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Changes in chosen immune system indicators and the level of HSP-70 after single whole-body cryostimulation in healthy men

BARBARA SZPOTOWICZ-CZECH[1], MAGDALENA WIECEK[2], JADWIGA SZYMURA[3], MARCIN MACIEJCZYK[2], ZBIGNIEW SZYGULA[4]

[1]Institute of Physical Culture, State University of Applied Sciences, Nowy Sacz, Poland
[2]Department of Physiology and Biochemistry, Faculty of Physical Education and Sport, University of Physical Education, Krakow, Poland
[3]Department of Clinical Rehabilitation, Faculty of Motor Rehabilitation, University of Physical Education, Krakow, Poland
[4]Department of Sports Medicine and Human Nutrition, Faculty of Physical Education and Sport, University of Physical Education in Krakow, Krakow, Poland

Abstract

Aim of the study: *The aim of our research was to examine the influence of single whole-body cryostimulation (WBC) on chosen immune system indicators including the heat shock protein HSP-70.*

Material and methods: *The study was carried out among ten young and healthy men (mean age 22.4 ±1.65, with a body mass index of 22.91 ±2.39 kg/m^2). The participants were subjected to single whole-body cryostimulation (at –130°C temperatures) in a special cryogenic chamber for 3 minutes. Blood samples were collected three times: before cryostimulation, 30 minutes and 24 hours after WBC. Immunoglobulins (IgA, IgG, IgM), interleukins (IL-6, IL-10, IL-1β) and the heat shock protein (HSP-70) were determined in the blood serum.*

Results: *As a result of a single exposure to cryogenic temperatures, a significant increase in the level of IL-6 was observed 30 minutes after the WBC ($p < 0.05$) and a decrease in the level of HSP-70 24 hours after the treatment ($p < 0.05$). There were no significant changes in the level of interleukins (IL-10, IL-1β) or immunoglobulins 30 minutes after a single WBC treatment or 24 hours later.*

Conclusions: *Detailed analysis of the issue shows that a single application of whole-body cryostimulation causes a small, modulating effect on the IL-6 level. Single whole-body cryostimulation treatment has also a slight silencing effect on the HSP-70 level in healthy, young men. Reduction in the concentration of HSP-70 24 hours after WBC may indicate lack of the damaging impact on the spatial structure of the protein due to cryogenic temperatures.*

Key words*: cold exposure, cryogenic temperatures, cold-stress response immunology, interleukin, immunoglobulins, heat shock proteins-70.*

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Introduction

Studies examining the effects of cryogenic temperatures on the human body were initiated more than thirty years ago. A short treatment time of 2-3 minutes in temperatures below –100°C, in a special cryogenic chamber, is used both in patients and athletes. It was noticed that physiological stress induced by short exposure to cryogenic temperatures influences a number of systems in the human body: nervous-muscular, hormone [1], cardiovascular [2] and immune system [3]. This also affects the prooxidant-antioxidant balance in healthy participants [3, 4] and patients with multiple sclerosis (MS) [5]. Whole-body cryostimulation (WBC) is also used in the biological regeneration of athletes (treatment of post-trauma changes, signs of overburdening, decreasing inflammation and muscle relaxation) [6-8].

One of the body's defence mechanisms against heat loss is contraction of the peripheral blood vessels which is regulated by the sympathetic nervous system (SNS). In contrast, activated SNS mediates and affects, among others, modulation of the immune response [9]. Various aspects of the impact of WBC on the human body were examined but unequivocal findings and lack of clarity in the results of individual authors are noticed in studies concerning the effects of WBC on the immune system. There is a general belief that cold stimulates the immune system [9]. Immunomodulatory actions of cold exposure confirm the

Correspondence: Prof. Zbigniew Szygula, Department of Sports Medicine and Human Nutrition, Faculty of Physical Education and Sport, University of Physical Education, 78 Jana Pawla II Ave., 31-571 Krakow, Poland, e-mail: wfszygul@cyf-kr.edu.pl
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work of several authors [3, 4, 10, 11]. Nonetheless, studies in which stimulation of the immune system did not occur were also conducted [6, 12].

There is also controversy regarding the effect of cold exposure on the level of selected interleukins which are regulators of human body responses to infection, immune responses, inflammation, and their main functions are T lymphocyte and macrophage activation, differentiation of B lymphocytes and modification of inflammatory response [13, 14]. Most of the available papers describe the effects of WBC on the level of selected interleukins (increased IL-6, IL-10, IL-1ra; reduction in the level of IL-1α, IL-1β, IL-2, IL-8, TNF-α, CRP [3, 4, 6-8], or no change in the level of IL-6, IL-1β, TNF-α (tumor necrosis factor-α) [7, 15]. However, in the vast majority of prior research, the effect of WBC was studied among athletes. For this reason, we decided to mark these selected interleukins in healthy, non-trained men in our research.

