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EFFECT OF STRENGTH EXERCISE AND N-ACETYLCYSTEINE ON METABOLISM OF GLUTATHIONE, OXIDATIVE DAMAGE AND ERYTHROPOIETIN PRODUCTION

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ABSTRACT

Introduction/Purpose: Glutathione is a ubiquitous thiol playing important role in cellular defense against oxidative stress. Earlier studies have shown, that glutathione metabolism and production of erythropoietin (EPO) may be modulated by N-acetylcysteine. The aim of this study was to determine whether a strength exercise induces oxidative stress and whether intensification of glutathione metabolism by oral treatment with N-acetylcysteine reduces plasma protein and lipid damage and results in changes of EPO level.

Methods: Ten healthy male volunteers participated twice in this study. Subjects performed an intensity resistance exercise (shoulder press, bench press, straightening up with weights). Subjects were given oral dose of 3 x 600 mg N-acetylcysteine (NAC) daily for three days before the second study. Venous blood samples were collected 5min before exercise, immediately post exercise and after 24 h rest. Protein carbonyls (PC), lipid peroxidation products (TBARS), erythropoietin (EPO) and glutathione (GSH) levels as well as γ-glutamyl transpeptidase (GGT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were evaluated.

Results: The applied exercise at maximal intensity (post-exercise lactate: 7.71 ± 2.13 and 8.00 ± 2.18 mmol $\cdot L^{-1}$) caused significant changes in the tested parameters. The pre- and post-exercise levels of oxidative damage (PC, TBARS) were lower after NAC administration. The glutathione metabolism (GSH, GGT, GPx, GR) was enhanced by NAC. No effect of NAC on EPO activity was observed.

Conclusions: Our study has shown that a strength exercise increases in oxidative damage level and initiates changes in glutathione metabolism whereas the N-acetylcysteine administration enhances antioxidative glutathione system and attenuates oxidative stress without any effect on erythropoietin level in subjects' blood.

Key words: thiols, oxidative stress, erythropoietin, strength test

Introduction

N-acetylcysteine (NAC), the acetylated form of the amino acid cysteine, is an excellent source of sulfhydryl groups, commonly called thiol groups (-SH), as well as precursor for the synthesis of proteins and other molecules of great biological importance, including glutathione. Tripeptide glutathione (g-glutamylcysteinylglycine) is the most abundant thiol in cells, often found in the milimolar range (1 to 10 mM, depending on tissue type), and ratio of reduced (GSH) to oxidized glutathione (GSSG) is responsible for redox status of both intracellular and extracellular medium (1).

Glutathione redox status plays a significant role in a cellular metabolism including mainly coordination of antioxidant defense network. GSH protects against reactive oxygen species (ROS) toxicity by direct and indirect reduction of these molecules. GSH, as a cofactor for glutathione peroxidase (GPx), participates in removing products of lipid peroxidation formed during attack of ROS on the cell membranes and lipoproteins. GSH also supports the recycling of other low molecular – weight antioxidants such as vitamin C and E. In addition to this crucial role in antioxidant defense, GSH/GSSG system is implicated in synthesis and structures of protein and nucleic acid. Oxidized glutathione facilitates folding of the polypeptide chain in endoplasmic reticulum whereas reduced glutathione stimulates reduction of ribonucleotides to deoxyribonucleotides. Many important enzyme protein (e.g., adenylate cyclase, glucose-6-phosphatase, pyruvate kinase, the transport Ca-ATPases), and at least eight enzymes participating in glucose metabolism, are regulated by glutathione redox status. GSH participates in detoxification of many xenobiotics *via* reaction catalyzed by glutathione S-transferase. Intracellular GSH is also required for the T-ell proliferative response to antigen stimulation, for the activation of cytotoxic T „killer" cells, and for the metabolism of interleukins (IL-2, IL-4, IL-5)

which are important for the immune response. Glutathione is involved in synthesis and transport of nitric oxide, synaptic transmission, programmed cell death, redox-sensitive signal transduction and functioning of membrane transporters e.g. glucose transporters (GLUT1, GLUT4). Moreover, GSH/GSSG system plays a pivotal role in the control and regulation of the expression of erythropoietin (EPO) gene by modulating the activity of transcription enhancer HIF-1 (1,2,3,4,5,6).

