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# Prooxidant–antioxidant balance in rats under hypothermia combined with modified hemoglobin–oxygen affinity

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**Abstract**

Deep hypothermia (fall of body temperature by more than 10°C) is associated with the serious perturbation of oxygen delivery and body prooxidant–antioxidant balance. The blood oxygen-binding properties determine the conditions of oxygen diffusion to tissues and the values of tissue $PO_2$ and have a specific role in the complex system of antioxidant defense. Our aim was to evaluate the prooxidant–antioxidant balance under the conditions of modified hemoglobin–oxygen affinity (HOA) in a rat experimental model of hypothermia (HOA was increased by sodium cyanate (NaOCN) and decreased by sodium *o*-iodobenzoate (OISB)). The preliminary shift of oxyhemoglobin dissociation curve (ODC) leftwards during the hypothermia was accompanied by the shift of prooxidant–antioxidant balance towards activation of the lipid peroxidation (LPO), and the ODC shift rightwards was accompanied by the LPO lowering. HOA decrease is favorable during the deep hypothermia due to the optimized oxygen flux to tissues and its lower fraction spent for the free radical reactions that is associated with the less marked distresses of prooxidant–antioxidant balance. 

*Keywords:* Hypothermia; Lipid peroxidation; Antioxidants; Hemoglobin–oxygen affinity; Sodium cyanate; Sodium iodobenzoate; Rat

## 1. Introduction

The development of hypothermia in homeothermic animals leads to complex changes in biochemical processes and physiological functions including tissue oxygen delivery (Schumaker et al., 1987). The temperature of body largely determines the body oxygen utilization: its lowering by 1°C decreases $O_2$ consumption by 11% (Wood, 1995). Deep hypothermia (fall of body temperature by more than 10°C) is characterized by the serious impairment of oxygen delivery and development of hypoxia (Coetzee and Swanepoel, 1990; Giesbrecht and Bristow, 1997). Hemoglobin–oxygen affinity (HOA) is one of the important determinants of oxygen flux to tissues (Hsia, 1998). The deep body cool-ing is accompanied by the prominent oxyhemoglobin dissociation curve (ODC) shift leftwards that can impair tissue $O_2$ supply and play an important role in the genesis of hypoxia, despite the decreased metabolic rate and oxygen demands (McArthur et al., 1992; Frappell, 1998).

Body lipid peroxidation (LPO) intensity is regulated by the ratio of prooxidant level to the antioxidant defense activity; these factors determine the body prooxidant–antioxidant state (McCord, 2000). A greater generation of active oxygen metabolites was noted during hypothermia (Langley et al., 2000). This prooxidant shift resulted from the switch of cellular oxygen metabolism from reduction by cytochrome oxidase to LPO (Halliwell, 1999). The antioxidant system opposes the mechanisms of enhanced free radical generation, but the deep cooling markedly weakens it (Nollert et al., 1999). Erythrocytes are the important component of the blood antioxidant capability associated with the specific intracellular enzymes such as superoxide dismutase and catalase and also with the glutathione system (Klatt and

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Lamas, 2000). The blood oxygen-binding properties determine the conditions of oxygen diffusion to tissues and the values of tissue $PO_2$ and take a specific part in the complex system of antioxidant defense (Zinchuk and Borisiuk, 1998). Methods to modify HOA may become useful for the development of strategies to strengthen the complex endogenous defense against the free radicals, such as was observed under hyperthermic conditions (Zinchuk, 1999a). Our aim was to evaluate the prooxidant–antioxidant balance under the conditions of modified HOA in the rat experimental model of hypothermia.

## 2. Materials and methods

The experiments were performed in 39 male laboratory rats (body 210–270 g) maintained in the standard conditions in vivarium. Hypothermia was created by the 90 min cooling of rats preliminary anesthetized by hexenal (50 mg/kg intraperitoneally). The animals were cooled in a special box with the circulating water (temperature 17°C). The rectal temperature was measured by an electronic thermometer. Blood samples were taken from rat right atrium, and then the tissue sampling was performed (liver, kidneys, heart, lungs). All surgical interventions were performed under analgesia.