Extracellular immunoglobulin (Ig) and T cell receptors take part in the recognition of foreign antigens and protection of the body against extracellular dangers. Immunoglobulins, comprising a group of glycoproteins, may be found in serum as well as body fluids and are an element which recognizes the humoral immune response. In the literature, very few studies evaluating the influence of WBC on the level of immunoglobulin can be found. Among the available publications, studies conclude a lack of changes in the group of athletes [16] or their reduction in patients with ankylosing spondylitis due to WBC usage [17]. In this study, it was decided to determine the level of three immunoglobulin classes among the five existing ones. Immunoglobulin M, which first appears after contact with an organism's antigen, IgG belonging to one of the most important serum antibodies and immunoglobulin A – the main antibody class secreted externally [18].

Heat shock proteins (HSP) function as protection of other proteins from abnormal changes, acting as chaperone proteins. The name of these proteins was established due to the conditions in which they were first described by the Italian geneticist Ferrucio Ritosso, discovering them accidentally in *Drosophila* (*Drosophila melanogaster*) at an elevated temperature in an incubator [19]. This resulted in increased synthesis of the HSP family of proteins, which all work to protect cells from harmful environmental conditions, silencing or weakening the effects of stressors [20-22]. Enhanced expression of HSP genes is also induced by physiological processes, as well as pathophysiological conditions such as ischemia, acidosis, oxidative stress, UV radiation, bacterial and viral infections, heavy metals and others [23, 24]. Proteins from the HSP-70 family are among the most well-known heat-shock proteins. In stressful conditions, these proteins combine with polypeptides of abnormal structure, take part in the development of abnormally collapsed or damaged proteins, prevent denaturation and aggregation, allowing denatured proteins to return to their normal structure [25]. These proteins protect the cardiac muscle, skeletal muscles, lungs and liver against damage caused by ischemia and reperfusion. In the literature, there are no reports on the effects of extreme cold (from –110°C to 130°C) on synthesis of the HSP-70 protein in the human body and to what extent it occurs at all. Given that exposure to very low temperatures is stressful for the body, it can be expected that there will also be variations in the level of the HSP-70 protein. However, the literature on the subject does not supply any reports of this nature.

The aim of the study was to investigate the early and late effects of a single cryostimulation treatment on the level of immunoglobulin classes A, G and M, interleukins (IL-6, IL-10, IL-1β) and the heat shock protein HSP-70 in healthy men.

Material and methods

Participants of the study comprised 10 young men aged 20-25 (mean age 22.40 ±1.65, with a body mass index of 22.91 ±2.39 kg/m^2). All participants were volunteers who did not use any stimulants during and two weeks before the experiment. They familiarized themselves with written instructions about the research goals and procedures, and signed written consent stating conscious and voluntary participation in the study. The proposed research methodology was accepted by the Bioethics Commission at the Regional Medical Chamber in Krakow.

The participants underwent medical screening prior to entering the cryochamber.

The screening involved measurements of systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR); an electrocardiography reading (EKG); and an interview that aimed to eliminate any contraindications to whole-body cryostimulation.

The following somatic traits were measured in all participants prior to the experiment: body height (BH), body mass (BM), fat percentage (FP), fat mass (FM) and lean body mass (LBM). Table 1 and 2 show the characteristics of the study participants.

Whole-body cryostimulation procedure

Ten men underwent a single WBC session at the Malopolskie Province Cryotherapy Center in Krakow. Before entering the cryochamber, the participants were asked to dry their skin thoroughly with a towel to remove sweat, since sweat could cause an acute feeling of cold. Each participant was provided with a surgical gauze mask, ankle-high woolen socks, warm knee protectors, gloves, a band or cap to protect the ears, wooden clogs and shorts. After preparing themselves, the participants entered the pre-chamber in groups (2-4 volunteers) for about 30 seconds in the temperature of –60°C. Next, they entered the main chamber, walking in a circle one behind the other for

Table 1. Characteristics of examined subjects

Variables	Mean ±SD	Min.	Max.
Age (years)	22.0 ±1.65	20.0	25.0
Height (cm)	179.0 ±4.62	170.0	187.0
BM (kg)	73.0 ±8.96	64.0	89.0
BMI (kg/m^2)	23.0 ±2.39	20.0	27.0
PF (%)	14.0 ±4.25	8.0	22.0
FM (kg)	11.0 ±3.96	5.0	16.0
LBM (kg)	63.0 ±6.44	55.0	73.0
SBP (mm Hg)	118.00 ±10.59	105.0	135.0
DBP (mm Hg)	74.00 ±7.38	60.0	85.0
HR (bpm)	73.20 ±8.65	60.0	88.0

The values are mean ±standard deviation
BM – body mass, BMI – body mass index, PF – percentage of fat, FM – fat mass, LBM – lean body mass, SBP – systolic blood pressure, DBP – diastolic blood pressure, HR – heart rate

three minutes in the temperature of about −130°C and under camera supervision. The three minutes later, the participants moved to a room with a temperature of about 19°C, where 30 minutes after WBC venous blood was collected.

