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EFFECT OF CRYOGENIC TEMPERATURES AND EXERCISE ON LIPID PEROXIDATION IN KAYAKERS

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Abstract. The aim of the study was to evaluate the influence of exercise and cryogenic temperatures on the conjugated dienes (CD) level and concentration of thiobarbituric acid-reactive substances (TBARS) in the blood plasma and erythrocytes of kayakers. The study was performed on 10 kayakers of the Polish Olympic Team who were training for 31 days. In the first 10 days of training sportsmen additionally had a cryogenic chamber session. The blood samples were taken before the study and after the 5th and 10th day of training with a cryostimulation and in the 7th, 14th and 21st day of training without cryostimulation. To facilitate an estimation of the influence of cryostimulation on measured parameters, the same kayakers had trained for 14 days, but without cryostimulation (control). The CD level in blood plasma significantly increased after the 10th day, while erythrocytes TBARS concentration increased after the 5th and 10th day of cryostimulation and training. The blood plasma CD level was lower in the 7th day of training without cryostimulation as compared to the 10th day of training with cryostimulation. However, erythrocytes TBARS concentration was lower in the 7th and 14th day of training as compared to the 10th day. Plasma TBARS concentration increased in the 7th and 14th day and decreased in the 21st day. Results are not univocal and require further investigation. The lower concentration of lipid peroxidation markers revealed during experimental studies in comparison to control studies, seems to suggest a profitable antioxidant influence of cryostimulation on oxidative stress.

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Key words: Conjugated dienes (CD) - Cryogenic chamber – Exercise - Thiobarbituric acid-reactive substances (TBARS)

Introduction

Cryotherapy is a method often used in contemporary medicine. The beneficial effects of the influence of low temperatures on organisms are often used in the treatment of sports trauma [9,14,17]. What is new in cryostimulation in sport is

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using this method for biological restoration and as a prophylaxis for over-training [22]. What have been observed as the effects of low temperature are: constriction of dermal blood vessels and then dilation and massive hyperaemia, a decrease of muscle tension and a decrease of the neuronal conductance of the feeling of pain, increase of the activity of some hormones (ACTH, cortisone, adrenaline, noradrenaline, testosterone in males), an increase of humoral and cell resistance and a concentration of endorphins. It has been suggested that the effects of cryotherapy are due to antioxidant action [18,22].

Exercise is one of the factors that stimulates oxygen metabolism and leads to an increasing level of oxygen derived free radicals [7,12]. A consequence of the generation of reactive oxygen species is the process of lipid peroxidation. Radicals which are produced in reaction to the oxidation of fatty acids undergo molecular rearrangement, forming conjugated dienes (CD) and then rapidly react with molecular oxygen. The final products of lipid peroxidation are ethane, pentane, lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS) - mainly malondialdehyde (MDA). Therefore, the intensity of this process may be measured by assaying the TBARS concentration [6,10]. The changes in CD level and TBARS concentration - markers of lipid peroxidation - indirectly confirm the increasing generation of oxygen derived free radicals.

The aim of the present study was to evaluate the influence of exercise and cryogenic temperatures on CD levels and the concentration of TBARS in the blood plasma and erythrocytes of kayakers. Additionally, creatine kinase (CK) – a marker of muscle cell injury - and the concentration of cortisol, which is a physical stress indicator, were assayed.

Materials and Methods

The study was performed on the Polish Olympic Team, which consists of 10 kayakers. The characteristic of the basic physical parameters of the people tested is shown in Table 1. The study was accepted by The Local Bioethic Committee at Ludwik Rydygier Medical University in Bydgoszcz. All sportsmen were informed about the aim of the study and gave their written consent. The kayakers were training (2 times per day) for 31 days. Each training session of the first 10 days was

