Scandinavian Journal of Clinical & Laboratory Investigation, 2013; 73: 315–325

informa
healthcare

ORIGINAL ARTICLE

Winter-swimming as a building-up body resistance factor inducing adaptive changes in the oxidant/antioxidant status

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Abstract

The aim of our research was to examine whether winter-swimming for five consecutive months results in adaptational changes improving tolerance to stress induced by exposure to cryogenic temperatures during whole-body cryostimulation (WBC). The research involved 15 healthy men, with normal bodyweight, who had never been subjected to either WBC or cold water immersion. During the experiment, the participants were twice subjected to WBC (3 min/− 130°C), namely before the winter-swimming season and after the season. Blood was taken seven times: In the morning before each cryostimulation, 30 min after each cryostimulation and the next morning. Additionally, control blood was collected in the middle of the winter season, in February. Our analysis concerned changes in hematological parameters as well as in reduced glutathione and oxidized glutathione, total oxidant status, total antioxidant status and in components of the antioxidant system: Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase and 8-Isoprostanes as a sensitive indicator of oxidative stress. We found significant changes in hemoglobin concentration, the number of red blood cells, the hematocrit index and mean corpuscular volume of red blood cell and the percentage of monocytes and granulocytes after the winter swimming season. The response to cryogenic temperatures was milder after five months of winter-swimming. The obtained results may indicate positive adaptive changes in the antioxidant system of healthy winter-swimmers. These changes seem to increase the readiness of the human body to stress factors.

Key Words: *Cold exposure, cold-stress response, oxidative stress, adaptation, antioxidant effects, cryotherapy*

Introduction

Critical water temperature, i.e. the lowest temperature at which a naked resting human is able to maintain stable body temperature without increased metabolism, is 32–35°C. The corresponding critical air temperature is 22–27°C. However, these values are not constant and may be lower in people that have better adapted to cold [1,2]. Immersion in cold water during winter-swimming season (4°C), accompanied by hydrostatic pressure, is undoubtedly a stress factor for the human body that induces significant heat and metabolic losses and results in a disturbance of thermal homeostasis. This leads to immediate and also long-term physiological and biochemical reactions, including both hormonal and metabolic reactions, and the response of the cardiovascular system, documented in experiments on animals and people [3–5]. Cold water immersion decreases skin, subcutaneous, muscle and rectal temperature [6]. Stimulation of the sympathetic nervous system and adrenal glands, the observed increase in the concentration of catecholamines (mainly norepinephrine, even by four times) and to a lesser extent in epinephrine, stimulates thermogenesis, control vasoconstriction, and together with cortisol are involved in energy metabolism [7,8].

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(Received 27 October 2012; accepted 3 February 2013)

ISSN 0036-5513 print/ISSN 1502-7686 online
DOI: 10.3109/00365513.2013.773594

Additionally, in cold water immersion, the metabolic rate is affected by the increased concentration of hormones, such as corticotropin (adrenocorticotropic hormone, ACTH), thyrotropin (thyroid-stimulating hormone, TSH), and vasopressin [9]. The metabolic rate in a cold environment is also increased by the mechanism of shivering thermogenesis, initiated to enhance the endogenous production of heat through increased residual tension of skeletal muscles and more intense shivers – frequent, low-amplitude muscle contractions (shivers). The work of muscles in cold water immersion, and also their shivering, increases the blood supply in body surfaces and lowers their insulating function, thus heat loss is even greater than before [10].

It is assumed that repeated immersions in cold-water (used to increase resistance) induce a physiological change of an adaptive character, associated with a complex cascade of molecular events, which involve the sympathetic nervous system pathways, ranging from the release of neurotransmitters to regulation of gene expression. Winter swimmers have been observed to have improved thermogenesis, especially an increase of non-shivering thermogenesis in adipose tissue, metabolic adaptation and adaptation of circulatory responses, where the insulative adaptation indicates an enhancement of the cutaneous vasoconstrictor response [3,11]. Only a few studies have confirmed that winter-swimming results in oxidative stress, and repeated immersions in cold water may enhance immune responses and improve antioxidant protection [12–14]. Previous research has concerned mainly the evaluation of thermal comfort [15], thermosensitivity and changes in body temperature during swimming, analgesic response [16], and resting concentrations of selected hormones [8,17,18], indicators of the pro-oxidant/antioxidant status after the winter-swimming season [12], well-being [19], or whole-body fluid regulation [20].

It is interesting whether winter-swimming results in habituation, improving antioxidant parameters, and if this is the case, whether these changes result in increased resistance to a strong stressor, i.e. cryogenic temperatures acting on the whole body, and whether it increases resistance to increased oxidative stress in the body. In order to verify this hypothesis we carried out research in which we evaluated the level of selected indicators of the pro-oxidant/antioxidant status in response to a single whole-body cryostimulation (WBC) applied before and after a five-month-long winter-swimming season in a group of healthy men. We examined the changes in concentration of 8-isoprostane (Iso-P) in plasma. Isoprostanes are non-classical eicosanoids and are considered the best available biomarkers of oxidative stress status and lipid peroxidation *in vivo* [21]. With regard to antioxidant status, our analysis concerned changes in reduced (GSH) and oxidized (GSSG) glutathione, total oxidant status (TOS), total antioxidant status (TAS) and components of the antioxidant system: superoxide dismutase (SOD, E.C.1.15.1.1), catalase (CAT, E.C.1.11.1.6), glutathione peroxidase (GPx, E.C. 1.11.1.9), glutathione reductase (GSSG-R, E.C.1.8.1.7) and glutathione S-transferase (GST, E.C.2.5.1.18).

