Whole-body cryostimulation and oxidative stress in rowers: the preliminary results

Alina Wozniak[1], Celestyna Mila-Kierzenkowska[1], Michał Szpinda[2], Jolanta Chwalbinska-Moneta[3], Beata Augustynska[4], Alicja Jurecka[1]

[1]The Chair of Medical Biology, *Collegium Medicum* of Nicolaus Copernicus University, Bydgoszcz, Poland
[2]Department of Normal Anatomy, *Collegium Medicum* of Nicolaus Copernicus University, Bydgoszcz, Poland
[3]Department of Applied Physiology, Medical Research Centre of the Polish Academy of Science, Warsaw, Poland
[4]Department of Psychiatry, *Collegium Medicum* of Nicolaus Copernicus University, Bydgoszcz, Poland

Corresponding author:
Celestyna Mila-Kierzenkowska PhD
The Chair of Medical Biology
Collegium Medicum
Nicolaus Copernicus University
24 Karlowicza St
85-092 Bydgoszcz, Poland
Phone: +48 52 585 37 37
Fax: +48 52 585 37 42
E-mail: celestyna@o2.pl

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Abstract

Introduction: The effect of whole-body cryostimulation (WBC) on the biomarkers of oxidative stress, lysosomal enzymes, creatine kinase and cortisol was studied.
Material and methods: The rowers underwent two 6-day training cycles: with pre-training daily WBC (temperature: from –125°C to –150°C) and without cryostimulation (control). Blood samples were taken before and after the third and sixth day of training.
Results: The activity of superoxide dismutase and glutathione peroxidase was lower (by 44% and 42%, respectively) after the third day of training with WBC than without WBC. The concentration of lipid peroxidation products was also lower after the training preceded by WBC. Moreover, the acid phosphatase activity was 50% lower after the third day of training with WBC than training without WBC. Considering the antioxidant enzymes activity during training without WBC, the increase of superoxide dismutase and glutathione peroxidase activity was observed after the third day of training (by about 74% and 100%, respectively). The level of lipid peroxidation products also increased after the training without WBC. No statistically significant changes were observed in creatine kinase activity after the training preceded with WBC, while after the training without WBC activity of this enzyme was two-fold higher than before the training.
Conclusions: The use of WBC prior to training may reduce the risk of oxidative stress and the extent of muscle fibre injuries provoked by intense exercise. The WBC seems to be an effective and safe method for limiting exercise-induced damage; thus it may be used in biological regeneration of sportsmen.

Key words: cryo-chamber, antioxidant enzymes, lipid peroxidation, creatine kinase, cortisol, lysosomal enzymes.

Introduction

At first the cooling of the organism in a cryo-chamber (temp. below –100°C) was used as a kind of therapy in the treatment and rehabilitation process of various disorders [1], mainly rheumatoid and those affecting the mobility system, such as arthritis, fibromyalgia and ankylosing spondyli-

tis [2]. Nowadays whole-body cryostimulation (WBC) has an increasingly greater application in sport medicine. It has become a method of choice to encourage recovery from muscular traumas [2], but first of all WBC is a new method used to accelerate regeneration of the organism in resting time and to stimulate greater strain-bearing capacity during training; thus it protects against over-use syndrome [3]. After cryostimulation, there occurs a reduced sense of pain and lowered muscle tension with concomitant increase in muscle strength; hence even acute exercise is made easier [3, 4].

Physical exercise may evoke the enhanced formation of reactive oxygen species (ROS), which is closely correlated with intensity of muscle action [5]. The effects of ROS are counteracted by nutritional antioxidants and by the endogenous enzymatic antioxidant system. The imbalance between ROS and antioxidant defence leads to oxidative stress, which is considered to have a role in the pathogenesis of various diseases [6]. Oxidative stress often occurs as an effect of different physical stresses, including exercise, because physical training can affect ROS formation either positively or negatively, depending mainly on training load and specificity [7]. One outcome of oxidative stress in the organism is for example the lipid peroxidation process, the products of which include malondialdehyde and conjugated dienes (CD) [8]. Oxidative stress is known to be involved in the process of muscle damage and to be heavily influenced by increased levels of stress hormones, such as cortisol [9]. The leakage of lysosomal enzymes from muscles under the influence of exercise was reported [3].

The antioxidant action of whole-body cryostimulation has been postulated by some authors [10, 11], but as yet not much is known about the effect of WBC on the generation of ROS and the activity of antioxidant mechanisms.