HOA was increased by sodium cyanate (NaOCN; 0.5% aqueous solution added to the diet of animals fed ad libitum for 8 weeks (Baer, 1992)). HOA was decreased by sodium ortho-iodobenzoate (OISB; 900.0 mg/kg intraperitoneally, for 3 weeks (Teisseire et al., 1979)). Six experimental groups were created: 1—control ($n = 7$); 2—with cooling of rats ($n = 8$); 3—with administration of NaOCN ($n = 7$), 4—with NaOCN administration and cooling ($n = 11$); 5—with iodobenzoate treatment ($n = 5$); 6—with iodobenzoate treatment and cooling ($n = 8$).

Acid–base balance: plasma concentration of bicarbonates ($HCO_3^-$), the concentration of total carbon dioxide ($TCO_2$), the actual excess of buffer bases (ABE), the standard excess of buffer bases (SBE) and the standard carbonate (SBC) were calculated by a program based on the formulas of Severinghaus (1966) and the nomograms of Siggaard-Andersen. $PO_2$ as measured with a micro gas analyzer (ABL-330, Radiometer) at 37°C and then as adjusted to the actual temperature value. HOA was assessed by P50 (blood $PO_2$ under its 50% saturation by $O_2$) as determined by a 'mixing method' (Scheid and Meyer, 1978) at 37°C, pH 7.4 and $PCO_2 = 40$ mm Hg (P50stand). The values of P50 at actual pH, $PCO_2$ and temperature (P50act) were calculated from P50stand by Severinghaus' equations (1966) and with the temperature coefficient $\Delta \lg P50/\Delta T = 0.024$; the ODCs were calculated according to Hill's equation with $n = 2.8$ from the measured P50. The amounts of hemoglobin (Hb) were determined spectrophotometrically.

Conjugated diene (CD) content was determined by UV absorption at 232–234 nm (indicating the conjugated double bonds of lipid hydroperoxides) (Rice-Evans et al., 1991). The results were expressed in relative units of optical density in 1 ml (ΔA233/ml). The level of Schiff bases (SB) was determined by fluorescence intensity of chloroform extracts at excitation and emission wavelengths of 344 and 440 nm, respectively, with an F-4010 spectrofluorimeter (Hitachi). The results were expressed in relative units of intensity in 1 ml of plasma or red blood cells (Aruoma and Cuppett, 1997).

Antioxidant system: catalase activity was determined by measurement of the rate of decomposition of hydrogen peroxide capable of generating a stable colored complex with molybdenum salts which was measured with an SF-46 spectrophotometer at 410 nm (Aruoma and Cuppett, 1997), α-tocopherol concentration was evaluated by fluorescence intensity of heptane extraction at excitation and emission wavelengths of 292 and 325 nm with an F-4010 spectrofluorimeter (Hitachi) using α-tocopherol (Sigma Chemical Co., St. Louis) as reference (Rice-Evans et al., 1991). Protein content was measured according to Layne (1957).

The data were statistically evaluated by Student's $t$-test with a significance level of $P < 0.05$. The results are presented as means ± SE. The analyses and graphs were performed using computer software packages.

## 3. Results

NaOCN and OISB injections to experimental rats resulted in the rise and fall of HOA, respectively, compared with a hypothermic control (Table 1). ΔP50 between experimental groups (4 and 6) was 10.93 ± 0.52 mm Hg. ODC was shifted leftwards with NaOCN and rightwards with OISB (Fig. 1). Rats with enhanced HOA had the maximal fall of rectal temperature at 90 min after the cooling (to 23.0 ± 0.13°C vs. 23.7 ± 0.24°C in hypothermic control, $P < 0.05$), and in rats with attenuated HOA this fall was minimal (to 25.5 ± 0.25°C; $P < 0.001$) (Fig. 2).