Materials and methods

The research involved 15 healthy men, aged 23 ± 1.47 years, with normal bodyweight (BMI $= 23.8 \pm 2.92$), who had never been subjected to either WBC or cold water immersion. The subjects were a homogeneous group with regard to age, level of daily physical activity. Each participant gave a written consent before participation in the study, and the Regional Bioethical Committee issued their formal consent, according to the Helsinki Declaration. Each participant was examined by a physician to test for any contraindications towards cryostimulation and cold water immersion. The participants were also subject to anthropometric measurements: Height and bodyweight, skinfold thickness at the right and left shoulder. Then the Body Mass Index (BMI) was determined. The bioimpedance method was used (Bodystat 1500) to determine the basic body composition of the participants, i.e. percentage content of Lean Body Mass (LBM), water and fat. The characteristics of the examined group are presented in Table I.

During the experiment the participants swam in a lake, on average 2–3 times a week for a prolonged period of five months, from November to March. Each immersion in water took from 2–5 min, covered the whole body, excluding the head. The mean water temperature ranged from 12–15°C to 0–7°C from the beginning to the end of the experimental period.

Additionally participants were subjected to cold-stress factor, as a WBC, twice, before and after the winter-swimming season (October and April). Each time, the participants were exposed to a 3-min session of extremely low temperature (-130°C) in a two-stage cryogenic chamber, where subjects were first introduced to the vestibule of the pre-chamber (-60°C) and then passed to the main chamber (-130°C). Before entering the cryogenic chamber, participants dried their bodies thoroughly to eliminate the sensation of cold. To protect the upper airways, all participants breathed through a surgical mask. For protective purposes, all participants wore gloves, socks, special footwear and head bands to protect the ears. While in the cryostimulation, the subjects were advised to move their fingers and legs slightly and to avoid holding their breath. The cryostimulations took place in the morning between 09:00 and 10:00 h. During the winter-swimming

Table I. Anthropometric characteristics and hematological indicators of the examined individuals, before (T_0), in the middle (T_M) and after ($T_{0'}$) the winter-swimming season (mean ± SD).

Parameters	T_0	T_M	$T_{0'}$
Age	23.0 ± 1.10	23.0 ± 1.47	23.0 ± 2.07
Height (cm)	181.5 ± 5.08	181.5 ± 5.08	181.5 ± 5.08
Bodyweight (kg)	79.1 ± 11.88	79.0 ± 10.67	80.2 ± 11.48
BMI (kg/m^2)	23.8 ± 2.92	23.9 ± 2.03	24.6 ± 3.02
Lean (%)	80.8 ± 2.08	83.0 ± 1.95	84.0 ± 2.65
Fat (%)	14.0 ± 3.69	14.1 ± 4.01	14.2 ± 3.30
Water (%)	59.5 ± 2.12	60.0 ± 0.57	60.5 ± 0.75
Skinfold thickness, total for arm and forearm(mm)	14.7 ± 3.01	15.0 ± 2.51	15.0 ± 2.51
RBC (10^12/L)	5.6 ± 0.71	5.2 ± 0.50	4,9 ± 0.26**T_0
Hb (mmol/L)	10.6 ± 1.11	9.9 ± 1.40*T_0	8.6 ± 1.36***T_0,T_M
Ht (L/L)	0.51 ± 0.09	0.44 ± 0.05**T_0	0.42 ± 0.03**T_0
MCV (fL)	87.4 ± 6.57	85.5 ± 6.88**T_0	83.8 ± 6.47***T_0,T_M
MCH (fmol)	1.7 ± 0.16	1.7 ± 0.09	1.70 ± 0.06
MCHC (mmol/L)	19.6 ± 1.16	20.1 ± 0.93	19.8 ± 0.78
RDW (%)	12.1 ± 1.06	13.1 ± 1.23**T_0	13.5 ± 1.32**T_0
WBC (10^9/L)	6.2 ± 1.15	6.0 ± 1.00	6.5 ± 1.22
LYM (%)	36.9 ± 6.56	36.7 ± 6.75	35.4 ± 3.76
MON (%)	12.9 ± 2.92	9.0 ± 1.71*T_0	8.6 ± 1.27**T_0
GRA (%)	47.6 ± 6.36	56.6 ± 8.60**T_0	57.9 ± 5.64**T_0
PLT (10^9/L)	276.0 ± 79.29	260.9 ± 45.62	269.1 ± 29.98
SBP (mmHg)	129 ± 7.5	127 ± 6.4	137 ± 6.4*T_0, T_M
DBP (mmHg)	77 ± 6.8	80 ± 6.3	78 ± 6.3

BMI, body mass index; RBC, red blood cells; Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; WBC, white blood cells; LYM, lymphocytes; MON, monocytes; GRA, granulocytes; PLT, thrombocytes; SBP, systolic blood pressure; DBP, diastolic blood pressure.
*Statistically significant difference at $P \leq 0.05$; **statistically significant difference at $P \leq 0.01$; ***statistically significant difference at $P \leq 0.001$.

season, the participants swam in cold water twice a week over a period of five months (from November to March), joining a group of experienced winter-swimmers. Figure 1 presents the scheme of the experiment.