The aim of this paper was to examine the effect of twice a day WBC on the level of lipid peroxidation products and on the activity of antioxidant enzymes in the peripheral blood of rowers during training. The study also evaluated whether stimulation by extremely low temperatures affects the activity of lysosomal enzymes, the activity of an indicator of damage to muscle fibres, creatine kinase (CK), and the concentration of the stress marker cortisol.

Table I. Subject characteristics (mean ± SD)

Variable	Rowers (n = 6)
Age [years]	26.7 ±3.6
Height [cm]	193.8 ±5.9
Mass [kg]	93.2 ±6.9
VO_2max [ml/min × kg]	64.4 ±3.1
Training experience [years]	10.7 ±2.4

Material and methods

Material

Six elite rowers (Table I) participated in this study after approval by the Bioethical Committee at the Ludwik Rydygier Medical University (Bydgoszcz) and the subjects provided written informed consent.

The rowers were subjected to a 6-day training cycle with training sessions twice a day. Each training session was preceded by WBC (two cryo-chamber treatments daily during six days). Before the training cycle with WBC (Table II), a control training session without WBC was conducted (Table II). Blood was obtained from an antecubital vein before training and after the third and sixth day of training without WBC and training with WBC. The fasting blood samples were taken in the morning, into two vacuum tubes: a serum one and a heparin one.

The training with WBC and without WBC differed in the type of training exercises and sometimes in their duration and intensity, because the training sessions were conducted during different training periods in which the athletes differed in their degree of fitness. The training protocol was adapted to the current functional capacity of the athlete. As fitness developed, intensity and duration of the exercise were gradually increased. Despite those differences, the comparative exercise volume (the proportion between oxygen demand during execution of the exercise and the maximum oxygen consumption by the organism) during both of the compared training cycles was similar. The exercise volume was controlled by the athlete's pulse. The current state of fitness was determined by measuring the maximum oxygen consumption and the lactate threshold, which provides information about the effectiveness of energy sources.

Cryostimulation

The cryogenic chamber type KN-1 (Kriotechnika Medyczna Sp. z o.o., Wroclaw, Poland) is a prefabricated wooden construction with walls filled with thermal insulation, which can admit up to 6 patients. In the presented experiment, the cryo-chamber was cooled with liquid nitrogen, and the temperature was monitored by a computer. In the cryo-chamber, the subjects were dressed in shorts, socks, gloves and a hat or headband covering the auricles. Wooden clogs were worn as protection against frostbite. The subjects entered the cryo-chamber after consultation with a doctor and were informed of the necessity of taking slow, shallow breaths. During WBC they remained at all times in visual contact with the person supervising the treatment. Each day, the first session of cryostimulation was performed in the morning, before the start of exercise, while the second one was performed at a specified time during the day, before the next training ses-

Table II. Six-day training without and with whole-body cryostimulation (WBC)

Day of the week	Type of training/time and intensity	
	Without WBC	With WBC
Monday	Gym/180 min I Rowing on water/300 min I, 10 min II Ergometer/60 min II	Volleyball/90 min II Gym – general strength/120 min I
Tuesday	The same as Monday	Running + stretching/35 min I Ergometer – continuous rowing/90 min II Outdoor run/walk/120 min I
Wednesday	The same as Monday	Basketball/35 min II Gym – stamina/120 min III Continuous swimming/45 min I
Thursday	Gym/80 min I Rowing on water/210 min I, 10 min II, 12 min III Running/80 min II	Ergometer – continuous rowing/90 min II Continuous running/105 min I
Friday	The same as Thursday	General training/35 min I Gym – stamina/120 min III Games/90 min I
Saturday	The same as Thursday	Treadmill – running/30 min III Ergometer/30 min I Games/90 min I

Band I – concentration of blood lactate < 4 mmol/l, band II 4-8 mmol/l, band III > 8 mmol/l

sion. During a single WBC treatment the subjects spent 3 min at a temperature between –125°C and –150°C. Each entry to the cryo-chamber was preceded by a 10-20 s adaptation in the vestibule at a temperature of –60°C.

The sportsmen after the WBC treatment perceived deep relaxation, calm and tension release with concomitant decline of feelings of tiredness. These effects of WBC are known to last at least for 3 h [12]; hence the training practice was performed under the direct reaction of the organism to extremely low temperature exposure.

