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ASSOCIATION OF PRO-ANTIOXIDANT STATUS WITH IMMUNOLOGICAL RESPONSE IN HEALTHY MEN AFTER ORAL N-ACETYL-L-CYSTEINE ADMINISTRATION

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Abstract

Introduction: N-acetyl-L-cysteine (NAC), as an antioxidant and precursor of glutathione, decreases in the capacity of neutrophils and macrophages to generate RONS, increases phagocytosis, reduces the extracellularly produced oxidative metabolites, modulates immune cells activity and interleukin level.

Aim of the study: This study was designed to assess the effect of N-acetyl-L-cysteine on plasma pro-antioxidant status, immune cells number, and to demonstrate the relationship between total antioxidant status and the immunological response in healthy men exposed to routine incremental exercise.

Materials and Methods: Fifteen healthy men were randomly assigned to one of two groups: control (CON, placebo) or N-acetyl-L-cysteine (NAC, 1200 mg·d^{-1} for 8 days prior to and 600 mg on the day of exercise trial). To measure the ergogenic effectiveness of NAC, subjects performed incremental cycle exercise until exhaustion.

Results: NAC administration significantly elevated the resting and post-exercise plasma total antioxidant status (TAS) by more than 30%. The lipid peroxidation (TBARS) and protein carbonylation (PC) products as well as creatine kinase (CK) and lactate dehydrogenase (LDH) were considerably reduced by NAC at rest and/or post-exercise period. Interleukin-6 (IL-6) did not change in response to NAC. The immune cells variously responded to NAC i.e. number of lymphocytes significantly decreased whereas neutrophils and monocytes increased compared with CON. Lymphocytes inversely correlated ($P<0.0001$) with TAS, and directly with TBARS and PC whereas monocytes demonstrated the opposite relations with pro-antioxidant parameters. NAC administration, in the contrary to expectations, did not significantly affect exercise performance.

Conclusions: These data show that modification of blood antioxidant status and reduction of oxidative damage by eight-day oral NAC application simultaneously influences on immune cells number, and suggest the possibility of N-acetyl-L-cysteine using to modulate immunological response in healthy men exposed to intense exercise.

Key words: *N-acetyl-L-cysteine, immune cells, muscle damage, interleukin-6*

Introduction

A number of studies have shown that reactive oxygen and nitrogen species (RONS) are physiological products of aerobic metabolism and are used by cells for variety of important metabolic processing which include gene expression, protein turnover and inflammatory reaction (1,2). However, the excess of RONS and low antioxidant capacity can lead to adverse effects such as oxidative damage which can impair cell metabolism. The immunological cells are exquisitely sensitive to RONS generation and, therefore, are favoured by relatively high levels of antioxidants molecules including tocopherols, ascorbic acid and glutathione (3).

N-acetyl-L-cysteine (NAC), as an antioxidant and precursor of glutathione, decreases in the capacity of neutrophils and macrophages to generate RONS, increases phagocytosis, reduces the extracellularly produced oxidative metabolites, modulates neutrophil activity and lymphocyte T proliferation as well as can effect on metabolism of interleukins which are important for the immune response (3-6). On the other hand NAC demonstrates beneficial effects at low concentration levels of glutathione which is observed in viral infection, cancer, chronic bronchitis and drug poisoning but never in physically active individuals (3,5,6). An excess of NAC can enhance tissue damage and disturb immune response, and also deteriorate exercise performance (3,7-9).

The aims of this study were to explain: 1) whether changes in pro-antioxidant status induced by N-acetyl-L-cysteine administration are simultaneously related to an immunological response, 2) whether there is an interaction between total antioxidant status (TAS) and oxidative stress markers, and immune cells numbers 3)

whether N-acetyl-L-cysteine (NAC) demonstrates the ergogenic effects in healthy men exposed to routine incremental exercise.

