



## The effects of whole-body cryotherapy on oxidative stress in multiple sclerosis patients

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### ABSTRACT

There is strong evidence that multiple sclerosis (MS) is characterized not only by immune mediated inflammatory reactions but also by neurodegenerative processes. Accumulated data indicate that oxidative stress (OS) plays a major role in this process. Generated in excess, reactive oxygen species (ROS) lead to oxidative stress and are involved in demyelination and axonal damage in MS. ROS generation may be inhibited partly by hypothermia, which is known as a potent putative neuroprotectant and may inhibit generating free radicals and oxidative stress. Whole-body cryotherapy (WBCT) treatment may improve both survival and neurological outcome in MS patients.

The aim of the study was to determine the effects of WBCT on oxidative stress by the level of total antioxidative status (TAS) in plasma and the activity of antioxidative enzymes: superoxide dismutase (SOD) and catalase (CAT) in the erythrocytes from MS patients. Moreover, we measured the combined effects of WBCT and melatonin on TAS and activity of antioxidative enzymes in MS patients. Sixteen MS patients were treated with 3 cycles of 10 exposures in a cryogenic chamber. The last cycle was accompanied by a 14-day-long supplementation of melatonin (10 mg daily). Healthy subjects as a control group had 1 cycle of WBCT.

Our preliminary results for the first time showed that WBCT treatment of MS patients resulted in the increase of TAS but had no effects on activity of antioxidative enzymes: SOD and CAT. Supplementation of melatonin and the treatment along with WBCT significantly increased the activity of SOD and CAT in erythrocytes of MS patients.

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### 1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelination disease of the central nervous system (CNS) with axonal degeneration and astrogliosis. Accumulating data indicate that oxidative stress plays a major role in this process. Reactive oxygen and nitrogen species (ROS/RNS), leading to oxidative stress, generated in excess primarily by macrophages, have been implicated as mediators of demyelination and axonal damage in MS. ROS cause damage to the main cellular structures of neurons

and components such as lipids, proteins and nucleic acids (e.g., RNA, DNA) and might be responsible for the neuronal dysfunction and result in cell death by necrosis or apoptosis contributing to pathogenesis of this disorder. In addition, weakened cellular antioxidant defense systems of CNS in MS, and its vulnerability to ROS effects may augment damage (Gilgun-Sherki et al., 2004).

Generation of ROS/RNS may partly be inhibited by hypothermia. Hypothermia has long been known as a potent putative neuroprotectant. It delays energy depletion, reduces intracellular acidosis and ischemia, related to the accumulation of excitotoxic neurotransmitters, and attenuates the influx of intracellular calcium. Additionally, hypothermia inhibits generating oxygen free radicals involved in the secondary damage associated with reperfusion. It also suppresses mechanisms of blood–brain barrier degeneration and post-ischemic remodeling (Gonsette, 2008; Liu and Yenari, 2007; Muniandy et al., 2009; Srinivasan, 2002). Thus, treatment with whole-body cryotherapy (WBCT) might theoretically prevent propagation of tissue damage and improve both survival and neurological outcome in MS patients.

Treatment with the total exposure of the body to extremely low temperatures was first introduced in Japan towards the end of

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the 1970s by Yamauchi, 1989, who constructed the first cryogenic chamber and successfully used cryotherapy to treat rheumatism.

The studies of WBCT effects were carried out on healthy subjects, sportsmen or rheumatic patients (Banfi et al., 2009; Lange et al., 2008; Leppäluoto et al., 2008; Lubkowska et al., 2008). There are no data concerning the treatment of MS patients with WBCT ( $-110$ – $-160$  °C); however cooling ( $7$ – $26$  °C) especially heat-sensitive MS patients as a factor improving functional performance of MS patients was applied (Grahn et al., 2008; Meyer-Heim et al., 2007).

Melatonin was used in this study as the antioxidant because it is effective in both aqueous and lipid phases, easily crosses the blood–brain barrier and can protect neurons from excitotoxicity (Reiter et al., 2005). Melatonin is twice as effective as vitamin E, at protecting cell membranes from lipid peroxidation and five times more effective than glutathione for neutralizing hydroxyl radicals (Pieri et al., 1994).

