Rodrigo et al., 2001). Lead exposed workers (Permpongpaiboon et al., 2011) and patients with coronary heart disease (Kotur-Stevuljevic et al., 2008) showed increased lipid peroxidation and decreased PON1 activity. In multiple sclerosis patients, PON1 activity does not change in the course of stable and progressive type of multiple sclerosis. However, PON1 activity in relapse was significantly lower in comparison to the other multiple sclerosis groups (Jamroz-Wisniewska et al., 2009). In the present study, paraoxonase activity was markedly decreased in the cortex of ethidium bromide treated rats and showed a further decrease following ISDN treatment. This occurred despite a decrease of in lipid peroxidation (MDA) and increased GSH by the nitric oxide donor. One intriguing possibility is that PON1 represents an early defense mechanism against oxidative stress, resulting in an initial sparing of GSH. With higher levels of oxidative stress, depletion of the antioxidant glutathione will ensue. Studies have also shown that incubation of myelin suspensions with the peroxynitrite donor 3-morpholinosydnonimine (SIN-1) (but not nitric oxide or superoxide alone) resulted in the formation of the lipid peroxidation product, MDA, indicating that peroxynitrite formation is required for myelin-lipid peroxidation. Nitric oxide actually inhibited lipid peroxidation in myelin, as demonstrated using simple nitric oxide donors (van der Veen and Roberts, 1999).

In the present study, ethidium bromide injection resulted in increased serotonin concentration in cortex. The increase in serotonin was partially restored by ISDN treatment which also decreased noradrenaline, compared with the ethidium bromide only treated group. Patients with multiple sclerosis were found to have increased cerebrospinal fluid noradrenaline and excitatory amino acid (glutamate and aspartate) levels (Barkhatova et al., 1998). It has been shown that a mouse model of EAE is associated with chronic deficits in spinal cord concentrations of noradrenaline, serotonin and γ-aminobutyric acid (GABA) (Musgrave et al., 2011) and that therapeutic agents that increase GABAergic and monoaminergic signaling can lessen the severity of EAE (Wang et al., 2008; Simonini et al., 2010). The findings of the current study also indicated that AChE activity decreased in the cortex early after ethidium bromide injection, suggesting alterations in cholinergic neurotransmission induced by the toxic agent (Taler et al., 2010). Moreover, AChE activity is increased by the higher dose of ISDN. Thus the administration of ISDN appears to correct the neurochemical alterations induced by the toxic agent in the cortex. Changes in neurotransmitter levels and AChE activity has been demonstrated in patients with multiple sclerosis and in experimental models of demyelination. AChE decreased in the cerebrospinal fluid of subjects with multiple sclerosis (and in Huntington's chorea patients). This suggested that cerebrospinal fluid AChE activity may globally reflect brain AChE, but pathology-induced changes may not be directly reflected (Ruberg et al., 1987). Alterations in butyrylcholinesterase activity, another enzyme capable of hydrolysing acetylcholine, were observed in multiple sclerosis white matter lesions diminished enzyme activity associated with myelin and an increased activity in cells with microglial

morphology (Darvesh et al., 2010). In ethidium bromide-treated rats, AChE activity was found to vary in all the brain structures in accordance with the day studied (Mazzanti et al., 2006; Abdel-Salam et al., 2011).

In summary, the present study indicated that the administration of the nitric oxide donor ISDN in a model of toxic demyelination in rats resulted in decreased lipid peroxidation, increased reduced glutathione. ISDN also lessened the elevation in nitric oxide and partially prevented the alterations in AChE activity induced by the toxic agent in the cortex. These findings suggest that nitric oxide donors might demonstrable therapeutic benefits in demyelinating conditions.

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