ORIGINAL ARTICLE

The effect of whole-body cryostimulation on lysosomal enzyme activity in kayakers during training

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Abstract Effects of whole-body cryostimulation on lysosomal enzyme activity: acid phosphatase (AcP), arylsulphatase (ASA) and cathepsin D (CTS D), as well as on the creatine kinase (CK), and the cortisol concentration in the serum of kayakers during training were studied. Additionally, the effect of a single cryostimulation treatment in untrained men was evaluated. The kayakers were subjected to a ten-day training cycle, in which training sessions were preceded by whole-body cryostimulation at a temperature ranging from -120 to $-140°C$, and to a control training without cryostimulation. Blood samples were taken from the kayakers before the training and after the sixth and tenth day of training and from untrained men before and after cryostimulation. The single cryostimulation caused a 30% ($P < 0.05$) decrease in the CK activity in untrained men. After the sixth day of training with cryostimulation, the activity of ASA was 46% ($P < 0.001$), AcP 32% ($P < 0.05$) and CK 34% lower ($P < 0.05$) than after the sixth day of training without cryostimulation. The results support that preceding training with whole-body cryostimulation alleviates exertion stress by a stabilisation of lysosomal membranes.

Keywords Sport · Cold · Cryo-chamber · Lysosomal hydrolase

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Introduction

Intense exercise or training injures skeletal muscles (Steinacker et al. 2004; Howatson et al. 2005; Weerapong et al. 2005). Injury to the cell and sarcoplasmic membranes leads to accumulation of intracellular calcium, impairment of muscle force generation and provides myofibrilar disruption (Kędziora 1998). Structural changes in muscle fibres are accompanied by the increased release of certain intracellular enzymes, including lysosomal hydrolases (McCully 1986).

Cryostimulation involves exposing the surface of the body to temperatures below $-100°C$ for 2–3 min (Yamauchi et al. 1981). Whole-body cryostimulation has applications in treating injuries in athletes, particularly the overuse syndrome. Low temperatures support rehabilitation and limit secondary damage to tissues (Swenson et al. 1996; Myer et al. 2001). Cryostimulation is also used for renewal and preventive treatment before training. Cryostimulation benefits the time it takes for the athlete to return to full fitness and may avoid surgery. Extremely low temperatures reduce pain, which curtails the efficacy of exercises (Long et al. 2005). Delayed tiredness or greater muscle performance in athletes during training is reported by cryostimulation (Wozniak et al. 2001).

This study evaluated the effect of whole-body cry-ostimulation on the activity of certain lysosomal hydro-lases: acid phosphatase (AcP), arylsulphatase (ASA) and cathepsin D (CTS D) during physical training in kayakers. In addition, the activity of CK was deter-mined as a marker of injury to muscle fibres, and the concentration of cortisol was determined as a marker of stress. The study also evaluated whether cryostimu-lation affects lysosomal enzymes and CK activity, and concentration of cortisol in untrained men.

Methods

The study was carried out in 21 kayakers from the Pol-ish Olympic Team and in ten untrained men (Table 1). The kayakers were subjected to a ten-day training cycle, in which training sessions were preceded by whole-body cryostimulation three times a day, for 3 min, at −120 to −140°C. Before the training, a con-trol training session without cryostimulation was con-ducted (Table 2). The untrained men had one treatment of the cryo-chamber at a temperature of −120°C for 3 min.

Table 1 Subject characteristics

	Kayakers ($N = 21$)	Untrained men ($N = 10$)
Age (years)	24.6 ± 4.3	26.9 ± 4.1
Height (cm)	181.5 ± 5.4	180.8 ± 8.9
Weight (kg)	82.9 ± 5.6	71.2 ± 4.4
VO_{2max} (ml min^{-1} kg^{-1})	66.2 ± 2.3	46.2 ± 2.4
Training experience (years)	12.7 ± 4.6	–

Mean ± SD

Venous blood was obtained from an antecubital vein before training and after the sixth and tenth day of training without cryostimulation and training com-bined with three times a day whole-body cryostimula-tion. Blood samples were obtained from the untrained men three days before cryostimulation and 20 min after cryostimulation.

