JOURNAL OF BASIC PHARMACOLOGY AND TOXICOLOGY

Original Research Article

Protection by methylene blue against haloperidol-induced neuronal and liver damage

Omar M.E. Abdel-Salam[1,*], Amany A. Sleem[2], Eman R Youness[3], Fatma A. Morsy[4]

[1]*Department of Toxicology and Narcotics, National Research Centre, Cairo, Egypt*

[2]*Department of Pharmacology, National Research Centre, Cairo, Egypt*

[3]*Department of Medical Biochemistry, National Research Centre, Cairo, Egypt*

[4]*Department of Pathology, National Research Centre, Cairo, Egypt*

Abstract

Haloperidol is a first-generation antipsychotic that induces oxidative stress and neuronal injury. The objective of this study was to examine the effect of the redox dye methylene blue (MethyB) on brain oxidative stress and neurodegeneration and on liver tissue damage in the rat after acute administration of high dose haloperidol. Rats were orally treated with haloperidol at a dose of 5 mg/kg alone or co-administered with MethyB at doses of 1, 5, 10, 20 mg/kg, intraperitoneally daily for two days. Biomarkers of oxidative stress including lipid peroxidation (malondialdehyde), nitric oxide, reduced glutathione, paraoxonase-1 activity (PON-1) and the level of the anti-apoptotic protein Bcl-2 were determined. Histopathological examination of brain and liver tissue was also done. Haloperidol treatment resulted in significant increases in malondialdehyde and nitric oxide compared with the vehicle group. Significant decreases were also observed in reduced glutathione, PON-1 activity and Bcl-2. Treatment with MethyB prevented the increase in lipid peroxidation and nitric oxide and restored PON-1 activity in a dose-dependent manner. Haloperidol resulted in spongiform changes, dead neurons in the cerebral cortex and degeneration of Purkinje cells and white matter vacuolation in cerebellum. The drug also caused distortion of liver architecture, massive vacuolation, necrosis and pyknosis of hepatocytes. These pathological changes were alleviated to great extent by MethyB at 20 mg/kg. Our findings suggest that oxidative stress is involved in the neurotoxic effects of high dose haloperidol and that MethyB affords protection. This protective action of MethyB involves decreased oxidative stress.

Key words: Haloperidol; Antipsychotic drugs; Methylene blue; Oxidative stress; Paraoxonase-1; B-cell leukemia/lymphoma 2; Neurotoxicity

1 Introduction

Haloperidol is a commonly used first-generation antipsychotic drug. The agent is employed in the pharmacological treatment of schizophrenia, a devastating mental illness with such severe symptoms as hallucinations, delusions, thought disorder, disturbances in social interaction, and cognitive impairment [1, 2]. It is also used to treat agitation and delirium in the intensive care unit [3], reduce agitated behaviors in mechanically ventilated patients [4] and following traumatic brain injury [5]. The drug is a classic neuroleptic, having high affinity for dopamine D2 receptors and blockade of these in the mesolimbic area ameliorates psychotic symptoms [6] but their blockade in the nigrostriatal pathway results in the liability for the emergence of extrapyramidal manifestations eg., parkinsonism [7] and tardive dyskinesia [8]. There is an evidence to suggest that haloperidol is neurotoxic [9]. Haloperidol induces caspase-dependent apoptosis in cultured cortical neurons by reducing cellular survival signalling [10]. In rats, single intraperitoneal (i.p.) injections of 4 or 12 mg/kg haloperidol increased the number of apoptotic neurons in the striatum and substantia nigra [11]. When given to mice at 5 mg/kg for 3 consecutive days, the drug resulted in disorganization of cerebral cortical layers, neuronal apoptosis, shrinkage and necrosis in the cortex and striatum [12]. It also exacerbated cognitive deficits in rats with traumatic brain injury [13].

Oxidative stress is the mechanism most implicated in the neurotoxic effects of haloperidol [9]. Oxidative stress ensues when the cell's antioxidant capacity is overwhelmed by free radicals due to either diminished cellular antioxidants or an increase in the generation of free radicals, with membrane lipids, proteins, nucleic acid, and other macromolecules becoming targets for oxidative modification [14, 15]. In the brain tissue, where there is paucity of cellular antioxidants, the redox balance might be tilted in favor of pro-oxidants. The latter comprises the reactive oxygen metabolites such as superoxide ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) generated by the mitochondrial respiratory chain as byproducts of oxygen metabolism.

*Corresponding author: Email: omasalam@hotmail.com

Other sources of free radicals are the oxidation of dopamine *via* auto-oxidation or its metabolism by the enzyme monoamine oxidase, activated phagocytes, nitric oxide synthases and the transition metals iron and copper undergoing redox-cycling reactions [14, 16]. In particular, the high rate of oxygen consumption and the high content of polyunsaturated lipids render the brain most susceptible to oxidative stress [16]. Haloperidol has been shown to increase the level of reactive oxygen metabolites in rat cortical neurons and mouse hippocampal neurons [17]. When given to rodents, haloperidol resulted in increased lipid peroxidation products [12, 18, 19], protein carbonyls [18], depletion of reduced glutathione, total antioxidant capacity [12, 20] and decreased activity of the antioxidant enzymes catalase [20], superoxide dismutase and glutathione peroxidase [21, 22]. Haloperidol has been shown to increase thiobarbituric acid reactive substances indicative of an increase in lipid peroxidation in plasma of healthy subjects [23]. There is also an accumulating evidence which suggests an important role for oxidative stress in subjects with schizophrenia [24]. First episode patients exhibited elevations in plasma oxidized glutathione [25], malondialdehyde [26], F2-isoprostane [27], and protein carbonyl levels [28]. These patients also had decreased total and reduced glutathione [25], serum arylesterase activity and whole blood glutathione peroxidase activity [26]. Hence, treatment with haloperidol could exacerbate oxidative stress in these patients.

Methylthioninium chloride or methylene blue (MethyB) is a synthetic redox dye with a number of medical applications such as treatment of cyanide poisoning and methemoglobinemia [29, 30]. It is also used to treat refractory hypotension unresponsive to intravenous fluids and vasopressors [31], possibly due to its nitric oxide synthase, and guanylate cyclase enzyme inhibitory effects [32, 33]. Methylene blue has come into attention in the recent years because of its neuroprotective potential which was demonstrated in experimental models such as amyotrophic lateral sclerosis [34], Huntington's disease [35], traumatic brain injury [36], Parkinson's disease [37], organophosphorus toxicity [38] and toluene toxicity [39]. These neuroprotective effects are considered to be mediated through the decreased production of $O_2^{\bullet-}$ by the mitochondria [40], decreased microglia activation and release of cytokines [34, 41], antioxidant [38, 39] and anti-apoptotic [37] effects.

This study therefore examines the potential of MethyB for ameliorating oxidative stress and neurodegeneration caused by high dose haloperidol. The drug has been shown to cause hepatic damage when given to mice at high doses [12]. The study was thus extended to investigate the effect of MethyB on the haloperidol-induced liver injury.