Measurements

Superoxide dismutase (SOD) activity in erythrocyte haemolysates was evaluated in accordance with the method based on the enzyme impeding the reaction of auto-oxidation of adrenalin to adrenochrome in an alkaline medium [13]. The unit of activity of SOD is the quantity of the enzyme that impedes the reaction by 50% at a maximum increase in absorption of 0.025 U/min on a rectilinear section of adrenochrome formation. Glutathione peroxidase (GPx) activity was measured in erythrocytes by detecting the changes in absorption caused by the change of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) into an oxidised form [14]. NADPH is a coenzyme in the reaction of the reduction of glutathione disulphide catalysed by glutathione reductase. The obtained oxidized glutathione is a product of the reaction catalysed by glutathione peroxidase. The substrate was hydrogen peroxide. The activity of SOD and GPx is expressed in Ug/Hb.

The Beers and Sizer method [15] was used to determine the catalase activity. This method is based on the measurement of the absorbance decrease of hydrogen peroxide which is decomposed by catalase, measured at a wavelength of 240 nm. CAT activity is expressed in IU/g Hb.

The level of conjugated dienes (CD) was determined in erythrocytes and in plasma according to Sergent *et al*. [16]. The basis of this method is the measurement of a characteristic peak of absorbance at a wavelength of 233 nm. The CD concentration was expressed in units of absorbency per millilitre of plasma (Abs/ml) and in units of absorbency per g Hb (Abs/g Hb).

The concentration of thiobarbituric acid reactive substances (TBARS) was determined in erythrocytes and in plasma using the Buege and Aust method [17]. The creation of a coloured complex between the products of lipid peroxidation and thiobarbituric acid at +100°C in an acidic medium was evaluated. The maximum absorption of this complex occurs at a wavelength of 532 nm. The majority of TBARS is MDA; therefore the TBARS concentration was expressed in nmol MDA/g Hb in erythrocytes and in nmol MDA/ml in blood plasma.

Activity of lysosomal enzymes was assayed according to the methods previously described by Wozniak *et al*. [3]. For determination of the activity of acid phosphatase (AcP), *p*-nitrophenylphosphate disodium (substrate) was used in a 0.5 M buffer of

citric-tartrate formaldehyde with a pH = 4.9. The activity of cathepsin D (CTS D) was determined using 2% denatured bovine haemoglobin diluted in 100 ml 0.1 M citric-phosphate buffer with a pH of 3.8 as a substrate. The measure of the arylsulphatase (ASA) activity was the quantity of 4-nitrocatechol (4-NC) released during the enzymatic hydrolysis of the substrate. 0.01 M of 4-*p*-nitrocatechol sulphate (NCS) in a 0.5 M acetate buffer with a pH of 5.6 was used as a substrate for the test. The activity of AcP, CTS D and ASA is expressed in nanokatals (nkat).

The reagents used for measurements were obtained from Sigma (Sigma-Aldrich Polska). The measurements of sample absorbance were made with a CARY 1E UV-Vis spectrophotometer controlled by the Cary WinUV software (Varian Inc., Palo Alto, USA). For evaluation of total CK activity, ready-made reagent kits from the Alpha Diagnostics Company (Warsaw, Poland) were used. The concentration of cortisol was determined by a method based on chemiluminescence using an automated device for immunological determinations – Immulite (Diagnostic Products Corporation, Los Angeles, USA).

Statistical analysis

All data were statistically analysed by means of an ANOVA test. Statistical significance was accepted at a value $p < 0.05$. Statistical analysis was performed using Statistica software, version 5.0 PL (StatSoft Polska Sp. z o.o., Krakow, Poland).

Results

Comparison of the studied parameters after training preceded by WBC and training without WBC

When training with WBC and without WBC was compared, the activity of SOD was found to be 44% lower ($p < 0.01$) and GPx 42% lower ($p < 0.05$) after the third day of training with WBC (Table III). The concentration of CD in the rowers' plasma after the third day of training with WBC was 35% lower than after the third day of training without WBC ($p < 0.05$). After the third day of training with WBC the level of CD in erythrocytes was twice as low ($p < 0.01$), and after the sixth day 34% lower ($p < 0.05$) than after training without WBC, while the concentration of TBARS in erythrocytes after the sixth day was more than 50% lower ($p < 0.001$) than after training without WBC. The activity of AcP in the rowers' blood serum was more than 50% lower after the third day of training with WBC than after the third day of the training cycle without WBC ($p < 0.05$).