The antioxidants and the antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) protect the organism against production of free radicals and different ROS/RNS (Siems et al., 1999, 1994). The different antioxidants such as glutathione, uric acid, bilirubin, vitamins C, D and other exogenous antioxidants including melatonin are responsible for total antioxidative status (TAS) (Miller et al., 2009).

The aim of our study was to evaluate the effects of WBCT on oxidative stress by the level of TAS in plasma and activity of antioxidant enzymes, SOD and CAT, in erythrocytes from MS patients. Moreover, we considered combining the effects of the WBCT together with melatonin supplementation. Therefore the effects of WBCT concomitant with supplementation of melatonin in MS patients were also evaluated.

## 2. Materials and methods

### 2.1. Patients presentation

Sixteen MS patients with secondary progressive disease course and sixteen healthy controls participated in the study including subjects with clinically diagnosed MS, according to the McDonald criteria. The MS samples consisted of 11 females and 5 males aged  $43.2 \pm 11.2$ ; the average disability status scale EDSS (expanded disability status scale) score reached  $4.5 \pm 1.83$  and average disease duration was  $9.0 \pm 6.45$  years. The MS subjects ( $n=16$ ) received no immunomodulators, immunostimulators, hormones,

vitamins, minerals or any other substitutions with antioxidative properties. Prior to the study, all the subjects had undergone medical check-ups including neurological and internist examination. Healthy control ( $n=16$ ) subjects were age matched to MS patients and selected according to sex, lifestyles and diet. Inclusion/exclusion criteria for this study were a diagnosis of MS and the ability to move independently. Patients suffering from circulatory or breathing insufficiency, clotting, embolism, inflammation of blood vessels, open wounds, ulcers, serious cognitive disturbances, fever, addictions, claustrophobia and over-sensitivity to cold were excluded from the study. MS patients were free from any clinical attacks and worsening symptoms for more than the last 5 years. The protocol and all the procedures were carried out in accordance with the Helsinki Declaration with the approval of the Medical University of Lodz, Poland. The study was performed in the Medical University, Department of Biochemistry and Neurorehabilitation, Division III General Hospital in Lodz, Poland.

### 2.2. Experimental design

An experimental trial with WBCT and melatonin involved 16 subjects treated with 3 cycles (with 3-month break after each cycle) of 10 exposures in a cryogenic chamber carried out daily from Monday to Friday (Fig. 1). The last cycle of WBCT was connected with 14 days supplementation of 10 mg of melatonin taken every evening at 6 p.m. Healthy control subjects underwent 1 cycle (10 exposures) of WBCT. The cryogenic chamber consisted of two rooms: the vestibule, with the temperature of  $-60$  °C, and the main chamber, with temperatures varying from  $-110$  to  $-160$  °C. Liquid nitrogen was used as the coolant agent. Sessions in the chamber lasted 2–3 min. We followed the guidance of Gregorowicz and Zagrobelny (2007) and on the appropriated duration of exposure and temperature for adult patients and a list of medical conditions in which WBCT is unsuitable. The study lasted from March to November, 2009. Observations were made in 3 groups: MS I—16 patients after the first cycle of WBCT (non-melatonin), MS II—16 patients immersed by 3 cycles of WBCT—including the last cycle with 14 days of melatonin supplementation and 16 healthy subjects immersed by 1 cycle of WBCT. All three groups were examined at 2 stages: at the beginning and at the end of the treatment. Blood samples were collected in cooled EDTA and centrifuged to isolate plasma and erythrocytes. In both MS groups blood samples were taken 1 h before the first 10 days' cycle of the therapy and 1 h after the last immersion.