Blood tests and biochemical analysis

The venous blood sampling procedure was performed three times: before the visit in the cryogenic chamber (I), 30 minutes after the WBC treatment (II) and 24 hours after the treatment (III). Blood samples were taken in accordance with applicable standards between 2:00-4:00 p.m. from a vein in the elbow joint in a volume of 6 ml by a sports physician or laboratory diagnostician, after a 10-minute rest in a sitting position. BD Vacutainer Systems Vacuum CAT 6.0 ml volume were used for the sampling of venous blood. The men taking part in the study could not perform any physical activity 24 hours prior to the blood collection.

IL-6, IL-1β, IL-10, HSP-70 were determined by enzyme immunoassay – high-sensitivity ELISA kits. Interleukins were determined using a kit from R&D Systems. Test sensitivity: IL-6 = 0.039 pg/ml; IL-10 = 0.09 pg/ml; IL-1β = 0.057 pg/ml. Coefficient of variation (CV) of intra-assay for IL-6 < 7.8%, IL-10 < 9.4%, IL-1β < 10.2% and between tests (inter-assay) IL-6 < 7.2%, IL-10 < 8.5% and IL-1β < 10.4%.

Determination of IgA, IgG and IgM was performed with the immunoradiometric method by the ARCHITECT *c* System analyzer using the tests: Immunoglobulin A (Limit of Quantitation (LOQ) ≤ 0.03 g/l; Total CV ≤ 4.1%), Immunoglobulin G (LOQ ≤ 0.061 g/l; Total CV ≤ 3.4%), Immunoglobulin M (LOQ ≤ 0.02 g/l; Total CV ≤ 4.4%); kits from Abbott Laboratories. This method consists in measuring the increasing turbidity of the sample caused by the formation of insoluble immune complexes after adding antibodies against IgA, IgG, IgM, respectively, to the sample.

Table 2. Morphological blood indicators of examined subjects before WBC

Variables	Mean ±SD	Min.	Max.
Leukocytes (10^9/L)	5.56 ±1.28	4.0	7.80
RBC (10^{12}/L)	5.35 ±0.28	4.90	5.90
Hb (g/dL)	16.06 ±0.74	14.50	17.40
Hct (%)	52.39 ±3.91	44.57	59.20
MCV (fL)	86.60 ±2.67	82.0	90.0
MCH (pg)	30.10 ±0.88	28.0	31.0
MCHC (g/dL)	34.61 ±0.84	33.20	35.90
PLT (10^9/L)	227.40 ±49.14	153.0	315.0
NEUT (10^9/L)	3.22 ±0.96	1.93	5.27
LYMPH (10^9/L)	1.61 ±0.51	1.24	1.87
MONO (10^9/L)	0.49 ±0.11	0.34	0.69

The values are mean ±standard deviation
RBC – red blood cells, Hb – hemoglobin, Hct – hematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, PLT – platelets, NEUT – neutrophils, LYMPH – lymphocytes, MONO – monocytes

HSP-70 was determined using the kits: WUHAN EIAB Science; test sensitivity = 0.039 ng/ml; detection range: 0.15-10.0 ng/ml.

Leukocytes, lymphocytes, monocytes, neutrophils and platelets were determined with the fluorescence flow cytometry method using a semiconductor laser. RBC, MCV, MCH and MCHC were determined using hydrodynamic focusing (HDF) and the DC impedance method (conductometric method). Hemoglobin was determined via the SLS method – this method utilizes the sodium lauryl sulphate (SLS: $C_{12}H_{25}SO_4Na$) surfactant. All the above indices were determined with the automated XT-2000i analyzer. Hematocrit was determined using the microhematocrit method immediately after the collection of blood from subjects.

In this paper, it was decided to correct all the examined indicators taking into account the changes in plasma volume after the treatment. The calculation of changes in plasma volume ΔPV was done on the basis of changes in concentration of total protein levels 30 minutes and 24 hours following WBC. Protein concentrations were determined using the biuret reagent (Roche reagent – Hitachi, Japan), Cobas 6000 analyzer.

The following formula was used for ΔPV calculation [26]:

$$\Delta PV = -100*[(P_f - P_i)/(P_f)]$$

P_i – initial protein level before WBC treatment

P_f – final protein level after WBC treatment

The formula by Kraemer and Brown [27] was used for final corrections of the examined parameters:

$$V_c = (\%\Delta PV*0.01*V_a) + V_a$$

V_c – corrected value

V_a – value after WBC

Statistical analysis

Statistical analysis of the obtained results were performed using an MS Excel spreadsheet and the STATISTICA 10 software package. Basic numerical characteristics of the analyzed variables – that is, arithmetic mean and standard deviations – were determined. After the normal distribution of the results was assessed, the significance of differences was determined using Friedman ANOVA and the significance of differences between each pair of data was determined using the Wilcoxon matched-pairs test. Statistical significance was accepted at $p < 0.05$.

Results

Initial values of blood morphological indicators were within normal limits (Table 2). We observed only a small increase in plasma volume 30 minutes after the first treatment (% ΔPV = 0.94) and 24 hours later (% ΔPV = 1.03), but these changes were not significant.

Table 3 shows a comparison of the results obtained before the WBC treatment and 30 minutes as well as 24 hours after its completion.