Materials and Methods

Fifteen healthy males (age 20.3 ± 2.3 yr, body mass 83.4 ± 14.4 kg, height 1.8 ± 0.1 m) participated in the double-blinded, placebo-controlled cross-over designed study. Subjects were instructed not to take any antioxidants supplements (vitamins or minerals) for 4 weeks prior to the study. All the subjects were informed of the aims of the study and gave written consent for participation in the project. The protocol of the study was approved by the local ethics committee in accordance with the Helsinki Declaration.

1200 mg of N-acetyl-L-cysteine (NAC group) and 700 mg lactose (placebo, CON group) were administered for eight days in two doses as powder dissolved in 50 ml of water. The participants took the first dose in the morning in a fasted state and the second dose was 2 hours before an evening meal. On day of exercise trial, 600 mg of NAC was taken 2 hours before the exercise test. The wash-out period between the trials with NAC and placebo was three weeks.

Subjects were advised to exercise until volitional exhaustion (also referred to as volition maximum), achieving maximal oxygen uptake ($\dot{V}O_2max$), on a calibrated cycle ergometer Monark E895 (Sweden). Breath-by-breath oxygen uptake was continuously recorded using a computerized on-line gas analysis system Ergo-oxyscreen Jaeger (Germany). Heart rate was continuously recorded during the test using a portable heart rate telemetry device Polar Sport Tester S-810 (Finland). The test was incremental and progressive, all subjects commenced at a 50 W workload and it was increased by 50 W every three minutes3 min until exhaustion at a constant pedal speed of 60 rpm. The lactate (LA) concentration in the capillary blood was assessed using LKM 140 Dr Lange kit (Germany).

Blood samples were obtained from the cubital vein with an anticoagulant ($EDTAK_2$) before exercise, immediately after completing exercise and after 24 h rest. The samples were immediately placed in 4°C temperature after collection. Within 10 min, the blood samples were centrifuged at 2500 g and 4°C for 10 min. Aliquots of plasma were stored at -20°C. Total antioxidant status (TAS) was determined immediately after plasma collecting and other parameters were analysed within 7 days.

Total antioxidant status (TAS) of the plasma was measured using a method developed by Randox laboratories (UK). The method is based on the formation of 2'-2'-azino-di-[3-ethylbenzthiazoline sulphonate] radical which is measured spectrophotometrically at 600 nm. Detection limit for the TAS kit was 0.21 mmol $\cdot l^{-1}$ and the intra-assay coefficient of variation (CV) was 2.77%.

Plasma lipid peroxidation products were estimated using the measurement of thiobarbituric acid – reactive substance (TBARS) level according to the method reported by Buege and Aust (10). To avoid further peroxidation, plasma samples were treated with 15% TCA containing 0.25M HCl immediately after separation of plasma. The TBARS level was expressed as nmol of malondialdehyde using 1,1,3,3-tetraethoxypropane as a standard. TBARS detection limit was 0.13 nmol $\cdot ml^{-1}$.

Plasma protein carbonyls (PC) were measured by the method of Levine et al. (11) using 2,4-dinitrophenyl hydrazine. The carbonyl content was calculated using an extinction coefficient of 22000 $M^{-1} \cdot l^{-1} \cdot cm^{-1}$ and expressed as nmol PC per mg of plasma protein. Protein concentration was determined by the method of Bradford (12). The intra-assay coefficient of variation (CV) for TBARS and PC procedures were <10%.

Plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activities were evaluated using the diagnostic kits for the kinetic enzyme analyser Konelap 60 BioMerieux (France). CK and LDH detection limits for the applied kits were 6 U $\cdot l^{-1}$ and 18 U $\cdot l^{-1}$ respectively. The intra-assay coefficient of variation (CV) for the CK assay was 1.85% and the LDH assay was 2.61%.

The plasma interleukin-6 (IL-6) level was measured with enzyme-linked immunosorbent ELISE kit, R&D Systems (USA). Detection limit for the IL-6 kit was 0.11 pg $\cdot ml^{-1}$ and the intra-assay coefficient of variation (CV) was 3.4%. Haemoglobin (Hb) and haematocrit (Hct) as well as number of leucocytes, lymphocytes, neutrophils and monocytes were assessed using ABX Micros OT 16 (France).