Blood was taken seven times: In the morning before the first cryostimulation (sample T_0), 30 min after the first cryostimulation (sample T_1) and in the next morning (sample T_2). The same scheme was used during the second cryostimulation, after the winter-swimming season, with the corresponding markings: $T_{0'}$, $T_{1'}$ and $T_{2'}$. Additionally, blood was taken in the middle of the winter season, in February (T_M).

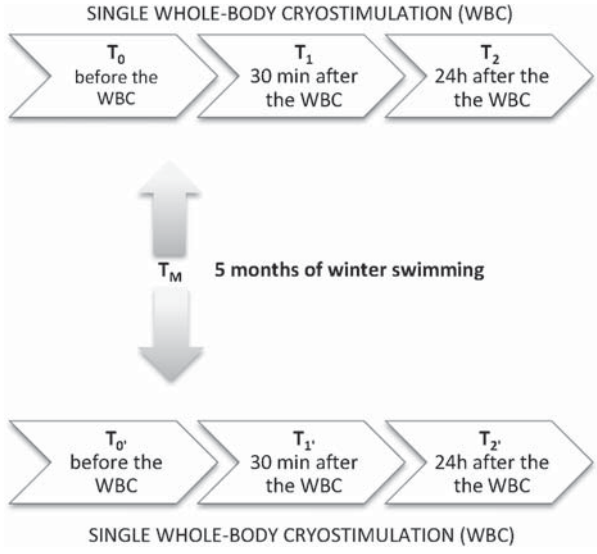

Figure 1. The scheme of the experiment. Blood sampling: T_0, before; T_M, in the middle; $T_{0'}$, after the winter-swimming season, in the morning after overnight fasting. T_1 and $T_{1'}$, blood sampling 30 min after the cryostimulation. T_2 and $T_{2'}$, blood sampling 24 h after the cryostimulation.

Each time ($T_{0,}$ $T_{0'}$ and T_M) venous blood samples were obtained after overnight fasting in the morning, between 08:00 and 09:00 h from an antecubital forearm vein, after 10-min rest in a sitting position, using vacutainer system tubes with appropriate anticoagulant (Sarstedt, Germany) for biochemical analysis of erythrocytes and plasma (7 mL; EDTA) and to determine peripheral blood cell parameters (1.2 mL; anticoagulated with 1 g/L EDTA): number of erythrocytes (RBC), hemoglobin concentration (Hb), hematocrit value (Ht), number of leukocytes (WBC), number of thrombocytes (PLT). Hematological parameters were measured by the use of a hematology analyzer (ABX Micros 60 HORIBA).Intra-assay precision and accuracy for all hematologic parameters were below 1.5% and inter-assay precision and accuracy for all hematologic parameters were below 2.0%.

The erythrocytes were separated by centrifugation (300 rpm, 1500 *g*, 10 min, 4°C), washed three time with buffered NaCl solution (PBS: 0.01 mol phosphate buffer 0.14 mol NaCl, pH 7.4) chilled to 4°C and finally frozen at − 70°C. Plasma was divided into aliquots and immediately deep-frozen at − 70°C until the time of analysis, however not longer than one month.

Total lipid peroxides as the total oxidant status (TOS) and the total antioxidant status (TAS) were measured by means of photometric tests (Immunodiagnostik AG, Bensheim, Germany). The limits of detection were 7 μmol/L for TOS and 130 μmol/L for TAS. ELISA kits were used for measuring plasma levels of 8-isoprostane (Cayman, MI, USA), according to the manufacturer's protocol.

Before the analysis, erythrocytes were thawed and the hemolysate of the washed red blood cells was diluted with distilled water and chilled to 4°C. GSH, GSSG level, as well as SOD, CAT, GPx and GSSG-R and GST activities were measured in hemolysate samples with a BIOXYTECH® kit (Oxis Research, Portland, OR, USA) using a UV/VIS Lambda 40 (Perkin-Elmer, Wellesley, MA, USA) spectrophotometer.

The enzyme activity and glutathione concentration were calculated per 1 g of erythrocyte hemoglobin. In all mentioned cases, hemoglobin levels were assayed using the Drabkin method [22]. Each sample was tested in duplicate.

Statistics

The obtained results were statistically analyzed. Data were checked for normal distribution using the Shapiro-Wilk test and tested by one-way ANOVA. The assessment of normality of distribution of continuous variables (Shapiro-Wilk test) was conducted and showed non-normal (log-normal) distributions of parameters. In order to normalize the distribution, logarithmic transformation was performed of the continuous variables tested. To assess the differences between the parameters tested the one-way ANOVA were used. For the parameters with normal distribution the results are expressed as the mean value with standard deviation (± SD), in other case the results are expressed as median. In order to demonstrate whether the observed correlations were statistically significant, we used the Spearman's rank correlation coefficient.

The coefficient of analytical variation (CV_A) was calculated from the mean and standard deviation of the control blood analyses, which were conducted at the beginning and end of each winter swimming season. Desirable analytical performance was assumed if the imprecision was such that $CV_A < 0.5 \times CV_I$ [23–25].