Cryostimulation

In the cryo-chamber, the subjects were dressed in shorts, socks, gloves and a hat or headband covering the auricles. Wooden clogs were worn as protection against frostbite of the feet. The subjects entered the cryo-chamber after consultation with, and qualification by, a doctor. Each entry to the cryo-chamber was pre-ceded by a 10–20 s adaptation in the vestibule at a tem-perature of −60°C. For the time the subjects were in the cryo-chamber, they remained in eye-and-hearing contact with the person operating the chamber.

The experimental procedures complied with the Helsinki declaration of 1975 and were accepted by the Bioethical Committee at the Ludwik Rydygier Medical University in Bydgoszcz (KB/132/2002). Each subject signed an informed consent before the study.

The training preceded by cryostimulation took place in the Centre for Olympic Preparations, while the untrained men underwent cryostimulation treatment in the Central Sports Centre in Warsaw.

Measurements

As the blood clotted, the serum was centrifuged for 10 min at 12,000g and kept at −20°C until tested. Acid phosphatase was determined according to Bessy (Krawczyński 1972). *P*-nitrophenylphosphate disodium

Table 2 Ten-day training without cryostimulation and preceded by ten-day, three times a day whole-body cry-ostimulation

Day of the week	Type of training	Time and intensity
Monday	General training	120 min II band
	Strength training	90 min I/II band
Tuesday	General training,	120 min II/III band
	Endurance training	120 min II/III band
	General training,	
	Endurance training	
Wednesday	Maximum strength training	90 min III band
	General training,	120 min I/II band
	Endurance training	
Thursday	Sports games and recreation	120 min II/III band
	Training on water	120 min I/II band
Friday	Training on water, running	120 min I/II band
	Strength training for strength	90 min II/III band
Saturday	Running	90 min I/II band
	Swimming, sports games and recreation	120 min II/III band
Sunday	Strength training	90 min II/III band

I band Concentration of blood lactate up to 4 mmol l^{-1}, *II band* 4–8 mmol l^{-1}, *III band* >8 mmol l^{-1}

(substrate) was used in a 0.5 M buffer of citric-tartrate formaldehyde at pH 4.9. Enzyme activity was quantited by p-nitrophenyl hydrolysis.

The activity of cathepsin D was determined according to Anson (1939). The substrate was 2% denatured bovine haemoglobin, diluted in 100 ml 0.1 M citric-phosphate buffer at pH 3.8.

Arylsulphatase activity was determined using Roy's method (Błeszyński and Działoszyński 1965). A quantity of 0.01 M of 4-p nitrocatechol sulphate (NCS) in a 0.5 M acetate buffer at pH 5.6 was used 4-nitrocatechol (4-NC) was released during the enzymatic hydrolysis of NCS. The activity of AcP, CTS D and ASA is expressed in nanokatal (nkat).

For the determination of CK, reagents were used (Alpha Diagnostics sp. z o.o., Warsaw) based on the Oliver method (1995) in the modification by Szasz et al. (1976).

Cortisol was determined by chemiluminescence (Diagnostic Products Corporation, Los Angeles, USA). The optical density of the samples was measured on a "Cary 100" spectrophotometre (Varian, USA). Reagents were from Sigma-Aldrich sp. z o.o. Polska and Przedsiebiorstwa Polskie Odczynniki Chemiczne S.A., Gliwice (Polish Chemical Reagents Enterprises).

Statistics

Statistical significance between data in the untrained men before and after cryostimulation was evaluated using Student's t-test. All other data were tested using the ANOVA test. A $P < 0.05$ was considered statistically significant. Correlations were performed with the Spearman test.

Results

Single whole-body cryostimulation treatment in untrained men did not change the activity of AcP, CTS

D and ASA (Table 3). However, there was a tendency for the ASA and CTS D activity to decrease 20 min after the cryo-chamber was observed.