In the present study, all immunoglobulin levels were within normal limits prior to the WBC, and all the studied males were healthy.

Venous blood collected 30 minutes after the single WBC treatment showed no significant changes in the level of the examined indicators (IgG, IgM, IgA, IL-10, IL-1β, HSP-70) – Table 3. One WBC treatment caused an increase in the level of IL-6 30 minutes after WBC ($p < 0.05$).

24 hours after the single WBC treatment, levels of the HSP-70 heat shock protein significantly decreased ($p < 0.05$). In blood taken 24 hours after the single WBC procedure, there were no significant changes in the level of the studied immunoglobulin classes: IgA, IgG, IgM and IL-6, IL-10, IL-1β (Table 3).

Discussion

In the present study, we try to answer the question of whether single exposure to cryogenic temperatures affects the level of individual immunological blood indicators and the level of heat shock proteins. In examining the effect of a single WBC treatment on the human body, we want to know whether the use of only a 3-minute treatment is beneficial and sufficient for safe systemic stimulation for healthy participants, as well as athletes.

Mainly three systems (and their mutual cooperation) take part in the body's response to cold: the nervous, endocrine and immune system. Cold-induced vasoconstriction is a protective insulating physiological response regulated by the sympathetic nervous system, which reduces heat loss [28, 29]. To maintain heat balance in a cold environment, the body releases heat generating hormones (catecholamines, triiodothyronine) [30] which may cause heat generation in a non-shivering manner. In order to stimulate metabolism, reflex stimulation of skeletal muscles and the development of muscle tremors are often also necessary. Exposure to cold causes, among others: increase in activity of the adrenergic neurotransmitter, i.e. norepinephrine, α2-adrenergic receptors [28, 29] and increased cortisol secretion [31, 32]. Lymphatic organs are also innervated by sympathetic noradrenergic nerve fibres, and α-adrenergic receptors can be found on a substantial majority of lymphoid cells (except for T-helper cells) [33-35].

IL-6, IL-10, IL-1β

The endocrine and immune systems are the two main systems which are involved in the body's response to cold. In this study, it was decided to designate selected pro- and anti-inflammatory cytokines because of the important role played by cytokines in mutual two-way interaction between the endocrine and the immune system [36]. The interaction of hormones and cytokines during thermal stress may affect homeostasis of the immune response and balance between pro- and anti-inflammatory cytokines [37-39].

IL-6 is a multi-directional, pleiotropically acting cytokine involved in B lymphocyte proliferation [40]. It participates not only in inflammatory reactions and infections but also in the regulation of metabolic processes and nerve regeneration [41]. Its most important functions are participation in the immune response, hematopoiesis and inflammatory response [42, 43]. IL-6, independent of TNF-α, also influences induction of the most important anti-inflammatory cytokine IL-10 [3, 4].

IL-10 is a cytokine which in effect, contributes to the inhibition of the cellular immunological cell type and inflammatory responses, and is produced by activated T cells, predominantly Th2 helper cells, B cells, monocytes

Table 3. Significant changes in chosen immune system indicators in response to single whole-body cryostimulation in young men

Variables	Before WBC	30 minutes after WBC	24 hours after WBC
IgG (g/l)	9.99 ±2.41	9.96 ±2.54	9.92 ±2.61
IgM (g/l)	0.77 ±0.24	0.74 ±0.23	0.77 ±0.23
IgA (g/l)	1.59 ±0.60	1.57 ±0.59	1.56 ±0.59
IL-6 (pg/ml)	0.62 ±0.12	0.73 ±0.15*	0.70 ±0.20
IL-10 (pg/ml)	0.95 ±0.26	0.93 ±0.21	0.80 ±0.30
IL-1β (pg/ml)	1.31 ±0.28	1.37 ±0.34	1.48 ±0.26
HSP-70 (ng/ml)	1.07 ±0.04	1.04 ±0.07	0.91 ±0.16**

The values are mean ±standard deviation
Ig – immunoglobulin, IL – interleukin, HSP – heat-shock protein
Statistically significant difference at $p < 0.05$
**significant differences between before WBC – after WBC*
***significant differences between before WBC – 24 hours after WBC*

and macrophages [44]. IL-1β, however, is an interleukin which acts systemically. It is secreted into the blood and synthesized similarly to IL-10 by monocytes and macrophages [39, 45].

Exposure to cold causes the release of catecholamines, and it was found that adrenaline and noradrenaline can reduce the inflammatory response, particularly through monocytes, macrophages and T lymphocytes, resulting in weakened synthesis of pro-inflammatory cytokines (TNF-α, IL-1β, IL-12) and an increased level of anti-inflammatory cytokines (IL-10, IL-1ra) [37]. According to our results, whole-body cryostimulation applied only once increase the IL-6 level 30 minutes after WBC and has no significant effect on modulating the levels of IL-10 and IL-1β in the blood serum of healthy young people. Similarly to our results, the level of IL-6 significantly increased both 30 minutes after a single treatment (–130°C/3`) and after the series, having a mobilizing effect on the immune system [3]. Similar changes were observed in males training tennis due to the use of the WBC twice a day for 5 days (–120°C/3`), causing a significant increase in IL-6 and decrease in TNF-α [8]. It is probable that the increased frequency of treatments had a significant impact on the level of secreted cytokines. Ziemann *et al.* [8] suggest that perhaps explanations for this should be sought in one of the thermoregulatory mechanisms which are involuntary contractions of working muscles, during which just like during exercise levels of IL-6 increase. Also it indicates that with the increase of IL-6 in the working muscles cortisol increases [8]. Cortisol is the main glucocorticoid in the human body, released in response to various stressful situations that affect inter alia the inhibiting of the immune response [46].