Relative changes in plasma volume were calculated according to the following equation:

$$\%\Delta PV = 100 \times \{([Hb]_1/[Hb]_2) \times [100 - (Hct_2 \times 0.874] / [100 - (Hct_1 \times 0.874)]-1\}$$

where $[Hb]_1$ (g $\cdot dl^{-1}$) and Hct_1 (%) are mean initial values, $[Hb]_2$ and Hct_2 are post-exercise values. The Hct was multiplied by 0.96 and 0.91 to correct for trapped plasma and peripheral sampling respectively (13).

In order to determine true physiological changes, post-exercise and post-supplementation values were corrected for changes in plasma volume according to Kraemer and Brown (14).

Statistical analysis was carried out using Statistica 8.0 (StatSoft, Poland). All data were graphed and tested for normality. To determine the effects of exercise and NAC as well as the interaction between exercise and NAC, statistical analysis was performed using two-way ANOVA and post-hoc Tukey's test. Correlations were calculated by the Pearson correlation coefficients. The accepted level of significance was defined as $P< 0.05$. Data are presented as mean ± SD.

Results

N-acetyl-L-cysteine administration significantly elevated resting and post-exercise TAS by about 30% compared to CON (tab.1). The majority of NAC is metabolized into small molecular weight protein-bound thiols are found in human plasma therefore an increase in TT level could be the main reason of significant increase in TAS. TAS presented statistically significant interaction (ANOVA) between NAC and exercise.

N-acetyl-L-cysteine revealed a distinct antioxidant action in relation to plasma lipid peroxidation and protein carbonylation products. TBARS and PC concentration reached over 30% declines in NAC compared to CON group (tab.1). Between oxidative stress markers and antioxidant status the high negative correlations were observed (ryc.1).

CK release from skeletal muscle was significantly reduced after exercise and during recovery whereas LDH activity was reduced by NAC administration at rest and post-exercise (tab.1). The present study did not demonstrate any direct relationships between CK, LDH and number of immune cells, and oxidative damage markers. However, both enzymes significantly ($P < 0.0001$) correlated with total antioxidant status (CK vs. TAS: r = -0.565; LDH vs. TAS: r = -0.557).

IL-6 is recently often analyzed parameter in sport biochemistry in related to various metabolic processes such as oxidative stress, immune response, carbohydrate metabolism etc. The present study did not demonstrate any changes in response of IL-6 to applied exercise and NAC. We only observed tendency to decrease following NAC, particularly at 24 h rest (tab.1).

As it was shown in figure 2, NAC caused a significant shift in white blood cells profile. The number of lymphocytes decreased while neutrophils and monocytes significantly increased over 40% following NAC. Lymphocytes and monocytes reacted to NAC at rest and post-exercise while neutrophils only when an incremental exercise was applied. Lymphocytes negatively correlated with TAS, and positively with TBARS and PC whereas monocytes demonstrated the opposite relations with pro-antioxidant parameters (tab.2). This means that enhancement of antioxidant barrier and attenuation of oxidative damage by NAC intake is associated with changes in immune cells numbers.

Table 1. *The plasma total antioxidant status (TAS), oxidative damage (TBARS, PC), muscle damage (CK, LDH) markers, interleukin-6 (IL-6) and calculated fluid shifts (ΔPV)*