No statistically significant change in the activity of AcP and ASA took place in the blood serum of the kayakers after 10 days of training without cryostimulation (Table 4). After the sixth day of training without cryostimulation, the activity of CTS D increased more than twofold ($P < 0.001$).

After the sixth day of training preceded by cryostimulation, the activity of ASA decreased by about 45% ($P < 0.001$). The activity of AcP also decreased and was 34% lower after the tenth day ($P < 0.05$). Cryostimulation treatments induced an almost twofold ($P < 0.001$) increase in the activity of CTS D after 6 days of training. After the tenth day, the activity of CTS D decreased slightly, remaining higher than before training.

After the sixth day of training with cryostimulation, the activity of ASA was 46% lower ($P < 0.001$), while that of AcP was 32% lower ($P < 0.05$) than after the sixth day of training without cryostimulation. A 47% lower ($P < 0.001$) activity of ASA was also demonstrated after the tenth day of training with cryostimulation. Comparing the training with and without cryostimulation did not reveal a statistically significant difference in the activity of AcP after the tenth day. The activity of this enzyme was, however, slightly lower when the training was preceded by the action of

Table 3 Serum values in untrained men after a 3 min whole-body cryostimulation

	Before	After 20 min
AcP (nkat)	13.2 ± 2.9	13.9 ± 2.9
ASA (nkat)	27.3 ± 10.4	6.5 ± 4.0
CTS D (nkat)	1305.3 ± 261.1	1266.5 ± 347.5
CK (IU l^{-1})	163.0 ± 53.2	114.5 ± 37.9[a]
Cortisol (µg dl^{-1})	16.4 ± 6.1	19.3 ± 4.8

Mean ± SD. In comparison with before cryostimulation

[a] $P < 0.05$, *AcP* acid phosphatase, *ASA* arylsulphatase, *CTS D* cathepsin D, *CK* creatine kinase

Table 4 Serum values in kayakers after training without and after training with whole-body cryostimulation

Parameter	Before training (control)	Without cryostimulation		With cryostimulation	
		After sixth day	After tenth day	After sixth day	After tenth day
AcP (nkat)	14.0 ± 4.0	15.1 ± 4.7	12.8 ± 2.6	10.2 ± 2.5[b]	9.3 ± 2.4[abb]
ASA (nkat)	39.3 ± 8.5	39.5 ± 9.8	40.0 ± 7.2	21.4 ± 3.6[aaabbccc]	21.1 ± 3.2[aaabbbccc]
CTS D (nkat)	703.4 ± 267.8	1588.2 ± 539.1[aaa]	1192.0 ± 380.7	1388.8 ± 361.4[aaa]	1124.1 ± 273.9
CK (IU l^{-1})	167.4 ± 56.8	491.2 ± 61.5[aaa]	412.2 ± 63.1[aaa]	322.1 ± 116.1[ab]	268.8 ± 91.6[bbb]
Cortisol (µg dl^{-1})	18.3 ± 5.1	22.6 ± 1.9[a]	22.3 ± 2.1	21.6 ± 4.1	19.2 ± 3.3

Mean ± SD. Difference to before training ([a] $P < 0.05$; [aaa] $P < 0.001$), difference in comparison with the test after the sixth day of training without cryostimulation ([b] $P < 0.05$; [bb] $P < 0.01$; [bbb] $P < 0.001$), difference in comparison with the test after the tenth day of training without cryostimulation ([ccc] $P < 0.001$), *AcP* acid phosphatase, *ASA* arylsulphatase, *CTS D* cathepsin D, *CK* creatine kinase

extremely low temperatures. No statistically significant differences were shown when comparing the activity of CTS D after training with and without cryostimulation, after the sixth day. The activity was, nevertheless, slightly lower when the training was preceded by whole-body cryostimulation.

After a single cryostimulation treatment, the activity of CK of the untrained men decreased by 30% ($P < 0.05$).

The activity of CK increased almost threefold ($P < 0.001$) after the sixth day of training without cryostimulation. After the tenth day of training, the activity of this enzyme decreased, but remained, nevertheless, higher than before training ($P < 0.001$).