For hematological parameters, the coefficient of intra-individual variation (CV_I) was estimated by the calculation $\delta/\mu \times 100$, where δ denotes the mean of the individual's results across seven samples and μ denotes the standard deviation of the seven measurements. As the calculated CV_I includes an analytical component we applied the Fraser's formula for each subject to remove the analytical variation (CV_A) from CV_I using the formula: $CV_{Ib} = (CV_I^2 - CV_A^2)^{0,5}$ [25,26], where CV_{Ib} denotes the CV_I without analytical variation. The total intra-individual coefficient of variation (CV_I) that was applied in RCV (reference change value) formula was calculated using the global mean of the CV_{Ib}. The coefficient of variation between individuals (CV_G) was calculated according to the mean and standard deviation of each hematological parameters obtained between the participants. The RCV calculation was based on the following formula: $RCV = 2^{0,5} \times Zp(CV_A^2 + CV_I^2)^{0,5}$, where $2^{0,5}$ denotes the probability of bidirectional change and Zp denotes the standard deviation corresponding to the level of statistical significance in bidirectional change (1.96 = 95%) [25,27]. Development of statistical results was performed using STATISTICA PL v 7.1 software (Statsoft, Krakow, Poland). The accepted level of significance was defined as $P < 0.05$.

Results

Anthropometric characteristics of the participants did not change significantly over the winter season, neither their weight nor proportions of body components. Consequently, BMI remained the same. There were no changes in the average skinfold thickness in the arm and forearm. It was found that winter-swimming for five months resulted in significant changes in the red blood cell system.

Swimming in winter influenced the CV_I values for all hematological parameters. RCVs were always greater in examined individuals than the values previously reported in the literature (Table II) [25].

Table II. Components of biological and analytical coefficients of variation with reference change values (RCV) for hematological parameters in examined individuals.

	Examined individuals					Healthy population (25)		
Parameters	CV_A (%)	CV_I (%)	CV_G (%)	II	$RCV_{95\%}$ (%)	CV_I (%)	CV_G (%)	$RCV_{95\%}$ (%)
RBC (10^{12}/L)	1.0	8.7	8.7	0.99	24.1	3.0	6.1	9.9
Hb (mmol/L)	1.0	13.0	12.5	0.96	34.8	2.8	6.8	8.7
Ht (L/L)	0.7	10.7	17.9	1.67	49.7	2.9	6.4	8.7
MCV (fL)	0.4	7.7	2.4	0.32	7.0	1.1	4.2	4.0
MCH (fmol)	0.8	5.6	4.0	0.71	11.0	1.3	5.2	5.0
MCHC (mmol/L)	0.6	3.6	5.3	1.44	14.8	1.6	2.8	5.3
RDW (%)	1.5	9.8	6.3	0.63	17.7	3.7	5.7	10.8
WBC (10^9/L)	1.2	18.5	10.5	0.57	29.9	10.8	17.4	33.8
LYM (%)	2.0	13.8	11.4	0.83	32.8	10.8	24.1	32.2
MON (%)	2.1	18.6	21.3	1.14	59.3	10.1	25.0	31.2
GRA (%)	0.8	10.7	12.0	1.12	33.3	17.3	23.1	49.9
PLT (10^9/L)	3.0	19.3	19.6	1.01	55.0	10.0	21.9	28.2

CV_A, analytical coefficient of variation calculated from blood control ($n = 20$); CV_I, intra-individual coefficient of variation; CV_G, between-subject coefficient of variation; II, individuality index ($II = CV_I/CV_G$); RCV, reference change value [%]. RBC, red blood cells; Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; WBC, white blood cells; LYM, lymphocytes; MON, monocytes; GRA, granulocytes; PLT, thrombocytes.

Seasonal changes for hemoglobin concentration, red blood cells count and hematocrit index were more marked in the examined subjects than in the healthy individuals – values previously reported in the literature (Table III) [28].

There was a reduction in the number of red blood cells ($P \leq 0.01$), although within physiological ranges. A decrease in hemoglobin, hematocrit index and MCV ($P \leq 0.01$) were observed in the middle of the season, after 3 months of regular swimming, albeit a decrease in the Hb concentration and MCV intensified at the end of the season ($P \leq 0.001$), reaching the lower limits of physiological standards for men (83.8 ± 6.47, $Hb = 8.6 \pm 1.36$ mmol/L). Other analyzed red cell indicators, i.e. MCH and MCHC, did not change significantly. There was an increase in red blood cell distribution width (RDW), from 12.1% before the season, to 13.5% at the end of the season.

Within the white blood cells, there was an increase in the percentage of granulocytes with a simultaneous decrease in the percentage of monocytes, not affecting the total number of white blood cells, remaining at a level comparable to baseline. There was also a significantly higher systolic blood pressure at rest after the season, compared to the level observed before the season (Table I).

Five months of winter-swimming had a significant effect on resting levels of many indicators of the pro-oxidant/antioxidant status of the participants (T_0 vs. T_M vs. $T_{0'}$) (Table IV). Already in the middle of the season we observed an increased total antioxidant status (TAS) and decreased total oxidant status (TOS), consequently increasing the TAS:TOS ratio. At the end of the season, there was a highly significant decrease in resting levels of 8-ISOP in plasma ($P \leq 0.001$) and oxidized glutathione ($P \leq 0.001$), which resulted in a reduction in GSH_{Total}, and the GSH:GSSG ratio increased two times. Considering the levels of antioxidant enzymes, an increase in SOD ($P \leq 0.05$), CAT ($P \leq 0.05$ and $P \leq 0.01$) and RGSSG ($P \leq 0.05$ and $P \leq 0.01$) activity in the mid-season was observed, lasting until the end of the season. There was a significant decrease in the activity of GPx ($P \leq 0.01$). There were no changes in GST activity.

