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Research report

The IFN-β 1b effect on Cu Zn superoxide dismutase (SOD1) in peripheral mononuclear blood cells of relapsing-remitting multiple sclerosis patients and in neuroblastoma SK-N-BE cells

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease leading to axonal injury. Even if the etiology of MS is still unknown the disease begins with inflammation involving autoreactive T lymphocytes activation in genetically susceptible subjects. Interferon beta-1b (IFN β 1b) is one of the most used drug in the MS therapy.

The results obtained in this study show that the concentration of SOD1 in CSF of relapsing-remitting MS (RR-MS) patients, evaluated by enzyme-linked immunosorbent assay (ELISA), is decreased compared to pathological controls. Moreover, the Western blotting analysis demonstrated that SOD1 in human peripheral blood mononuclear cells (PBMC) in healthy controls was significantly higher compared to MS subjects before starting DMT therapy. In addition IFN β 1b therapy causes an increase of intracellular SOD1 protein as well as mRNA levels in PBMC. Moreover, the treatment of neuroblastoma SK-N-BE cells with IFN β 1b increased SOD1 protein and mRNA levels; these data also suggest that neuroprotective effect of this physiological molecule is, at least in part, carried out through its effect on SOD1. This study demonstrate that DMT therapy is able to increase SOD1 expression in PBMC of RR-MS patients. Therefore, the effectiveness of DMT therapy can be ascribed, at least in part, to an increased levels of this antioxidant enzyme as further confirmed by *in vitro* studies in SK-N-BE cells.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder of the Central Nervous System (CNS) characterized by the infiltration of CNS white matter by myelin-reactive lymphocyte T leading to primary or secondary axonal injury. The etiology of MS is not yet known; it has been reported that genetic factors could play important roles in the pathogenesis of this disease (Ebers and Sadovnick, 1994; Oksenberg et al., 1993; Reboul et al., 2000; Kantarci et al., 2002; Tolide-Ie et al., 2014). It is also known that environmental factors, such as smoking (Russo et al., 2011), viral infections and vitamin D status have been linked to the occurrence of MS (De Lorenze et al., 2006; Marrie, 2004).

Although the initiating event of MS is a matter of debate, the disease begins with inflammation involving autoreactive T lymphocytes (Dhib-Jalbut et al., 2002) that could be triggered by viral infection in genetically susceptible subjects (Marrie, 2004). In addition, activated monocytes and macrophages can also release a variety of neurotoxic molecules like nitric oxide, reactive oxygen species (ROS) that contribute to the destruction of the myelin

Abbreviations: SOD1, superoxide dismutase; MS, multiple sclerosis; ELISA, enzyme-linked immunosorbent assay; CSF, cerebrospinal fluid; CNS, central nervous system; P Ctr, pathological controls; HD, healthy donator; SK-N-BE, human neuroblastoma cells; PBMC, peripheral blood mononuclear cells; BFA, brefeldin A; DMT, dimethyltryptamine; IFN, interferon; CDP, chronic inflammatory demyelinating polyneuropathy; EAE, autoimmune encephalomyelitis; RR-MS, relapsing-remitting multiple sclerosis.

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Table 1
Demographic and clinical characteristics of patients.

Characteristic	RR-MS patients (23)	ONID (20)	HD (10)	*p* Value
Female, N (%)	18 (78.3)	13 (65)	5 (50)	$p = 0.26$
Age, mean ± SD (years)	32.6 ± 8.9	34.4 ± 8.2	37.6 ± 6.9	$p = 0.69$
Disease duration, mean ± SD (months)	35.82 ± 55.86			
EDSS, mean ± SD	2.51 ± 0.58			

sheath (Dhib-Jalbut et al., 2002). Moreover histological data from the spinal cord of mice affected by autoimmune encephalomyelitis (EAE), that represents a good experimental animal model of MS, confirm a strong correlation of inflammation and axonal injury with perivascular lymphocytes infiltration that spread into the parenchyma (Giuliani and Yong, 2003).