Cryostimulation caused a twofold increase in the activity of CK after the 6 days of training ($P < 0.05$). The activity of CK after the sixth day of training with cryostimulation was 34% lower than after the sixth day of training without cryostimulation ($P < 0.05$). After the tenth day of training, no statistically significant differences were shown in the activity of CK. The activity of this enzyme was, nevertheless, slightly lower when the training was preceded by cryostimulation.

After a single treatment of the untrained men in the cryo-chamber, cortisol did not change. The concentration of cortisol increased by 23% after the first 6 days of training without stimulation in the cryo-chamber ($P < 0.05$) and remained at that level after the tenth day. Cryostimulation during the day did not result in significant changes in the concentration of cortisol after 6 days of training. There was a tendency for cortisol to increase after the sixth day of training with cryostimulation and to decrease slightly after the tenth day.

After training, with and without cryostimulation, no significant differences were found in cortisol, but it was, nevertheless, lower during training preceded by cryostimulation.

Correlations were found between the level of cortisol and the activity of AcP ($r = 0.66$; $P < 0.05$) and between cortisol and the activity of CTS D ($r = -0.80$; $P < 0.01$) after the sixth day of training without cryostimulation.

Negative correlations were observed between the activity of CTS D and AcP in the kayakers ($r = -0.77$; $P < 0.01$) after the sixth day of training with whole-body cryostimulation, as between the activity of ASA and AcP ($r = -0.79$; $P < 0.05$) after the tenth day of training with cryostimulation.

Discussion

After 6 days of training, the activity of CTS D increased. Also a tendency for the activity of AcP and

ASA to increase in the serum was found. The increase in the activity of lysosomal enzymes may be the effect of micro-injury to the muscle fibres by physical exercise. Intensive physical exercise is accompanied by micro-injury to the muscles (Howatson et al. 2005). Lysosomal enzymes are released from the injured muscle fibres. Micro-injury to the muscle fibres after training without cryostimulation is demonstrated by an increase in the activity of CK in serum of the kayakers.

Despite the fact that there is evidence for muscle injury during exercise, it is not known what is responsible for this damage. The difficulty is a consequence of the complexity of the reactions and processes that take place in the organism during exercise. Muscle-fibre injury may be caused by lipid peroxidation reactions provoked by intensified generation of oxygen-free radicals (OFR). The role of OFR in micro-injury to myocytes and the increase in the activity of lysosomal enzymes after exercise are adressed (Child et al. 1998, 1999). After a half-marathon run on a treadmill, there is an increase in the activity of β-glucuronidase, accompanied by a greater concentration of malondialdehyde (Child et al. 1998). The labilisation of, and injury to, lysosomal membranes by OFR is supported by studies (Karla et al. 1988).

The increase in the activity of lysosomal enzymes after training might aid their participation in the hydrolysis of proteins from the injured muscle fibres (Dohm et al. 1980). Dohm et al. observed a reduction in the synthesis of proteins in muscles and their increased degradation in rats after exercise, with a concurrent increase in the activity of cathepsin D in the muscles. Physical exercise causes an increase in the total (free and bounded) activity of cathepsin D and other lysosomal enzymes in the muscles (Pilström et al. 1978; Vihko et al. 1978). The increased activity of lysosomal enzymes in muscles with micro-injury to the muscle fibres manifests as increased activity of these enzymes in the blood serum.

The greater activity of lysosomal enzymes in the muscles after exercise is also a consequence of the accumulation of phagocytes, which contain lysosomes (Fehr et al. 1989). Monocytes and macrophages participate in the repair of damaged tissue and release lysosomal enzymes, including AcP and β-glucuronidase.

The greater activity of ASA and AcP after exercise was found in the serum of kayakers and rowers after supramaximal exercise was carried out at high altitude (Drewa et al. 2000). The greater activity of ASA and the tendency for CTS D activity to increase was found in untrained men an hour after supramaximal exercise (Wozniak et al. 2002).