2 Materials and methods

2.1 Animals

Experiments were conducted on Sprague Dawley rats (180-200 g). Animals were group housed on a 12-h light/dark cycle and allowed free access to standard laboratory rodent chow and tap water. All experiments were conducted in accordance with the recommendations of the Institutional Ethics Committee and the United States National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2 Drugs and chemicals

Haloperidol was obtained from Kahira Pharm and Chem. IND (Cairo, Egypt) and dissolved in sterile physiological saline. Methylene blue was purchased from Sigma (St. Louis, USA) and dissolved in normal saline to obtain the necessary dose. Other chemicals and reagents were of analytical grade (Sigma, St. Louis, USA). The doses of haloperidol and MethyB were based on previous studies [12, 38, 39].

2.3 Study design

Rats were randomly divided into six equal groups (n=6/group). Group 1 was treated with i.p. saline and served as negative control. Groups 2-5 were orally treated with haloperidol at the dose of 5 mg/kg on two successive days either alone (group 2) or together with MethyB i.p. at doses of 1, 5, 10, or 20 mg/kg (groups 3-6). Rats were euthanized by decapitation under light ether anaesthesia and their brains and livers were quickly dissected out on an ice-cold plate, washed with ice-cold saline at pH 7.4, weighed, and stored at −80 °C until biochemical assays. Tissue homogenization was done with 0.1 M phosphate-buffered saline (pH 7.4) to give a final concentration of 10% weight/volume. Parts of the tissues were kept in 10% formol saline for subsequent histopathological examination.

2.4 Biochemical analyses

2.4.1 Determination of lipid peroxidation

Malondialdehyde (MDA), an end product of lipid peroxidation was measured according to the method described by Nair and Turne [42] based on the principle that thiobarbituric acid reactive substances (TBAS) react with thiobarbituric acid forming TBA-MDA adduct and the absorbance is read at 532 nm using spectrophotometer.

2.4.2 Determination of nitric oxide

Nitric oxide was measured using Griess reagent according to Moshage et al. [43]. Nitrate is converted to nitrite by nitrate reductase. Griess reagent then converts nitrite to a deep purple azo compound. The absorbance is read at 540 nm using spectrophotometer. Nitrite, a stable end-product of nitric oxide radical, is mostly used as indicator for the production of nitric oxide.

2.4.3 Determination of reduced glutathione

The Ellman's assay was used for the determination of the free sulfhydryl (thiol, SH) groups, such as free cysteine and

glutathione, the most abundant form of non-protein thiols. Ellman's reagent (5, 5'-dithiobis-2-nitrobenzoic acid) is reduced by sulfhydryl groups to form 2-nitro-5-mercaptobenzoic acid. The latter has an intense yellow color and is determined colorimetrically at 412 nm [44].

2.4.4 Determination of paraoxonase-1 activity

Paraoxonase-1 arylesterase activity was determined using phenylacetate as a substrate and the formation of phenol was measured by monitoring the increase in absorbance at 270 nm and 25 °C with a spectrophotometer. One unit of arylesterase activity is defined as 1 μM of phenol produced per minute. Enzyme activity is calculated based on the extinction coefficient of phenol (1310 M^{-1} cm^{-1} at 270 nm, pH 8.0 and 25 °C) and expressed as kilo International Units/Liter (kU/L) [45].

2.4.5 Quantification of Bcl-2

The level of B-cell leukemia/lymphoma 2 (Bcl-2) was determined by double-antibody sandwich enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Wuhan Fine Biotech Co., Ltd., China).

2.5 Statistical analyses

Results are expressed as mean ± SE. Statistical significance was determined by using one-way ANOVA, followed by Duncan's multiple range test using SPSS software (SAS Institute Inc., Cary, NC). A probability value of less than 0.05 was considered statistically significant.

2.6 Histopathological studies

The brain and liver specimens of different groups were removed and fixed in 10% formol saline for at least 72 hr. Specimens were then washed in tap water for half an hour, dehydrated in ascending grades of alcohol, cleared in xylene and finally embedded in paraffin. Sections of 5 μm thick were cut and stained with haematoxylin and eosin for histopathological investigations and investigated by light microscope.

3 Results

3.1 Biochemical results

3.1.1 Effect of methylene blue on oxidative stress in haloperidol-treated rats

Rats treated with haloperidol alone exhibited significant elevations in brain malondialdehyde and nitric acid contents by 40.4% (25.38 ± 1.36 vs. 18.08 ± 1.26 nmol/g tissue) and 48.2% (35.62 ± 1.43 vs. 20.26 ± 1.0 μmol/g tissue) compared with the saline control group. Haloperidol also resulted in -34% decrease in brain reduced-glutathione content (2.21 ± 0.13 vs. 3.35 ± 0.29 μmol/g tissue) (Figure 1). In haloperidol-treated rats, the administration of MethyB at 20 mg/kg produced a significant attenuation of brain malondialdehyde level by 27.2% (18.47 ± 0.71 vs. 25.38 ± 1.36 nmol/g tissue) compared with the haloperidol only group (Figure 1A). In addition, there was a significant decrease in brain nitric oxide by 22.6%, 31.3%, and 44.9% after treatment with MethyB at 5, 10 or 20 mg/kg, compared to the haloperidol control group (27.56 ± 1.65, 24.46 ± 1.8, 19.61 ± 1.08 vs. haloperidol control 35.62 ± 1.43 μmol/g tissue) (Figure 1B). Meanwhile, the administration of MethyB had no significant effect on brain reduced-glutathione in haloperidol-treated rats (Figure 1C).

3.1.2 Effect of methylene blue on paraoxonase-1 in haloperidol-treated rats

In haloperidol-only-treated rats, paraoxonase-1 (PON-1) activity showed a 54.8% decrease (4.57 ± 0.2 vs. 10.11 ± 0.62 kU/l) compared with the saline control value. The administration of MethyB at doses of 1, 5, 10 or 20 mg/kg resulted in significant increase in PON-1 activity by 33.5%, 50.3%, 85.5% and 113.3%, respectively. Values were 6.10 ± 0.4, 6.87 ± 0.28, 8.48 ± 0.27 and 9.75 ± 0.6 for the MethyB-treated groups compared with the haloperidol control value of 4.57 ± 0.2 kU/l (Figure 1D).

3.1.3 Effect of methylene blue on Bcl-2 in haloperidol-treated rats

Significantly decreased level of Bcl-2 by 23.5% was observed in the brain of haloperidol-treated rats as compared with the saline group (2.41 ± 0.1 vs. 3.15 ± 0.16 ng/ml). MethyB had no significant effect on Bcl-2 in haloperidol-treated rats (Figure 2).