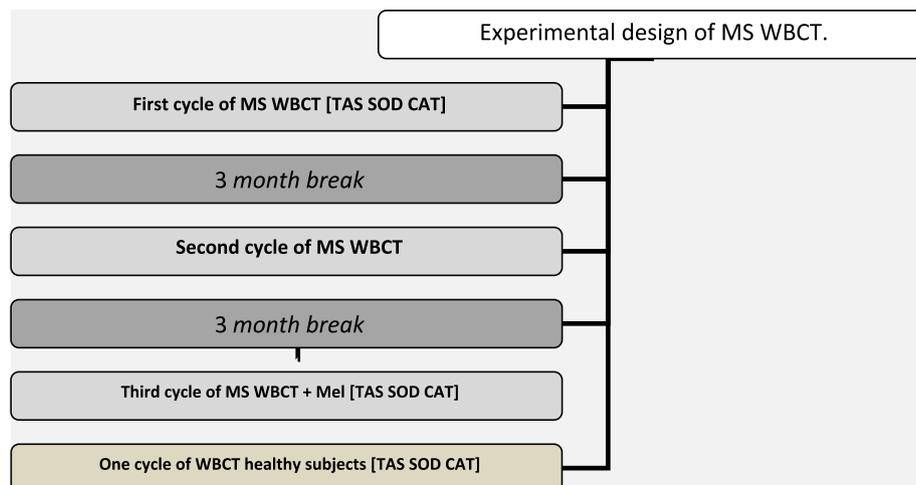


Fig. 1. Illustration of the study design.

### 2.3. Biochemical investigations

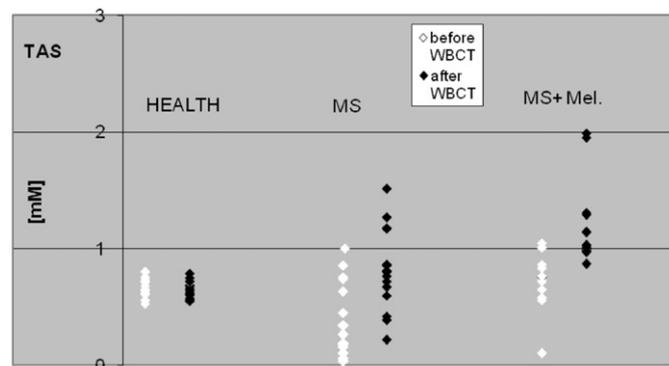
TAS was measured in plasma samples (healthy group and MS patients) using the kit by Randox Laboratories Ltd. (Cat. no. NX 2332). The plasma volume taken to estimation was 5  $\mu$ l and the total assay volume was 305  $\mu$ l. The reaction was carried out for 3 min. and the absorbance was measured spectrophotometrically at 600 nm. Activity of antioxidative enzymes, SOD and CAT, was determined in erythrocytes obtained from blood of MS patients and control subjects. Superoxide dismutase activity in erythrocytes was measured according to method of Misra and Fridovich (1972). The absorbance of the examined samples was estimated at 380 nm (using the Beckman spectrophotometer) at the temperature of 37 °C. The activity was expressed as U/gHb. Catalase activity in erythrocytes was determined according to the method of Beers and Sizer (1952). Absorbance was measured at 240 nm using the Beckman spectrophotometer. Enzymatic activity was expressed as Berg Mayer units, U/gHb.

### 2.4. Statistical analysis

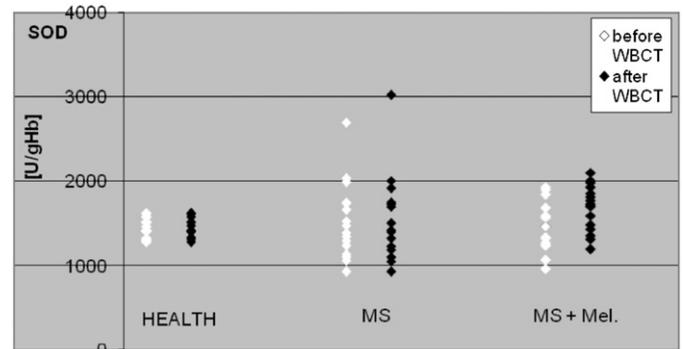
Results were statistically elaborated. Differences were considered significant when the significance was  $p < 0.05$ . Due to non-parametric distribution, the Wilcoxon test was applied to analyze changes.