Parameter	Pre-exercise	CON vs. NAC	Post- exercise	CON vs. NAC	Rest 24 h	CON vs. NAC	ANOVA Ex. vs. Suppl.
TAS mmol · l^{-1}							
CON	1.19 ± 0.12	*P*<0.01	1.13 ± 0.09	*P*<0.01	1.18 ± 0.10	*P*<0.01	0.001
NAC	1.79 ± 0.18		1.56 ± 0.12**		1.57 ± 0.15		
TBARS μmol · l^{-1}							
CON	1.89 ± 0.39	*P*<0.01	1.99 ± 0.33	*P*<0.01	1.55 ± 0.44	*P*<0.01	
NAC	1.17 ± 0.37		1.20 ± 0.36		1.27 ± 0.30		
PC μmol · mg^{-1}							
CON	1.42 ± 0.28	*P*<0.01	1.63 ± 0.30	*P*<0.01	1.52 ± 0.36	*P*<0.01	
NAC	0.98 ± 0.13		1.15 ± 0.21		1.01 ± 0.15		
CK U · l^{-1}							
CON	314.33 ± 114.42	ns	400.83 ± 102.82	*P*<0.05	476.91 ± 173.68*	*P*<0.01	
NAC	217.14 ± 91.11		258.23 ± 102.64		261.69 ± 148.23		
LDH U · l^{-1}							
CON	359,53 ± 59,37	*P*<0.01	381.38 ± 76.09	*P*<0.05	355.90 ± 93.59	*P*<0.01	
NAC	286.71 ± 57.83		319.71 ± 58.99		264.40 ± 34.44		
IL-6 ng · l^{-1}							
CON	1.90 ± 0.23	ns	1.81 ± 0.17	ns	2.60± 1.20	ns	
NAC	1.94 ± 0.17		2.13 ± 0.96		2.41 ± 1.17		
ΔPV %							
CON			-7.80 ± 2.41	ns	4.74 ± 2.33	ns	
NAC			-7.60 ± 2.87		4.00 ± 2.86		

