INTRODUCTION

Mitoxantrone (MX) is approved for the treatment of aggressive relapsing-remitting, secondary-progressive and progressive-relapsing form of multiple sclerosis (MS). The mechanism of its action is multiaxial, however, it is not free from side effects. The causes of the side effects are still unknown and require further investigation. The aim of this study was to investigate the influence of MX therapy on enzymatic parameters of endogenous antioxidative status: manganese and copper/zinc superoxide dismutase (MnSOD, Cu/ZnSOD), catalase (CAT), glutathione peroxidase (GSH-Px) and lipid peroxidation marker - malondialdehyde (MDA) in blood serum and cerebrospinal fluid (CSF) in patients suffering from MS. After the MX therapy serum and the CSF MDA concentrations increased significantly. We reported that MnSOD activities decrease in serum and the CSF, while, surprisingly, the serum Cu/ZnSOD activity increases after the MX therapy. We also noted a marked decrease in CSF CAT and GSH-Px activity after the MX treatment. Our results strongly suggest the influence of MX therapy on oxidation/antioxidation status of serum and the CSF. These findings open up new opportunities for a better understanding of underlying physiopathological events in MS and provide a new insight into MX’s mechanisms of action, especially its potent side effects.

Key words: multiple sclerosis, antioxidative enzymes, superoxide dismutase, malondialdehyde, mitoxantrone, reactive oxygen metabolites

ANTIOXIDATIVE ENZYMES ACTIVITY AND MALONDIALDEHYDE CONCENTRATION DURING MITOXANTRONE THERAPY IN MULTIPLE SCLEROSIS PATIENTS

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Mitoxantrone (MX) is approved for the treatment of aggressive relapsing-remitting, secondary-progressive and progressive-relapsing form of multiple sclerosis (MS). The mechanism of its action is multiaxial, however, it is not free from side effects. The causes of the side effects are still unknown and require further investigation. The aim of this study was to investigate the influence of MX therapy on enzymatic parameters of endogenous antioxidative status: manganese and copper/zinc superoxide dismutase (MnSOD, Cu/ZnSOD), catalase (CAT), glutathione peroxidase (GSH-Px) and lipid peroxidation marker - malondialdehyde (MDA) in blood serum and cerebrospinal fluid (CSF) in patients suffering from MS. After the MX therapy serum and the CSF MDA concentrations increased significantly. We reported that MnSOD activities decrease in serum and the CSF, while, surprisingly, the serum Cu/ZnSOD activity increases after the MX therapy. We also noted a marked decrease in CSF CAT and GSH-Px activity after the MX treatment. Our results strongly suggest the influence of MX therapy on oxidation/antioxidation status of serum and the CSF. These findings open up new opportunities for a better understanding of underlying physiopathological events in MS and provide a new insight into MX’s mechanisms of action, especially its potent side effects.

Key words: multiple sclerosis, antioxidative enzymes, superoxide dismutase, malondialdehyde, mitoxantrone, reactive oxygen metabolites
MATERIAL AND METHODS

Patients

Fifty-seven patients observed in Department of Neurology in Zabrze, Medical University of Silesia, were accepted into the study. The study was approved by the local Ethics Committee of the Medical University of Silesia.

The patients were divided into four groups:

Group M: twenty-one patients with secondary-progressive or progressive-relapsing form of MS, diagnosed according to McDonald criteria (17). All of the patients received quarterly 5 doses of mitoxantrone i.v. (12 mg/m²/dose). The results were separated into two subgroups: before therapy (M1) and after (M2).

Group C1: seventeen healthy controls diagnosed in our Department due to previously undiagnosed headaches. Controls were matched for gender and age with the study group.

Group C2: nineteen patients with de novo diagnosed relapsing-remitting MS form, without any MS immunomodifying treatment.

All patients received no antioxidative substances, vitamins, anti-inflammatory or hormonal treatment for at least 3 months prior to the study. Demographic characteristics of the patients are presented in Table 1.