Table 1
Mean value (±SD) of basic characteristics of sportsmen

Age (years)	24.8±2.9
Body height (cm)	183.4±5.6
Body mass (kg)	82.4±4.6
Training experience (years)	10.7±2.7
VO_2max (ml/min./kg)	64.1±4.7

preceded by a session in the cryogenic chamber in The Victoria Centre of Rehabilitation in Radzyń. Each sportsman was treated 20 times in the cryogenic chamber (2 times per day), in temperature ranging from - 120˚C to -160˚C with each treatment lasting for 3 minutes (in days 1. and 2. in -120˚C, in days 3.-6. -130˚C, day 7. -140˚C, days 8. and 9. -150˚C, day 10. -160˚C). Each time the treatment was preceded by 1 minute of adaptation at - 60˚ C in an ante-chamber. Each participation in a cryogenic session was preceded by a medical examination with measurement of blood pressure. During treatment in the cryogenic chamber the sportsmen were dressed only in underwear and in face and feet protectors. After the first 10 days of training, the kayakers attended a training camp for an additional 21 days where the training was similar to that in Radzyń, but without the cryogenic chamber session. The training protocol is shown in Table 2.

Table 2
Training protocol

Day of week	Type of training	Intensity of training
Monday		
Afternoon	kayakers specialist training	pulse rate 140-150 lasting 50 min
Tuesday		
Morning	kayakers specialist training	pulse rate 140-160 lasting 30 min, 150-170 lasting 24 min, 160-180 lasting 8 min
Afternoon	kayakers specialist training	pulse rate 120-140 lasting 20 min, 140-160 lasting 30 min
	power training executed by repetition method	22 tones
Wednesday		
Morning	kayakers specialist training	pulse rate 110-130 lasting 10 min, 130-150 lasting 25 min, 150-170 lasting 40 min
Afternoon	kayakers specialist training	pulse rate 130-150 lasting 34 min, 160-180 lasting 16 min
	running	pulse rate 110-130 lasting 20 min, 130-150 lasting 12 min, 160-180 lasting 18 min
Thursday		
Morning	cycle ergometer test	pulse rate 160-180 lasting 12 min
Afternoon	power endurance training	18 tones

Friday			
	Morning	kayakers specialist training	pulse rate 110-130 lasting 30 min, 140-160 lasting 16 min, 160-180 lasting 21 min
	Afternoon	kayakers specialist training	pulse rate 110-130 lasting 10 min, 140-160 lasting 30 min, 160-180 lasting 20 min
		running	pulse rate 110-130 lasting 10 min, 130-150 lasting 24 min, 160-180 lasting 18 min
Saturday			
	Morning	kayakers specialist training	pulse rate 110-130 lasting 25 min, 140-160 lasting 10 min, 160-180 lasting 25 min
	Afternoon	kayakers specialist training	pulse rate 140-160 lasting 10 min, 150-170 lasting 15 min, 170-190 lasting 10 min
		power training executed by repetition and stationary method	25 tones
Sunday			
	Morning	kayakers specialist training	110-130 lasting 54 min, 140-160 lasting 12 min, 150-170 lasting 18 min,170-190 lasting 6 min
	Afternoon	running	pulse rate 130-150 lasting 30 min

To facilitate an estimation of the influence of cryostimulation on the measured parameters, the same kayakers had trained for 14 days at a very similar intensity, but without cryostimulation (control study). This training session was performed a few months before the start of the cryogenic chamber session.

Blood samples for the study were taken in the morning before eating, from the cubital vein. Samples were taken before the start of the study, and after the 5^{th} and 10^{th} days of the cryogenic session and during the 7^{th}, 14^{th} and 21^{st} days of training without the cryogenic stimulation. In the control study the samples were taken before the start of the study, and during the 7^{th} and 14^{th} day of training.

Concentration of TBARS, CD level, concentration of cortisol and CK activity in the obtained blood samples were assayed. The full blood with the addition of 3.2% sodium citrate was centrifuged to obtain an erythrocyte suspension. After removing the blood plasma, cells were washed three times with a buffered phosphate saline solution (PBS) in 1:3 proportion and centrifuged after each washing. The haemoglobin concentration in haemolysates was assayed by the colorimetric standard method using Drabkin's reagent and expressed as g/l.