As stated in the work of Pournot *et al.* [7], cryostimulation can be effective in reducing the inflammatory process caused by exercise. They reported that an hour after application of WBC there was a decrease in IL-1β and increase in IL-1ra compared to the control group, while TNF-α, IL-6 and IL-10 remained unchanged. In turn, the use of the WBC over a 12-week period (–110°C/2`), 3 times a week in non-trained persons did not cause any change in the level of IL-1β, and similarly in the levels of IL-6 and TNF-α [46]. Similarly, in the work of Selfe *et al.* [15], there were no significant changes in the level of IL-6 in 14 rugby players in 1`, 2` or 3` treatments at –135°C. Perhaps the differences in the results of the said authors should be associated with the particular participant's different adoptive abilities to adapt to the low body temperature and their response to the situation in which they are, often "stressful situations". As observed by Hausswirth *et al.* [47], the thermal comfort of the participants during WBC (–110°C/3`) was described as "uncomfortable" and maintained for 20 minutes post the applied treatment [47].

A series of experiments indicating changes in the level of individual interleukins were carried out in a sports environment, in which WBC is used to treat post-traumatic changes, signs of overburdening, in order to speed up tissue healing, reduce inflammation and facilitate muscle relaxation [6, 16].

In the study by Rhind *et al.* [31], on the first day of the experiment, exposure to cold (cold water immersion – CWI) caused changes in the level of IL-1β and TNF-α, suggesting a dominant influence of α-adrenergic mechanisms. After 7 days of physical activity, on the 8th day of the experiment, TNF-α and IL-1β secreted by monocytes decreased and the expression of IL-1ra increased, indicating the predominance of β-adrenergic mechanisms. The authors postulate that these changes were induced by exposure to cold, but preceded by 7-day strenuous physical activity. Therefore, it is extremely difficult to compare works exploring the effects of WBC on the level of individual indicators in healthy, non-trained individuals with studies devoted to evaluation of the efficacy of WBC in the process of recovery after exercise in athletes or with studies researching the influence of WBC on ill subjects.

IgG, IgM, IgA

Immunoglobulins play a very important role in the body's defence mechanisms. IgM, IgG and IgA are three classes of immunoglobulins which share a common idiotype. They have an identical variable domain of the light and heavy chain against the same antigen [48]. The antigens that appear in the body are recognized by Th cells which proliferate, release and stimulate B cells to produce antibodies. Attention should be paid to the close cooperation of T as well as B lymphocytes and other cells in humoral type response [49].

In the studied group of healthy individuals, as a result of single WBC treatment, no significant changes in the levels of the tested IgG, IgM and IgA were noticed. Perhaps there is a need for long-term stimulus to induce significant changes. In the literature, there are no studies on the effect of cold cryogenic action on immunoglobulin levels in healthy and/or non-trained subjects. In a group of athletes, Banfi *et al.* [6] investigated the influence of 5 repeated WBC treatments (–110°C/2`) and similarly, did not notice any changes in the level of IgG, IgM, IgA. Whereas Sieroń *et al.* [17] studied the effect of 10 WBC treatments (–130°C/2`) in patients with ankylosing spondylitis. They found a significant reduction in the level of IgA and IgG, explaining that the decrease in the concentration of acute phase proteins and immunoglobulins in these patients may indicate the anti-inflammatory effects of WBC. Decreases in the levels of IgA and IgM were recorded during the first 4 months of a year-long Antarctic expedition. These changes were associated with stress in connection with participation in the expedition, while no infections of the upper respiratory tract were found [50]. Due to lack of papers describing the impact of single WBC treatments on

the level of immunoglobulins, no references to the results of other authors can be made. The increase in IgG after a single immersion in cold water up to the chest in athletes for 1 hour and in the water temperature of 14°C was only examined by Jansky *et al.* [51].

HSP-70

To the best of our knowledge, this is the first study to evaluate the influence of cryogenic temperatures on the level of the heat shock protein (HSP-70) in the human body. So far, there have been only a few research papers on the effect of cold on the level of heat shock proteins, mainly in fish, mice and insects [52-55]. Most authors examine the effect of hypothermia on prokaryotic and eukaryotic organisms. In contrast, it should be emphasized that whole-body cryostimulation does not cause hypothermia of the human body [15].