Changes of oxidative stress markers after training without WBC

The activity of GPx in the erythrocytes of the rowers increased almost twofold ($p < 0.01$) and SOD by 74% ($p < 0.01$) after the first 3 days of training without WBC as compared with the activity before training started (Table III). After the sixth day of training without WBC the activity of both enzymes had

Table III. The activity of antioxidant and lysosomal enzymes, and creatine kinase as well as the concentration of lipid peroxidation products and cortisol in serum of rowers after training without and preceded by whole-body cryostimulation (WBC) (mean ± SD)

Parameter	Before training	Without WBC		With WBC	
		After third day	After sixth day	After third day	After sixth day
SOD [10^2 U/g Hb]	11.4 ±2.4	19.8 ±0.5^{aa}	16.7 ±4.1^{a}	11.0 ±0.4^{bbc}	12.5 ±2.4^{b}
CAT [10^4 IU/g Hb]	65.0 ±10.0	81.8 ±6.9	75.0 ±17.1	81.0 ±20.5	83.0 ±24.2
GPx [U/g Hb]	11.0 ±3.9	22.0 ±3.9^{aa}	23.4 ±3.8^{aaa}	12.8 ±1.5^{bccc}	19.3 ±3.2^{aa}
CD in plasma [10^{-1} Abs/ml]	13.6 ±1.5	19.6 ±0.8	14.0 ±3.4	12.7 ±4.2^{b}	13.1 ±1.9
CD in eryth. [10^{-2} Abs/g Hb]	10.3 ±2.0	19.9 ±0.7^{aa}	18.0 ±3.7^{aa}	10.0 ±2.2^{bbcc}	11.8 ±3.4^{bc}
TBARS in plasma [10^{-1} nmol MDA/ml]	4.4 ±0.6	4.7 ±0.5	4.6 ±0.8	4.6 ±0.6	4.2 ±0.8
TBARS in eryth. [nmol MDA/g Hb^{-1}]	29.1 ±6.4	33.2 ±4.1	45.5 ±8.7^{aa}	17.3 ±4.9^{accc}	20.3 ±6.8^{ccc}
AcP (nkat)	16.6 ±5.6	23.2 ±0.5	15.1 ±9.2	10.1 ±2.1	10.0 ±1.1^{b}
ASA (nkat)	23.2 ±5.2	33.9 ±8.8	20.6 ±7.0	25.1 ±7.8	26.6 ±3.2
CTS D (nkat)	654.4 ±98.3	669.1 ±56.8	783.2 ±132.5	665.2 ±179.2	660.1 ±102.7
CK [IU/l]	62.0 ±17.7	138.3 ±33.2^{a}	155.0 ±15.9^{aaa}	103.7 ±28.1	107.0 ±37.8
Cortisol [µg/dl]	17.9 ±0.8	22.4 ±0.2^{aa}	20.3 ±0.3	19.8 ±1.4	20.3 ±1.6^{a}

Difference versus before training: $^{a}p < 0.05$; $^{aa}p < 0.01$; $^{aaa}p < 0.001$. Difference versus after third day of training without cryostimulation: $^{b}p < 0.05$; $^{bb}p < 0.01$; $^{bbb}p < 0.001$. Difference versus after sixth day of training without cryostimulation: $^{c}p < 0.05$; $^{cc}p < 0.01$; $^{ccc}p < 0.001$
SOD – superoxide dismutase, CAT – catalase, GPx – glutathione peroxidise, CD – conjugated dienes, TBARS – thiobarbituric acid reactive substances, AcP – acid phosphatise, ASA – arylsulphatase, CTS D – cathepsin D, CK – creatine kinase

not changed and was still higher than before training. The activity of GPx increased by 65% ($p < 0.01$) after the sixth day of training with WBC as compared with the activity before training started. The concentration of CD in erythrocytes increased by 93% ($p < 0.01$) after the first three days of training without WBC and TBARS by 56% after 6 days ($p < 0.01$). The CD content in erythrocytes after 6 days, on the other hand, decreased slightly, but it was still 75% higher than the pre-training value ($p < 0.01$). WBC led to a 41% decrease in the level of TBARS in erythrocytes in the rowers' blood after the first 3 days of training exercises ($p < 0.05$).

Values of CK activity and cortisol level in studied subjects

The CK activity in the serum increased more than twofold after the first three days of training without WBC ($p < 0.05$). After the sixth day of training the activity of CK increased slightly and was 2.5 times higher than before training started ($p < 0.001$). No statistically significant changes in CK activity were found after the training with WBC. The concentration of cortisol in the serum after the first 3 days of training without WBC increased by 25% ($p < 0.01$) and after the sixth day of training with WBC was 13% higher than before training ($p < 0.05$).