It has been also recently demonstrated that autoimmune process affects not only the white matter but also the grey matter in the course of the disease (Derakhshan et al., 2013).

Many experimental data suggest a significant role of ROS in the pathogenesis of myelin destruction in MS as well as in other neurodegenerative diseases. Mitochondria constantly produce ROS, which have important signalling functions (Damiano et al., 2012, 2015). When the rate of ROS production exceeds the cellular antioxidant capacity it can cause oxidative stress and extensive damage to essential cell components (Lin and Beal, 2006; Witte et al., 2013). However so far it is not completely clear whether the correlation between ROS and MS is due to a decrease of enzymatic antioxidant defence mechanisms or to an overproduction of ROS.

In previous researches we showed that the antioxidant enzyme Cu–Zn superoxide dismutase (SOD1) is secreted by many cellular lines including human neuroblastoma SK-N-BE cells through a microvesicles pathway (Mondola et al., 2003) and increases calcium intracellular levels through an activation of phospholipase C-protein Kinase C pathway (Mondola et al., 2004, 1996). It has also been shown that an impaired secretion of mutant superoxide dismutase 1 is associated with neurotoxicity in familial amyotrophic lateral sclerosis (ALS) (Turner et al., 2005; Turner and Atkin, 2006); moreover an endoplasmic reticulum (ER) stress, associated with an unfolded protein response and Golgi apparatus fragmentation in mutant SOD, is present in familial ALS (Mondola et al., 2004). Recent data demonstrated that SOD1 is able to activate, through muscarinic M1 receptor, a trasductional pathway involving P-ERK and P-AKT in human neuroblastoma SK-N-BE cells (Damiano et al., 2013).

The aim of the present research was to evaluate the SOD1 amount in plasma and cerebrospinal fluid (CSF) of relapsing-remitting multiple sclerosis (RR-MS) subjects, pathological and healty controls; we also evaluated how interferon β 1b (IFN) could modify the expression of SOD1 in neuroblastoma SK-N-BE cells and in peripheral blood mononuclear cells (PBMC) of RR-MS patients.

A further goal of this study was to investigate whether IFN beta-1b can affect export of SOD1 in neuroblastoma SK-N-BE cells.

2. Material and methods

2.1. Patients

We enrolled 23 consecutive outpatients of the MS Centre of Federico II University Hospital (Naples Italy), from May to November 2010 with clinically definitive RR-MS according to the Mc Donald criteria (Polman et al., 2005), aged 20–50 years without relapse nor corticosteroids treatment in the previous 3 months naïve to any disease modifying therapy. Patients were treated with 250 μg/ml IFN beta-1b (8.000.000 IU) on alternate days with subcutaneous injection. Visits were scheduled with monthly neurological evaluation and blood samples were collected at baseline and after 3 months of therapy. Committee approval was obtained and each patient gave their signed informed consent to participate in this study.

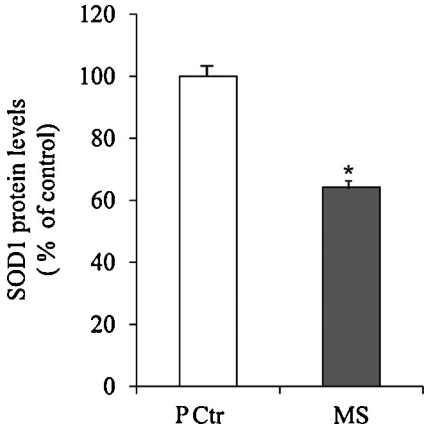

Fig. 1. SOD1 expression decreases in CSF of MS patients.
Enzyme-linked detection of SOD1 in cerebrospinal fluid of control versus MS patients; the values of SOD1 are expressed as% value. Error bars indicate ±SE (*$P < 0.001$). Statistical analysis has been performed by using *t*-test study.

