

Impact of cold adaptation on cardiac tolerance to ischemia/reperfusion and glucocorticoid, thyroid hormone levels

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ABSTRACT

We have established that the continuous cold exposure (CCE, 4°C, 4 weeks) causes cold adaptation, increases systolic blood pressure, exerts infarct-limiting effect during coronary artery occlusion (45 min) and reperfusion (2 h). The CCE increases adrenal weight, heart weight and T₃ level but does not change thymus, spleen weight, serum cortisol, corticosterone and T₄ levels. The long-term (4°C, 8 h/day, 4 weeks) intermittent cold exposure (LICE) induces adaptation to the cold and increases T₄ level. The brief (4°C, 1.5 h/day, 4 weeks) intermittent cold exposure (BICE) also evokes adaptation to the cold but had no effect on the blood pressure, the cardiac tolerance to ischemia/reperfusion, and does not change thymus, spleen weight, serum cortisol, corticosterone, T₃ and T₄ levels.

Keywords: cold adaptation, heart, ischemia, reperfusion

Short title: cold adaptation and cardiac ischemia

Introduction

It is known that prolonged exposure of alien population in the conditions of the Arctic leads to an increase in the incidence of cardiovascular disease (Maslov et al. 2014). The incidence of hypertension among migrants in the Far North is increased with their length of stay in the Arctic, reaching a level of 61% in people who have lived in this region for over 15 years (Skavronskaya et al. 2005). The rate of acute myocardial infarction (AMI) among the migrants to the far north increases dramatically after 7 - 10 years in the Arctic (Turchinskiy 1979). An unexpected AMI

statistic is that 24% of AMI cases in the polar city of Norilsk Russia occur in individuals under 44 years of age (Turchinskiy and Sakharova 1979).

Experimental studies have shown that prolonged exposure to the cold can have a negative effect on the functional state of the cardiovascular system in the experimental animals. As early as 1956, it was shown that the 20-day continuous adaptation of rats to cold (+5°C) led to an increase in their blood pressure (BP) (Adolph 1956). In 1990, Shechtman et al. (Shechtman et al. 1990) found that hypertensive effect of adaptation (+5°C, 39 days) was maintained, at least for 30 days after placing the rats in the room temperature. However, this data applies only to the long-term continuous exposure to the cold. Meanwhile, the people working in the Far North are subject to the periodic exposure to low temperatures, which evokes in them essential hypertension (Skavronskaya et al. 2005). There is no evidence that the continuous or intermittent exposure to the cold modifies the heart resistance to ischemia and reperfusion. Based on epidemiological data it was possible to hypothesize that the adaptation to the cold will reduce cardiac resistance to the pathogenic impact of ischemia and reperfusion.

However, as early as 1946, Selye established (Selye 1946), that a state of resistance occurred after exposure to extreme stress and also after exposure to excessively strong irritants. Selye called this phenomenon cross-resistance. Later studies indicated that after the adaptation to various extreme effects, cross-resistance to coronary artery occlusion and reperfusion could appear (Meerson and Malyshev 1989, Meerson et al. 1989, Maslov et al. 2013, Maslov et al. 2014). In 1984, Kuroshima et al. (Kuroshima et al. 1984) found that the adaptation by means of short-term immobilizations increases the body's tolerance to the cold stress. However, it was unknown whether the adaptation to the cold could have cross effects which increase cardiac tolerance to the damaging effect of coronary artery occlusion and reperfusion.