3.2 Histopathological analysis of brain tissue

3.2.1 Cerebral cortex

Sections from the saline control group showed a normal appearance (Fig. 3A). Rats treated with only haloperidol demonstrated spongiform changes consisting of relatively small delicate vacuoles in the cerebral cortex. Lewy bodies could be seen in some neurons. There were some dead neurons seen as red neurons with vacuolation of neuropil and congestion of cerebral blood vessel (Figs. 3B & 3C). Sections of rats treated with haloperidol and MethyB at 1 mg/kg revealed no improvement of the histopathological changes. Vacuolation in majority of sections, referred to as spongiform degeneration was still present. Some degenerated neurons and light stained cells with pale nuclei and prominent nucleoli were seen (Fig. 3D). Sections from rats treated with haloperidol and MethyB at 5 mg/kg showed some improvement in the histopathological changes in the form of no vacuoles in the cortex but most of neurons appeared degenerated and some dead neurons were occasionally seen. Others neurons were with bright nuclei

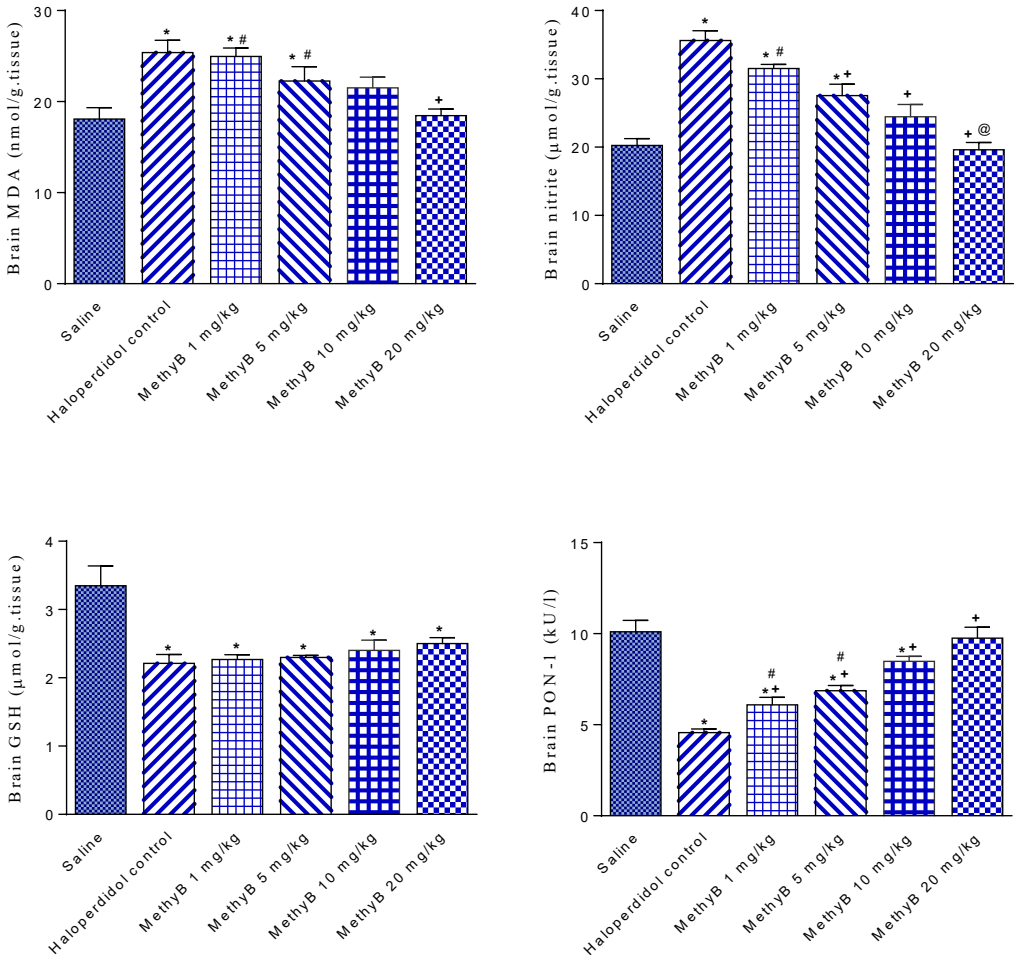

Figure 1. Effect of methylene blue (MethyB) on brain malondialdehyde (MDA), nitric oxide, reduced glutathione levels and paraoxonase-1 (PON-1) in haloperidol-treated rats. *:p<0.05 vs. saline control group. +:p<0.05 vs. haloperidol control group. @: p<0.05 vs. MethyB 10 mg/kg. #: p<0.05 vs. MethyB 40 mg/kg (malondialdehyde). #: p<0.05 vs. MethyB 20 or 40 mg/kg (nitrite and PON-1).

and cytoplasm and there was formation of numerous capillaries (Fig. 3E). Rats treated with haloperidol along with MethyB at 10 mg/kg showed some cortical neurons and astrocytes that appeared normal, pyramidal shaped with distinct nuclei and nucleoli. Neuroglia cells were also seen (Fig. 3F). Sections from rats treated with haloperidol and MethyB at 20 mg revealed that most of neurons returned to normal appearance, but some degenerated neurons and congestion of cerebral blood vessel were seen (Fig. 3G).

3.2.2 Cerebellum

The cerebellum from the saline control group showed a normal appearance (Fig. 4A). Rats treated with only haloperidol showed degeneration in most of Purkinje cells and vacuolation in white matter (Fig. 4b). Rats treated with haloperidol along with MethyB at 1 mg/kg showed no improvement with degeneration in Purkinje cells and vacuolation in white matter ((Fig. 4C). Rats treated with haloperidol and MethyB at 5 mg/kg showed degeneration in most of Purkinje cell (Fig. 4D). Following MethyB at 10 mg/kg, there was degeneration in some of Purkinje cell and vacuolation in white matter (Fig. 4E). Following MethyB at

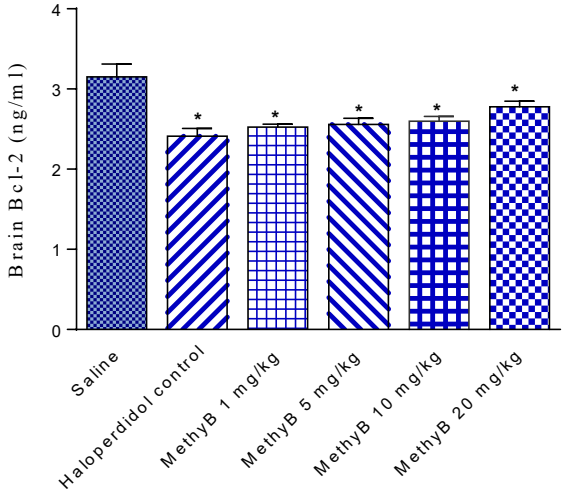

Figure 2. Effect of methylene blue (MethyB) on brain Bcl-2 in haloperidol-treated rats. *:p<0.05 vs. saline control group.