Low temperatures led to the development of metabolic acidosis on the background of moderate hypercapnia and to the considerable decreases of ABE and SBE (Table 1), but their values at real temperature were different. pHact was 7.331 ± 0.005 in cyanate-treated and hypothermic rats and 7.377 ± 0.008 in OISB-treated and hypothermic ($P < 0.001$). In rats with increased

Table 1
Blood oxygen transport indices in rats under hypothermia combined with a modification of hemoglobin–oxygen affinity (M ± m)

| Parameter | Control | Hypothermia | Sodium cyanate | Sodium cyanate + hypothermia | Sodium iodobenzoate | Sodium iodobenzoate + hypothermia |
|---|---|---|---|---|---|---|
| P50stand, mm Hg | 30.48 ± 0.56 | 32.78 ± 0.72* | 21.62 ± 0.44* | 20.88 ± 0.80*,# | 36.59 ± 0.59* | 36.46 ± 0.91*,#,$ |
| P50act, mm Hg | 33.23 ± 0.70 | 20.63 ± 0.47* | 24.50 ± 0.57* | 12.72 ± 0.57*,# | 41.65 ± 0.60* | 23.65 ± 0.59*,#,$ |
| Hb, g/L | 120.85 ± 0.44 | 117.62 ± 0.23 | 122.71 ± 0.26 | 120.4 ± 0.23 | 115.80 ± 0.20 | 113.38 ± 0.38 |
| $PO_2$, mm Hg | 27.36 ± 0.81 | 23.31 ± 0.63* | 27.96 ± 0.77 | 22.99 ± 0.37* | 25.52 ± 0.20 | 24.60 ± 0.41*,$ |
| $PO_2$act, mm Hg | 27.34 ± 1.01 | 8.95 ± 0.36* | 28.31 ± 0.82 | 8.58 ± 0.14* | 26.41 ± 0.29 | 10.82 ± 0.23*,#,$ |
| $PCO_2$, mm Hg | 59.20 ± 1.15 | 65.79 ± 0.95* | 51.71 ± 1.00* | 69.66 ± 0.82*,# | 59.72 ± 1.18 | 67.93 ± 0.93* |
| $PCO_2$act, mm Hg | 59.15 ± 1.31 | 36.50 ± 0.48* | 52.10 ± 0.98* | 38.10 ± 0.60* | 61.06 ± 1.53 | 41.03 ± 0.72*,#,$ |
| pH, units | 7.333 ± 0.017 | 7.148 ± 0.010* | 7.293 ± 0.022 | 7.125 ± 0.004*,# | 7.315 ± 0.007 | 7.207 ± 0.010*,#,$ |
| pHact, units | 7.333 ± 0.015 | 7.343 ± 0.009 | 7.291 ± 0.022 | 7.331 ± 0.005 | 7.308 ± 0.006 | 7.377 ± 0.008*,$ |
| $HCO_3^-$, mM | 31.49 ± 1.35 | 21.10 ± 0.65* | 24.99 ± 1.04* | 23.45 ± 0.42*,# | 30.32 ± 0.91 | 26.08 ± 0.56*,#,$ |
| $TCO_2$, mM | 33.49 ± 1.45 | 22.94 ± 0.66* | 26.54 ± 1.04* | 25.35 ± 0.42*,# | 31.92 ± 0.85 | 23.39 ± 0.61*,#,$ |
| ABE, mM | 4.4 ± 1.42 | −8.49 ± 0.79* | −1.13 ± 0.94* | −6.45 ± 0.43*,# | 2.98 ± 0.80 | −2.86 ± 0.79*,#,$ |
| SBE, mM | −0.21 ± 1.93 | −4.89 ± 1.08* | 3.81 ± 2.25 | −2.83 ± 0.41 | −3.76 ± 0.77 | −6.06 ± 0.90*,$ |
| SBC, mM | 21.6 ± 1.41 | 17.55 ± 0.77* | 22.9 ± 0.69 | 17.9 ± 0.36* | 19.36 ± 0.62 | 16.69 ± 0.72* |

Abbreviations used: $P_{50}$stand, blood $pO_2$ under its 50% saturation by $O_2$ as determined at 37°C, pH 7.4 and $pCO_2 = 40$ mm Hg; $P_{50}$act, $P_{50}$ at actual pH, $pCO_2$ and temperature; Hb, hemoglobin; $PO_2$, oxygen pressure; $PCO_2$, carbon dioxide pressure; $HCO_3^-$, plasma concentration of hydrocarbonate; $TCO_2$, concentration of total carbon dioxide; ABE, the actual excess of buffer bases; SBE standard excess of buffer bases; SBC, standard hydrocarbonate.