Study protocol

Before the therapy and after its completion, a clinical neurological examination was performed using the Kurtzke’s Expanded Disability Status Scale (EDSS) (18). The 10 mL samples of venous blood were collected, centrifuged and serum samples were frozen until laboratory measurements. Cerebrospinal fluid (CSF) samples (5 mL) were taken from lumbar puncture in all groups at the baseline. Moreover, in group M another puncture was performed after MX therapy. Antioxidative enzymes activity of MnSOD, Cu/ZnSOD, CAT, GSH-Px and MDA concentration in blood serum and CSF were assayed.

All laboratory analyses were performed at the Department of Biochemistry in Zabrze, Medical University of Silesia.

Enzymatic assays

The MnSOD and Cu/ZnSOD (EC 1.15.1.1) activities were estimated according to Oyanagui and expressed in nitrite units per ml (NU/ml) (19). The GSH-Px (EC 1.11.1.9) activities were measured according to Paglia and Valentine using enzymatic conjection with glutathione reductase (µmol NADPH/µl medium) (20). The CAT (EC 1.11.1.6), activities were measured according to kinetic method of Aebi and expressed in IU/ml of medium (21). MDA concentrations were determined according to the colorimetric methods by Ohkawa et al. using reaction with thiobarbituric acid (22).

Statistical analysis

All results are expressed as means ±S.E.M. Normal data distribution was tested with Kolmogorov-Smirnov’s test. Comparisons between two related groups were performed using the Wilcoxon test. Due to the different number of subjects, differences among the subgroups of MS patients and controls were assessed by the Kruskal-Wallis one-way ANOVA tests. The differences between means were considered statistically significant at p<0.05. The results were statistically analysed using Statistica v. 8.0 (StatSoft, Poland).

RESULTS

Effects on malondialdehyde

We observed a significant increase in MDA concentration (lipid peroxidation marker) in MS patients (M1 group, C2 group) as compared to controls (C1) in both the serum and the CSF. No differences were observed between M1 and C2 group (MX untreated MS patients) both in serum and CSF. The serum MDA concentrations were higher than in the CSF in all tested groups; p<0.05. After the MX treatment, the MDA concentration increased significantly in the serum and the CSF; however, the serum increase was more noticeable (about 33% vs. 16%) (Fig. 1).

![Fig. 1. Malondialdehyde concentrations in MS patients, before (M1) and after (M2) mitoxantrone treatment, healthy control group (C1) and control MS patients (C2) in both serum and cerebrospinal fluid (CSF). * p<0.05 serum vs. CSF; # p<0.05 C2, M1 vs. C1; • p<0.05 M2 vs. M1. Data are presented as mean ±S.E.M.](image)
Effects on superoxide dismutase

The activity of mitochondrial isoenzyme of SOD (MnSOD) was higher in the serum than in the CSF in all studied groups; p≤0.05 (Fig. 2). On the contrary, the activities of cytosolic isoenzyme SOD (Cu/ZnSOD) were significantly lower in the serum than in the CSF (Fig. 3). It is remarkable that the Cu/ZnSOD activity in the CSF is significantly higher than the MnSOD activity in all tested groups (Fig. 4). The MnSOD activities were significantly higher in MS patients (C2 and M1 group) than in controls (C1) in the CSF, whereas the serum levels of MnSOD activities were higher only in M1 group (Fig. 2). The M2 patients (after the MX treatment) exhibited an essential reduction in MnSOD activities in comparison with M1 (before MX), in both the serum and the CSF; p≤0.05 (Fig. 2).

The values of MnSOD activity in the CSF after the MX therapy were similar to those in healthy control group, C1, but in serum the activity was significantly lower than in the controls. The Cu/ZnSOD activities were significantly lower in C2 and M1 group than in C1 controls in the CSF, while the serum activity of this enzyme was statistically lower only in M1 group (Fig. 3).