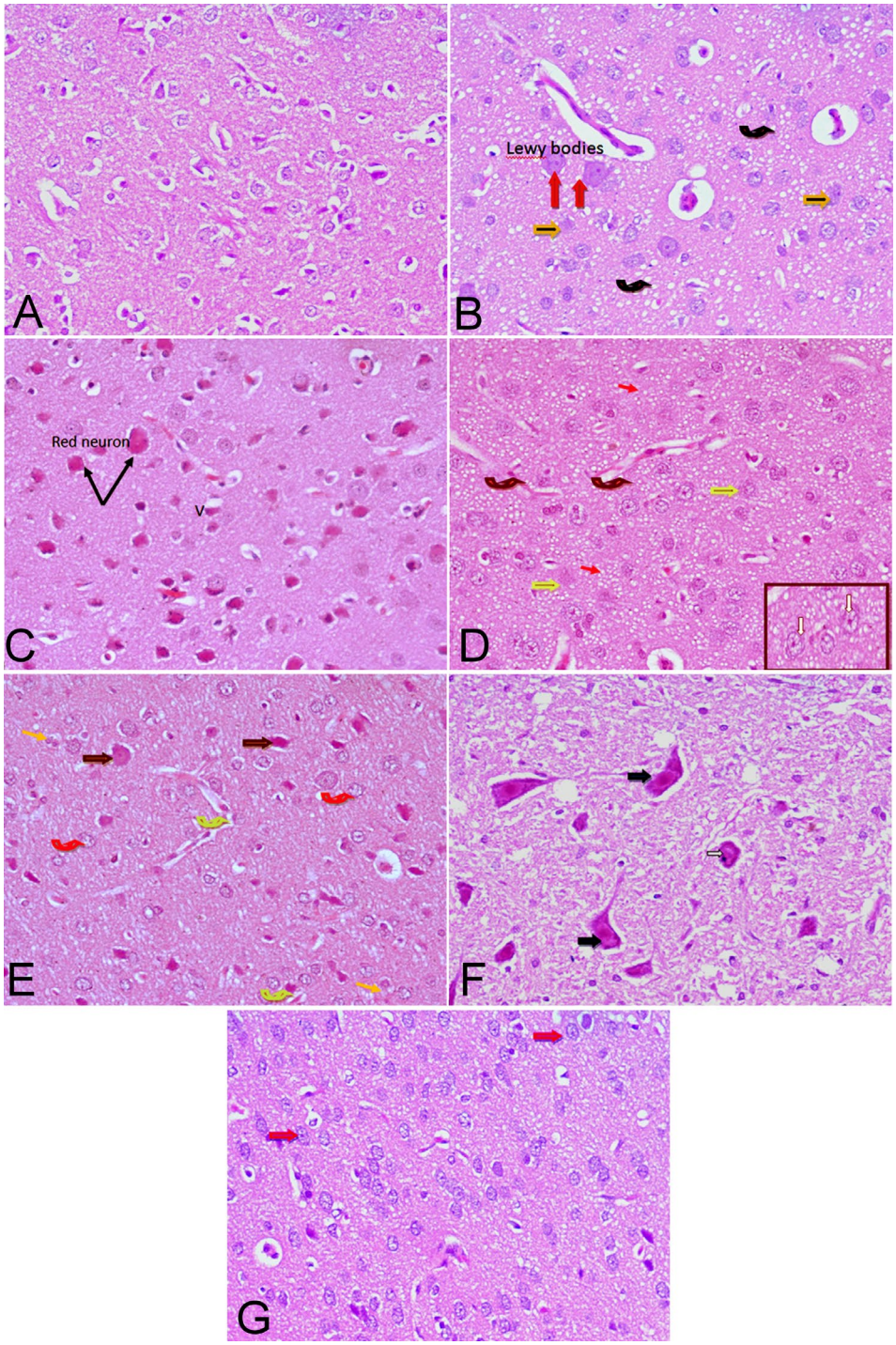

Figure 3. Representative photomicrographs of the cerebral cortex. (A) Saline control shows normal histological structure. (B) Haloperidol only showing Lewy bodies (red arrow), small delicate vacuoles in the cortex (black arrow) and degenerated neurons (yellow arrow). (C) Haloperidol only showing red neurons, shrunken neurons with vacuolation of neuropil (V) and formation of numerous capillaries. (D) Haloperidol + MethyB 1 mg/kg showing small vacuoles in most of the section (spongiform degeneration) (red arrow), some degenerated neurons (light green arrow), light stained cells (white arrow) with pale nuclei and prominent nucleoli. (E) Haloperidol + MethyB 5 mg/kg showing no vacuoles but degeneration and shrinkage of neurons (yellow arrow), some dead neurons characterized by red neurons (black arrow), neurons with bright nucleus and cytoplasm (red arrow) and formation of numerous capillaries. (F) Haloperidol + MethyB 10 mg/kg showed some cortical neurons, normal astrocytes (black arrow), neuroglia cells (white arrow). (G) Haloperidol + MethyB 20 mg/kg showed that most neurons returned to normal appearance (H & E x 400).

20 mg/kg, degeneration of some Purkinje cells was seen (Fig. 4F).

3.2.3 Histopathological analysis of liver tissue

Sections from control rats showed normal hepatic architecture with distinct hepatocytes, central vein and sinusoidal spaces (Fig. 5A). Haloperidol-treated rats exhibited distortion in hepatocyte architecture, massive vacuolation and pyknosis in hepatocytes, fibrosis, inflammatory cell infiltration and necrotic cells especially in periportal area. Dilatation and congestion in sinusoidal space were seen (Figs. 5B & 5C). In rats treated with haloperidol and MethyB at 1 mg/kg, the liver still suffered