*P<0.05 and **P<0.01 significantly different from the pre-exercise value

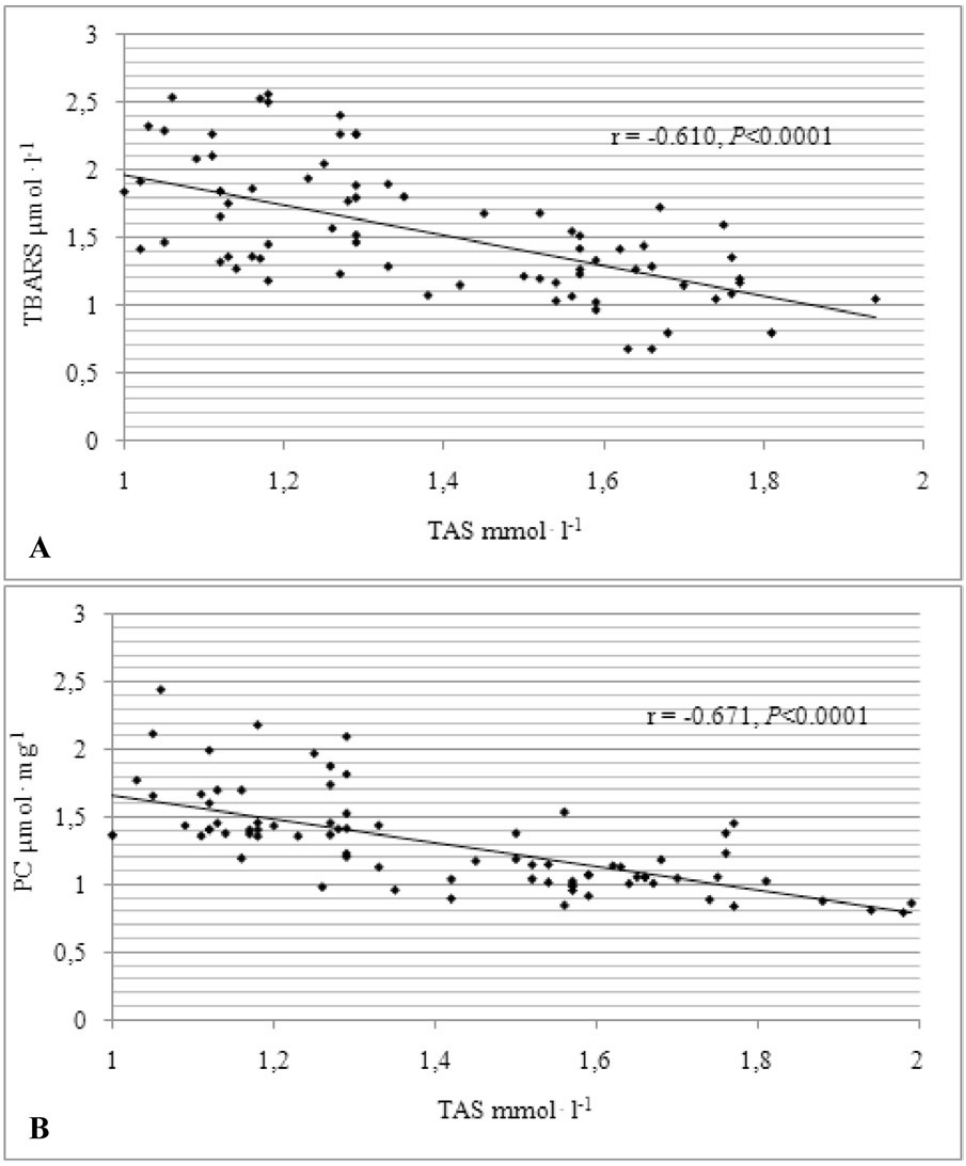

Figure 1. *Relationships between total antioxidant status (TAS), and (A) lipid peroxidation (TBARS) and (B) protein carbonylation (PC) products*

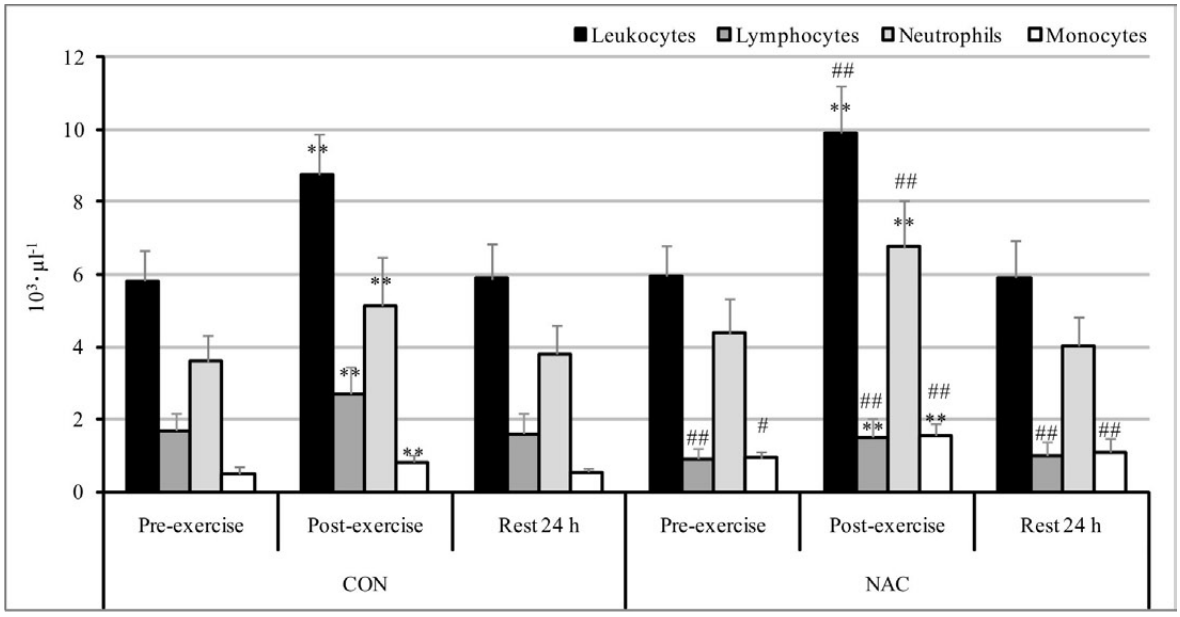

Figure 2. *Effect of exercise and NAC administration on number of immunological cells in peripheral blood. *P<0.05 and **P<0.01 significantly different compared the pre-exercise value; #P<0.05 and ##P<0.01 significantly different compared the CON group.*