It has been demonstrated that HSP mRNA was actively produced in the erythrocytes of brown trout which were subjected to heat shock [56,57] and in rainbow trout red blood cells after exposure to 25°C for 2 hours, after previous month-long adaptation to 9-11°C temperatures [52]. In mice subjected to whole body cooling (2-3°C temperature for 8 hrs) a cellular response to stress was induced. This resulted in an increased expression of HSP-72 mRNA in the brain, heart, kidneys, liver and lungs, with a decrease in body temperature by 2.5°C [53]. In addition, in Drosophila larvae [54] and Leuconostoc esenteroides [55], in response to the cold, the thermal-shock protein is synthesized during the period of thermal regeneration after hypothermia, which is necessary in order for the maximum stimulation of the HSP expression. Also, in mice after 1 hour at normal temperature, there was a large increase in the synthesis of HSP-72 mRNA. It has been suggested that induction of heat shock proteins can occur as a result of the transition from 0°C to 25°C rather than as a result of the reaction to applying 0°C temperature [53]. In addition, exposure of 5-day-old larvae to 0°C for less than 8 hours did not cause any induction of heat shock proteins, however, 11-16 hours proved to be optimal to increase HSP synthesis [54]. Liu *et al.* [58] studied the induction of HSP genes in human diploid fibroblast cells previously incubated at 4°C. HSF (heat shock factor) activation, increase in the number of HSP transcripts and increased synthesis of this protein were all results of increasing the temperature to 37°C. Not only exposure to low temperature but also its increase activates this response. It is postulated that perhaps during exposure to cold, metabolism slows down but after exposure, the body of the animal regenerates itself and begins to produce new proteins, or in order to obtain induction of HSP, a specific type of cell damage is needed [53, 54]. The authors indicate that the effects of cold cause denaturation of proteins and denatured proteins can lead to the induction of HSP, which in response to cold, is manifestation of damage to the cells caused by the low temperature. HSP will be helpful in unfolding improperly folded proteins.

The mechanism by which the effects of cold induce HSP genes is not well understood, however, it is described that there are two mechanisms that may be responsible for induction of HSP genes: the damaging effect of cold temperature on cells or an increase in oxygen free radicals [58]. The HSF1 protein mediates in stress induced by cold but does not exhibit the characteristic hyperphosphorylation of protein observed for the HSF1 protein activated by heat stress [59], and thus mechanisms of inducing cellular stress as a result of cold or heat may differ [53].

The results obtained in this study are different from the results of the research described above. It is difficult to refer to the works of other authors because of applied designation methodology, study material and interspecies differences. Perhaps a 3-minute treatment at –130°C is not a sufficient amount of stress on the body to activate chaperone proteins in order to prevent the formation of defects in the spatial structure of the protein. What is puzzling is the reduction in the level of HSP-70 observed 24 hours after treatment. Perhaps the effects of cold caused the "silencing" or "extinguishing" of the HSP gene transcription. It is also possible that under the influence of the cryogenic cold, cortisol levels increased in subjects resulting in suppression of HSP-70, which was also found in studies on fish that were exposed to a temperature of 12°C [60]. Referring to the studies of other authors, regeneration and increased synthesis of chaperones should occur after exposure to cold. The opposite direction of changes was observed in this study.

Due to the different results of individual authors and no clear conclusion, it is still not apparent what effects the modulation the immunological response under the influence of WBC, whether it impacts the use of cryogenic temperatures and direct effect of cooling and if the stress factor plays a significant role in the procedure itself, which may also affect the immune system. No changes in the synthesis of heat shock protein HSP-70 after the WBC treatment and the decrease in its level a day later may have double meaning. Firstly, the results may indicate a lack of damaging effect of cryogenic temperatures on the spatial structure of proteins, and as a result of this, lack of increased HSP-70 synthesis. Secondly, the use of the WBC treatment to induce physiological stress may not be effective in increasing the acceleration and regeneration of the cell cytoskeleton.

Conclusions

Single whole-body cryostimulation treatment causes a small, modulating effect on the IL-6 level. Single whole-body cryostimulation treatment has also a slight silencing effect on the HSP-70 level in healthy, young men. However, further studies with a larger number of participants and similar methodology to examine the impact of a series of

WBC treatments and delayed response of the immune system after repeated WBC treatments are needed to confirm the research results.

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The authors declare no conflict of interest.