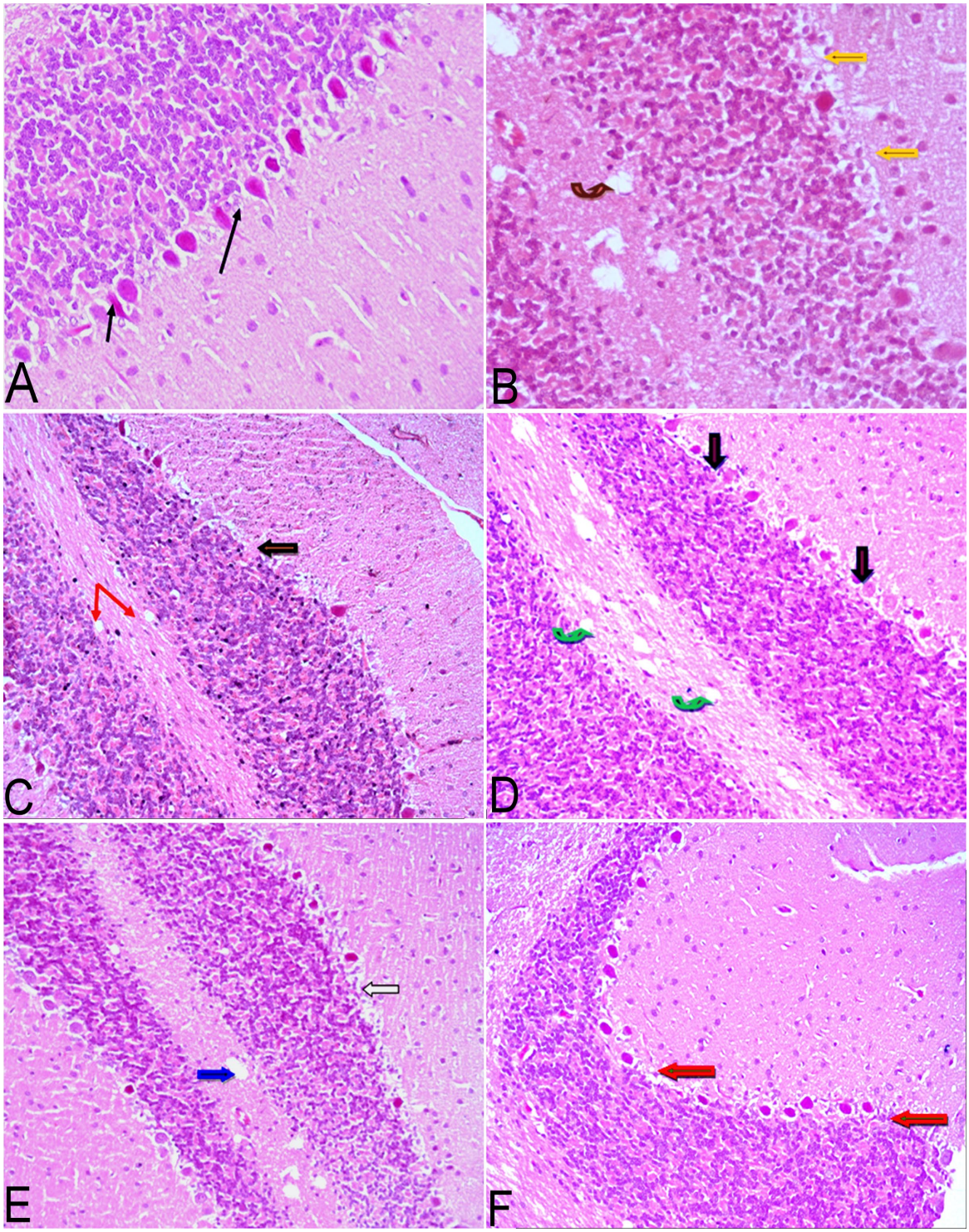

Figure 4. Representative photomicrographs of the cerebellum. (A) Saline control shows normal histological structure of Purkinje cells (black arrow) (x 400). (B) Haloperidol only showing degeneration in the most of Purkinje cell (yellow arrow) and vacuolation in white matter (black arrow) (x 400). (C) Haloperidol + MethyB 1 mg/kg showing degeneration in the most of Purkinje cells (black arrow) and vacuolation in white matter (red arrow) (x 200). (D) Haloperidol + MethyB 5 mg/kg showing degeneration in some Purkinje cells (black arrow), vacuolation of white matter (green arrow) (x 200). (E) Haloperidol + MethyB 10 mg/kg showing degeneration in the most of Purkinje cells (white arrow) and vacuolation in white matter (blue arrow) (x 200). (F) Haloperidol + MethyB 20 mg/kg showing degeneration in some of Purkinje cells (red arrow). (x 200)

from massive vacuolation and congestion of sinusoidal space. Signs of degeneration were evident in most hepatocytes in the form of pyknotic nuclei, karyolysis and karyorrhexis (Fig. 5D). Treatment with MethyB at 5 mg/kg resulted in normalization of some hepatic cells with dilatation, congestion of central vein and sinusoidal space. Vacuolation was still present and also hypertrophy of Kupffer cells (Fig. 5E). The administration of MethyB at 10 mg/kg resulted in improvement in pathological changes in the form of most hepatocytes appearing normal although slight dilatation in the portal vein, minimal fibrosis and few vacuoles were seen. Activation of Kupffer cells was also observed (Fig. 5F). Sections from rats treated with haloperidol and MethyB at 20 mg/kg revealed normalization

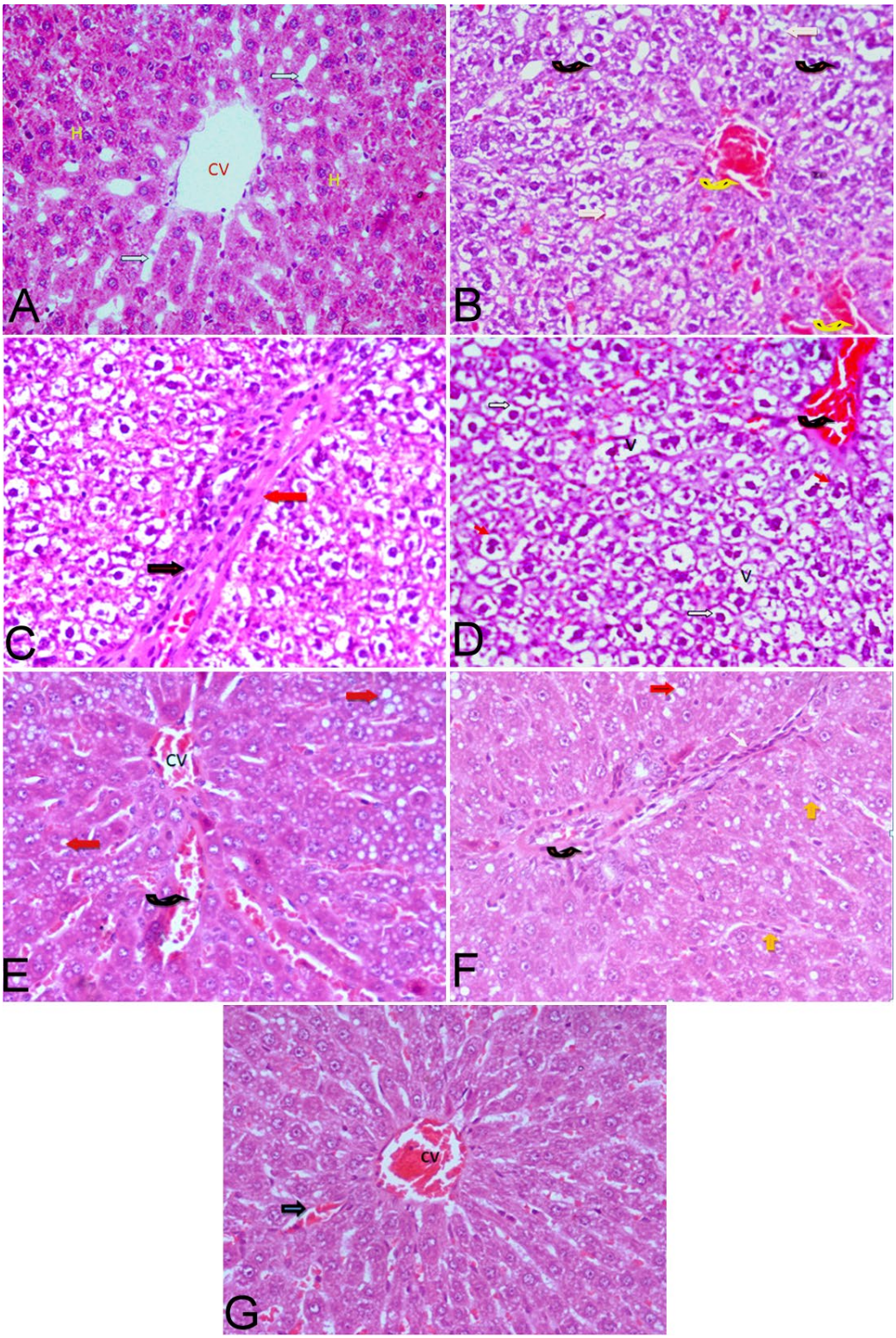

Figure 5. Representative photomicrographs of the liver. (A) Saline control showing normal histological structure of hepatic lobules and central vein (CV), surrounded by hepatocytes (H) and sinusoids (arrow) (x 200). (B) Haloperidol only showing distortion of hepatic architecture, vacuolation (white arrow), pyknotic nuclei (black arrow), dilatation and congestion of the sinusoidal space (yellow arrow) (x 400). (C): Haloperidol only showing fibrosis (black arrow), and inflammatory cell infiltrate in periportal area (red arrow) (x 400). (D) Haloperidol + MethyB 1 mg/kg showing distortion of hepatic architecture, vacuolation (V), congestion of sinusoidal space (back arrow), degeneration in most of hepatocytes in the form of pyknotic nuclei (white arrow) and karyorrhexis (red arrow) (x 400). (E) Haloperidol + MethyB 5 mg/kg showing congestion in central vein (CV) and blood sinusoids (black arrow) and vacuolation (red arrow) (x 400). (F) Haloperidol + MethyB 10 mg/kg showing dilatation, thickening in wall of portal vessel (black arrow), minimal fibrosis (white arrow), few vacuoles (red arrow), activation of Kupffer cells (yellow arrow) (x 400). (G) Haloperidol + MethyB 20 mg/kg showing normal hepatic cells with congestion of central vein (CV), and sinusoidal space (x 400).

of the liver tissue and hepatic architecture although congestion in the central vein and sinusoids was still present (Fig. 5G).