The TBARS concentration in haemolysates obtained from the erythrocyte suspensions and in the blood plasma were assayed according to the Buege and Aust

[4] with the latest modifications [8]. This method is based on the formation of a coloured complex of lipid peroxidation process products and thiobarbituric acid at 100˚C in acidic conditions. This complex has maximum absorbance at a wavelength of λ=532 nm. The substance that reacts most with thiobarbituric acid is MDA thus TBARS concentration has been expressed as nmol MDA/gHb in erythrocytes and as nmol MDA/ml in blood plasma. The level of conjugated dienes in haemolysates and blood plasma were assayed according to Sergent *et al.* [16]. The basis of this method is the measurement of a characteristic peak of absorbance at a wavelength of λ=233 nm. The level of conjugated dienes has been expressed as units of absorbance in a millilitre of blood plasma (Abs./ml) and, in erythrocytes, as units of absorbance in a gram of haemoglobin (Abs./gHb).

The activity of CK was measured in blood plasma at 37º C by using commercial kits (POCh Gliwice) and expressed as IU/l. Cortisol concentration was assayed by the chemiluminescence method with Imonlite-TPC (USA) kits and expressed as ng/ml of plasma.

Statistical analyses: The values obtained were statistically analysed by the one-way ANOVA test. The correlation coefficients between chosen biochemical parameters for an evaluation of relationships were also estimated.

Table 3
Concentration of CD, TBARS, cortisol and CK activity in kayakers (n=10) measured before the start and in
7th and in 14th day of training of the control study

	CD in erythrocytes (Abs./gHb)	CD in blood plasma (Abs./ml)	TBARS in erythrocytes (nmol MDA/gHb)	TBARS in blood plasma (nmol MDA/ml)	CK (IU/l)	Cortisol (ng/ml)
Before start of study	5.92 ±0.44	0.81 ±0.04	16.32 ±2.01	0.45 ±0.11	252 ±39.41	169.4 ±10.54
7th day of training	9.1 aaa ±0.27	1.01 ±0.1	47.12aaa ±5.2	0.75aaa ±0.07	292.2 ±55.43	302.2aaa ±25.94
14th day of training	8.08aaa ±0.28	1.24aaa ±0.15	43.38aaa ±2.32	0.63aaa ±0.08	272.7 ±100.55	270.5aaa ±28.97

Values are means ±SD; differences statistically significant as compared to the value measured before the start
of the study (aaa - p<0.001)

Results

A significant increase of all studied parameters was noticed except for CK activity in the 7th and 14th day and CD level in blood plasma in the 7th day of training during the control study without cryostimulation (Table 3).

A statistically significant higher concentration of lipid peroxidation markers (except TBARS concentration in erythrocytes) in the 7th day of training was revealed in the control study in comparison to the experimental study (Table 4). In 14th day of training, CD level and TBARS concentrations (except TBARS concentration in blood plasma) were also significant higher in the control study than in the experimental study. Cortisol concentration and CK activity were significantly higher in the control study than in the experimental study, both in 7th and in 14th day of training (Table 4).

A significant increase in CD levels in the erythrocytes of approximately 10% ($p<0.05$) was observed when comparing the values of the 5th day to the 10th day of the experimental study (Table 4). A non-significant increase in the CD level in blood plasma after the 5th day and a significant increase after the 10th of training and cryogenic sessions was found (Table 4). After the 10th day of training and cryogenic chamber sessions the observed CD level in blood plasma was about 47% ($p<0.001$) higher in comparison to the value before cryostimulation started. In the 7th day of training, after cryostimulation, CD levels in blood plasma decreased significantly by about 29% ($p<0.001$) and in the 14th day increased non-significantly by about 23%. In the 21st day of training, the CD level in blood plasma also increased. This increase was not significant when compared to the value observed in the 14th day, but significant as compared to the values reached in the 7th day and those reached before the start of training (respectively about 38% $p<0.01$ and 45% $p<0.001$).