References

1. Smolander J, Leppaluoto J, Westerlund T, et al. (2009): Effects of repeated whole-body cold exposures on serum concentrations of growth hormone, thyrotropin, prolactin and thyroid hormones in healthy women. Cryobiology 58: 275-278.
2. Bonomi FG, Nardi M, Fappani A, et al. (2012): Impact of different treatment of whole-body cryotherapy on circulatory parameters. Arch Imunol Ther Exp 60: 145-150.
3. Lubkowska A, Szygula Z, Klimek AJ, Torii M (2010): Do sessions of cryostimulation have influence on white blood cell count, level of IL6 and total oxidative and antioxidative status in healthy men? Eur J Appl Physiol 109: 67-72.
4. Lubkowska A, Szyguła Z, Chlubek D, Banfi G. (2011): The effect of prolonged whole-body cryostimulation treatment with different amounts of sessions on chosen pro- and anti-inflammatory cytokines levels in healthy men. Scand J Clin Lab Invest 71: 419-425.
5. Miller E, Mrowicka M, Malinowska K, et al. (2010): Effects of the whole body cryotheraphy on a total antioxidative status and activities of some antioxidative enzymes in blood of patients with multiple sclerosis - preliminary study. J Therm Biol 57: 168-173.
6. Banfi G, Melegati G, Barassi A, et al. (2009): Effects of whole-body cryotherapy on serum mediators of inflammation and serum muscle enzymes in athletes. J Therm Biol 34: 559.
7. Pournot H, Bieuzen F, Louis J, et al. (2011): Time-Course of changes in Inflammatory Response after Whole-Body Cryotherapy Multi Exposures following Severe Exercise. PLOS ONE 6: e22748.
8. Ziemann E, Olek RA, Kujach S, et al. (2012): Five-day whole-body cryostimulation, blood inflammatory markers, and performance in high-ranking professional tenis players. J Athl Train 47: 664-672.
9. Walsch NP, Whitman M (2006): Exercising in enviromental extremes: a greater threat to immune function? Sports Med 36: 941-976.
10. Brenner IK, Castellani J, Gabaree C, et al. (1999): Immune changes in human during cold exposure: effect of prior heating and exercise. J Appl Physiol 87: 699-710.
11. Dybek A, Szyguła R, Klimek A, Tubek S (2012): Impact of 10 session of whole-body cryostimulation on aerobic and anaerobic capacity and on selected blood count parameters. Biol Sport 29: 39-43.
12. Lubkowska A (2012): Cryotherapy: Physiological Considerations and Applications to Physical Therapy. In: Physical Therapy Perspectives in the 21st Century – Challenges and Possibilities. Bettany-Saltikov J (Ed.). InTech 155-176.
13. Dinarello CA (2000): Proinflammatory cytokines. CHEST 118: 503-508.
14. Ganong WF, Lange L, Lange J (2005): Review of Medical Physiology. 22nd ed. The McGraw-Hill Companies Inc., New York.
15. Selfe J, Alexander J, Joseph T, et al. (2014): The effect of three different (-135°C) whole body cryotherapy exposure durations on elite rugby league players. PLOS ONE 9: 1-9.
16. Banfi G, Lombardi G, Colombini A, Melegati G (2010): Whole-Body Cryotherapy in Athletes. Sports Med 40: 509-517.
17. Sieroń A, Stanek A, Jagodziński L, et al. (2003): The influence of whole-body cryotherapy on some selected parameters of inflammation in patients with ankylosing spondyitis – preliminary report. Acta Biooptica Inf Med 9: 39-43.
18. Stryer L (1998): Biochemistry. 4th ed. W.H. Freeman and Company, New York.
19. Ritossa F (1962): A new puffing pattern induced by temperature shock and DNP in Drosophila melanogaster. Experientia 18: 571-573.
20. Craig EA, Gambill BD, Nelson RJ (1993): Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol Rev 57: 402-414.
21. Hendrick JP, Hartl FU (1993): Molecular chaperone functions of heat shock proteins. Annu Rev Biochem 62: 349-384.
22. Morimoto RI, Tissieres A, Georgopoulos C (1990): Stress Proteins in Biology and Medicine. Cold Spring Harbor Laboratory, New York.
23. Tkáčová J, Angelovičová M (2012): Heat shock proteins (HSPs): a review. Animal Sci Biotechnologies 45: 349-353.
24. Morton JP, Kayani AC, McArdle A, Drust B (2009): The exercise-induced stress response of skeletal muscle, with specific emphasis on humans. Sports Med 39: 643-662.
25. Aufricht C (2005): Heat-shock protein 70: molecular supertool? Pediatr Nephrol 20: 707-713.
26. Harrison MH, Graveney MJ, Cochrane LA (1982): Some sources of error in the calculation of relative change in plasma volume. Eur J Appl Physiol Occup Physiol 50: 13-21.
27. Kraemer RR, Brown BS (1984): Alterations in plasma-volume-corected blood components of marathon runners and concomitant relationship to performance. Eur J Appl Physiol Occup Physiol 55: 579-584.
28. Flavahan NA (1991): The role of alpha2-adrenoceptors as cutaneous thermosensors. NIPS 6: 251-255.
29. Vanhoutte PM (2011): Physical Factors of Regulation. Compr Physiol Suppl. 7: Handbook of Physiology, The Cardiovascular System, Vascular Smooth Muscle: 443-474. First published in print 1980.
30. Pääkkönen T, Leppäluoto J 2002: Cold exposure and hormon secretion: a review. Int J Circ Health 61: 265-276.
31. Himms-Hagen J (2011): Neural and Hormonal Responses to Prolonged Cold Exposure. Compr Physiol Suppl. 14: Hand-