4 Discussion

Haloperidol is a widely prescribed classic antipsychotic drug which demonstrated neurotoxicity in vitro and in vivo studies [10-13]. In this study, we aimed to investigate the neuroprotective potential of the redox dye MethyB in rats acutely treated with high dose haloperidol. Our results demonstrated increased oxidative stress in the brain of rats treated with haloperidol. The drug significantly decreased the level of the anti-apoptotic protein Bcl-2 and induced severe neurodegenerative changes in the cerebral cortex and cerebellum. There were in addition distortion of hepatic architecture, and apoptosis and necrosis of hepatocytes. Here, the administration of MethyB reduced the extent of brain lipid peroxidation, nitric oxide, restored PON-1 activity and afforded protection against the histopathological changes induced by the antipsychotic drug in the brain and liver.

Oxidative stress is considered an important mechanism underling the neurotoxic effects of haloperidol [9]. In this study, neurotoxicity was associated with significantly increased malondialdehyde level in the brain of rats given haloperidol. Malondialdehyde is an end product of lipid peroxidation and increased level is an indicative of increased production of reactive oxygen metabolites and consequent attack on membrane lipids [46]. There was also a significant decrease in reduced glutathione in brain of haloperidol-treated rats. Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is the major non-protein thiol in cells and is synthesized by both neurons and astrocytes. Glutathione protects cells against reactive oxygen metabolites and reactive nitrogen species *via* non-enzymatic reduction or enzymatic reactions involving glutathione peroxidase. The resultant glutathione disulfide (GSSG, or oxidized glutathione) is then reduced back to GSH by glutathione reductase in a reaction that uses NADPH from the pentose phosphate shunt. Glutathione is important in maintaining the redox-balance of the cell and decreased levels occurs when there is increased generation of free radicals [47, 48]. The increase in brain lipid peroxidation and the depletion of reduced glutathione observed herein suggest the involvement of oxidative stress in haloperidol-induced neurodegeneration. Other studies also indicated increased oxidative stress in the brain of rats after treatment with haloperidol where increased lipid peroxidation and decreased cellular antioxidants e.g., reduced glutathione, catalase and total antioxidant capacity were observed [12, 19, 20]. Haloperidol also resulted in increased reactive oxygen metabolites in the rat whole blood [49]. There is also an evidence for increased formation of reactive oxygen metabolites from the mitochondria of cerebral and hippocampal neurons after exposure to haloperidol [17]. The decrease in reduced glutathione in the brain of haloperidol-treated rats is therefore likely to be due to its consumption by the increased generation of reactive oxygen metabolites and other free radicals. A decrease in brain glutathione has been demonstrated in patients with mood disorders and schizophrenia [50, 51]. Schizophrenic patients also showed significantly lower levels of reduced glutathione and the antioxidant enzymes superoxide dismutase and catalase in blood, especially those who are untreated [50]. It follows that haloperidol treatment might increase the already impaired redox-homeostasis in brain of these patients and increases the neurodegeneration. In this study, the increase in brain lipid peroxidation after haloperidol challenge was reduced by MethyB. The ability of the dye to cycle between its oxidized and reduced (leucoMethyB) is thought to block the production of superoxide by mitochondria and therefore protects against mitochondrial damage [40].

In addition, our results indicate significant elevation in brain nitric oxide level after haloperidol treatment which is consistent with previous observations in mice following high dose haloperidol [12]. Nitric oxide is an important signalling molecule in brain cells and controls vasodilator tone. Nitric oxide is formed from L-arginine by the action of three isoforms of the enzyme nitric oxide synthase (NOS); these are the endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) isoforms [52]. The excessive production of nitric oxide, for example by iNOS expressed in astrocytes and microglia during inflammatory and toxic conditions results in impairment of cellular respiration with consequent neuronal energy failure and cell death [53]. This occurs through the reaction of the excess nitric oxide with superoxide resulting in the formation of the highly oxidant peroxynitrite ($ONOO^-$) and the oxidation of thiols or nitration of protein tyrosine residues. Other reactive nitrogen oxides e.g., nitrogen dioxide ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3) could also be produced by the reaction of nitric oxide and molecular oxygen and cause nitrosation of thiols or protein cysteine residues (52, 54). The increase in brain nitric oxide could be an important contributor to the haloperidol neurotoxicity *via* oxidative and/or nitrosative modification of cellular biomolecules. Moreover, the demonstrated ability of MethyB to inhibit the increase in brain nitric oxide after haloperidol and to afford neuroprotection lends support to the involvement of nitric oxide in the haloperidol-induced neurodegeneration. MethyB inhibits nitric oxide synthases [32, 33] and inhibition of brain nitric oxide was proposed as a mechanism underlying the neuroprotective effect of MethyB as the dye was shown to decrease the number of iNOS and eNOS activated cortical cells in ischaemic/reperfusion brain injury [55, 56]. Other studies have also indicated inhibition of brain or serum nitric oxide by MethyB together with protection against the toxicant-induced neuronal injury [37-39].

The results of the present study are also consistent with reports of significantly decreased paraoxonase-1 (PON-1) activity in brain of mice after haloperidol administration [12]. Paraoxonase-1 which possesses an esterase and lactonase activities is involved in the hydrolysis of a number of organophosphorus insecticides, nerve agent, and lipid hydroperoxides and in metabolism of many other xenobiotics [57]. The enzyme is endued with an antioxidant and anti-inflammatory properties, inhibiting the release of cytokines e.g., tumour necrosis factor-alpha, interleukin-6 and reactive oxygen species from macrophages [58]. In addition, deficiency of PON-1 was associated with increased oxidative stress in serum and macrophages [59].

Moreover, mice deficient in PON-1 exhibited downregulation of a number of brain proteins involved in the antioxidant defense such as superoxide dismutase, DJ-1 and Park-7 [60]. Paraoxonase-1 thus modulates oxidative stress and inflammation [61] and is considered to have an important role in neurodegenerative diseases where a decrease in its activity was found [62]. The activity of PON-1 is also decreased in drug-naive schizophrenic patients compared with healthy subjects [63] most probably due to increased levels of oxidative stress. The latter has been shown to inactivate PON-1 [64]. The inhibition of PON-1 by haloperidol could therefore result in increased oxidative stress and/or render the cell more vulnerable to oxidative stress and consequently neurodegeneration. Meanwhile, treatment with MethyB resulted in increase in the activity of the enzyme which is probably due to decreased level of oxidative stress and/or a consequence of neuroprotection.

The Bcl-2 family of proteins controls the apoptotic pathway in the mitochondria. It includes both anti-apoptotic or pro-survival Bcl-2 proteins and pro-apoptotic effector proteins Bcl-2-associated X protein (Bax), Bcl-2 antagonist/killer-1 (Bak) and BH3 domain only proteins which mediates permeabilization of the outer mitochondrial membrane, an essential step in apoptosis. Expression of Bcl-2 or other anti-apoptotic proteins can inhibit the activation of Bax and Bak following a death signal [65]. The present study indicates significantly decreased level of the anti-apoptotic protein Bcl-2 in brain of haloperidol-treated rats. There is evidence that Bcl-2 protects cells against metabolic oxidative stress [66]. Moreover, the increase in Bcl-2 expression was found to protect hippocampal cells against haloperidol toxicity in vitro [67]. The decrease in Bcl-2 by haloperidol therefore should increase the cell vulnerability to apoptotic signals and which could have an important role in haloperidol toxicity.