*—Significant difference from control group;

#—Significant difference from hypothermic group;

$—Significant difference between the groups of NaOCN + hypothermia and OISB + hypothermia.

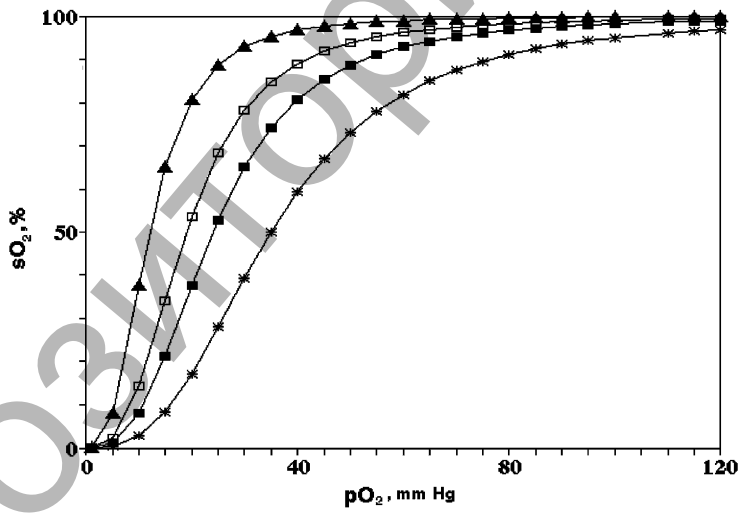


Fig. 1. Actual ODCs in rats of the following groups: control (∗), 0.9% NaCl + hypothermia (□), NaOCN + hypothermia (▲) and OISB + hypothermia (■).

HOA hypothermia was accompanied by more marked metabolic acidosis and the least changes in acid–base balance, respectively.

The preliminary OISB administration suppressed the peroxidative processes, activated during the acute deep hypothermia (Table 2). Thus, in rat erythrocytes of group 4 (NaOCN + hypothermia) CD and SB contents were higher than in control by 110% and 32.5%, respectively (both $P < 0.001$), and in group 6 (OISB + hypothermia) these contents were higher than in control by 49.7% and 8.5% ($P < 0.001$ and $P < 0.05$, respectively). During the hypothermia hepatic CD and SB contents were higher than in control by 49.8% and 32.9% ($P < 0.01$ and $P < 0.001$, respectively); in group 4