book of Physiology, Environmental Physiology: 439-480. First published in print 1996.
32. Marino F, Sockler JM, Fry JM (1998): Thermoregulatory, metabolic and sympathoadrenal responses to repeated brief exposure to cold. Scand J Clin Lab Invest 58: 537-545.
33. Felten DL, Felten SY, Bellinger DL, et al. (1987): Noradrenergic sympathetic neural interactions with the immune system: structure and function. Immunol Rev 100: 225-260.
34. Kohm AP, Tang Y, Sanders VM, Jones SB (2000): Activation of antigen- specific CD4+ Th2 cells and B cells in vivo increases norepinephrine release in the spleen and bone marrow. J Immunol 165: 725-733.
35. Ramer-Quinn DS, Baker RA, Sanders VM (1997): Activated T helper 1 and T helper 2 cells differentially express the b2-adrenergic receptor. J Immunol 159: 4857-4867.
36. Gailland R (1994): Neuroendocrine-immune system interactions. The immune-hypothalamo-pituitary-adrenal axis. Trends Endocrinol Metab 5: 303-309.
37. Rhind SG, Castellani JW, Brenner IK, et al. (2001): Intracellular monocyte and serum cytokine expression is modulated by exhausting exercise and cold exposure. Am J Physiol Regul Integr Comp Physiol 281: 66-75.
38. Dugue B, Lepannen E (2000): Adaptation related cytokines in man: effects of regular swimming in ice-cold water. Clin Physiol 20: 114-121.
39. Dinarello CA (1997): Role of pro- and anti-inflammatory cytokines during inflammation: experimental and clinical findings. J Biol Regul Homeost Agents 11: 91-103.
40. Spooren A, Kolmus K, Laureys G, et al. (2011): Interleukin-6, a mental cytokine. Brain Research Rev 67: 157-183.
41. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S (2011): The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 1813: 878-888.
42. Salgado R, Junius S, Benoy I, et al. (2003): Circulating interleukin-6 predictors survival in patients with metastatic breast cancer. Int J Cancer 103: 642-646.
43. Culig Z, Steiner H, Bartsch G, Hobisch A (2005): Interleukin-6 regulation of prostate cancer cell growth. J Cell Biochem 95: 497-505.
44. Opal SM, DePalo VA (2000): Anti-Inflammatory cytokines. Chest 117: 1162-1172.
45. Simms JE, Smith DE (2010): IL-1 family: regulators of immunity. Nature Rev Immunol 10: 89-102.
46. Leppaluoto J, Westerlund T, Huttunen P, et al. (2008): Effects of long-term whole-body cold exposures on plasma concentrations of ACTH, beta-endorphin, cortisol, catecholamines and cytokines in healthy females. Scand J Clin Lab Invest 68: 145-153.
47. Hausswirth C, Shaal K, Le Meur Y, et al. (2013): Parasympathetic activity and blood catecholamine responses following a single partial-body cryostimulation and a whole-body cryostimulation. PLOS ONE 8: e72658.
48. Murray RK, Granner DK, Rodwell VW (2000): Harper's Illustrated Biochemistry, 27th ed. The McGraw-Hill Companies Inc., New York.
49. Janeway CA Jr, Travers P, Walport M, Shlomchik MJ (2001): Immunobiology: The Immune System in Health and Disease. 5th ed. Garland Science, New York.
50. Gleeson M, Francis JL, Lugg DJ, et al. (2000): One year in Antarctica mucosal immunity at three Australian stations. Immunol Cell Biol 78: 616-622.
51. Janský L, Pospísilová D, Honzová S, et al. (1996): Immune system of cold-exposed and cold-adapted humans. Eur J Appl Physiol Occup Physiol 72: 445-450.
52. Currie S, Tufts BL (1997): Synthesis of stress protein 70 (Hsp70) in rainbow trout (Oncorhynchus mykiss) red blood cells. J Exper Biol 200: 607-614.
53. Cullen KE, Sarge KD (1997): Characterization of Hypothermia-induced Cellular Stress Response in Mouse Tissues. J Biol Chem 272: 1742-1746.
54. Burton V, Mitchell HK, Young P, Petersen NS (1988): Heat shock protection against cold stress of Drosophila melanogaster. Mol Cell Biol 8, 3550-3552.
55. Salotra P, Singh DK, Seal KP, et al. (1995): Expression of DnaK and GroEL homologs in Leuconostoc esenteroides [sic] in response to heat shock, cold shock or chemical stress. FEMS Microbiol Lett 131: 57-62.
56. Lunal SG, Phillips MC, Moyes CD, Tufts BL (2000): The effect of cell ageing on protein synthesis in rainbow trout red blood cells. J Exp Biol 203: 2219-2228.
57. Jonak C, Klosner G, Trautinger F (2006): Heat shock protein in the skin. Int J Cosmetic Sci 28: 233-241.
58. Liu A, Bian H, Huang E, Lee YK (1994): Transient cold shock induces the heat shock response upon recovery at 37°C in human cells. J Biol Chem 269: 14768-14775.
59. Sarge KD, Murphy SP, Morimoto RI (1993): Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol Cell Biol 13: 1392-1407.
60. Basu N, Nakano T, Grau EG, Iwama GK (2001): The effects of cortisol on heat shock protein 70 levels in two fish species. Gen Comp Endocrinol 124: 97-105.