Our histopathological data demonstrated severe neurodegenerative changes in the cerebral cortex of haloperidol-treated rats. In addition, the cerebellum showed degeneration in most of Purkinje cells. Other researchers reported increased number of apoptotic neurons in the substantia nigra and striatum of rats following single i.p. dose of 4 or 12 mg/kg of haloperidol [11]. In mice, haloperidol (5 mg/kg, i.p.) given for three consecutive days resulted in severe neuronal damage in the form of shrunken, apoptotic and necrotic and red neurons in the cerebral cortex and striatum [12]. Consistent with other studies, haloperidol also evoked massive vacuolation and degeneration of hepatocytes [12]. The drug was shown to increase malondialdehyde and nitric oxide and to decrease PON-1 activity in liver of mice, suggesting a role for oxidative stress in the haloperidol-induced liver injury [20]. The histopathological changes evoked by the antipsychotic were preventable by MethyB co-administration which clearly proved both a neuro- and hepato-protective effects for the dye against the damaging effects of high dose haloperidol.

In summary, the present study demonstrated that treatment with the redox dye MethyB reduces brain oxidative stress and prevents neuronal cell death and liver injury induced by haloperidol in the rat. The study provides evidence that MethyB might be a potential candidate for the prevention of the progression of neurodegeneration during treatment with classic antipsychotic drugs.

Acknowledgements

This work is not supported by any research grants.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

1. Wong AH, Van Tol HH. Schizophrenia: from phenomenology to neurobiology. Neurosci Biobehav Rev. 2003;27:269-306.
2. Hartling L, Abou-Setta AM, Dursun S, Mousavi SS, Pasichnyk D et al. Antipsychotics in adults with schizophrenia: comparative effectiveness of first-generation versus second-generation medications: a systematic review and meta-analysis. Ann Intern Med. 2012;157:498-511.
3. Trompeo AC, Vidi Y, Ranieri VM. Sleep and Delirium in the Critically Ill: Cause or Effect?. In: Vincent JL (eds) Yearbook of Intensive Care and Emergency Medicine. Yearbook of Intensive Care and Emergency Medicine, 2006. Springer, Berlin, Heidelberg.
4. Milbrandt EB, Kersten A, Kong L, Weissfeld LA, Clermont G et al. Haloperidol use is associated with lower hospital mortality in mechanically ventilated patients. Crit Care Med. 2005;33:226-9.
5. Anderson RL, Birrer KL, Liu-DeRyke X. Haloperidol use in acute traumatic brain injury: A safety analysis. J Intensive & Crit Care. 2016;2:2.
6. Kane JM, Correll CU. Past and present progress in the pharmacologic treatment of schizophrenia. J Clin Psychiatry. 2010;71:1115-24.
7. Shin HW, Chung SJ. Drug-Induced Parkinsonism. J Clin Neurol. 2912;8:15–21.
8. Soares-Weiser K, Fernandez HH. Tardive dyskinesia. Semin Neurol. 2007;27:159-69.
9. Nasrallah HA, Rush SJ. First generation antipsychotics are neurotoxic and impair neuroplasticity via multiple mechanisms. Biol Psychiatry. 2013;73:61S.
10. Ukai W, Ozawa H, Tateno M, Hashimoto E, Saito T. Neurotoxic potential of haloperidol in comparison with risperidone: implication of Akt-mediated signal changes by haloperidol. J Neural Transm. 2004;111:667–681.
11. Mitchell IJ, Cooper AC, Griffiths MR, Cooper AJ. Acute administration of haloperidol induces apoptosis of neurones in the striatum and substantia nigra in the rat. Neuroscience. 2002;109:89-99.
12. Abdel-Salam OME, Sleem AA, Youness ER, Mohammed NA, Omara EA. Bone marrow-derived stem cells protect against haloperidol-induced brain and liver damage in mice. Biomed Pharmacol J. 2018;11:11-22.
13. Wilson MS, Gibson CJ, Hamm RJ. Haloperidol, but not olanzapine, impairs cognitive performance after traumatic brain injury in rats. Am J Phys Med Rehabil. 2003;82:871–879.
14. Halliwell B. Biochemistry of oxidative stress. Biochemical Society Transactions. 2007;35;1147-1150.
15. Sies H. Oxidative stress: a concept in redox biology and medicine. Redox Biol. 2015;4:180–183.
16. Halliwell B. Oxidative stress and neurodegeneration: where are we now? J Neurochemistry. 2006;97;1634–1658.