Objective: To evaluate the effect of the cold adaptation on the cardiac tolerance to the pathogenic impact of ischemia-reperfusion and glucocorticoid, thyroid hormone levels

Materials and Methods

Animals and protocols. The experimental protocol was approved by the Ethical Committee of the Cardiology Research Institute and it was conformed to the European Union Directive 2010/63/EU. Male Wistar rats weighing 230 - 260 g were housed at $24 \pm 1^\circ\text{C}$ with a relative humidity of 60-70% and a light and dark cycle of 12 h with free access to water and standard rat chow. We used three research protocols. Rats were exposed to continuous normoxic conditioning for four weeks (28 days) in a cold chamber equipped with a generator Bio-Nova-204G4R1 (NTO Bio-Nova Company, Moscow, Russia). Concentrations of O_2 and CO_2 in the chamber were continuously measured by TCO₂-IR and OLC 20 sensors, and maintained at 20.95% and 0.03%, respectively, by a MX32 controller (Oldham, France). The first protocol (protocol 1) included animals with continuous cold exposures (CCE), they resided inside the refrigerator chamber (23 m³) at +4°C two rats in a cage for four weeks (Kvetnansky et al. 2012). The second protocol (protocol 2) included rats with long intermittent cold exposures (LICE) which were exposed at +4°C for 8 h daily for 4 weeks (van Bergen et al. 1992). The third protocol (protocol 3) included animals which were subjected to brief intermittent cold exposures (BICE), which were in a cold chamber at +4°C for 1.5 h daily for 28 days (4 weeks) (Bozhko and Gorodetskaya 1994). The control group consisted of rats which were at a temperature of 24°C in vivarium of Laboratory Experimental Cardiology. All rats were included in the experiment 24 h after termination of the cold exposure.

Cold tolerance test. Cold tolerance was assessed after 24 h after finish of cold adaptation. Animals were placed in a cooler at -18°C for 4 h. The temperature was measured using an electronic thermometer DT – 501 (A&D company Ltd, Japan) at every hour after initiation of exposure to the cold. The number of animals in the experimental groups was 32. There were 8 rats in each group.

Myocardial ischemia/reperfusion. The rats were anesthetized with α -chloralose (50 mg/kg, i.p.; Sigma-Aldrich, St. Louis, USA). A tracheotomy was performed, and the lungs were ventilated by a rodent ventilator model SAR-830 Series (Central Wisconsin Engineers Inc., Schofield, USA) with room air. Atelectasis was prevented by maintaining a positive end-expiratory pressure of 5 – 10 mm H₂O. Arterial blood pH, PCO₂, and PO₂ were determined throughout the experiment by a gas analyzer model Stat Profile M (Nova Biomedical Corporation, Waltham, MA, USA) and maintained within a normal physiological range by adjusting the respiratory rate and tidal volume. The rectal temperature was maintained between 36.5 and 37.5 °C during the experiment by using a non-invasive PhysioSuite heating pad (Kent Scientific Corporation, Torrington, USA) during the experiment. The left descending coronary artery was occluded 1 -2 mm below the atrial appendage and reperfused as described previously Schultz *et al.* (Schultz *et al.* 1997). The right carotid artery was cannulated for the measurement of blood pressure (BP), which was detected by a pressure transducer model SS13L (Biopac System Inc., Goleta, California, USA) coupled with the device for electrophysiological studies MP35 (Biopac System Inc., Goleta, USA). The same device was used to record the ECG. After 45 minutes of ischemia the ligature was loosen and reestablished blood flow was confirmed by the epicardial hyperemic response. The duration of reperfusion was 2 h. ECG recording was performed during the period of ischemia and 10 min after the resumption of the coronary circulation with an apparatus for electrophysiological studies MP35. Arrhythmias were quantified during the first 10 min of ischemia (phase 1a), following 35 min of ischemia (phase 1b), and the first 10 min of reperfusion. According to Russell (Russell *et al.* 1984), arrhythmogenesis of heart rhythm disturbance in the first 10 min of coronary artery occlusion is different from the mechanism of arrhythmia development after 10 min of ischemia. Therefore, in this study, we analyzed the incidence of arrhythmias during phase 1a and phase 1b and following reperfusion. Recording and processing of the data was performed using the software INSTBSL-W company Biopac System Inc., (Goleta, USA). In assessing cardiac arrhythmias, only the ventricular arrhythmias were taken into account: single premature ventricular beats (sPVC), multiple PVCs, ventricular tachycardia, ventricular fibrillation.