Correlating the level of individual isoprostanes with antioxidant status indicators, we observed a positive correlation between isoprostane concentration and GPx activity ($r = 0.63$, $P = 0.014$) before the

Table III. Seasonal variations of some hematological parameters in examined subjects and healthy young men (control group) in autumn and spring (Means ± SD).

	Examined group			Control group (28)		
Parameters	Autumn	Spring	% drop	Autumn	Spring	% drop
Hb (mmol/L)	10.6 ± 1.1	8.6 ± 1.4	18.9	9.7 ± 0.1	9.6 ± 0.1	1.0
RBC (10^{12}/L)	5.6 ± 0.71	4.9 ± 0.26	12.5	4.9 ± 0.05	5.1 ± 0.04	3.2
Ht (L/L)	0.51 ± 0.09	0.42 ± 0.03	20.0	0.437 ± 0.004	0.452 ± 0.003	3.3

% drop = [(higher concentration − lower concentration)/higher concentration] × 100. Hb, hemoglobin; RBC, red blood cells; Ht, hematocrit.

Table IV. Changes in pro-oxidant/antioxidant mediators level in serum following cryostimulation before (T_0, T_1, T_2), in the middle of the winter-swimming session (T_M) and after the five-month-long winter-swimming season ($T_{0'}$,$T_{1'}$, $T_{2'}$).

	Cryostimulation before the winter-swimming season			The middle of the season	Cryostimulation after the 5-month-long winter-swimming season		
Parameters	T_0	T_1	T_2	T_M	$T_{0'}$	$T_{1'}$	$T_{2'}$
TAS (µmol/L)	184.30 ± 15.79	175.20 ± 16.94^{*T_0}	192.30 ± 14.83^{*T_0}	190.00 ± 12.06^{*T_0}	192.70 ± 12.32^{***T_0}	183.70 ± 9.57$^{*T_{0,}}$	190.90 ± 9.40
TOS (µmol/L)	136.00 ± 22.42	141.10 ± 21.46^{**T_0}	127.60 ± 15.41	129.10 ± 12.93^{*T_0}	128.70 ± 17.33^{*T_0}	133.20 ± 15.2$^{**T_{0,}}$	127.60 ± 22.96
TAS:TOS	1.39 ± 0.28	1.26 ± 0.20^{*T_0}	1.52 ± 0.21^{*T_0}	1.48 ± 0.20^{*T_0}	1.52 ± 0.24^{**T_0}	1.39 ± 0.17$^{**T_{0,}}$	1.55 ± 0.35
8-Isoprostane (pg/mL)	591.28 ± 62.27	824.74 ± 77.55^{**T_0}	480.58 ± 78.16^{**T_0}	491.27 ± 67.71^{**T_0}	242.25 ± 90.37^{***T_0}	214.28 ± 96.90	223.21 ± 77.26
GSH_{Total} (µmol/g Hb)	1.14 ± 0.28	1.01 ± 0.25	1.06 ± 0.18	1.02 ± 0.20	0.96 ± 0.24^{*T_0}	0.90 ± 0.15	0.93 ± 0.21
$GSH_{Reduced}$ (µmol/g Hb)	1.01 ± 0.28	0.81 ± 0.21	0.84 ± 0.18	0.91 ± 0.19	0.90 ± 0.23	0.82 ± 0.16	0.87 ± 0.26
GSSG (µmol/g Hb)	0.13 ± 0.03	0.19 ± 0.08^{*T_0}	0.21 ± 0.08^{**T_0}	0.11 ± 0.05$^{**T_{0,}}$	0.05 ± 0.01^{***T_0}	0.07 ± 0.02$^{*T_{0,}}$	0.08 ± 0.02$^{*T_{0,}}$
GSH:GSSG	7.12 ± 3.25	3.54 ± 1.73^{***T_0}	3.49 ± 2.21^{***T_0}	11.21 ± 14.27$^{*T_0**T_{0,}}$	16.10 ± 5.41^{***T_0}	11.19 ± 6.07$^{**T_{0,}}$	9.24 ± 3.52$^{**T_{0,}}$
CAT (U/g Hb)	1.47 ± 0.26	1.47 ± 0.19	1.52 ± 0.23	1.63 ± 0.21^{*T_0}	1.75 ± 0.14^{**T_0}	1.75 ± 0.12	1.75 ± 0.14
SOD (U/mgHb)	2.18 ± 0.83	1.90 ± 0.54^{**T_0}	2.99 ± 0.42^{**T_0}	2.54 ± 0.52^{*T_0}	2.58 ± 0.55^{*T_0}	2.35 ± 0.49	2.48 ± 0.61
SOD:CAT	1.51 ± 0.60	1.32 ± 0.48	1.98 ± 0.33^{*T_0}	1.58 ± 0.36	1.48 ± 0.35	1.34 ± 0.28	1.42 ± 0.36
GST [U/g Hb]	1.65 ± 0.63	1.31 ± 0.5	4.54 ± 1.56^{***T_0}	1.04 ± 0.40	1.88 ± 0.81	1.44 ± 0.51	1.79 ± 0.58
GPx [U/g Hb]	3.84 ± 1.66	3.85 ± 0.87	4.76 ± 0.85^{*T_0}	2.33 ± 0.56^{**T_0}	2.28 ± 0.78^{**T_0}	2.24 ± 0.65	2.42 ± 0.82
GPx:CAT	2.70 ± 1.34	2.69 ± 0.86	3.18 ± 0.73^{*T_0}	1.45 ± 0.37^{**T_0}	1.29 ± 0.39$^{**T_0,**T_{0,}}$	1.28 ± 0.36	1.38 ± 0.46
GSSG-R [U/g Hb]	0.26 ± 0.07	0.25 ± 0.10	0.26 ± 0.07	0.34 ± 0.09 ^{*T_0}	0.38 ± 0.08^{**T_0}	0.38 ± 0.08	0.39 ± 0.09