We also enrolled 20 patients with other neurological inflammatory disease (ONID) such as LES, Systemic Sclerosis, Vasculitis and CIDP (Chronic Inflammatory Demyelinating Polyneuropathy) used as pathological controls (P Ctr). Patients and pathological controls were matched by age and gender. CSF was collected from 20 RR-MS patients and from 23 ONID controls. Written informed consent was obtained from each patient after a detailed explanation of all procedures. The study was approved by the "Federico II" University Ethical Committee (No 197/11/E1) and was conducted in concordance with the Declaration of Helsinki.

In addition we enrolled 10 healthy donators (HD) to evaluate the amount of intracellular SOD1 in PBMC. Demographic and clinical characteristics are summarized in Table 1.

2.2. Blood collection

Blood samples were obtained between 07:00 and 09:00 A.M. from 23 RR-MS patients and 10 healthy donators. Blood testing was performed in the morning after a 12-h fast either in patients or in healthy donators**.** The samples were centrifuged after collection at 1500 rpm for 10 min. and cell-free supernatants were stored at −80 °C for further analysis.

2.3. PBMC isolation

PBMC cells were isolated from 12 ml of blood drawn by vein puncture from HD and RR-MS patients; diluted (1:1 with PBS) samples were layered on the top of Ficoll solution (Ficoll-GE Healthcare) and spun at 3000 rpm for 25 min. PBMC cells were collected, two time washed in PBS and then spun at 3000 rpm for 4 min; the pellet fraction was then dissolved in RIPA buffer before polyacrylamide gel electrophoresis (PAGE) determination.

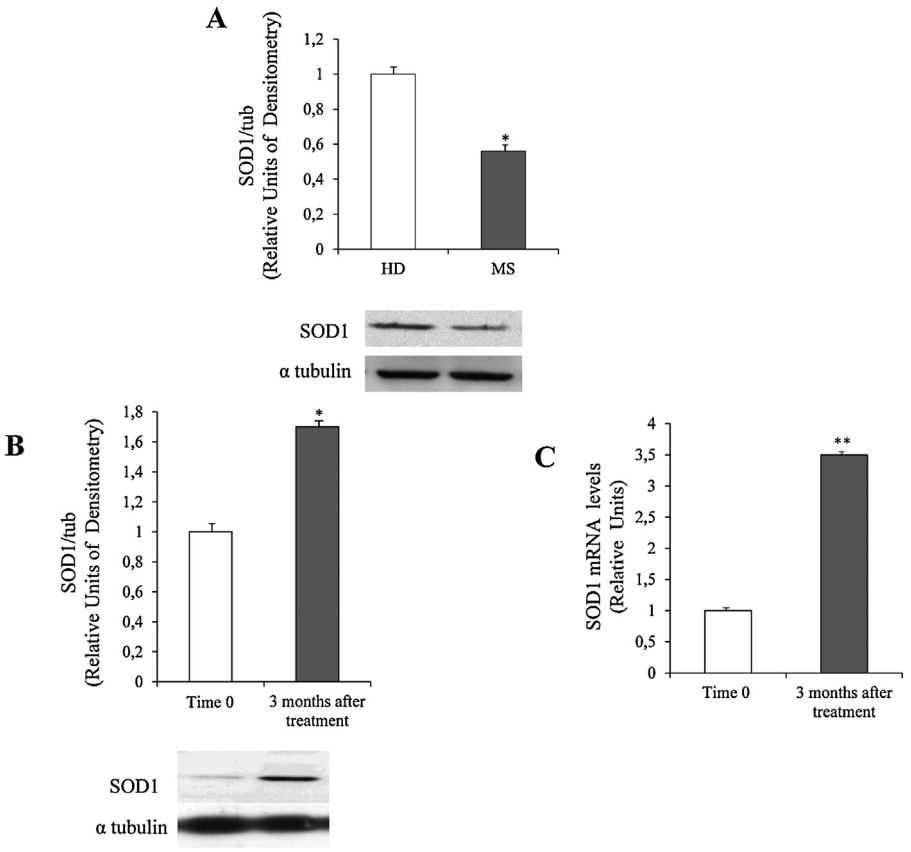

Fig. 2. Protein and mRNA SOD1 expression increase in MS patients after Interferon treatment.
Panel A shows the Western blotting evaluation of SOD1 in PBMC in healthy voluntary subjects (CTR) and in subjects affected by MS before starting the interferon treatment. In panel B the different amount of SOD1 in MS patients before and after three months of interferon treatment is showed. Panel C indicates the semi-quantitative RT-PCR of SOD1 before and after three months of interferon treatment. $^{*}P < 0.001$ *vs* HD and *vs* Time 0 (Panel A and B). $^{**}P < 0.005$ *vs* Time 0 (Panel C).