In this study, the changes of the activity of enzymes involving an increase in the activity of one of the enzymes at the same time as the absence of change in the activity of other enzymes, both after exercise without cryostimulation and after exercise combined with whole-body cryostimulation, may be the result of the selective release of lysosomal enzymes. During exercise, pH in lysosomes increases and the degree of aggregation of only certain enzymes decreases, which enables them to be released (Tsuboi et al. 1993). Confirmation of the theory of selective release of lysosomal enzymes may also be the negative correlation between the activity of CTS D and AcP in the blood serum of kayakers after the sixth day of training with cryostimulation and between the activity of ASA and AcP after the tenth day of training with cryostimulation.

After training preceded by cryostimulation, the activity of AcP, ASA, CTS D and CK was lower after the sixth day of training than after the sixth day of training without the stimulating effect of extremely cold temperatures. Yet, these differences were not always statistically significant.

A single, 3 min stay in the cryo-chamber for untrained men did not cause significant changes in the activity of lysosomal hydrolases, indicating that a low temperature per se is not a labiliser of lysosomal membranes.

The whole-body effect of cryogenic temperatures is a hormonal response, including an increase in the secretion of the adrenocorticotropic hormone—ACTH (Biały et al. 1999) to stabilise lysosomal membranes. Providing rats adrenocorticotropic hormone during training causes a stabilisation of the membranes of liver cell lysosomes (Toncsev and Frenkl 1984). Under the influence of ACTH, the cells of the adrenal cortex demonstrate an increased concentration of cAMP, which initiates steroidogenesis. On the other hand, the level of cholesterol, the main precursor of steroid hormones, decreases, which testifies to its utilisation for the synthesis of these compounds. The increased secretion of steroid hormones stabilises lysosomal membranes (Adams and Parker 1979).

A low surrounding temperature ($-10°C$) does not affect the activity of β-glucuronidase in the muscles of rats after submaximal exercise on a treadmill and there are no morphological changes in the muscles of the hind limbs (*m. soleus* and *m. tibialis anterior*) (Mäkinen et al. 1998). However, there was an increase in the activity of CK and lactate dehydrogenase in comparison with the activity of these enzymes after exercise at a temperature of $+22°C$. Exercise carried out at a low temperature causes tiredness of the muscles, but not injury.

After training without cryostimulation, there was an increase in the concentration of cortisol in the serum of the kayakers. After training preceded by treatment in the cryo-chamber, changes in the concentration of cortisol were not statistically significant. Cortisol is a stress marker, and in response to stress factors its concentration increases in the plasma (King and Hegadoren 2002). Factors conditioning physical stress and the reaction of the adrenal cortex to this stress include the intensity and duration (Brenner et al. 1998). An increase in the concentration of cortisol in sportsmen testifies to the fact that physical exercise is a stress factor. At the same time, the lack of changes in the level of cortisol after training combined with cryostimulation demonstrates that whole-body stimulation in the cryo-chamber relieves exertion stress. This finding may be refuted by the tendency of cortisol in the serum of untrained men to increase after a single 3 min whole-body cryostimulation treatment. An increase in the level of cortisol can also take place as a result of factors other than exercise, e.g. infection, pain caused by trauma (Butcher and Lord 2004) or by emotions connected with competition (Brenner et al. 1998). A single treatment in a cryo-chamber must be linked with certain anxieties for untrained men. An increase in the concentration of cortisol in the serum of those suffering from rheumatoid inflammation of the joints was found after the seventh and fourteenth day of whole-body cryostimulation, at a temperature of between -110 and $-160°C$ (Zagrobelny et al. 1992). However, after a 2 min cryostimulation treatment, the patients were also given kinesitherapy, which could have affected the increase in the level of cortisol in the serum.

The changes found in this study in the activity of lysosomal enzymes after training preceded by whole-body cryostimulation testify to the fact that cryogenic temperatures stabilise lysosomal membranes. Cryostimulation may reduce micro-injury to muscle fibres caused by exercise. The alleviation of exertion stress following the combination of training with whole-body cryostimulation is supported by the changes in the activity of CK and the concentration of cortisol.

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