Glutathione redox status perturbation may be dangerous for cell metabolism therefore following compounds or methods with the aim to maintain GSH on physiological level were used: glutathione monoesters (methyl-, ethyl-, propyl-), methionine analogs (S-adenosyl methionine, SAM), oxoproline analogs (2-oxothiazolidine-4-carboxylate, OTC), cysteine analogs (NAC, taurine), glutamylcysteine or glutamylcystine, lipoic acid and transfer gene of glutamylcysteine synthetase (1,6,7,8).

N-acetylcysteine is probably the least flawed and the most effective compound maintaining the glutathione redox status. Earlier studies have shown that oral, intravenous or intraperitoneal supplementation of NAC prevented the decline in muscle, lung and blood glutathione level and weakened exercise-induced GSH oxidation (9,10,11). Beside the stimulation of glutathione synthesis and reduction, NAC reduces extracellular cystine to cysteine and affects directly reactive oxygen species as well as corrects activities of catalase, mitochondrial superoxide dismutase, glutathione S-transferase and different forms of glutathione peroxidase and eventually results in significant preservation of membrane lipid and protein (7). Moreover, it was observed that NAC can improve exercise performance (11,12,13).

The aim of this study was to determine whether a strength exercise induces oxidative stress and thus effects on antioxidant glutathione system and whether intensification of glutathione metabolism by oral treatment with N-acetylcysteine reduces plasma protein and lipid damage and results in change of EPO level.

Material and Methods

Ten healthy male volunteers regularly weight trained for at least 3 years participated twice in this study. The characteristics of the athletes are presented in table 1.

All the subjects were informed of the aim of the studies and gave their consent for taking blood samples. The protocol of the study was approved by the Local Bioethical Committee in Gorzów Wlkp.

Subjects performed an intensity resistance exercise (strength test): shoulder press, bench press, straightening up with weights. Each exercise element was repeated five-fold. The initial weight was 60 kg and then was increased by 10 kg until exhaustion.

N-acetylcysteine (NAC) has been administered at a dose of 1800 $mg \cdot d^{-1}$ (each pill was 600 mg) with the morning, afternoon and evening meals for 3 days before the second study.

Blood samples were collected from cubital vein with an anticoagulant ($EDTAK_2$) before exercise, immediately after completing exercise and after 24h rest. Plasma was obtained by centrifugation at 2500 x g for 10 min, then frozen and stored at –20°C. Erythrocyte fraction was resuspended and washed three times with cold isotonic saline solution. Washed erythrocytes were stored at –20°C until analysis. All the samples were analysed within 7 days.

In the erythrocytes, activities of glutathione reductase (GR, EC 1.6.4.2) and glutathione peroxidase (GPx, EC 1.11.1.9) using Randox kit (UK) were estimated. The enzyme activities were expressed relatively to the Hb concentration measured by the Drabkin method.

In the plasma protein carbonyls (PC) as a marker of protein oxidation were measured by the method of Levine et al. (14). The carbonyl content was expressed as nmol PC per mg of plasma protein. Protein concentration was estimated by the Bradford method (15). Lipid peroxidation was estimated using the measurement of thiobarbituric acid – reactive substance (TBARS) level according to the method of Buege and Aust (16). The TBARS level was expressed as nmol of malondialdehyde using 1,1,3,3- tetraethoxypropane as standard.

Moreover, in the plasma erythropoietin (EPO) and γ-glutamyl transpeptidase (GGT, EC 2.3.2.2) activities were estimated. The EPO level was measured using chemiluminescent immunoassay system Immulite (USA) and GGT level with Emapol kit (Poland).

In the whole blood the reduced glutathione (GSH) concentration was estimated by the method of Beutler et al. (17) and lactate (LA) concentration using Dr Lange kit (Germany).

Statistical analysis was carried out using Statistica 6.0 program. Data were tested by two – way ANO-

Table 1. *The characteristic of the subjects under study. Data are means (SD).*

Age (years)	Body mass (kg)	Height (cm)	Period of training (years)	Exercise lactate ($mmol \cdot L^{-1}$)	
				Before NAC	After NAC
21.3 (1.4)	84.5 (10.4)	181.1 (8.8)	7.0 (3.2)	7.71 (2.13)	8.00 (2.18)

VA to determine exercise or N-acetylcysteine related effects. When a significant F- value was found, Newman – Keul post-hoc was performed. The accepted level of significance was defined as $p< 0.05$.