TAS, total antioxidant status; TOS, total oxidant status; GSH, glutathione; GSSG, oxidized glutathione; CAT, catalase; SOD, superoxide dismutase; GST, glutathione S-transferase; GPx, glutathione peroxidase; GSSG-R, glutathione reductase.

winter season, and after the season there was a highly significant negative correlation with the GSH: GSSG ratio ($r = -0.88$, $P = 0.004$) and GST activity ($r = -0.76$, $P = 0.016$).

The course of reaction to WBC changed after the period of winter-swimming, but in only a few cases (Table V). In response to the first cryostimulation, before the season, we observed a very significant increase in 8-ISOP concentration at 30 min after treatment (T_1), and after 24 h it fell below baseline. After the winter-swimming season, there were no changes in this stress marker (T_1 and T_2).

TAS decreased at 30 min after the first WBC and then increased significantly after 24 h above the baseline value ($P \leq 0.05$), similar to SOD and GST activity ($P \leq 0.01$ and $P \leq 0.001$). After a period of adaptation to cold, the reaction in TAS was similar although less intense (reduction at 30 min after the procedure), and 24 h later the values were comparable to the initial ones ($T_{1'}$, $T_{2'}$). There were no significant changes in the activity of SOD and GST in response to WBC. During the second WBC there was also a slightly less significant increase in the concentration of the oxidized form of glutathione (GSSG): $\Delta(T_0–T_1) = -0.06 \pm 0.08$ and $\Delta(T_0–T_2) = -0.08 \pm 0.08$ vs. $\Delta(T_{0'}–T_{1'}) = -0.02 \pm 0.02$ and $\Delta(T_{0'}–T_{2'}) = -0.03 \pm 0.02$ and therefore we observed a positive development in the GSH: GSSG ratio.

Discussion

The use of immersion in cold water and whole-body cryotherapy is widely used in sport in recovery [29–33]. Winter swimming involves taking a dip in ice-cold natural waters, regularly throughout the winter season. It is regarded as a readily available and inexpensive method to gain resistance to adverse conditions, a natural type of athletic recovery, and as an adaptive stimulus against respiratory tract infections and musculoskeletal pains, especially in northern countries. Epidemiological reports show a decrease in respiratory tract infections by 40% in regular winter swimmers [34]. However, its effects on health have been debated.

Being in water with a temperature below 10°C is clearly a threat to the positive heat balance of the human body and causes systemic backlash in the form of stress. Stress can be very severe and can lead to 'collapse', or it can be light, but repeated regularly may cause activation of the body leading to hardening (a form of hormesis). It is assumed that if the impact of a stressor (in this case, cold-stress) is repeated, the body can 'get used to' the stimulus and becomes more resistant, although the mechanisms of this phenomenon are not yet fully understood. It is important to dose cold gradually and at appropriate levels, as mismatched hardening

Table V. Trends in response to cryogenic temperatures during cryostimulation before ($T_0–T_1$, $T_0–T_2$, $T_1–T_2$) and after ($T_{0'}–T_{1'}$, $T_{0'}–T_{2'}$, $T_{1'}–T_{2'}$) swimming season.