There was a crucial induction of Cu/ZnSOD activities after the MX treatment (M2 vs. M1) in the serum, but not in the CSF; p≤0.05 (Fig. 3). After the MX therapy Cu/ZnSOD activity in the CSF was reduced, as compared to C1, likewise in C2 and M1 groups, but in serum the activity was significantly higher than in all tested groups, as well as in the controls.

Effects on catalase and glutathione peroxidase

The CAT activities of C2 and M1 groups were significantly higher than C1 group in the CSF. After the MX therapy, the CAT activity decreased considerably; p≤0.05 (Fig. 5). The GSH-Px activity in the CSF decreased considerably after the MX treatment (M2 vs. M1) with the noted decrease by almost 90% (Fig. 6).

Both CAT and GSH-Px activities in the CSF in M2 group were lower than in the controls. The results of CAT and GSH-Px

![Fig. 2. The activity of MnSOD in MS patients, before (M1) and after (M2) mitoxantrone treatment, healthy control group (C1) and control MS patients (C2) in both serum and cerebrospinal fluid (CSF). * p≤0.05 serum vs. CSF; # p≤0.05 C2, M1 vs. C1; • p≤0.05 M2 vs. M1. Data are presented as mean ±S.E.M.](image1)

![Fig. 3. The activity of Cu/ZnSOD in MS patients before (M1) and after (M2) mitoxantrone treatment, healthy control group (C1) and control MS patients (C2) in both serum and cerebrospinal fluid (CSF). * p≤0.05 serum vs. CSF; # p≤0.05 C2, M1 vs. C1; • p≤0.05 M2 vs. M1. Data are presented as mean ±S.E.M.](image2)
**Fig. 5.** The activity of CAT in MS patients before (M1) and after (M2) mitoxantrone treatment, healthy control group (C1) and control MS patients (C2) in cerebrospinal fluid (CSF). * p ≤ 0.05 M2 vs. M1; # p ≤ 0.05 C2, M1 vs. C1. Data are presented as mean ± S.E.M.

**Fig. 6.** The activity of GSH-Px in MS patients before (M1) and after (M2) mitoxantrone treatment, healthy control group (C1) and control MS patients (C2) in cerebrospinal fluid (CSF). * p ≤ 0.05 M2 vs. M1. Data are presented as mean ± S.E.M.
Activities in the serum were inconsistent, and as such, difficult to interpret, requiring additional studies (data not presented).

**Effects of mitoxantrone**

The MX therapy induced a 20.3% increase of MDA concentration and reduction of MnSOD (51.9%), Cu/ZnSOD (2.4%), CAT (77.8%), GSH-Px (91.1%) activities in the CSF (Fig. 7). Similarly, in the serum the MDA concentration increased (by about 37%) and MnSOD activity decreased (by about 44.7%) after the MX therapy. Contrary, serum activity of Cu/ZnSOD after MX treatment increased about 3 times (Fig. 8).

**DISCUSSION**

Our results show the influence of MX therapy on oxidation/antioxidation status of serum and the CSF in MS patients. MX’s mechanisms of action are based on the inhibition of DNA replication and DNA-dependent RNA synthesis in both proliferating and non-proliferating cells, T-cells, B-cells, and macrophages. Moreover, MX impairs antigen presentation by causing apoptosis in the cells that respond to the antigen. Additionally, in MS, MX inhibits the secretion of pro-inflammatory cytokines *e.g.*, IFN-gamma, tumor necrosis factor (TNF) and IL-2 and increases anti-inflammatory response. Furthermore, MX generates ROS (23). Finally, these
mechanisms inhibit macrophage-mediated myelin degradation (12-14).

The results of our study strongly confirm a prooxidative effect of MX. After the MX therapy, serum and CSF MDA concentrations (membrane lipid peroxidation marker - MDA) increased significantly. Our work also showed that proportional MDA production after the MX therapy was higher in serum than in the CSF.