Identification of area of necrosis and area at risk (AAR) was performed by the method of Neckar *et al.* (Neckar *et al.* 2003). The area at risk is the myocardium subject to ischemia-reperfusion. After reperfusion hearts were removed from the chest and washed with a syringe through the aorta cannulated with saline containing 125 IU/ml heparin and then the ligature was tightened again and myocardium was stained through aorta with 5% potassium permanganate to determine the AAR. After washing heart sections were made of 1 mm thick perpendicular to the longitudinal axis with slicer SHARA001-1 (Zivic Instruments, Pittsburgh, USA). The area at risk was not stained with potassium permanganate. Infarct size (IS) was determined by staining with 1% solution of 2,3,5-triphenyl tetrazolium chloride for 30 min at 37°C. Necrotic myocardium does not stain because the dead cardiomyocytes do not contain dehydrogenases. After completing, coloring slices were placed in 10% formaldehyde solution for 1 day. The next day, after staining, the right ventricle was removed and slices were scanned on both sides with the scanner HP Scanjet G4050.

The AAR and infarct size was determined by a computerized planimetric method. The infarct size was expressed as a percentage of the area at risk as the IS/AAR. The number of animals in the experimental groups was 48. In each group there were 12 rats.

Evaluation of cold adaptation. In a separate series of experiments, the rats were used to determine the level of hormones, adrenal weight, spleen, thymus, heart and brown fat. We did not perform coronary artery occlusion in these rats. One index of cold adaptation of animals is a relative increase in brown fat weight (Lim et al. 2012) therefore interscapular brown fat was weighed in rats. The number of animals in the experimental groups was 51.

Evaluation of the stress response to cold exposure. Rats were decapitated 24 h after completion of exposure to the cold. Blood was centrifuged during 10 min at 3000 g and at a temperature 4°C. The obtained blood serum was frozen and stored at -18°C for not more than 2 weeks. Evaluation of stress damage to the stomach was determined by the number of gastric ulcers and their total area (Scoto and Parenti, 1993; Yigiter et al., 1998). The number of animals in the experimental groups was 51.

Determination of hormones. The determination of corticosterone content in blood serum was carried out with EIA (Enzyme immunoassay kit) «Corticosterone (Human, Rat, Mouse) ELISA» RE52211 (IBL International GmbH, Hamburg, Germany) using Microplate Reader Infinite 200 PRO (Tecan Austria GmbH, Grodig, Austria). The content of cortisol, T₃ and T₄ in blood serum was performed with radioimmunoassay kit “Cortisol RIA KIT” IM1841, “T₃ RIA KIT” IM1669 and “T₄ RIA KIT” IM1447 (Beckman Coulter, Immunotech, Prague, Czech Republic). The radioactivity of samples was measured with a multichannel radiometer RIG-12 Progress-RIA (NTC Amplitude Company, Moscow, Russia). The number of animals in the experimental groups was 51.

Statistical analysis. Results were expressed as means ± SEM. One-way analysis of variance with Newman-Keuls post hoc test was used to detect differences in parametric variables among adapted and control rats. The Chi squared test was used to detect differences in the incidence of ventricular arrhythmias among groups. Differences were considered significant at p<0.05.

Results

Our studies indicate that continuous exposure to the cold (4°C, 4 weeks) causes a twofold increase in brown fat weight in comparison with the intact animals (Table 1). We have also found that LICE (4°C, 8 h/day, 4 weeks) causes a 50% an increase in weight of the interscapular brown fat in comparison with the intact animals. We found that BICE (4°C, 1.5 h/day, 4 weeks) does not cause a change in the weight of brown fat. It has been documented that brown fat plays an important role in the thermogenesis and cold acclimatization and that an increase in the weight of brown adipose tissue is an indicator of exposure to cold adaptation (Cannon and Nedergaard 2004). Therefore it is a reasonable hypothesis that resistance to cold would occur after both continuous exposure to the cold and intermittent prolonged exposure to the cold.