## 3. Results

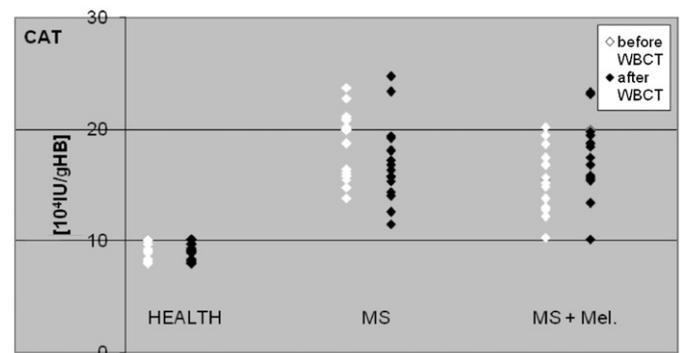
Our studies have shown that WBCT distinctly changes the level of TAS in MS patients (Fig. 2). CAT activity was significantly (2-fold) higher in erythrocytes of MS patients than in erythrocytes of healthy volunteers (Fig. 4). Activities of SOD and CAT in erythrocytes of MS patients after treatment with WBCT were not changed. Melatonin supplementation increased SOD and CAT activity compared with the “non-melatonin” group ( $p < 0.05$  and  $< 0.0001$ , respectively) (Figs. 3 and 4). Melatonin caused statistically significant increase of SOD activity in erythrocytes of MS patients ( $p < 0.05$ ), contrary to “non-melatonin” MS patients, where SOD activity showed no changes (Fig. 3). We have observed that the level of TAS in both MS groups (with and without melatonin) after WBCT was considerably higher than before WBCT  $p < 0.0001$  and  $< 0.0005$ , respectively.



**Fig. 2.** Total antioxidative status (TAS) level in plasma before and after 10 exposures of whole-body cryotherapy (WBCT) in 3 groups: healthy subjects; multiple sclerosis patients non-supplemented (MS) and multiple sclerosis patients supplemented with melatonin (MS+MEL).



**Fig. 3.** Superoxide dismutase (CuZnSOD) activity in erythrocytes before and after 10 exposures of whole-body cryotherapy (WBCT) in 3 groups: healthy subjects; multiple sclerosis patients non-supplemented (MS) and multiple sclerosis patients supplemented with melatonin (MS+MEL).



**Fig. 4.** Catalase (CAT) activity in erythrocytes before and after 10 exposures of whole-body cryotherapy (WBCT) in 3 groups: healthy subjects; multiple sclerosis patients non-supplemented (MS) and multiple sclerosis patients supplemented with melatonin (MS+MEL).

## 4. Discussion

Thermoregulation is a complex process, involving many levels of control and interaction with other physiological processes in the central and peripheral nervous systems. It enables to maintain a stable temperature of internal tissue and organs (Gordon, 2001). During exposure to cold, the thermoregulatory system with surface cold receptors attempts to maintain a constant core temperature (around 37 °C) by means of skin vasoconstriction and by increase in the metabolic rate through shivering. Being exposed to cold, the skin temperature decreases rapidly due to vasoconstriction and direct skin cooling, especially in the unprotected extremities. In our study the lowest local skin temperatures were recorded in the forearm (12 °C) and in the calf (14 °C), which are similar to the results observed by Westerlund et al. (2003). Immediately after WBCT, all skin temperatures increased rapidly within 14 min. According to Knight (1976) and McMeeken et al. (1984), to reduce nerve velocity by approximately 10%, the temperature of 12.5 °C is required. To obtain the lower metabolic enzyme activity by approximately 50%, skin temperatures between 10 and 11 °C are necessary. Vasoconstriction is followed by vasodilatation connected with increase in the blood flow and return to normal skin temperatures (after about 14 min). Vasodilatation occurs about 4 min after WBCT and reaches 4-times higher value than before WBCT and can remain for a few hours, increasing the blood flow and stimulating elimination of metabolic products (Bauer and Skrzek, 1999).

Moderate hypothermia protects the brain, heart and other organs (Gordon, 2010). The reduction in temperature by 1 °C will lead to a 9.6% reduction in the rate of cellular respiration, oxygen demand, carbon dioxide production, etc. Reduced demand for oxygen during hypothermia is especially critical in highly aerobic organs such as brain and heart subject to ischemia. Reduced temperature slows the rate of lipid peroxidation and protects ischemic cell membranes by stabilizing potassium efflux. The mechanisms of action of hypothermic protection are not entirely understood. It seems that lower temperatures protect tissues deprived of oxygen by slowing the rate of cellular damage due to the formation of free radicals, chemical metabolites, and tissue edema (Gordon, 2001).