2.4. SOD1 immunoblotting assay of PBMC

Western blotting was performed as previously described (Kantarci et al., 2002). Briefly PBMC cells, washed twice with cold phosphate-buffered saline were homogenized in buffer containing 10 mM Tris–HCl, pH 7.4, 4 mM 2-mercaptoethanol, 5 mM EDTA adding a mix of protease inhibitors. Nuclei and cell debris were eliminated by slight centrifugation at 3.000 rpm for 5 min; the supernatant was then centrifuged at 100,000 × *g* for 1 h at 4 °C. After centrifugation the pellet was discarded and 50 μg of total proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions; then the proteins were transferred onto a nitrocellulose filter membrane (GE-Healthcare, UK) with a Trans-Blot Cell (Bio-Rad Laboratories, UK) and transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. Membranes were placed in 5% non-fat milk in phosphate-buffered saline, 0.5% Tween 20 (PBST) at 4 °C for 2 h to block the non specific binding sites. Filters were incubated with specific antibodies before being washed three times in PBST and then incubated with a peroxidase-conjugated secondary antibody (GE-Healthcare, UK). After washing with PBST, peroxidase activity was detected with the enhanced chemiluminescent (ECL) system (GE-Healthcare, UK).

SOD1 was detected by a rabbit polyclonal antibodies purchased by Santa Cruz Biotechnology (USA) diluted 1:1000 in 0.1% Tween 20 PBS.

The filters were also probed with an anti α-tubulin antibody (Sigma, USA). Protein bands were revealed by ECL and, when specified, quantified by densitometry using Scion Image software. Densitometric values were normalized to α-tubulin.

2.5. Neuroblastoma SK-N-BE cells culture

Human neuroblastoma SK-N-BE cells (American Type Culture Collection, ATCC, USA) and were grown in monolayer in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/ml streptomycin and 50 IU/ml penicillin. The cells were kept in a 5% CO_2 and 95% air atmosphere at 37°C. SK-N-BE and grown to semi-confluence in 60-mm dishes, then were washed twice with PBS and incubated for 18 h in medium containing 0.2% FBS before the experiments. The cells were then incubated for 3 h with 100.000 U/ml of IFN β 1a in presence of 5 μg/ml of the inhibitors of SOD1 protein secretion Brefeldin A (BFA) and in presence and in absence of 3 μl of 20 μM DPI. SK-N-BE and cell lysates were obtained in RIPA buffer, containing 50 mM.

Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS,2.5 mM Na-pyrophosphate, 1 mM β-glycerophosphate, 1 mM $NaVO_4$, 1 mM NaF, 0.5 mM PMSF and a cocktail of protease inhibitors (Roche, USA). The cells were kept for 15 min at 4 °C and disrupted by repeated aspiration through a 21-gauge needle. Cell lysates were centrifuged for 15 min at 13,000 rpm and the pellets were discarded. Fifty micrograms of total proteins was subjected to SDS-PAGE under reducing conditions.