The concentration of TBARS in erythrocytes increased significantly after both the 5th and 10th day of training and cryostimulation (Table 4). After the 5th day, TBARS concentration had increased over three fold ($p<0.001$) and after the 10th day had increased about five-fold ($p<0.001$). A significant two-fold decrease in TBARS concentration in erythrocytes as compared to the value observed after the 10th day training with cryostimulation, was observed after 7 days of training without cryostimulation. In the 21st day TBARS concentration in erythrocytes had increased about 95% ($p<0.001$) as compared to the value reached in the 14th day. The training and cryogenic sessions, did not have a statistically significant influence on the TBARS concentration in the blood plasma of kayakers (Table 4).

Table 4

Concentration of CD, TBARS, cortisol and CK activity in kayakers (n=10) measured before the start of the experimental study, after the 5th and 10th day of training preceded by cryogenic chamber session and in 7th 14th and 21st day of training after the ending of the cryogenic sessions

	CD in erythrocytes (Abs./gHb)	CD in blood plasma (Abs./ml)	TBARS in erythrocytes (nmol MDA/gHb)	TBARS in blood plasma (nmol MDA/ml)	CK (IU/l)	Cortisol (ng/ml)
Before start of study	6.57 ±1.36	0.83 ±0.23	17.86 ±8.72	0.45 ±0.14	249.8 ±90.9	173.2 ±18.7
After 5th day of training and Cryogenic chamber session	6.23 ±0.84	0.90 ±0.08	58.86^{aaa} ±12.79	0.41 ±0.05	384.5^{a} ±174.7	181.7 ±26.8
After 10th day of training and cryogenic chamber session	6.91^{b} ±0.88	1.22$^{aaa\ bb}$ ±0.26	86.80$^{aaa\ bbb}$ ±6.68	0.42 ±0.06	402^{a} ±103.1	200.8 ±21.2
7th day of training after ending cryogenic session	7.81$^{b\ f}$ ±1.40	0.87$^{ccc\ ff}$ ±0.08	44.04$^{aaa\ bb\ ccc}$ ±7.20	0.54$^{b\ fff}$ ±0.06	210.1$^{bb\ ccc\ fff}$ ±67.5	239.7$^{aaa\ bbb\ fff}$ ±41
14th day of training after ending cryogenic session	7.31^{f} ±1.32	1.07^{f} ±0.18	5.67$^{bbb\ ccc\ ddd\ fff}$ ±6.74	0.6$^{a\ bbb\ ccc}$ ±0.07	170.7$^{bbb\ ccc\ f}$ ±52.6	226.5$^{aa\ b\ ff}$ ±33.6
21st day of training after ending cryogenic session	7.64^{b} ±0.96	1.20$^{aaa\ bb\ dd}$ ±0.26	49.96$^{aaa\ ccc\ eee}$ ±12.7	0.5 ±0.1	262.5^{c} ±78.6	238.4$^{aaa\ bbb}$ ±32.9

a - differences statistically significant as compared to the value measured before the start of the study
(^{a} - $p<0.05$; ^{aa} - $p<0.01$; ^{aaa} - $p<0.001$)
b - differences statistically significant as compared to the value measured after the 5th day of training and cryogenic chamber session (^{b} - $p<0.05$; ^{bb} - $p<0.01$; ^{bbb} - $p<0.001$)
c - differences statistically significant as compared to the value measured after the 10th day of training and cryogenic chamber session (^{c} - $p<0.05$; ^{ccc}- $p<0.001$)
d - differences statistically significant as compared to the value measured in the 7th day of training after end of cryogenic chamber session (^{dd} - $p<0.01$; ^{ddd} - $p<0.001$)
e - differences statistically significant as compared to the value measured in the 14th day of training after end of cryogenic chamber session (^{eee} - $p<0.001$)
f - differences statistically significant as compared to the value of control study (^{f} - $p<0.05$;^{ff} - $p<0.01$;^{fff} - $p<0.001$)