Results

Table 2 and figure 1 show the results of exercise and N-acetylcysteine effects on tested parameters. The applied exercise at maximal intensity (post-exercise lactate: 7.71 ± 2.13 and 8.00 ± 2.18 mmol $\cdot L^{-1}$) caused significant changes in glutathione metabolism and protein an lipid oxidative damage. The GSH concentration increased during exercise and after 24h rest by about 13% and 38%, respectively. The GGT activity increased after 24 h by 21% whereas GPx activity decreased significantly below the baseline. The GR activity elevated by 23% following exercise but returned to the pre-exercise state during recovery.

The glutathione metabolism (GSH, GGT, GPx, GR) was remarkably enhanced by NAC administration. Elevation in GSH concentration (20%) and GR activity (45%) before and immediately after exercise was observed. The GGT activity was significantly higher before exercise compared to the first trial. The GPx increased by 26% during exercise and remained on high level after 24 h rest. Apart from GPx activity, GSH concentration and GGT and GR activities were not significantly different compared to first trial (before NAC) after 24 h rest.

The exercise test triggered protein and lipid damage. The PC and TBARS concentration significantly increased during exercise by 22% and 17% and then declined to initial level after 24 h rest. Oral treatment with NAC significantly attenuated oxidation damage of proteins and lipids. The plasma PC and TBARS concentrations decreased by 20% to 46% during the second study.

Neither exercise nor NAC administration had any influence on EPO level in subjects.

The NAC supplementation did not affect the maximal number of repetitions in performed strength test.

Table 2. *Pre-, post- exercise and 24 h rest carbonyls (PC), lipid peroxidation products (TBARS), glutathione (GSH) concentrations and erythropoietin (EPO), γ-glutamyltransferase (GGT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities in subjects. Data are means (SD).*

	Before NAC (n=10)			After NAC (n=10)		
	Pre-exercise	Post-exercise	Rest 24 h	Pre-exercise	Post-exercise	Rest 24 h
GSH mg·ml^{-1}	212.3 (30.8)	238.9 (26.6)*	291.7(41.1)**	254.9(29.4)$^{\#\#}$	285.5(27.2)*$^{\#\#}$	283.4(31.1)*
GGT U·L^{-1}	13.19 (2.45)	14.97 (3.79)	16.06 (2.31)*	19.08 (5.94)$^{\#}$	19.47 (5.61)	15.47 (3.27)*
GR U·gHb^{-1}	10.89 (2.06)	13.40 (2.52)*	13.17 (4.62)	12.74 (1.27)$^{\#}$	15.38 (2.51)*$^{\#}$	14.58 (2.90)
GPx U·gHb^{-1}	48.93 (6.09)	49.75 (6.17)	41.73(2.78)**	50.28 (7.79)	56.56 (7.22)*$^{\#}$	52.55 (7.42)$^{\#\#}$
PC nmol·mg^{-1}	1.67 (0.26)	2.03 (0.25)*	1.53 (0.31)	0.99 (0.11)$^{\#\#}$	1.26(0.14)**$^{\#\#}$	1.22 (0.27)*
TBARS nmol·ml^{-1}	2.13 (0.32)	2.49 (0.38)*	2.22 (0.46)	1.15 (0.36)$^{\#\#}$	1.38 (0.49)$^{\#\#}$	1.44 (0.37)$^{\#\#}$
EPO mU·ml^{-1}	8.15 (3.67)	8.18 (3.43)	9.89 (4.28)	8.56 (2.88)	8.45 (2.60)	10.04 (3.27)

*p<0.05 and **p<0.01 significantly different from the pre-exercise value
$^{\#}$p<0.05 and $^{\#\#}$p<0.01 significantly different from before NAC