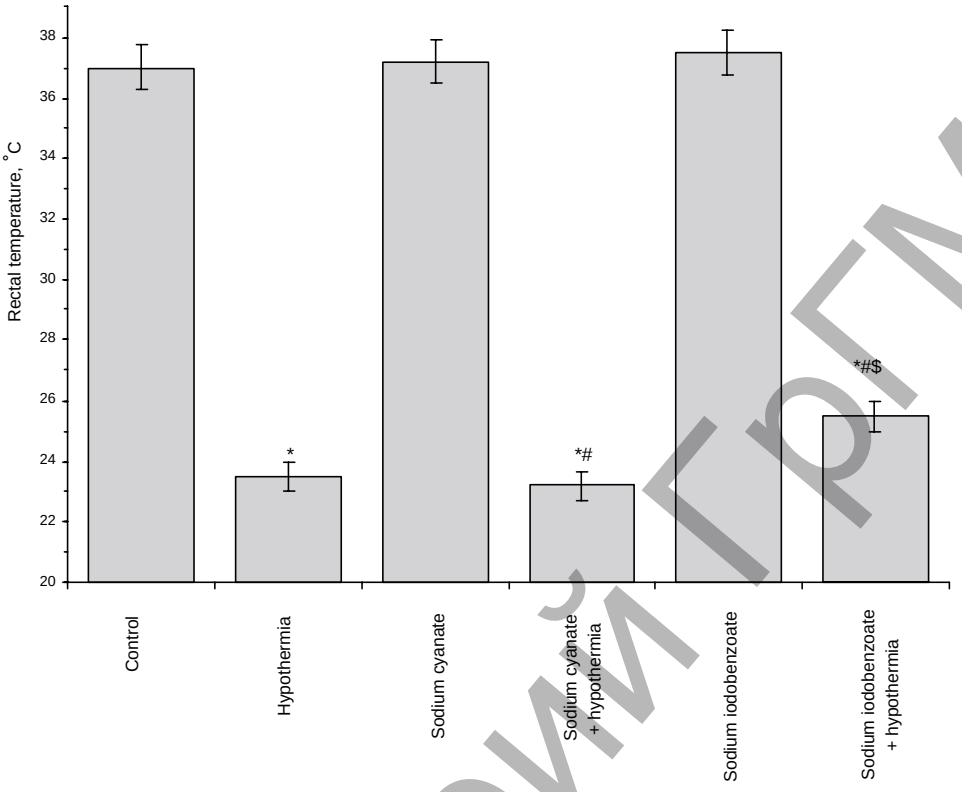


Fig. 2. Rectal temperature in rat after the hypothermia combined with a modification of HOA. *—significant difference from control group; #—significant difference from hypothermic group; $—significant difference between the groups of NaOCN + hypothermia and OISB + hypothermia.

Table 2
Lipid peroxidation indices in rat blood and tissues under hypothermia combined with modified hemoglobin–oxygen affinity (M ± m)

| Parameter | | Control | Hypothermia | Sodium cyanate | Sodium cyanate + hypothermia | Sodium iodobenzoate | Sodium iodobenzoate + hypothermia |
|---|---|---|---|---|---|---|---|
| CD, $\Delta A_{233}$/ml | Erythrocytes | 3.82 ± 0.24 | 6.49 ± 0.43* | 3.89 ± 0.24 | 8.04 ± 0.18*,# | 3.81 ± 0.21 | 5.72 ± 0.27*,$ |
| | Liver | 9.63 ± 0.94 | 14.43 ± 1.14* | 10.48 ± 0.34 | 15.98 ± 0.92* | 10.10 ± 0.55 | 12.63 ± 0.33*,# |
| | Kidneys | 8.04 ± 0.53 | 10.73 ± 0.58* | 8.17 ± 0.11 | 14.18 ± 0.56*,# | 8.40 ± 0.35 | 9.07 ± 0.34#,$ |
| | Lungs | 6.33 ± 0.56 | 9.89 ± 0.69* | 6.06 ± 0.30 | 9.51 ± 0.43* | 5.63 ± 0.36 | 7.22 ± 0.12#,$ |
| | Heart | 6.45 ± 0.60 | 9.85 ± 0.63* | 6.10 ± 0.37 | 14.29 ± 0.78*,# | 7.19 ± 0.30 | 9.08 ± 0.24*,$ |
| SB, units/ml | Erythrocytes | 67.19 ± 1.19 | 79.27 ± 1.25* | 64.32 ± 1.00 | 89.02 ± 1.01*,# | 64.82 ± 1.21 | 72.88 ± 1.78*,#,$ |
| | Liver | 182.07 ± 3.12 | 241.99 ± 4.38* | 172.82 ± 3.39 | 263.11 ± 4.76*,# | 180.80 ± 3.21 | 199.85 ± 2.51*,#,$ |
| | Kidneys | 141.47 ± 4.35 | 208.94 ± 7.16* | 137.02 ± 6.52 | 242.19 ± 9.37*,# | 144.02 ± 6.52 | 175.43 ± 1.96*,#,$ |
| | Lungs | 229.16 ± 4.04 | 282.66 ± 5.35* | 232.06 ± 6.26 | 284.61 ± 6.44* | 224.60 ± 3.23 | 257.97 ± 10.07*,#,$ |
| | Heart | 177.0 ± 8.09 | 255.59 ± 5.72* | 178.42 ± 4.0 | 257.62 ± 5.41* | 169.46 ± 13.53 | 208.88 ± 7.16*,#,$ |

Abbreviation used: CD—conjugated diene content; SB—Schiff base content.
*—Significant difference from control group;
#—Significant difference from hypothermic group;
$—Significant difference between the groups of NaOCN + hypothermia and OISB + hypothermia.

rats—by 65.9% and 44.5% ($P<0.001$ and $P<0.01$, respectively); and in group 6 rats—by 31.7% and 9.8% ($P<0.05$ and $P<0.01$, respectively). Renal, pulmonary and myocardial tissues had the similar pattern of LPO gain. The contents of primary (CD) and final (SB) LPO products in rats that received OISB before the cooling

were significantly lower than after NaOCN injection accompanied by a considerable rise of LPO activity. The administration of these HOA modifiers without hypothermia did not result in any changes in LPO products.