17. Sagara Y. Induction of reactive oxygen species in neurons by haloperidol. J Neurochemistry. 1998;71:1002-12.
18. Agostinho FR, Jornada LK, Schröder N, Roesler R, Dal-Pizzol F et al. Effects of chronic haloperidol and/or clozapine on oxidative stress parameters in rat brain. Neurochem Res. 2007;32:1343-50.
19. Martins MR, Petronilho FC, Gomes KM, Dal-Pizzol F, Streck EL et al. Antipsychotic-induced oxidative stress in rat brain. Neurotox Res. 2008;13:63-69.
20. Abdel-Salam OM, Youness ER, Khadrawy YA, Sleem AA. Brain and liver oxidative stress after sertraline and haloperidol treatment in mice. J Basic Clin Physiol Pharmacol. 2013;24:115-23.
21. Pillai A, Parikh V, Terry AV Jr, Mahadik SP. Long-term antipsychotic treatments and crossover studies in rats: differential effects of typical and atypical agents on the expression of antioxidant enzymes and membrane lipid peroxidation in rat brain. J Psychiatr Res. 2007;41:372-86.
22. Perera J, Tan JH, Jeevathayaparan S, Chakravarthi S, Haleagrahara N. Neuroprotective effects of alpha lipoic acid on haloperidol-induced oxidative stress in the rat brain. Cell Biosci. 2011;1:12.
23. Dietrich-Muszalska A, Kontek B, Rabe-Jabłońska J. Quetiapine, olanzapine and haloperidol affect human plasma lipid peroxidation in vitro. Neuropsychobiology. 2011;63:197-201.
24. Sawa A, Sedlak TW. Oxidative stress and inflammation in schizophrenia. Schizophr Res. 2016;176:1-2.
25. Raffa M, Atig F, Mhalla A, Kerkeni A, Mechri A. Decreased glutathione levels and impaired antioxidant enzyme activities in drug-naive first-episode schizophrenic patients. BMC Psychiatry. 2011;11:124
26. Sarandol A, Sarandol E, Acikgoz HE, Eker SS, Akkaya C et al. First-episode psychosis is associated with oxidative stress: Effects of short-term antipsychotic treatment. Psychiatry Clin Neurosci. 2015;69:699-707.
27. Lee EE, Eyler LT, Wolkowitz OM, Martin AS, Reuter C et al. Elevated plasma F2-isoprostane levels in schizophrenia. Schizophr Res. 2016;176:320–326.
28. Young J, McKinney SB, Ross BM, Wahle KW, Boyle SP. Biomarkers of oxidative stress in schizophrenic and control subjects. Prostaglandins Leukot Essent Fatty Acids. 2007;76:73–85.
29. Clifton J, 2nd, Leikin JB. Methylene blue. Am J Ther. 2003;10:289–91.
30. Bradberry SM. Occupational methaemoglobinaemia: mechanisms of production, features, diagnosis and management including the use of methylene blue. Toxicol Rev. 2003;22:13–27.
31. Van Berkel MA, Fuller LA, Alexandrov AW, Jones GM. Methylene blue, midodrine, and pseudoephedrine: a review of alternative agents for refractory hypotension in the intensive care unit. Crit Care Nurs Q. 2015; 38:345–58.
32. Mayer B, Brunner F, Schmidt K. Inhibition of nitric oxide synthesis by methylene blue. Biochem Pharmacol. 1993;45:367–374.
33. Volke V, Wegener G, Vasar E, Rosenberg R. Methylene blue inhibits hippocampal nitric oxide synthase activity in vivo. Brain Res .1999;826:303–305.
34. Dibaj P, Zschüntzsch J, Steffens H, Scheffel J, Göricke B et al. Influence of methylene blue on microglia-induced inflammation and motor neuron degeneration in the SOD1(G93A) model for ALS. PLoS One. 2013;7:e43963.
35. Sontag EM, Lotz GP, Agrawal N, Tran A, Aron R et al. Methylene blue modulates huntingtin aggregation intermediates and is protective in Huntington's disease models. J Neurosci. 2012;32:11109–19.
36. Miclescu A, Sharma HS, Cécile M, Wiklund L. Methylene blue is neuroprotective against mild traumatic brain injury. J Neurotrauma. 2014;31:1063–1071.
37. Abdel-Salam OME, Omara EA, Youness ER, Khadrawy YA, Mohammed NA et al. Rotenone-induced nigrostriatal toxicity is reduced by methylene blue. J Neurorestoratol. 2014; 2:65–80.
38. Abdel-Salam OME, Youness ER, Esmail RSE, Mohammed NA, Khadrawy YA et al. Methylene blue as a novel neuroprotectant in acute malathion intoxication. Reactive Oxygen Species. 2016;1:165–177.
39. Abdel-Salam OME, Youness ER, Morsy FA, Yassen NN, Mohammed NA et al. Methylene blue protects against toluene induced brain damage: involvement of nitric oxide, NF-κB, and caspase-3. Reactive Oxygen Species. 2016;2:371–387.
40. Atamna H, Nguyen A, Schultz C, Boyle K, Newberry J et al. Mthylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways. FASEB J. 2008;22:703–712.
41. Fenn AM, Skendelas JP, Moussa DN, Muccigrosso MM, Popovich PG et al. Methylene blue attenuates traumatic brain injury-associated neuroinflammation and acute depressive-like behavior in mice. J Neurotrauma. 2015;32:127–138.
42. Nair V, Turner GA. The thiobarbituric acid test for lipid peroxidation: structure of the adduct with malondialdehyde. Lipids. 1984;19:804–805.
43. Moshage H, Kok B, Huizenga JR, Jansen PL. Nitrite and nitrate determinations in plasma: a critical evaluation. Clin Chem. 1985;41:892–896.
44. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82:70–77.
45. Haagen L, Brock A. A new automated method for phenotyping arylesterase (EC 3.1.1.2) based upon inhibition of enzymatic hydrolysis of 4-nitrophenyl acetate by phenyl acetate. Eur J Clin Chem Clin Biochem.1992;30:391–395.
46. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. 3rd ed. 1999, Clarendon Press, Oxford, UK.
47. Dringen R. Metabolism and functions of glutathione in brain. Prog Neurobiol. 2000;62:649-671.
48. Dickinson DA, Forman HJ. Cellular glutathione and thiols metabolism. Biochem Pharm. 2002;64:1019–1026.
49. Heiser P, Sommer O, Schmidt AJ, Clement HW, Hoinkes A et al. Effects of antipsychotics and vitamin C on the formation of reactive oxygen species. J Psychopharmacol. 2010;24:1499-504.
50. Raffa M, Mechri A, Othman LB, Fendri C, Gaha L et al. Decreased glutathione levels and antioxidant enzyme activities in untreated and treated schizophrenic patients. Prog Neuropsychopharmacol Biol Psychiatry. 2009;33:1178-83.
51. Gawryluk JW, Wang JF, Andreazza AC, Shao L, Young LT. Decreased levels of glutathione, the major brain antioxidant, in post-mortem prefrontal cortex from patients with psychiatric disorders. Int J Neuropsychopharmacol. 2011;14:123-130.
52. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J. 2012;33:829-37, 837a-837d.
53. Brown GC. Nitric oxide and neuronal death. Nitric Oxide. 2010;23:153-165.
54. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315–424.
55. Wiklund L, Basu S, Miclescu A, Wiklund P, Ronquist G et al. Neuro- and cardioprotective effects of blockade of nitric oxide action by administration of methylene blue. Ann N Y Acad Sci. 2007;1122:231–244.
56. Miclescu A, Sharma HS, Martijn C, Wiklund L. Methylene blue protects the cortical blood-brain barrier against ischemia/reperfusion-induced disruptions. Crit Care Med. 2010;38:2199–206.
57. La Du BN. Human serum paraoxonase: arylesterase. In: Pharmacogenetics of Drug Metabolism (W Kalow). Pergamon Press, New York, NY, 1992, pp. 51–91.

58. Aharoni S, Aviram M, Fuhrman B. Paraoxonase 1 (PON1) reduces macrophage inflammatory responses. Atherosclerosis. 2013;228:353–361.
59. Rozenberg O, Rosenblat M, Coleman R, Shih DM, Aviram M. Paraoxonase (PON1) deficiency is associated with increased macrophage oxidative stress: studies in PON1-knockout mice. Free Radic Biol Med. 2003;34:774–784.
60. Suszyńska-Zajczyk J, Luczak M, Marczak L, Jakubowski H. Inactivation of the paraoxonase 1 gene affects the expression of mouse brain proteins involved in neurodegeneration. J Alzheimers Dis. 2014;42:247-260.
61. Furlong CE, Marsillach J, Jarvika GP, Costa LG. Paraoxonases-1, -2 and -3: What are their Functions? Chem Biol Interact. 2016;259:51–62.
62. Menini T, Gugliucci A. Paraoxonase 1 in neurological disorders. Redox Rep. 2014;19:49–58.
63. Brinholi FF, Noto C, Maes M, Bonifácio KL, Brietzke E et al. Lowered paraoxonase 1 (PON1) activity is associated with increased cytokine levels in drug naïve first episode psychosis. Schizophr Res. 2015;166:225-230.
64. Nguyen SD, Sok DE. Preferential inhibition of paraoxonase activity of human paraoxonase 1 by negatively charged lipids. J Lipid Res. 2004;45:2211–20.
65. Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci. 2009;122:437-441.
66. Lee YJ, Chen JC, Amoscato AA, Bennouna J, Spitz DR et al. Protective role of Bcl2 in metabolic oxidative stress induced cell death. J Cell Sci. 2000;114:677-684.
67. Lezoualc'h F, Rupprecht R, Holsboer F, Behl C. Bcl-2 prevents hippocampal cell death induced by the neuroleptic drug haloperidol. Brain Res. 1996;738;176-179