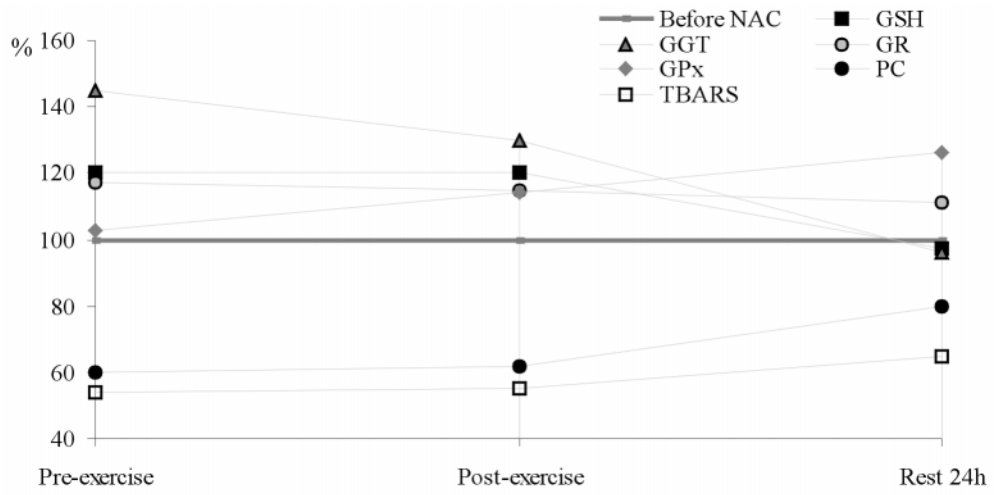

Fig. 1. *Percent changes of glutathione parameters and oxidative damage after 3-day NAC supplementation*

Discussion

Physical exercise affects the level of the reduced and oxidized glutathione and disturbs the thiol redox status. The tissues tend to maintain the adequate GSH/GSSG ratio by enhancing the glutathione synthesis or releasing the glutathione disulfide from the cells. This activity is essential to minimize the deleterious effects of oxidative stress. The increase in GSH amounts protects the cell membrane against ROS toxicity and irreversible damage which appear in enhancement of protein carbonylation and lipid peroxidation, whereas the pumping out of intracellular GSSG excess prevents formation of protein-SSG disulfides and temporary inactivation of enzymes, transporters and receptors. The reactivity of these molecules may occur only by over-physiological GSH concentration (6).

In present study strength test elevated GSH level immediately after exercise and 24 h rest. Similar effect was obtained by Ji et al. (18) who observed 40% increase in GSH concentration after 120 min submaximal exercise. On the contrary, Vigue et al. (19) (90 min, 68% $\dot{V}O_2max$) and Dufaux et al. (20) (19-26 km run) found the decline in reduced glutathione until by 50%. Gohil et al. (21) also observed decrease in GSH level during prolonged submaximal exercise, however, GSH increased overshooting pre-exercise level during recovery. According to Rajguru et al. (22) the increase of blood thiols compensates for lipid and protein oxidative damage and aids the repair of damaged cells.

The elevated blood GSH level is a result of enhanced glutathione biosynthesis predominantly in the liver. This organ synthesizes GSH from endogenous or dietary amino acids *de novo* and supplies most of circulating GSH. During exercise, hepatic GSH efflux is increased due to the stimulation of elevated plasma catecholamines, glucagon, vasopressin levels (23). Studies in rats estimated that active transport of GSH from hepatocytes into blood rises from 12.4 ± 1.4 $nmol \cdot min^{-1} \cdot g^{-1}$ to 26.4 ± 1.2 $nmol \cdot min^{-1} \cdot g^{-1}$ tissue following exercise. This ensures blood GSH homeostasis despite increased use of tissue glutathione (6).

The other reason for increased GSH content in blood is intensification of glutathione biosynthesis in erythrocytes. It was estimated that the erythrocytes can synthesize the entire GSH content in about 10 min. Dass et al. (24) have speculated that RBCs participate in inter-organ glutathione homeostasis. Authors have proposed that glutathione might be extracted from erythrocytes by those tissues that have been identified as sites of the most utilization GSH including lung, heart, gut, brain and skeletal muscles. The precise mechanism by which the erythrocyte GSH or its constituents are made available to other cells are not presently known. This process could involve intra-erythrocyte catabolism of GSH followed by the release of glycine, cysteine and γ-carboxyglutamic acid or export of GSSG followed by its extra-erythrocyte catabolism to absorbable intermediates. The mean transport rate of GSSG from red cells was found to be 6.7 nmole GSSG/ml RBC/h (25).

Skeletal muscles are the major consumption site of glutathione during physical exercise where its content depends on muscle fiber type. Type I muscle (slow twitch oxidative), such as the soleus, contains six-fold higher GSH content (about 3 mM) than the type IIb muscle, white vastus lateralis (fast-twitch glycolytic) (26). The several studies have demonstrated that the enhancement of oxidized glutathione formation and release of GSSG from muscles affect the decrease of total glutathione pool and increase of sensitivity for oxidation damage (27,28). Recovery of the muscle GSH concentration was slow in the post-exercise period. However, this recovery may be sped up by administration of selective enzyme inhibitors, and also by giving compounds that increase glutathione synthesis (1).