Indeed, further experiments showed that a 2 h exposure to cold (-18°C) causes a decrease in the body temperature in the non-adapted rats to 37.4°C after 4 h temperature drops to 34°C (Fig. 1). Cold tolerance occurs in animals subjected to brief (4°C, 8 h/day, 4 weeks) intermittent exposure to the cold (Fig. 1).

We have found that after the CCE adrenal weight increased by 40% as compared to the intact animals (Table 1). The heart weight after the CCE has increased by 17%. The weight of the left ventricle increased by 18% while the weight of the right ventricle did not change. The left

ventricle mass index was increased by 16% in CCE group (Table 1). We have found no changes in the weight of thymus and spleen in these rats. We found that after the CCE the T₃ level in rat serum increased by 49% as compared to the intact animals (Table 2). Cortisol, corticosterone and T₄ levels in rat serum after CCE were unchanged (Table 2). We have found that intermittent exposure to the cold (4°C, 8 h/day, 4 weeks) increased T₄ level in rat serum increased by 56% as compared to intact animals (Table 2) but did not affect the weight of the heart, thymus, spleen, adrenal glands (Table 1). Cortisol, corticosterone and T₃ levels in rat serum after cold adaptation were unchanged (Table 2). We have found that brief intermittent exposure to the cold (+4°C, 1.5 h/day, 4 weeks) did not affect the weight of the heart, thymus, spleen, adrenal glands (Table 1). Cortisol, corticosterone, T₃ and T₄ levels in rat serum after cold adaptation were unchanged (Table 2). We also could not detect gastric ulcers after continuous and intermittent exposures to the cold.

We have demonstrated that the cold adaptation had no effect on the incidence of ischemic and reperfusion of ventricular arrhythmias (Table 3).

We have found an increase in systolic blood pressure from 127 mm Hg in the intact rats up to 146 mm Hg in rats exposed to continuous cold (Table 4). At the same time, the diastolic blood pressure and heart rate did not change. After the intermittent exposure to the cold we were not able to detect changes in blood pressure and heart rate.

As shown in Figure 2 in rats which were subjected to continuous exposure to the cold the IS/AAR ratio was significantly lower by 33% than that of the control group. We found no reduction of myocardial infarct size in rats after LICE and BICE.

Discussion

Our data indicates that after CCE and LICE in rats actually formed adaptation to the cold as evident by the increase in brown fat weight and increased resistance to the effects of low temperature (-18°C). In animals after BICE we found no change of brown fat weight but increased tolerance to the impact of low temperature (-18°C). Our studies indicate that there are different degrees of cold adaptation in the three groups. Despite finding no change in brown fat weight in BICE, there could have been changes in the mitochondrial components of the brown fat, leading to the cold tolerance seen in this group (Kuroshima et al., 1984; Ricquier et al., 1979).

We have shown that continuous exposure to the cold causes cardiac hypertrophy, the left ventricle mass index was increased by 16% in CCE group. The cold induced hypertrophy of the myocardium has been demonstrated by other authors (Hamilton et al. 1972, Harri et al. 1984, van Bergen et al. 1992). Apparently this hypertrophy in the heart is the result of an increase in blood pressure.

Continuous exposure to the cold promoted an increase in adrenal weight but had no effect on the weight of the thymus and spleen. Increased adrenal weight in our view indicates an increase in the adaptive capacity of the organism. After LICE and BICE we found no weight change of these organs. The presented data demonstrates the exposure to the cold used in these studies is not a chronic stress.

We found an increase of the levels in rat blood serum of the hormone T₃ after CCE and an increase in T₄ after LICE.

The increase in thyroid hormone levels in rat blood serum is consistent with earlier findings of other investigators (Bojko et al. 2008, McAllister et al. 2000, Vezyraki et al. 2000).