Chronic WBCT reduces oxidative stress by increasing the activity of antioxidative enzymes, especially in immunoactive disorders. Acute cold temperature on a regular basis during a period of several months represents an obvious stress, which could lead to some adaptive mechanisms. This is one of a few tentative explanations of hardening the body after cold treatment. It has been suspected that an adaptation to cold stimuli and the improvement in body hardening could be related to an increase of protection against oxidative stress. Siems et al. (1999) reported a higher enzymatic protection (i.e. in the increased activity of red blood cells enzymes) for those who regularly practiced winter swimming activities or after heavy endurance physical exercise in comparison with the control group. It may suggest that stimuli such as acute exercises and cold stress activate antioxidant defense of the body (Miller et al., 2010; Siems and Brenke, 1992). This activation can be viewed as an adaptive defensive mechanism to cope with increased oxidative stress.

We observed that in MS patients the activities of erythrocyte antioxidative enzymes, especially SOD, are evidently reduced (Fig. 3). Antioxidants, whether synthesized endogenously or exogenously administered, act as reducing agents that neutralize the oxidative compounds (ROS) before they can cause any damage to different biomolecules. The measurement of TAS in plasma represents the body Redox status better than the measurement of the single circulating antioxidant does (Duqué et al., 2005; Miller et al., 2010). Therefore in our study to establish the effects of the therapy, we estimated the level of TAS in plasma. After strenuous physical exercise when ROS are produced, a significant increase of TAS may occur (MacKinnon et al., 1999). We also observed that after WBCT, just like after exercises done by MS patients TAS in plasma was increased compared to untreated patients (Miller et al., 2010). WBCT was applied for the first time in the treatment of rheumatic patients with inflammation and oxidative stress background. The measurement of specific oxidative stress markers such as TAS and the activity of antioxidative enzymes are commonly used to estimate oxidative stress (Duqué et al., 2005; Miller et al., 2010).

Our present data demonstrate that plasma TAS was found to be significantly lower in MS patients than in the healthy group (Fig. 2). These findings indicate that in MS patients the impaired antioxidant defense system may be dependent partly on the lower activity of SOD. Along with the reduction of oxidative stress, the improvement of symptoms e.g., statistically considerable decrease of fatigue, increase of muscle strength and lower disability were observed.

Melatonin treatment enhances the antioxidative potential of the cell by stimulating the activity of antioxidative enzymes like superoxide dismutase (Srinivasan, 2002). We observed that supplementation of melatonin together with WBCT treatment causes a significant increase of the activities of SOD and CAT, contrary to the treatment of MS patients with WBCT alone, where SOD and CAT activities in erythrocytes of MS patients are not changed. WBCT evidently increased the level of TAS after the first

cycle of 10 exposures from 0.35 to 0.81 (Fig. 2). After the last immersion together with melatonin supplementation the TAS level got higher and reached 1.13.

Melatonin (n-acetyl-5-methoxy-tryptamine) is a well known antioxidant that plays a crucial role in the genesis of neurodegenerative diseases (Reiter et al., 2005). It interacts with highly toxic hydroxyl radicals, hypochlorous and hydrogen peroxide, singlet oxygen, nitric oxide and peroxyxynitrite. Despite its antioxidative properties, melatonin stimulates several antioxidative enzymes, which increase their efficiency as antioxidants: SOD, GPx and glutathione reductase. Our results indicate that melatonin essentially stimulates SOD and to a lesser extent CAT activity. Melatonin affects the function of the mitochondrial complex (I and IV) involved in oxidative phosphorylation and may stimulate gene expression to antioxidative enzymes or to increase their activity. SOD is considered as a critical enzyme (Srinivasan, 2002). It seems that in MS patients melatonin has a positive effect on antioxidative status although in our studies supplementation with melatonin had no effects on TAS level. Our results showed that WBCT of MS patients reduces oxidative stress via a significant increase in TAS but has no effects on the activity of antioxidative enzymes like SOD and CAT. Supplementation of melatonin during the treatment of MS patients with WBCT increases SOD and CAT activity but has no effects on TAS level in MS patients. Further studies of oxidative stress in MS patients are required to explain the role and antioxidative mechanisms of WBCT in MS patients' treatment.

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