Our study demonstrated that strength exercise did not induce the glutathione deficit. Nevertheless, 3-day NAC supplementation elevated blood GSH concentration before and during exercise by 20%. It was established that NAC is actively transported into cells, deacylated and increases cysteine availability for GSH synthesis.

Bioavailability of oral NAC has been shown to be the highest when fast – dissolving effervescent tablets, as used in this study. Studies on pharmacokinetics of N-acetylocysteine revealed that after an oral dose of 200 – 400 mg the peak plasma concentration is achieved in 1-2 h, after which NAC is cleared from plasma and is not detectable in blood at 10–12 h post-administration (7). This information explains the decline in GSH concentration after 24 h to the level without NAC. We suppose that supply of NAC to athletes before and during exercise could support the high glutathione level until 24 h after strength test. Medved et al. (12) reported intravenous NAC infusion before and during cycling exhaustive exercise did not affect reduced, oxidized and total glutathione in blood. This result is in contrast to previous studies. Medved et al. (10) observed earlier that pre-exercise NAC infusion increased GSH and decreased GSSG during sprint test and recovery. Sen et al. (11) and Sastre et al. (29) also found that oral NAC administration is effective in preventing oxidation of the blood glutathione after physical exercise. Child et al. (30) obtained controversial results. Authors recognized that post-exercise supplementation with vitamin C and NAC transiently enhanced oxidative stress in spite of increased total antioxidant status and antioxidant enzyme activity. Furthermore, Kleinveld et al. (31)

observed enhancement of low-density lipoprotein oxidation and decline in GSH concentration after 6-week supplementation with NAC.

Nevertheless, the above study does not exclude NAC administration in athletes but further studies are required to explain effect of NAC on glutathione, especially under conditions of exercise-induced oxidative stress.

The elevated GSH level by exercise and supply of substrates to its synthesis stimulates changes in g-glutamyl cycle enzymes activity catalyzing degradation, translocation, synthesis, oxidation and regeneration glutathione.

In our research γ-glutamyl transpeptidase (GGT) activity increased during the first trial, however, during the second one GGT activity decreased to the level without NAC. The GGT activity has altered similarly to GSH concentration.

The GGT enzyme is bound to cell membranes, hydrolyzes γ-peptide bond and transfers the γ-glutamyl moiety of GSH (and GSSG) to amino acid acceptor to form γ-glutamyl amino acids and cysteinylglycine. Cleavage of cysteinylglycine is catalyzed by dipeptidase. The products formed by transpeptidase and dipeptidase are transported into cells and used for glutathione biosynthesis (6). It was known that increase in plasma GGT activity may be a marker of tissues damage and also oxidative stress (32,33). On the other hand, the elevation of GGT activity may be the result of GGT biosynthesis induction and glutathione anabolism enhancement (27,28,34). The information regarding the effect of exercise on GGT activity is scanty. The studies on GGT activity changes were performed by Sen et al. (28) who found increase in GGT activity in muscles of trained dogs. The activation of GGT was also observed by Leeuwenburgh et al. (34) in rat tissues after exhaustive swim exercise.

It is possible that post-exercise increase in GGT activity in our first trial involves membrane damage especially muscle cells. However, pre-exercise high GGT activity after NAC administration could have affected by increased GSH level. As above mentioned GGT initiates a series of reactions for tissues GSH uptake. We also suppose that high GSH concentration with NAC in conjunction with GGT activation might diminish cell damage and decrease in plasma GGT activity in the next period.

The strength test increased in erythrocyte GR activity. The exercise-induced rise in erythrocyte GR activity was also observed by Hübner – Woźniak et al. (35) and Vesovic et al. (36). GR is responsible for the high GSH/GSSG ratio by reduction of GSSG at the expense of NADPH i.e. GR regenerates glutathione. According to kinetic studies the elevation of GSSG level activates GR whereas elevation of GSH to the physiological level decreases the activity of the enzyme (37). Taking this into account we expected the decline in GR activity due to the high glutathione concentration. Probably, parallel to the increase in GSH level, increase in GSSG may occur. In our previous study we demonstrated decrease in GSH/GSH+GSSG ratio following strength exercise which is a proof the rise of glutathione oxidation (38).