We have found an increase in systolic blood pressure after CCE. This data is in agreement with the experimental results of other researchers who have found an increase in blood pressure in

conscious rats after acclimatization to the cold (+6°C, 4 weeks) (Fregly et al 1989). Blood pressure was measured 1 h after exposure to cold in rats at room temperature (25°C) (Fregly et al 1989). Intermittent exposure to cold had no effect on blood pressure.

We have found that only CCE causes a decrease in the IS/AAR ratio. The mechanism of cardioprotective effect of cold adaptation remains unknown, we hypothesize that it can be associated with an increase in the density of β_3 adrenergic receptor (β_3 AR) in the myocardium in response to long-term continuous exposure to cold (Benes et al. 2012). Indeed, there is evidence that the administration of adrenomimetics in animals prior to the coronary occlusion may be the cause of the infarct-limiting effect (Khaliulin et al. 2011, Salie et al. 2012). It has also been shown that pretreatment with β_3 AR agonist BRL37344 can increase cardiac tolerance to ischemia and reperfusion (Aragon et al., 2011; García-Prieto et al., 2014). Thyroid hormones also can be involved in the regulation cardiac resistance to ischemia and reperfusion. It has also been demonstrated that the thyroid receptor agonist DITPA can ameliorate ischemic/reperfusion heart injury (Abohashem-Aly et al., 2011). The long-term administration of T₄ also enhanced cardiac resistance to ischemia and reperfusion (Kumar et al., 2012).

It has been shown that migrants living long-term in the Far North have a greater frequency of myocardial infarction than in the population of Russians living in the mid-latitudes (Turchinskiy 1979, Turchinskiy and Sakharova 1979). It is now apparent to us that our findings contradict the results of epidemiological studies. In this regard, it should be noted that the increase in the incidence of cardiovascular diseases in the alien population occurs only after living a few years in the Arctic Circle (Turchinskiy 1979). Perhaps after a few years of living in the conditions of the cold and the polar night, a person develops disadaptation. We hypothesize that after a few months in the refrigerator chamber, that the phenomena of disadaptation will develop in rats and a high tolerance to cardiac ischemia and reperfusion will be replaced by low tolerance.

Thus, we have established that the continuous exposure to the cold (4°C, 4 weeks) causes cold adaptation, increases systolic blood pressure, exerts infarct-limiting effect but does not induce a chronic stress. The intermittent exposure to the cold had no effect on cardiac tolerance to ischemia/reperfusion and is not a chronic stress. The continuous exposure to the cold promotes an increase of T₃ level in serum but intermittent exposure to the cold (8 h/day, 4 weeks) contributes to an elevation of serum T₄ levels. The continuous and intermittent exposure to the cold had no effect on the glucocorticoid levels. We believe that the study of the molecular mechanisms of the cardioprotective effect of adaptation to cold will not only expand the horizons of our knowledge of heart tolerance to the action of ischemia / reperfusion, but also in the future will contribute to the development of new approaches to the prevention of human ischemic and reperfusion injuries.

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Conflict of Interest

There is no conflict of interest.

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Figure 1. Cold tolerance test.