After electrophoresis, the proteins were transferred onto a nitrocellulose filter membrane (GE-Healthcare, UK) with a Trans-Blot Cell (Bio-Rad Laboratories, UK) and transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were placed in 5% non-fat milk in tris-buffered saline, 0.1% Tween 20 (TBST) at 4 °C for 2 h to block the nonspecific binding sites. Filters were incubated with specific antibodies before being washed three times in TBST and then incubated

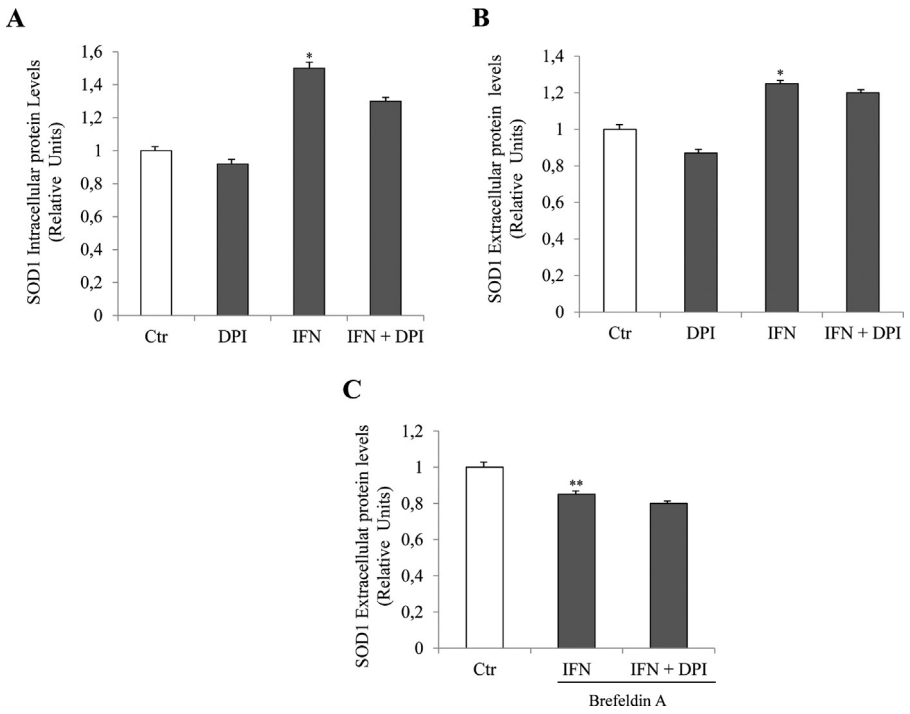

Fig. 3. SOD1 protein levels determined by ELISA assay increase after interferon treatment in SK-N-BE cells.
Panel A shows the value of intracellular SOD1 in neuroblastoma SK-N-BE cells after interferon treatment in absence and in presence of reactive oxygen species inhibitor DPI. Panel B indicates the value of extracellular SOD1 after the same treatment as indicated in Panel A. In Panel C the impairment of interferon-induced SOD1 secretion after Brefeldin A pretreatment in absence and in presence of DPI is illustrated. $^{*}P < 0.005$ *vs* DPI $^{**}P < 0.001$ *vs* control.

with a peroxidase-conjugated secondary antibody (GE-Healthcare, UK).

After washing with TBST, peroxidase activity was detected with the ECL system (GE-Healthcare, UK). SOD1 was detected with rabbit polyclonal antibodies; SOD1 antibodies were from Santa Cruz Biotechnology Inc. (USA), Protein bands were revealed by ECL and, when specified, quantified by densitometry using ScionImage software. Densitometric values were normalized to α-tubulin antibodies. Each experiment was three times repeated.

2.6. RNA preparation

Total RNAs of cell lines were extracted with High Pure RNA isolation kit (Roche), according to the manufacturer's instructions, using 2×10^6 cells. Traces of contaminated DNA were removed with DNAse I treatment.

2.7. Semi-quantitative RT-PCR

To improve the accuracy and sensitivity of the procedure, we performed a one-step quantitative reverse transcription polymerase chain reaction, in which the reverse transcriptase enzyme and a Taq DNA polymerase blend, possessing a proofreading activity were combined in the one tube, and a single, non-interrupted thermal cycling program. Quantification was achieved in a single reaction by using the housekeeping beta-actin gene as internal standard. 100 ng of RNA template was reverse-transcribed in 50 ll reaction mix containing 200 lM dNTPs, 100 mM DTT, 0.25 ll RNase inhibitor, 1.5 mM MgCl2, and 1 ll enzyme mix (Titan One-tube RT-PCR kit, Roche). The solution was incubated for 30 min at 50 °C in an automated DNA thermal cycler (GeneAmp 2400 Perkin.Elmer). To perform RT-PCR at optimal conditions and to stay within the logarithmically linear product formation, 40 cycles were chosen (30 s at 94 °C, 30 s at 59 °C, 60 s at 68 °C) and were followed by a final extension for 7 min at 68 °C. b-actin, SOD1, primers pairs were designed to yield PCR products of different sizes, 587 bp for b-actin, 290 bp for SOD1.