Another mechanism of GR activity regulation is known as well. Brady et al. (39) suggested that low pH results in a reduced affinity of GR for NADPH and activates GR i.e. the GR elevation appears to be due to an activation of preexisting inactive enzyme. We noted increase in lactate concentration in whole blood after strength test but Hildebrand et al. (40) showed that generation of lactate occurs also in red cells, which could activate the erythrocyte GR.

The next enzyme involved with g-glutamyl cycle is GPx. It is a selenium enzyme removing hydrogen peroxide and organic peroxides in presence of GSH as cofactor. The analysis of K_m showed that GPx is more effective at reducing hydrogen peroxide than CAT at lower levels of substrate (41). Therefore GPx activity depends on H_2O_2 and GSH concentrations. The limitation of glutathione availability and ROS generation might decrease GPx activity.

In the present first study decrease in erythrocyte GPx activity after 24 h rest was observed. The previous works have showed no change, decrease or increase in this enzyme activity in blood after various physical exercises (36,42,43,44,45). It means that GPx activity depends on type of performed exercise. It was also stated that exercise alters the kinetics and catalytic activity of antioxidant enzymes by allosteric or covalent modification (26).

In the study with NAC activity of GPx was significantly higher compared to the first trial. We suppose that NAC could have indirect effects on GPx activity by supporting GSH biosynthesis and increasing its availability.

In our study common markers of oxidative stress were applied such as protein carbonyls (PC) and lipid peroxidation products (TBARS). The changes in PC and TBARS concentration proceeded parallel i.e. we observed increase in both parameters following exercise. The exact reason of plasma protein and lipid oxidative damage was explained by others. Reid et al. (46) stated that ROS are produced intracellularly in muscle cells and then released to the extracellular medium. Ashton et al. (47), using electron spin resonance spectroscopy, showed the high ROS level in serum after exhaustive aerobic exercise. Liu et al. (48) observed a positive correlation between lipid peroxidation products and carbonyls in muscle, brain, liver after exercise. This indicates that ROS are produced and released from muscle into plasma and

attack proteins and lipids. Additionally, products of peroxidation and carbonylation are also released from tissues into blood and cause damage to other molecules. However, PC and TBARS levels decreased at 24 h rest after strength test, which is presumably related to protein proteolysis and removing of peroxidation products in liver during recovery.

The exercise-induced increase in PC concentration in blood and muscles was also reported by Alessio et al. (49), Lee et al. (50), Smolka et al. (51) whereas the enhancement of lipid peroxidation was observed in many studies e.g. by Ashton et al. (47), Cavas (42), McBride et al. (52), Marzatico et al. (44), Palazzetti et al. (53).

Peroxidation and carbonylation were markedly attenuated by NAC administration indicating that this supplement could be used as antioxidant. However, we suppose that decrease in PC and TBARS concentration was caused not directly by NAC action but by GSH. It suggest that other thiols could induce similar effect.

Erythropoietin is a hormone produced mainly in kidney and liver and is an essential growth factor for erythrocytic progenitors in the bone marrow. The earlier study showed that the regulation of the ventilatory response and erythropoietin production by the respective oxygen sensors involves redox–sensitive signaling pathways, which are triggered by ROS. Authors stated that oxygen–dependent ROS production may be involved in down-regulation of the transcription factor HIF-1 activity (4). Fandrey et al. (54) observed that EPO production was more susceptible to H_2O_2 –induced inhibition by low level of GSH.

The effects of physical exercise on circulating EPO levels have been studied in various disciplines including cross – country skiing, cycling, long–distance running and biathlon. The results of these studies indicate that EPO level is not significantly affected by single bouts of strenuous exercise (55). Similarly, strength test applied in our study, despite induction of oxidative stress, did not increase in EPO activity.

We also did not observed any effect of NAC on EPO concentration in contrary to Hildebrandt et al. (4) who recognized about 45% increase in serum EPO level as well as 30% increase in plasma thiol after 3-6 days oral treatment with NAC. Authors have concluded that thiols can modulate EPO production.

Our study has shown that a strength exercise increases in oxidative damage level and initiates changes in glutathione metabolism whereas N-acetylcysteine administration enhances antioxidative glutathione system and attenuates oxidative stress without any effect on erythropoietin level in subjects' blood. Nevertheless, positive effect of NAC on metabolism glutathione was observed, we suggest that NAC requires more studies mainly in other sport disciplines.

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