Table 2. *Relationship between number of lymphocytes and monocytes, and total antioxidant status (TAS), lipid peroxidation (TBARS) and protein carbonylation (PC)*

	TAS	TBARS	PC
Lymphocytes	-0.521 $P < 0.0001$	0.661 $P < 0.0001$	0.634 $P < 0.0001$
Monocytes	0.524 $P < 0.0001$	-0.502 $P < 0.0001$	-0.539 $P < 0.0001$

Oral treatment with NAC did not significantly affect exercise performance. The mean $\dot{V}O_2max$ in CON and NAC groups were 48.50 ± 5.16 and 47.74 ± 5.47 ml · kg^{-1} · min^{-1}, respectively. The maximal heart rates were 191.9 ± 6.9 and 189.9 ± 8.8 beats · min^{-1}, post-exercise lactate concentrations were 10.85 ± 2.31 and 11.63 ± 2.28 mmol·l^{-1}. The time to fatigue (CON 17.24 ± 1.73 min, NAC 17.64 ± 1.65 min) and maximal workload (CON 168.63 ± 14.84 W, NAC 171.31 ± 14.94 W) tended to higher values, however, the differences were not statistically significant.

Discussion

Total antioxidant status (TAS) was introduced by Ingold et al. (15) as a marker of antioxidant capacity. The plasma TAS has depended on many factors such as intensity of cell RONS generation, concentration of stress hormones and antioxidants, cysteine availability and glutathione synthesis etc. Therefore, it has been difficulty observed the distinct shift in total antioxidant status (TAS) following a single intense exercise. The present study has not shown any effect of exercise on TAS, similarly to others (8,9). Nevertheless, NAC administration significantly elevated TAS which could be associated to high concentration of plasma thiols such as cysteine and glutathione. The study on the pharmacokinetics of NAC revealed that following an oral NAC intake the peak in plasma concentration is achieved in 1-2 h, after which it is transported into cells, deacylated and involved in the small thiol compounds (5). Previously, Nielsen et al. (16) observed a high cysteine level in plasma after three-day NAC supplementation (6 g · d^{-1}) in rowers. Sen et al. (8) found that oral NAC administration is effective in preventing oxidation of blood glutathione after physical exercise. Also our earlier study showed that NAC supplementation in athletes causes increase in blood glutathione (17).

The detection of lipid peroxidation and protein carbonylation products using thiobarbituric acid and dinitrophenyl hydrazine have been the most widely used markers of oxidative damage. The improvement of plasma antioxidant status by NAC considerably weakened lipid peroxidation and protein carbonylation. It has been interesting that the antioxidant effect of NAC persisted to 24 h after the last NAC dose. TBARS and PC negatively correlated with antioxidant status which confirms antioxidant action of N-acetyl-L-cysteine. Contrary to our results, Kleinveld et al. (7) observed increase in oxidative damage markers after NAC administration. However, researchers applied the long-term supplementation with high dose of N-acetylcysteine, exactly at dose of 1200 mg·d^{-1} and 2400 mg·d^{-1} for 28 days and 14 days. This excess of NAC caused the thiol auto-oxidation which is potential source of reactive oxidants and contributed to the cytotoxicity of reactive thiols such as cysteamine (18). It should be stressed that the present study has shown the opposite results i.e. the applied dose of NAC considerably reduced peroxidation and carbonylation in subjects. The observed antioxidant action of NAC was even more effective than vitamin E which was previously used in our studies (19). This means that 1200 mg NAC daily for eight days could be recommended to athletes to avoid risk of oxidative stress during competitions or high-mountain training when RONS generation could been exceptionally enhanced (20,21).