Additionally, our work shows that serum and the CSF MDA concentrations are significantly higher in MS patients, in both de novo diagnosed RRMS (C2) and in worsening MS form (M) than in the controls, which is in accordance with recent studies of Tavazzi et al., where serum MDA of MS patients showed a tremendous increase in comparison with controls (24). Also, Sybrurra et al. have noted an increased concentration of lipid oxidation markers in MS patients’ serum (25). Other studies reported that lipid peroxidation products MDA and 4-hidroxialkenals increased significantly in the serum of subjects with RRMS in comparison with those of healthy controls (26). What is more, further data revealed an increase of lipid peroxidation markers in the CSF of patients during MS exacerbations (27).

Nervous tissue is susceptible to ROS damage because it has a high rate of oxygen consumption, an abundant supply of the transition metals, a high content of unsaturated lipids and a relatively lower regenerative capacity. Oxidative stress triggers peroxidation of the lipid membrane that leads to the generation of oxidized phospholipids and reactive aldehydes, which increases blood-brain barrier permeability, possibly resulting in disease exacerbation (28). It is thought that the ROS reactions and highly oxidised mitoxantrone metabolites form covalent complexes with DNA and inhibit DNA replication enzymes, which may be responsible for the cytotoxic effects of the drug (29, 30).

The results from previous studies prove that toxic MX mechanisms are a result of oxidative stress reactions. Simultaneously, a cardioprotective treatment with dexrazoxane (iron-chelating agent) reversed toxic actions of MX by decreasing the synthesis of superoxide radicals (31). However, some data indicates a cytotoxic, antioxidative activity of MX (32). Mitoxantrone was able to protect myocardial cells against iron toxicity by increasing ferritin expression. The iron catalyzes free radical reactions that overrule the antioxidative defenses of cardiomyocytes (33).

Several reports indicate that oxidative stress plays a major role in the pathogenesis of MS (34-38). In MS, ROS enhance monocyte adhesion and migration across brain endothelial cells. As such, the ROS are generally thought to be derived from activated inflammatory cells, and to play a role in demyelination and axonal damage in MS.

The oxidative stress activates antioxidants, as well as the transcription of genes encoding antioxidant and detoxification enzymes. Those adaptive mechanisms protect the cells against ROS-mediated toxicity and maintain tissue redox balance (39). The antioxidative enzymatic systems include superoxide dismutases, hydroxyperoxidase, CAT and other hemoprotein peroxidases (5, 9, 10, 37).

The results of our research show an increase in serum and the CSF MnSOD activities in MS patients. On the other hand, the serum and CSF Cu/ZnSOD activities are lower in MS group vs. healthy. It is interesting that in our study, in the CSF, the cytosolic form of SOD (Cu/ZnSOD) revealed a much higher activity than its mitochondrial form (MnSOD). Acting like a scavenger of superoxide, the SOD catalyzes its dismutation to the molecular oxygen and hydrogen peroxide in the matrix. The SOD exists as Cu/ZnSOD isoform located in the cytoplasm and in the intermembrane space and MnSOD isoform located in the mitochondrial matrix.

The Cu/ZnSOD and MnSOD are abundantly expressed in the CNS. The Cu/ZnSOD is primarily expressed in astrocytes, and, to a lesser extent, in neurons, whereas the MnSOD is mainly found in neurons, and less in astroglial cells. In microglia, oligodendrocytes, and brain endothelial cells the basal expression of both SODs is low (5).

An enhanced expression of MnSOD, but not the Cu/ZnSOD, was found in guinea pigs suffering from EAE. This research indicates an increase in the MnSOD activity in astroglial cells and microglial/phagocytic cells, contributing to the relative sparing of these cells from injury in EAE. Whereas the low level of MnSOD in oligodendroglial cells and axons may increase their vulnerability to the effects of superoxide-induced oxidative injury that results in demyelination (5, 40).