The forward and reverse b-actin primers were 5′-CC AAGGCCAACCGCGAGAAGATGAC-30, 50-AGGGTACATGGTGGTGCCGCCAGAC-3′, respectively; the forward and reverse SOD1 primers were 5′-CAGTGCAG GTCCTCACTTTA-3′ and 5′-CCTGTCTTTGTACTTTCTTC-3′, respectively. To rule out genomic DNA contamination we performed a negative control which contained RNA instead of cDNA. The signal intensities of PCR products were separated on a 1.2% agarose gel and were visualized by ethidium bromide staining. The products' signal intensities were determined by computerized densitometric analysis using Fotoplot software. The expression of SOD1 was normalized to b-actin mRNA levels.

2.8. DNA sequencing

To check the specificity of the amplified products, DNA bands were eluted from the gel and purified; sequence analysis was determined by the Big Dye Terminator Cycle Sequencing method (ABI-PRISM Sequencer 310 PerkinElmer).

2.9. Enzyme-linked immunosorbent assay (ELISA)

The quantitative detection of CuZn superoxide dismutase (SOD1) in the cerebrospinal fluid was determined by the Bender Med system kit (Bender Med System Diagnostic,Vienna, Austria) as previously described (Mondola et al., 2003). The concentration of SOD1 on the liquor was determined in undiluted samples calculating the average absorbance (450 nm) values for each sample and for standard curve in triplicate. The reproducibility of the method was evaluated in an independent experiment; the detection limit of the assay was 0.07 ng/ml and the intra-assay coefficient of variation was 4.8%.

2.10. Statistical analysis

All results shown are mean ± SE of at least three separate experiments. Statistical differences were evaluated using a Student's *t* test; for unpaired samples.

3. Results

23 RR-MS patients, 20 age and gender matched ONID patients and 10 age and gender matched HD were enrolled.

3.1. SOD1 detection in CSF

SOD1 determination in cerebrospinal fluid in 10 pathological controls and in 10 RR-MS patients is shown in Fig. 1. As can be noticed in RR-MS subjects, a statistical significant decrease of SOD1 concentration ($p < 0.001$) was observed respect to pathological controls suggesting an impaired secretion of this antioxidant enzyme in RR-MS patients.

3.2. Detection of SOD1 in PBMC

In Fig. 2 (Panel A) the Western blotting analysis of SOD1 in PBMC of voluntary healthy donators (HD) and in RR-MS patients was shown. As can be noticed, before starting DMT therapy, the SOD1 protein amount was strongly decreased compared to healthy donators. EDSS and disease duration were not related to SOD1 level.

IFN beta-1b therapy modifies SOD1 protein levels in PBMC of RR-MS; as shown in Fig. 2, after three months of Interferon β 1b treatment an increase of both intracellular SOD1 (Panel B) protein and mRNA (Panel C) levels in PBMC were observed.

3.3. Effect of IFN beta-1b in SK-N-BE cells

In Fig. 3 (Panel A) the increase of intracellular SOD1 protein amount after incubation with 100 ng/ml of IFN beta 1b 3 h is shown. The effect was not dependent on ROS because the preincubation of the cells for 15 min with 20 μM DPI, an inhibitor of ROS, did not impair interferon effects on SOD1. In addition, in presence of BFA, that impairs SOD1 secretion, the export of SOD1 strongly decreases (panel C) suggesting an involvement of BFA on SOD1 export.