	$\Delta(T_0–T_1)$	$\Delta(T_0–T_2)$	$\Delta(T_1–T_2)$	$\Delta(T_{0'}–T_{1'})$	$\Delta(T_{0'}–T_{2'})$	$\Delta(T_{1'}–T_{2'})$
TAS	9.07 ± 24.69	−8.00 ± 17.40*$^{\Delta(T_0–T_2)}$	−17.07 ± 23.55	9.00 ± 6.05	1.78 ± 9.07*$^{\Delta(T_0–T_2)}$	−7.21 ± 9.76
TOS	−5.14 ± 3.65	8.35 ± 18.77	13.50 ± 19.09	−4.50 ± 3.39	1.14 ± 17.19	5.64 ± 17.55
TAS:TOS	0.13 ± 0.18	−0.13 ± 0.18	−0.26 ± 0.12	0.12 ± 0.09	−0.02 ± 0.24	−0.15 ± 0.27
GSH_{Total}	0.13 ± 0.35	0.07 ± 0.34	−0.05 ± 0.20	0.05 ± 0.33	0.00 ± 0.25	−0.05 ± 0.37
$GSH_{Reduced}$	0.19 ± 0.37	0.16 ± 0.39	−0.03 ± 0.21	0.08 ± 0.32	0.03 ± 0.24	−0.04 ± 0.36
GSSG	−0.06 ± 0.08	−0.08 ± 0.08**$^{\Delta(T_0–T_2)}$	−0.02 ± 0.07	−0.02 ± 0.02	−0.03 ± 0.02**$^{\Delta(T_0–T_2)}$	0.00 ± 0.01
GSH:GSSG	3.57 ± 4.30	3.62 ± 4.15*$^{\Delta(T_0–T_2)}$	0.05 ± 2.94	4.90 ± 5.77	6.85 ± 4.19*$^{\Delta(T_0–T_2)}$	1.94 ± 7.34
CAT	−0.06 ± 0.27	−0.05 ± 0.29	−0.04 ± 0.16	0.00 ± 0.20	0.00 ± 0.20	0.00 ± 0.18
SOD	0.27 ± 1.30	−0.80 ± 0.83**$^{\Delta(T_0–T_2)}$	−1.08 ± 0.79**$^{\varnothing(T_1–T_2)}$	0.23 ± 0.40	0.10 ± 0.54**$^{\Delta(T_0–T_2)}$	−0.13 ± 0.31**$^{\Delta(T_1–T_2)}$
SOD:CAT	0.18 ± 0.98	−0.47 ± 0.73	−0.66 ± 0.41**$^{\Delta(T_1–T_2)}$	0.13 ± 0.30	0.06 ± 0.37	−0.07 ± 0.23**$^{\Delta(T_1–T_2)}$
GPx	−0.05 ± 1.93	−0.91 ± 1.86	−0.90 ± 1.15	0.04 ± 0.40	−0.13 ± 0.60	−0.17 ± 0.67
GPx:KAT	0.01 ± 1.61	−0.47 ± 1.70	−0.48 ± 0.70	0.00 ± 0.21	−0.08 ± 0.34	−0.09 ± 0.45
GSSG-R	0.00 ± 0.09	0.00 ± 0.09	0.00 ± 0.08	0.00 ± 0.10	0.00 ± 0.08	0.00 ± 0.04

TAS, total antioxidant status; TOS, total oxidant status; GSH, glutathione; GSSG, oxidized glutathione; CAT, catalase; SOD, superoxide dismutase; GST, glutathione S-transferase; GPx, glutathione peroxidase; GSSG-R, glutathione reductase.
*Statistically significant difference at $P \leq 0.05$; **statistically significant difference at $P \leq 0.01$; ***statistically significant difference at $P \leq 0.001$.

conditions can cause irreversible changes and damage in the body [35].

In our study, the winter swimmers swam on average 2–3 times a week for a period of five months, from November to March. Each immersion in water took from 2–5 min, covered the whole body, excluding the head. In this study we attempted to identify the extent to which winter-swimming induced adaptive changes in the body, in relation to the pro-oxidant/antioxidant status. Additionally, it was suspected that adaptation to cold stimuli and the improvement in body hardening could be related to an increase in the protection against oxidative stress which occurs during exposure to cold. Literature data confirm that regular winter swimmers have an increased resting number of white blood cells, monocytes and plasma interleukin-6 [36,37].

While the previously published studies also show regular winter-swimmers to have an increase in the baseline concentration of reduced glutathione and a decrease in the concentration of oxidized glutathione in the erythrocyte, higher activities of erythrocytic catalase glutathione peroxidase and superoxide dismutase [12,38], there are still no reports in literature which would describe any change in response to stress factors (in this case cryogenic temperatures) after a period of adaptation to the cold, that would testify to improved resistance to stress factors and could explain the widely recognized mechanism of improved resistance by the cold hardening process. We have managed to show just this in our experimental scheme. We observed an improvement in reaction to cold-induced stress after a period of adaptation, with prolonged immersion in water at a temperature in the range of 4–15°C used as a hardening factor (cold-wet) to the reaction to whole-body exposure to cryogenic air at − 130°C in a cryogenic chamber.

Our previous studies showed that a single WBC is a stress to the body that disrupts the balance of the body's pro-oxidant-antioxidant balance [39]. Subsequent studies confirmed that the repetition of these treatments in a series of 10, 15, or 20, affects the lipid profile [40], the level of inflammation markers [41] and increases the antioxidant capacity of the system [42]. For financial reasons, WBC is usually limited to 10 up to 20 daily treatments, often as complement to primary therapy or in sports injuries, which means an average of 4 weeks of exposure. However, it should be noted that in most cases beneficial changes typically occur after 20 sessions and its stability depends on the duration of the WBC treatment. Therefore, it seems reasonable to seek long-term methods that are less expensive than WBC. Although it could be expected that exposure to dry cold in a cryochamber would be better tolerated by the body and less noticeable than immersion in cold water, research conducted by Smolander et al. [15] showed that regular short-term cold stimuli (both WBC and winter swimming) lead to habituation, especially concerning thermal sensation and comfort. Additionally, Leppäluoto et al. [43] showed that sustained cold-induced stimulation of norepinephrine was remarkably similar between WBC and winter swimming.