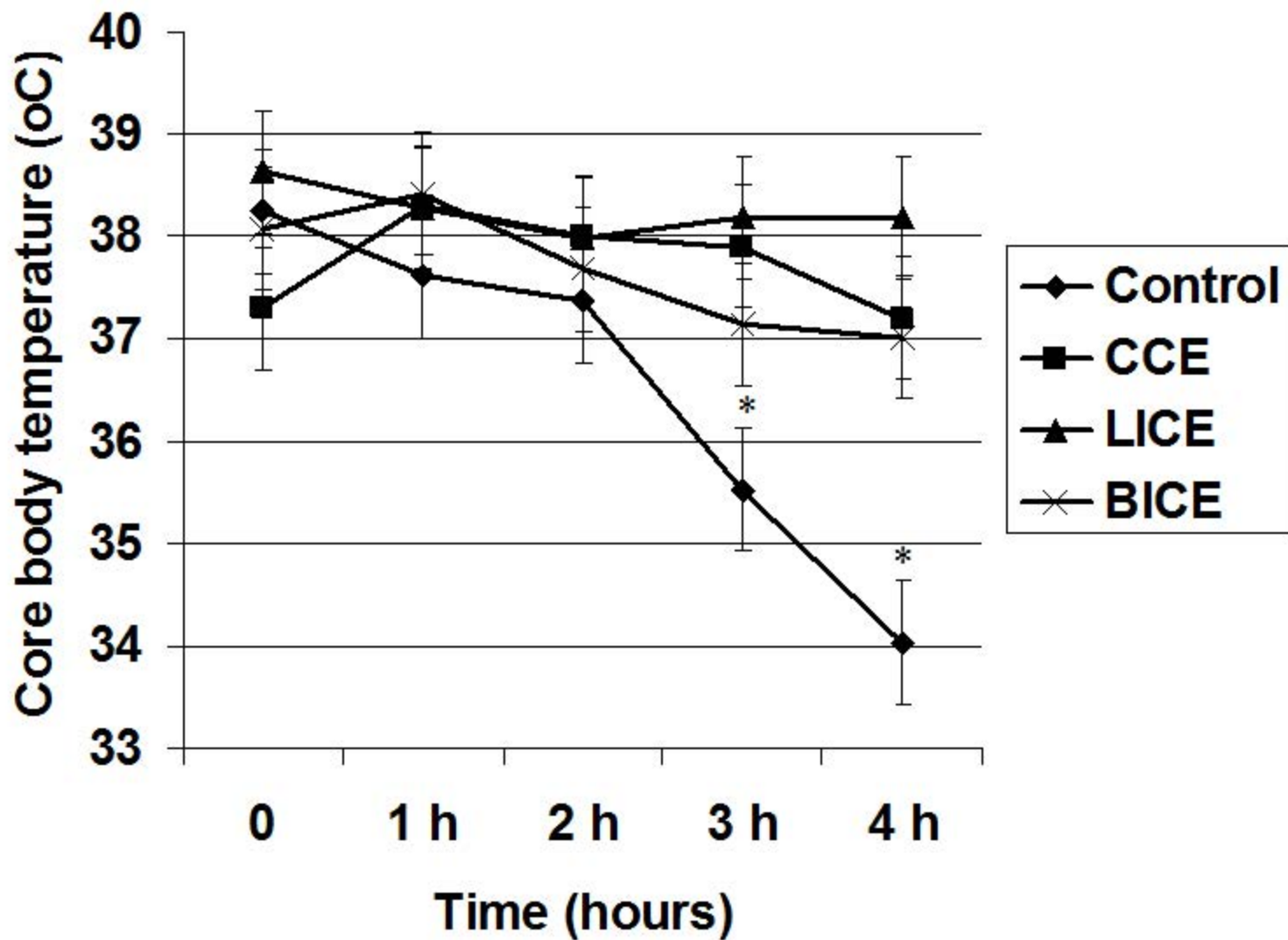
number of animals was 32; 0 - the moment when the animal was placed in a cooling chamber; CCE - +4°C, 4 weeks; LICE - +4°C, 8 h/day, 4 weeks; BICE - +4°C, 1.5 h/day, 4 weeks;

* - P < 0.05 versus all cold exposure groups

Figure 2. Infarct-limiting effect of cold exposure.