The largest antioxidant potential depletion was noted in the animals with increased HOA, with a smaller fall in rats with decreased HOA (Fig. 3). Thus, in cyanate-treated animals the amounts of α-tocopherol in erythrocytes, liver, kidneys, lungs and myocardium were by 24.8%, 20.96%, 23.12%, 14.1%, and 34.9% (all $P<0.001$) lower than in control rats, respectively. In OISB-treated rats these amounts were by 8.7% ($P<0.05$), 9.8% ($P<0.05$), 9.5% ($P<0.01$), 10.6% ($P<0.01$), and 28.5% ($P<0.001$) lower than in control, respectively. Retinol amounts and catalase activities had the similar dynamics.

## 4. Discussion

Our experiments have shown that a shift of prooxidant–antioxidant balance towards LPO activation and weakening of the antioxidant defense were accompanied by an ODC shift leftwards combined with an exposure to cold, and the inverse changes were accompanied by an ODC shift rightwards.

ODC position during the whole body hypothermia is largely determined by the influences of temperature, pH and $PCO_2$. ODC position under real values of temperature, pH and $PCO_2$ is a compromise between these differently acting determinants. The evaluation of acid–base state should take into account the intrinsic temperature effect (in our investigations pH and $pCO_2$ changes under standard conditions (37°C) correspond to acidosis and hypercapnia, but their values at real