Discussion

When both types of training are compared, a lower activity of SOD and GPx in the erythrocytes of the rowers was found after the third day with a simultaneous lower concentration of lipid peroxidation products when training was preceded by two WBC sessions per day. This suggests that the action of extremely low temperatures facilitates the maintenance of the prooxidant-antioxidant balance. Similar results were obtained in earlier research on kayakers [4] who performed a ten-day training cycle with two training sessions and three WBC procedures per day. The WBC is supposed to stimulate the generation of ROS *per se* [4, 18, 19]. Extensive formation of oxygen free radicals after exposure to a short-term intensive cold stimulus was previously reported in healthy volunteers [20, 21]. Wozniak *et al.* [4] imply that oxidative stress induced by the action of extremely low temperatures brings about beneficial adaptive changes in the organisms of kayakers and protects against disturbance of the prooxidant-antioxidant balance during training. Such an adaptive response to different stress factors seems to be common and for instance various noxious stimuli are known to be able to induce tolerance in the brain against subsequent deleterious stimulus of the same or even another modality [22]. Considering the results presented in this paper we can state that application of two sessions in the cryo-chamber per day is sufficient to obtain similar results to those after three exposures to extremely low temperatures per day.

In the present studies we revealed lower concentration of both investigated lipid peroxidation products, TBARS and CD, in erythrocytes after the training with whole-body cryostimulation as compared to the training cycle without WBC, while in blood plasma only the CD level was lower when the exercise was preceded by exposure to extremely low temperatures. Erythrocytes transport oxygen to tissues and exercise-induced oxidative stress increases erythrocyte damage for example by erythrocytes' membrane lipid peroxidation [23]. Decreased levels of lipid peroxidation products may testify to the fact that WBC diminishes the erythrocyte oxidative damage. The literature data about changes in concentrations of plasma lipid peroxidation products are unequivocal, because the concentration of TBARS and CD in blood plasma depends not only on their production but also on the rate of their removal from damaged tissues and the level of their decomposition. TBARS for example is supposed to be metabolized in the liver and in trained muscle tissue [23]. These facts may also explain the differences in erythrocyte and plasma concentrations of lipid peroxidation products under the influence of WBC.

When the training was preceded by WBC no statistically significant changes in the activity of CK were found. The obtained results indicate that WBC reduces the extent of injury to the muscle fibres. This fact is also confirmed by lower activity of AcP after the third day of training with two WBC procedures per day than after training without WBC. The beneficial effect of stimulation using extremely low temperatures on the maintenance of prooxidant-antioxidant balance that has been demonstrated in this paper may be responsible for the stabilizing effect of WBC on membranes of both muscle cells and lysosomes. Hypothermia is also known to protect the blood-brain barrier integrity, thus reducing the passage of potentially harmful substances across the endothelial barrier [24]. Possible mechanisms of this neuroprotective action probably include the decreased generation of reactive oxygen species that may partly ensue from attenuation of leukocyte accumulation due to hypothermia [24].

The decreased leakage of intracellular enzymes into the blood after training with WBC may also be the result of increased synthesis of heat shock proteins (HSP). These proteins protect cells against damage caused by ROS [25]. In response to the stress caused by a low temperature an increase in the level of HSP70 occurred in the brown adipose tissue and in the aorta [26]. The expression of HSP is probably induced by adrenalin, which is released during exposure to cold. An increase in the concen-

tration of adrenalin is one of the responses of the organism after WBC [1].

When training was associated with WBC an increase in the concentration of cortisol was noted only after the sixth day. Intensity and duration of training contribute significantly to changes in cortisol concentration in circulating blood [12]. The delayed reaction of the organism to exercise after training with WBC confirms that the action of extremely low temperatures alleviates stress.

In conclusion, preceding rowers' training sessions with WBC reduces the risk of oxidative stress induced by exercise. Extremely low temperatures alleviate physical stress and reduce the extent of injury to muscle fibres. The results obtained in this paper indicate that whole-body cryostimulation is an effective and safe method for limiting exercise-induced damage and practical introduction of WBC to sport medicine may widely enrich the treatment applied so far, especially focused on biological regeneration. Yet the study was performed on limited number of elite rowers and those results must be confirmed.

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