A significant reduction of Cu/ZnSOD activity was also found in the erythrocytes of the MS patients, which may suggest lower enzymatic defense against oxidative stress (41). Proinflammatory cytokines such as IL-1, IL-4, IL-6, and TNF-α are potent MnSOD activators (42). On the other hand, in MS, a significantly enhanced Cu/ZnSOD gene expression has been observed in active demyelinating lesions (43). Some data suggests that the state of endogenous protection system and blood content of antioxidant enzymes (CAT, SOD) in MS patients could play a significant role for early progression from RRMS into SPMS (44). Interestingly enough, our results indicate a similar SODs CSF and serum trend of tested values when MnSOD serum values are strongly expressed. On the other hand, Cu/ZnSOD CSF activity was predominant to serum and unexpected increase of serum activity of this enzyme after MX therapy was observed.

Similarly to previous reports, our work shows the reduction of antioxidative enzymes activities after MX treatment (33). We reported that MnSOD activities decrease in serum and the CSF, while surprisingly the serum Cu/ZnSOD activity increases after MX therapy. It is worthy of noting that the values of MnSOD activity in the CSF after the MX therapy were similar to those in healthy controls, but in serum the activity was significantly lower than in the controls.

After the MX therapy, the Cu/ZnSOD activity in the CSF was reduced in comparison with the controls, similar to the untreated MS patients, but in serum the activity was significantly higher as compared to all tested groups as well as the control group.

These findings prove that MX acts by altering the existing antioxidative status.

The endogenous H2O2 is one of the principal mediators involved in the alteration of vascular permeability and demyelination. The conversion of hydrogen peroxide into water and molecular oxygen is performed by CAT and alternatively by GSH-Px. CAT is an intracellular antioxidant enzyme, mainly located in cellular peroxisomes. In the CNS, an expression of CAT has been demonstrated for all cell types, both in vitro and in vivo (45).

Our work demonstrates a significantly higher CSF activity of CAT in MS patients than in the controls. Previous data also revealed that CAT and Cu/Zn, MnSOD are markedly upregulated in active demyelinating MS lesions as compared to normal-appearing white matter and white matter tissue from non-neurological control brains (46).

Conversely, a decreased peroxisomal function accompanied by reduced gene expression and activity of CAT in brain homogenates of rats suffering from EAE was observed (47). The results from our research do not show significant CSF differences in GSH-Px activities between studied groups, contrary to other reports. We also observed reduced CAT and GSH-Px activities in the CSF after the MX therapy as compared to the pretreated group the controls alike.
The GSH-Px is found mainly in the cytosol and mitochondria, where large amounts of superoxide are generated. In the brain tissue, the GSH-Px activity is higher than CAT. In addition to selenium-dependent GSH-Px, the brain cells contain a selenium-independent GSH-Px and the level of this enzyme is elevated in neurologic diseases (7). It has been demonstrated that GSH-Px gene expression is significantly increased in active demyelinating MS lesions (37). In addition, the GSH-Px activity is decreased in MS-affected cerebrospinal fluid (27). Reduced concentrations of antioxidants have been measured in serum of MS patients and in MS plaques (11, 48).

In fact, the differences between our results and data from previous reports may arise from different forms of MS in subjects accepted into the study. We also noted a marked decrease in CSF CAT and GSH-Px activity after the MX treatment. It is consistent with the reduction of antioxidative enzymes activities described previously (7, 23). The results obtained from our research strongly suggest that MS pathophysiology is related to alterations in oxidative defense system. Activities of MnSOD and CAT are increased in patients with MS, in both the RRMS and the worsening MS form. The MX treatment provokes the reduction of antioxidative enzymes activities and marked increase in MDA concentration.

These findings open up new opportunities for a better understanding of underlying pathophysiological events in MS. Finally, on the basis of current findings, the simultaneous use of potent antioxidative drugs together with MX treatment in MS needs to be considered as a therapeutic option.

Conflict of interests: None declared.

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