In the 14th day of training without cryostimulation TBARS concentration in blood plasma increased about 42% ($p<0.001$) as compared to the value reached after the 10th day of the study. The TBARS concentrations in blood plasma observed in the 7th, 14th and 21st day of training were higher than the value observed before the start of the study. In the 14th day of training the difference was significant ($p<0.05$) as compared to the value noticed before the start of training and cryogenic chamber sessions. A significant correlation was found between TBARS concentration in erythrocytes and blood plasma ($r=0.645$, $p<0.05$) and CD level in erythrocytes and blood plasma ($r=0.830$, $p<0.001$) in the 14th day of training without cryogenic session.

Creatine kinase activity increased significantly after the 5th and 10th day of training in conjunction with cryostimulation as compared with the activity of this enzyme before the start of the study (Table 4). After the 5th day of training CK had increased about 54% ($p<0.05$) and after the 10th day by about 61% ($p<0.01$). During the training without cryostimulation, CK activity was significantly lower (except in the 21st day of the training period where it was non-significantly lower) in each case than the activity of this enzyme measured during training preceded by cryostimulation. The cortisol concentration increased non-significantly after the 5th and 10th day of training with cryostimulation as compared to the value measured at the beginning of the study. Concentration of cortisol during the training without cryostimulation was in each case significantly higher than values observed prior to the start of the study.

Discussion

The influence of cryogenic temperature and training caused a significant increase of the CD level after 10 days of training and cryostimulation. In the opinion of Jenkins [12] an increasing CD level is an oxidative stress marker. The intensified oxygen derived free radicals generation is an effect of the action of cryogenic temperature and the increased oxygen consumption during exercise. A number of studies have found an increased generation of oxygen derived free radicals after exercise [5,12,15]. Low temperatures cause many reactions in organism, which may intensify the generation of reactive oxygen species. Some of them are vessel constriction and dilation and massive hyperaemia after leaving a cryogenic chamber [22]. The source of reactive oxygen species in reperfusion conditions is a reaction that is catalysed by xanthine oxidase [2].

The significant increases of TBARS concentration in the erythrocytes of kayakers, after both 5th and 10th day of training and cryostimulation, confirms the intensified lipid peroxidation process in cell membranes of erythrocytes. Erythrocytes are subject to the action of oxygen derived free radicals generated both inside cells and originating outside of them [10]. The source of reactive oxygen species in the interior of erythrocyte is the oxidation of oxyhaemoglobin into methaemoglobin. The carriage of an electron from the ferrous ion of

haemoglobin to molecular oxygen leads to the formation of superoxide anionradical ($O_2^{-\cdot}$). Erythrocytes have a limited antioxidant defence mechanism [1]. The consequence of inadequate deactivation of reactive oxygen species is an over four-fold increase of TBARS concentration in erythrocyte after 10 days of training and cryogenic chamber sessions.

The training effect after the end of the cryogenic sessions caused a non-significant increase of CD levels in erythrocytes as compare to the value obtained after 10 days of training in conjunction with cryostimulation. The TBARS concentration in erythrocytes was significantly lower in the 7th, 14th and 21st day of training as compared to that observed after 10 days of training accompanied with cryostimulation. These results were statistically higher (except in the 14th day) than the TBARS concentration in erythrocytes at the beginning of the study. The above mentioned data may suggest that cryogenic temperature action intensifies erythrocytes cell membrane lipid peroxidation. It can also be confirmed by the control study in which lower TBARS concentration in erythrocytes in both the 7th and 14th day of the control training were found as compared to the value of TBARS measured after the 5th and 10th day of training with cryostimulation.