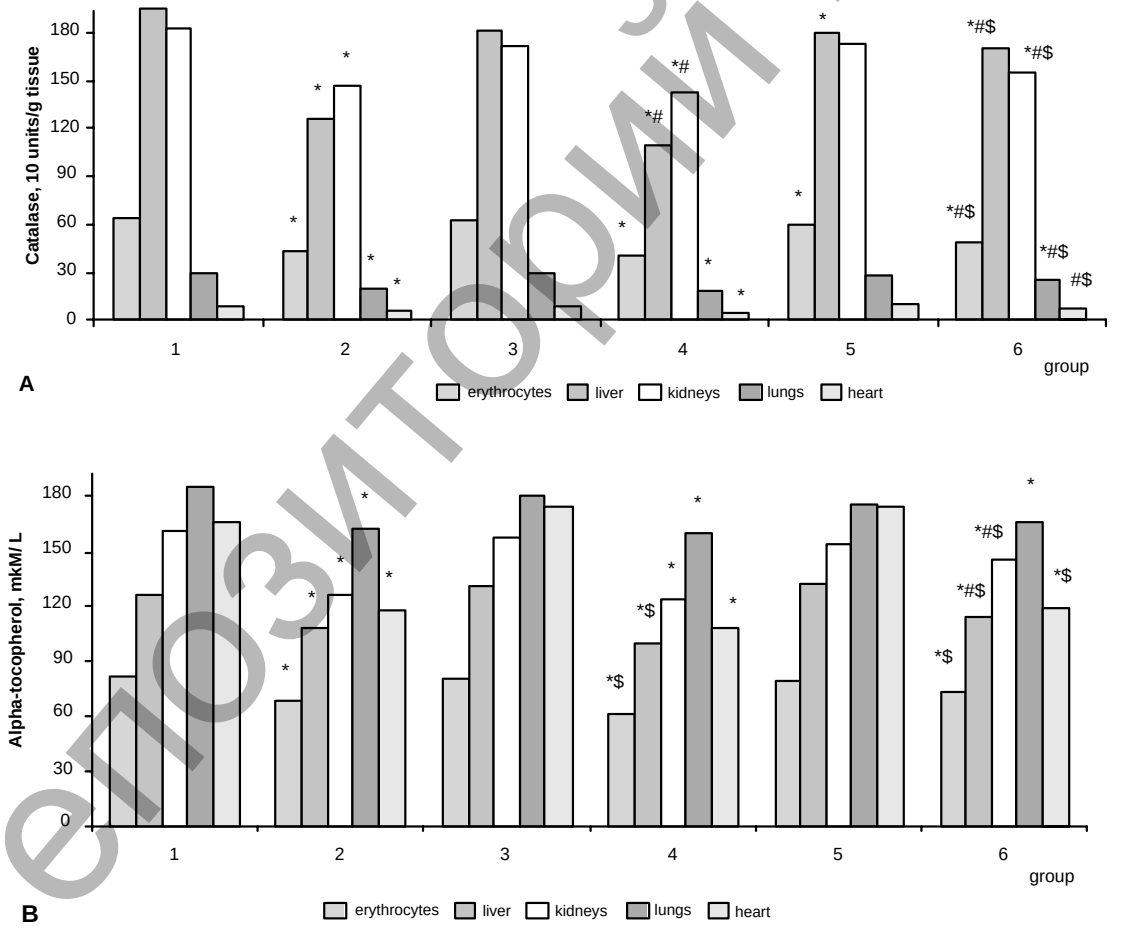


Fig. 3. Changes in antioxidant defense indices (A—catalase activity, and B—α-tocopherol content) in rat tissues during hypothermia combined with the modified HOA. 1—control; 2—hypothermia + 0.9% NaCl; 3—NaOCN; 4—NaOCN + hypothermia; 5—OISB; 6—OISB + hypothermia. *—significant difference from control group; #—significant difference from hypothermic group; $—significant difference between the groups of NaOCN + hypothermia and OISB + hypothermia.

temperatures were different). The hypothermia differs between hibernating and non-hibernating mammals and due to the time of cold exposure and velocity of hypothermia appearance. In the investigation of McArthur et al. (1992) the time of hypothermia was 24 h in rats and 72 h in hibernating Richardson's ground squirrels. In our experiments the hypothermia was deeper and quicker.

The temperature is the most significant determinant. Hb oxygenation is an exothermic reaction; hence, oxyhemoglobin dissociation consumed heat (Hsia, 1998). Such a pattern of temperature influence on Hb–oxygen interaction is typical for the majority of Hbs (Samaja, 1997). The temperature effect on ODC position, expressed by the temperature ratio $\Delta \lg P50/\Delta T$, is different between the poikilothermic and homeothermic animals. Willford and Hill (1986) considered the dependence between P50 and temperature as the mechanism for prevention of unbalance between oxygen demands and delivery. The temperature influences may be compensated either by a higher synthesis of thermotolerant Hbs or as the result of changes in HOA modulator synthesis (Wood, 1980). The last mechanism appears to be more realistic for the homeothermic animals. Rats are known to have the higher ATP and 2,3-diphosphoglycerate (DPG) concentrations during the hypothermia (Mairbaurl and Humpeler, 1980), resulting in a weaker temperature influence on the ODC position (weaker shift leftwards).

HOA is known largely to determine $O_2$ flux to tissues and to participate in the development of hypoxia (Osborne and Milson, 1993). During the hypothermia HOA increases, and P50 decreases. Thus, in anesthetized patients the fall in body temperature from 35.5°C to 32.0°C was associated with a decrease of P50 from $23.8 \pm 1.7$ to $20.0 \pm 0.9$ mm Hg (Bacher et al., 1997). In the experiments with a decrease of canine body temperature from 37.7°C to 30.5°C hypothermia also shifted ODC leftwards (P50 changed from $32.4 \pm 0.7$ to $19.8 \pm 0.7$ mm Hg ($P < 0.001$) (Gutierres et al., 1986). Moderate hypothermia may be useful under the hypoxic states, because of the moderate ODC shift leftwards that improves the pulmonary blood oxygenation, decreases tissue oxygen consumption and reduces the energy costs of all the body system functions (Wood, 1995). ODC shift leftwards may have an important role in the depression of energy metabolism in the patients with fever during body cooling (Manthous et al., 1995). Under the deep hypothermic conditions the positive effects of the adaptive responses to the low body temperature are compromised resulting in serious metabolic distress and beginning of hypoxia (Alfaro et al., 1995). In our experiments with NaOCN the temperature of body and P50 decreased more considerably, with a corresponding larger ODC shift leftwards and rather negative effect. In such conditions the HOA modification directed into the less ODC shift leftwards and temperature influence was the most profitable (Fig. 4).