The present study shows that the single exposition to cryogenic temperatures during WBC after the season of winter-swimming caused much smaller changes in prooxidant/antioxidant status than WBC without adaptation. Winter-swimming significantly lowered the resting plasma levels of 8-ISOP, which was observed already in the middle of the season, and WBC after the season did not cause a significant increase in their concentrations, as was the case before the season. Isoprostanes are not only the indicators of increased free radical reactions – they also play a role of mediators of oxidant damage both in physiological and pathophysiological processes. Our previous studies showed that 8-ISOPs are a sensitive indicator of oxidative stress in exposure to cold, which has also been confirmed in this study [42]. According to Roberts and Morrow [21], there are a number of attributes confirming the importance of measurement of isoprostanes as a reliable indicator of oxidative stress *in vivo*. Their formation is modulated by antioxidant status and their levels are not affected by the lipid content of the diet. They are stable compounds, specific products of lipid peroxidation.

Particularly significant changes in our study were WBC-induced changes in the glutathione system and glutathione-dependent enzymes. The changes in GSH level are one of the earliest signs of oxidative stress, and blood and tissue levels of GSH are sensitive to oxidative stress. We observed a significantly reduced resting level of glutathione disulfide (in favor of the GSH fraction in total GSH), and also the nature of the concentration changes in response to WBC after the season of winter-swimming indicates the beneficial adaptive changes in the body. Erythrocytes contain glutathione as an antioxidant, mainly to prevent against the oxidation of hemoglobin. Intracellular glutathione is rapidly oxidized to GSSG in response to an increase in free radicals (e.g. the presence of H2O2 and hydroperoxides enzymatically with glutathione peroxidase) but is rapidly reduced back to GSH if the oxidative stress is not severe and the antioxidant system is efficient enough. If the stress exceeds the capacity of the cell to reduce GSSG to GSH, the increase in GSSG in the body may be used as a marker of oxidative stress [44]. A smaller WBC-induced increase in GSSG after a period of adaptation (winter-swimming) indicates favorable changes in the antioxidant system, which was further confirmed by an increase in the resting activity of GSSG-R in this period, a flavoprotein whose function is to maintain the correct concentration of GSH in cells thanks to the ability

to convert GSSG into GSH with NADPH as a co-enzyme.

The increased GSSG after the first WBC, before the season of winter-swimming, resulted in a lower activity of GST at 30 min after WBC and increased GST activity after 24 h, which can be explained by the necessity of removing the oxidized glutathione outside the cell, i.e. glutathione produced during the reduction reaction of hydrogen peroxide with GSH as a proton donor. Glutathione S-transferase inactivates endogenous unsaturated aldehydes, epoxides and peroxides, or reactive products of oxidative stress [45]. Reduced proportions of oxidized glutathione in the total amount of glutathione after the period of hardening is very beneficial for the body, because glutathione, apart from scavenging ROS and regenerating other antioxidants, is also involved in restoring damaged cell components, mainly proteins and lipids of cell membranes, and is involved in maintaining a proper redox potential of cells [46] which is important in the regulation of intracellular metabolism [47] and also in the processes of growth and cell differentiation and apoptosis [48,49].

From a clinical point of view, it is important to mention the unfavorable changes in the red blood cell system that appeared during the mid-winter swimming season. In the case of some indicators these changes even intensified during the rest of the season, which may be a factor detracting the use of winter bathing. Of particular note is the significant progressive decrease in the number of red blood cells, hemoglobin and hematocrit, which was accompanied by an increase in red blood cell distribution width. Perhaps prolonged exposure to low temperatures in winter-swimming could have led to changes in iron metabolism or increased blood hemolysis, but this is difficult to interpret without the determination of parameters of iron metabolism. The simultaneous increase in RDW red blood cell distribution width can suggest the appearance of a greater number of juvenile red blood cells in peripheral blood. It should be noted that the lack of a control group is a weakness in our study; although changes in hematologic indices in the study group were analyzed with regard to their initial level of output, which was a type of control. However this is not enough to exclude the possibility of changes resulting from wintertime cold acclimatization responses.

The biological variation could complicate the interpretation of our results, performed consecutive for the same participants during the 5-month period of the experiment.

In order to make sure whether the changes in hematological parameters did not result only from the biological or seasonal variation, we calculated intra-individual coefficient of variation (CV_I), between-subject coefficient of variation (CVG), reference change value (RCV) and the percent drop between autumn and spring in examined subjects (Tables II and III). The CV_I values and RCV_S were higher for all red blood cells parameters, monocytes and PLT than the values for healthy population previously reported in the literature [25], also higher % drop was observed in subjects exposed to winter swimming compare to reference value for seasonal variation [28]. The obtained results indicate that prolonged winter swimming contributed to a decrease in hemoglobin concentration, hematocrit and erythrocyte counts. Although it was possible to refer the results for erythrocyte to reference values in the literature, the biological variability of pro-oxidant/antioxidant mediators is still not established, whereas our previous results do not concern a sufficiently large and comparable population that could serve as reference in terms of age and sex of subjects.

On the contrary, the observed increase in the resting activity of superoxide dismutase and catalase, the main endogenous antioxidant enzymes, after the season of winter-swimming, should be seen as a beneficial effect for the human body. The results indicate beneficial adaptive changes in the antioxidant system in healthy individuals who decide to use winter-swimming to increase their resistance. Therefore they confirm the hypothesis that regular winter-swimming may increase the capacity and efficiency of the antioxidant system. In addition, winter-swimming may also increase the resistance and improve the body's defensive reaction to the factors increasing oxidative stress, thereby increasing tolerance of the human body to stressors.

Acknowledgements

This paper was supported by the Polish Ministry of Science and Higher Education (Grant no. N N404 312940).

***Declaration of interest*:** The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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