The elevation of TBARS concentration in blood plasma after exercise is probably due to the peroxidation of LDL fraction lipoproteins of blood plasma and oxygen mediated injuries of myocytes membranes. The significant increase of CK activity during training preceded by cryostimulation may indicate muscle cells injuries or an increased permeability of biological membranes. However, this increase is not accompanied by an elevation of TBARS concentration in blood plasma which may suggest that injuries of myocytes during training preceded by cryostimulation are not due to an increased peroxidation of muscle cells membranes. The activity of CK measured during training without cryostimulation was significantly lower than during training preceded by a cryogenic chamber session. This may suggest some protective influence of cryostimulation against injuries of myocytes during training after 10 days of cryogenic chamber sessions. However, it may also suggest that cryostimulation may enlarge the microinjuries of the muscle cells. Changes in cortisol concentration negate this ascertainment. The lower cortisol concentration found during training preceded by cryostimulation than during training without cryogenic chamber sessions proves that cryostimulation softens physical stress caused by exercise. Also, during the control study without cryostimulation higher cortisol concentration in the 7th and 14th day of training as compared to values observed after the 5th and 10th day of training with cryostimulation and in the 7th, 14th and 21st day of training after the ending of the cryogenic sessions was shown. An increase of cortisol concentration in serum was observed by Zagrobelny *et al.* [21] in patients with rheumatoid arthritis after 7 and 14 days after 2 minutes of a cryogenic chamber session in temperature from -110ºC to -160ºC. A not significant tendency of cortisol concentration to increase after a 2 minutes cryogenic chambers session in - 110ºC was also shown in healthy volunteers by Zagrobelny *et al.* [20]. The authors of those studies explained that

the lack of non-significant changes in cortisol concentration could be due to the fact that the blood samples were taken about 10 minutes after the end of the cryostimulation. An increase in cortisol concentrations may have occurred later, since an increase in ACTH was observed. A beneficial influence of cryostimulation on post-exercise muscle damage has also been shown by Eston and Peters [9]. These authors observed a lower activity of CK in blood plasma in persons who had immersed their exercised arm in cold water after eccentric exercise compared to the level in a control group.

Kawabata and Hata [13] have shown a significant increase in lipid superoxides in rats and mice heart tissue after being influenced of low temperatures. These authors have not observed an increase in lipid superoxides in the blood plasma of the tested animals. Xie *et al.* [19] has shown an increase of MDA concentration in rat blood serum after the influence of cryogenic temperature.

The differences observed in the types of changes in TBARS blood plasma concentration during training preceded and not preceded by cryostimulation may suggest some antioxidant effect of a cryogenic chamber session. Since changes caused by whole body cryostimulation last about 3 hours and they can have an effect during a whole exercise period. The antioxidant action of a cryogenic chamber is also confirmed by the fact that the TBARS concentration in blood plasma measured during the control study without cryostimulation was higher when compared to the values observed during the 31 days of training, the first 10 days of which were accompanied by cryostimulation. The antioxidative effect of cryostimulation may rely on antioxidant enzyme activation in muscle cells. As well, cryostimulation may improve the TBARS elimination mechanism. MDA, which is the major component of TBARS, is metabolised in the liver and probably in trained skeletal muscle [11]. It is difficult to explain changes in TBARS concentration only by oxygen mediated tissue injury.

Physiological adaptations of sportsmen's organisms to exercise and training after whole-body cryostimulation was revealed not only on the level of prooxidant-antioxidant balance. Biały *et al.* [3] noticed decrease of Ca ions level in blood serum of sportsmen what is in author's opinion an effect of calcium ions movement to cellular area and it can influences on physical ability improvement. The authors also point to changes in hormones status or in nervous system. This, in turn, can lead to profitable influence on sportsman's organism during ordinary training process.

The results presented in this work are not univocal and require further investigation. Nevertheless, the lower concentration of lipid peroxidation markers revealed during experimental studies (occurring in kayakers as a result of training performed after the end of cryogenic chamber sessions), in comparison to control studies, seems to suggest a profitable antioxidant influence of cryostimulation on oxidative stress. Whole-body cryostimulation is a source of controlled generation of reactive oxygen species. An adaptation of sportsman's organism to increased reactive oxygen species generation as an effect of cryostimulation can lead to

improvement of antioxidant capacity in response to oxidative stress induced by exercise.

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