AAR – area at risk; CCE - +4°C, 4 weeks; LICE - +4°C, 8 h/day, 4 weeks; BICE - +4°C, 1.5 h/day, 4 weeks;

* P < 0.01 versus control



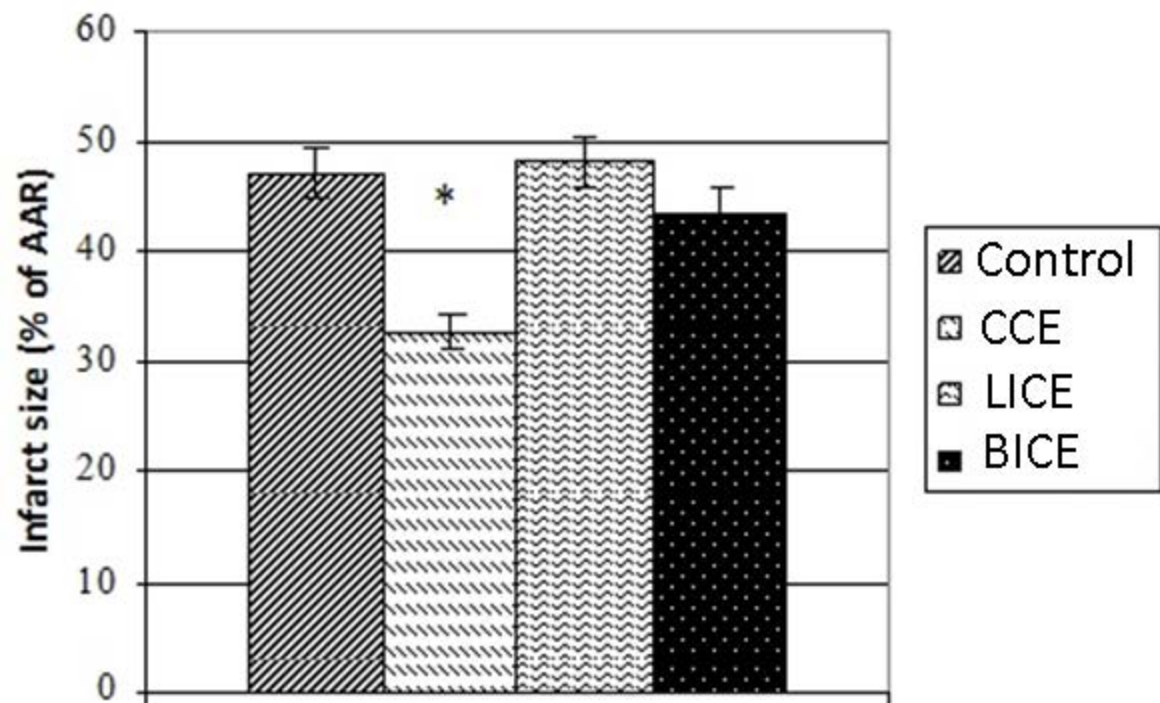


Table 1

Body, organ and tissue weight after exposure to cold in rats

	Body weight, g	Thymus, mg	Adrenal glands, mg	Spleen, mg	Heart weight, mg	left ventricle weight, mg	right ventricle weight, mg	The left ventricle mass index, %	Brown fat, mg
Control n = 15	282.6 ± 9.3	240.5 ± 13.2	35.6 ± 2,1	1059.5 ± 96.1	956.2 ± 31.4	808.6 ± 36.2	148.5 ± 11.4	0.31 ± 0.013	300.4 ± 38.2
CCE n = 12	264.8 ± 3.56	217.0 ± 12.9	49.7 ± 2*	933.8 ± 45.2	1123.3 ± 46.7*	957.8 ± 41.7*	166.3 ± 14.7	0.36 ± 0.015	764.6 ± 30.5*
LICE n = 12	266.7 ± 28.9	272.5 ± 13.7	33.1 ± 1.1	1052.8 ± 58.6	984.2 ± 31.7	846.3 ± 17.7	138.7 ± 13.5	0.32 ± 0.006	566.66 ± 28.9*
BICE n = 12	271.7 ± 21.1	244.5 ± 15.2	34.2 ± 1.9	1060.2 ± 64.3	1003.4 ± 58.7	869.6 ± 21.5	134.5 ± 15.1	0.32 ± 0.007	311.7 ± 21.02