The same increase (about 50%) of SOD1 mRNA levels was observed in neuroblastoma SK-N-BE cells after incubation with interferon β 1b (data non shown).

4. Discussion

Multiple sclerosis in one of the most common cause of disability in young adults, mainly in female subjects. Despite an elevated number of studies, the pathogenesis of this disease still remains obscure. Many studies indicate that the disease risk is more elevated in family members with MS disease compared to general populations (Sadovnick et al., 1997); in addition several epidemiological data indicate that specific bacteria or virus infections, associated to oxidative stresses, can be involved in this chronic demyelinating inflammation. The imbalance between ROS production and antioxidant defense mechanisms are associated to many neurodegenerative diseases, included MS. To this respect the antioxidant superoxide dismutase (SOD) isoenzymes could carried out a key role in the MS disease. We previously showed for the first time that CuZn superoxide dismutase (SOD1) is exported by many cellular lines including human neuroblastoma SK-N-BE cells (Mondola et al., 2003, 2004). Subsequently, observations from several authors also confirmed the presence of SOD1 in culture medium from different cell types (Turner et al., 2005; Urushitani et al., 2006; Gomes et al., 2007).

In this preliminary study we evaluated the effect of DMT therapy for a short period of three months on SOD1 protein amount and its gene expression in peripheral blood mononuclear cells (PBMC) of asymptomatic RR-MS patients. We found that after DMT therapy with IFN β 1b, intracellular concentration of SOD1 in PMBC of RR-MS patients increased compared to the basal levels (before treatment); in addition, likewise raised the mRNA SOD1 levels.

A further goal of our study has been either to investigate, by *in vitro* experiments on neuroblastoma SK-N-BE cells, the effects of IFN β 1b on intracellular content and export of SOD1 in order to point out the role of SOD1 in the effectiveness of IFN β 1b treatment.

The *in vitro* studies on neuroblastoma SK-N-BE cells showed that IFN β 1b, increases SOD1 intracellular amount as well as its secretion indicating that constitutive SOD1 secretion is modulated by this molecule (Fig. 3).

This effect cannot be ascribed to reactive oxygen species (ROS) production by IFN β 1b treatment because the ROS inhibitor diphenyleneiodonium-chloride (DPI) did not affected IFN β 1b effects on intracellular and extracellular SOD1 levels. Moreover IFN β 1b treatment did not increase ROS levels measured by 5,6-carboxy-2',7'-dichlorofluorescein-diacetate, DCHF-DA, (Molecular Probes, The Netherlands) (data not shown).

The absence of ROS production in neuronal cells after IFN β incubation for a short period was also confirmed by others authors (Alboni et al., 2013).

In CSF the presence of the three SOD isoenzymes has been previously demonstrated; CuZn–SOD accounts for ~75% of the SOD activity, EC-SOD for 25% and Mn-SOD for <5% (Jacobsson et al., 2001).

The concentration of CuZn–SOD in CSF could be correlated with the amount of this enzyme in the cytosol of cells in the CNS. In our study, the higher level of SOD1 detected in cerebrospinal fluid of control subjects compared to untreated RR-MS patients suggests an impaired SOD1 secretion that, decreasing antioxidant defences, could impair neuroimmunoregulatory responses and therefore, could have a role in the pathogenesis of multiple sclerosis.

Some studies demonstrated the suppression of the Lipopolysaccharide-induced *in vitro* production of two proinflammatory cytokines, tumor necrosis factor α (TNFα) and interleukin β (IL-1β) in subjects receiving spinal manipulative therapy (SMT) (Teodorczyk-Injeyan et al., 2006). It could be of interest to further explore the possible effects of SMT on the SOD1 and other cellular antioxidant defences in multiple sclerosis patients.

Therefore, this preliminary study indicates that the effectiveness of DMT therapy can be ascribed, at list in part, to an increase of intracellular SOD1 levels in addition to an induction of its gene expression that can be finalized to improve antioxidant defence mechanisms. Finally our data on SK-N-BE cells pointed out on a new physiological mechanism involved in neuroprotection by interferon β 1b.

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