Muscle proteins such as CK and LDH have been released during and after exercise as a result of muscle damage and have attributed to membrane peroxidation and/or inflammatory process (9,22-24). The incremental exercise led to increase in CK activity during recovery but enzyme did not correlate with markers of oxidative damage and immune cells levels. Nevertheless, administration with NAC reduced activities of CK and LDH. Both enzymes inversely correlated with total antioxidant status. It could be indirect evidence that exercise-induced muscle damage could be limited by thiol supplementation.

IL–6 is produced by many different cells in response to infection, damage, neoplasia and intense physical exercise. The main sources of IL-6 during exercise are stimulated skeletal muscle where IL-6 is released in response to high levels of TNFα, IL-1β, Ca^{2+} and RONS as well as low level of glycogen (MAP-kinase activation). The magnitude of the change in IL-6 concentration depends on the intensity and duration of exercise and the type of the muscle contraction i.e. endurance vs. eccentric, systemic vs. local, aerobic vs. anaerobic exercise (25). In the present study, IL-6 concentration did not change after incremental exercise. The prescribed exercise could be insufficient to provoke a significant change in factors necessary for IL-6 production for example, RONS. Another reason for the unchanged IL-6 content in plasma might be the elevated level of LA above 10 mmol · l^{-1} in both groups. Suzuki et al. (26) proposed that intense exercise with high lactate production may impair the ability of cells to produce IL-6 during exercise. This very interesting suggestion requires further explanation.

As shown in several studies, antioxidants can modulate the production of various cytokines. Can-

non et al. (27) observed the suppressive action of α-tocopherol on the release of IL-6 after downhill running while Childs et al. (9) did not find any effect of vitamin C and NAC on plasma cytokines in subjects performing eccentric exercise. In the present studies IL-6 concentration only tended to decrease after NAC administration.

The key results of our study were changes in immune cells numbers caused by thiol antioxidant NAC and interaction between pro-antioxidant status and immune response. The applied dose of NAC induced decrease in lymphocytes and increase in neutrophils and monocytes numbers which indicates that immune response is sensitive to antioxidant level. However, the reason of these alternations is still unclear. It is known that the extracellular supply of cysteine influences strongly the intracellular level of glutathione in phagocyte cells and also the activity of the transcription factor NF kappa B that regulates the expression of several immunologically relevant genes (3). Moreover, in vitro experiments with monocytes/macrophages and lymphocytes revealed that cysteine plays an important role as a regulatory mediator between these cell types (24).

Further, we observed that high TAS directly correlated with monocytes which play important role in removing of cell debris and stimulation of proliferation after intense exercise. In NAC group, the activities of CK and LDH were significantly lower than CON. Previously, Öhman et al. (4) showed in vitro study that NAC enhances receptor-mediated phagocytosis by protecting Fc(IgG)-receptors from oxidative damage mediated by myeloperoxidase and H_2O_2. The increase in circulating neutrophils and monocytes numbers, and reduction of muscle damage related to interaction between TAS and monocytes suggest a novel function thiol compounds. NAC is not only antioxidants, but also important determinant of number of phagocytes and stimulant to the repair processes in muscle.

However, the question arises whether the changes in pro-antioxidant status, immune cells numbers and reduction of muscle damage can induce some ergogenic effects. Reid et al. (28) were the first researchers who have shown that NAC improves muscle performance. Then, Medved et al. (29) and Matuszczak et al. (30) have revealed that NAC application during incremental and isometric exercises delays muscle fatigue. Our study did not confirm an ergogenic action of NAC in healthy men performed routine incremental exercise. We observed only tendency to increase physical efficiency.

Conclusions

In conclusion, eight-day administration with 1200 mg N-acetyl-L-cysteine had the following effects: 1) led to improvement of antioxidant status and immunological response, 2) revealed the relationships between pro-antioxidant parameters and immune cells, 3) indicated the possibility of thiol using to modulation of immunological response and limitation on exercise-induced muscle damage in physically active men.

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