Oxygen involvement in LPO initiation and propagation suggests that HOA may affect the mechanisms of the free radical process activation during the hypothermia. Hb can regulate the oxygen flux to tissues according to their demands by changes in its oxygen affinity, thereby preventing excessive $O_2$ delivery with its following redistribution from cytochrome oxidase to LPO reactions (Zinchuk, 1999a). During the lypopolysaccharide-induced fever the value of ODC shift correlated with indices of the free radical oxidation (Zinchuk, 1999b); therefore one can consider HOA as the factor participating in the body prooxidant–antioxidant balance maintenance. Oxyhemoglobin is a component of defensive mechanisms during the hydroperoxide-initiated oxidative stress; its antioxidant properties depend both on its oxygen-binding ability and the intrinsic state of the molecule. The stronger HOA decreases the velocity of oxyhemoglobin autooxidation in methemoglobin, and weaker HOA facilitates the generation of met-Hb (Stepuro et al., 1994). However, Hb can also express the prooxidant activity mediated by hydroxyl radical generation. Such prooxidant potential may be decreased by Hb modifications (conjugate of piridoxalated Hb and polyoxyethylene) (Privalle et al., 2000). ODC shift rightwards protected the myocardium (its high-energy phosphates and mechanic function) after low-flow ischemia (Woods et al., 1998). The synthetic allosteric Hb modifier RSR-13 (2-[[[4-(3,5-dimethylanilino)carbonyl]methyl]-phenoxy]-2-methylpropionic acid, shifting the ODC rightwards) improved the restoration of myocardial mechanic and metabolic functions in dogs with the

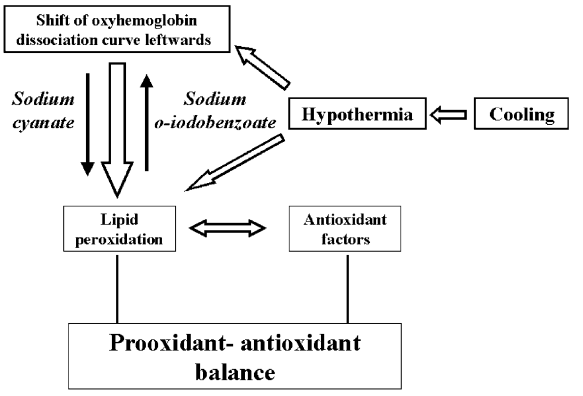


Fig. 4. The role of HOA in the mechanisms of body prooxidant–antioxidant balance during the hypothermia: the ODC shift leftwards by sodium cyanate provides the LPO activation, and its shift rightwards by sodium *o*-iodobenzoate decreases LPO.

hypothermic cardiopulmonary bypass after the surgical procedures (Kilgore et al., 1999). In our experiments the preliminary shift ODC rightwards might help the maintenance of optimal prooxidant–antioxidant balance through the better equilibrium between electron donors and acceptors; without such equilibrium the respiratory chain cannot properly reduce oxygen, resulting in the free radical process activation (gain of LPO products and antioxidant system depletion). Nitric oxide also can participate in the genesis of prooxidant–antioxidant unbalance during hypothermia (Zinchuk and Dorokhina, 2002), because by its reaction with superoxide this free radical can form the powerful oxidant peroxynitrite (Lee et al., 2000), also able to modify the Hb properties (Minetti et al., 1999).

Thus the directed decrease of HOA during the hypothermia reduced the prooxidant–antioxidant unbalances. Weakening of HOA is more favorable during the deep hypothermia due to the optimization of oxygen flux to tissues and lowering of the fraction spent in the free radical reactions. This finding allowed us to consider HOA as one of the physiological mechanisms participating in maintaining the body prooxidant–antioxidant balance.

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