Note: Data is expressed as M ± SEM. n – number of rats in group; * p < 0.01 versus control

animals; CCE - +4°C, 4 weeks; LICE - +4°C, 8 h/day, 4 weeks; BICE - +4°C, 1.5 h/day, 4 weeks;

Table 2

Hormonal indices of stress response in rats

	Corticosterone nmol/l	Cortisol nmol/l	T3 nmol/l	T4 nmol/l
Control n = 15	2568.5 ± 93.3	58.3±12.2	1.04±0.17	45.6±4.6
CCE n =12	2587.3 ± 91.4	47.1±6.1	1.55± 0.1 *	49.9±2.4
LICE n =12	2571.5 ± 98.5	49.7±5.5	0.84±0.08	71.1±5.9 *
BICE n =12	2589.7 ± 99.8	41.9±6.1	0.71±0.09	64.2±4.1

Note: Data is expressed as M± SEM. n – number of rats in group; * p < 0.01 versus control animals; Protocol 1 - +4°C, 4 weeks; CCE - +4°C, 4 weeks; LICE - +4°C, 8 h/day, 4 weeks; BICE - +4°C, 1.5 h/day, 4 weeks;

Table 3

The incidence of ventricular arrhythmias

	Ischemia								Reperfusion (10 min)			
	Phase 1a (1-10 min)				Phase 1b (10-20 min)				sPVC (%)	PVCs (%)	VT (%)	VF (%)
	sPVC (%)	PVCs (%)	VT (%)	VF (%)	sPVC (%)	PVCs (%)	VT (%)	VF (%)				
Control n = 15	2 (13)	13 (87)	10 (67)	6 (40)	6 (40)	9 (60)	6 (40)	2 (13)	13 (77)	2 (13)	0 (0)	0 (0)
CCE n =12	2 (17)	10 (83)	9 (75)	5 (42)	5 (42)	7 (58)	5 (42)	1 (8)	11 (92)	1 (8)	0 (0)	0 (0)
LICE n =12	1 (8)	11 (92)	8 (67)	4 (33)	4 (33)	8 (67)	6 (50)	1 (8)	10 (83)	2 (17)	0 (0)	0 (0)
BICE n =12	0 (0)	12 (100)	8 (67)	5 (42)	5 (42)	7 (58)	6 (50)	1 (8)	9 (75)	3 (25)	0 (0)	0 (0)

Note: n- number of rats; sPVC is a single premature ventricular complexes; PVCs is premature

ventricular complexes; VT is ventricular tachycardia; VF is ventricular fibrillation.

CCE - +4°C, 4 weeks; LICE - +4°C, 8 h/day, 4 weeks; BICE - +4°C, 1.5 h/day, 4 weeks;

Table 4

Effect of cold exposure (or adaptation) on systolic blood pressure during ischemia and reperfusion

	Systolic BP before ischemia	Systolic BP Ischemia	Systolic BP start of reperfusion	Systolic BP 5 minutes of reperfusion	Systolic BP 15 minutes of reperfusion	Systolic BP 45 minutes of reperfusion
Control n = 15	127 ± 6	127 ± 8	125 ± 6	124 ± 6	119 ± 7	110 ± 7
CCE n=12	146 ± 5 *	145 ± 7 *	142 ± 6 *	140 ± 7 *	136 ± 6 *	135 ± 6 *
LICE n=12	126 ± 5	123 ± 5	126 ± 7	125 ± 7	123 ± 6	120 ± 7
BICE n=12	123 ± 5	125 ± 7	122 ± 6	120 ± 7	115 ± 6	113 ± 6

Note: Data is expressed as M ± SEM. n – number of rats in group;* P < 0.05 versus control

CCE - +4°C, 4 weeks; LICE - +4°C, 8 h/day, 4 weeks; BICE - +4°C, 1.5 h/day, 4 weeks