Effect of N-Acetylcysteine Supplementation on the Oxidative Damage and Inflammatory Response after Eccentric Physical Exercise

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Abstract

The objective of the study was to verify the effect of N-Acetylcysteine (NAC) supplementation on parameters of oxidative damages and inflammatory response after high-intensity eccentric exercise (EE). 29 subjects with a mean age of 21.3 ± 4 yr, weight of 74.5 ± 7.7 kg and height of 177.2 ± 6.9 cm were selected and divided randomly into 3 groups: Placebo (21 days of placebo; n=8), NAC (21 days of NAC; n=9); NAC plus placebo (14 days of NAC + 7 days of placebo; n=8). Four subjects withdrew from the groups for personal reasons. 14 days after starting supplementation, the subjects performed EE: 3 sets until exhaustion (elbow flexion and extension on the Scott bench, 80%-1RM). Blood samples were collected before and on the 2nd, 4th and 7th day after EE. Muscle soreness (MS), lipoperoxidation, protein carbonylation, Tumor Necrosis Factor-α (TNF-α) and interleukin 10 (IL-10) were determined. Results showed a significant increase in MS in all the groups on the 2nd day after EE and a decrease in the following days. A significant increase was observed in MDA and carbonyl levels on the 4th and 7th days after EE in all groups. TNF-α increased significantly on the 2nd day after eccentric exercise and decreased in the following days irrespective of NAC supplementation; concentration of IL-10 increased significantly on the 4th in all groups. Only the supplemented groups maintained high levels of IL-10 on the 7th day after EE. The results suggest that treatment with NAC represents an important factor in the defense against muscle soreness and presents different effects on oxidative damage and pro and anti-inflammatory cytokine.

Introduction

High intensity eccentric exercise increases the production of Reactive Oxygen Species (ROS) altering the antioxidant defense system (Childs, Jacobs, Kaminski, Halliwell, & Leeuwenburgh, 2001; Goldfarb, Bloomer, & Mckenzie, 2005). This can lead to oxidative stress and contribute to a decrease in performance, fatigue, muscle damage and muscle soreness (MS) (Ji, 1996; Mcbride, Kraemer, Triplett, & Sebastianelli, 1998; Avery, Kaiser, Sharman, Scheett, Barnes, Gomez, Kraemer, & Volek, 2003).

During the performance of eccentric physical exercise, neutrophils and macrophages migrate to and infiltrate the lesioned muscular tissue, activating pro-inflammatory cytokines and producing additional ROS (Childs et al., 2001; Goldfarb et al., 2005). However, ROS is also produced in other ways such as activation of xanthine oxidase, production of NADPH oxidase, ischemia-reperfusion, increase of phagocyte activity, protein breakdown and excessive accumulation of calcium (Goldfarb et al., 2005).

Several studies have suggested antioxidants supplementation to decrease ROS production during physical exercise, as well as to improve the body's defense systems against the attack of free radicals (Avery et al., 2003; Ji, 1996; Bloomer, Goldfarb, McKenize, You, & Nguyen, 2004). Therefore, while many studies have pointed to antioxidant supplementation as essential in fighting oxidative damage induced by eccentric physical exercise, it remains unclear which biological mechanisms are involved in this process, and which supplementation model can improve the response of oxidative defense (Beaton, Allan, Tarnopolsky, Tiidus, & Phillips, 2002).

N-acetylcysteine (NAC) is a donor of antioxidant thiols used in clinical practice to facilitate glutathione biosynthesis, improving the enzymatic defense system, as well as decreasing the harmful effects of ROS (Pinho , Silveira, Silva, Streck, Dal-Pizzol, & Moreira, 2005). NAC acts as scavenger of ROS including hypochlorous acid, hydroxyl radical and hydrogen peroxide (Aruoma, Halliwell, Hoey, & Butler 1989; Medved, Brown, Bjorksten, Murphy, Petersen, Sostaric, & Mckenna, 2004). However, in high doses, in case of homeostasis breakdown or after reacting with transition metals, it can cause a cytotoxic effect and produce additional ROS such as superoxide, hydrogen peroxide and hydroxyl radical (Neal, Cooper, Gurer, & Ercal, 1998; Halliwell, & Gutteridge, 2007).

Thus, it is possible that antioxidants supplementation can reduce the oxidative responses and muscle soreness induced by eccentric exercise. The objective of the study was to verify the effect of NAC supplementation on the development of muscle soreness, parameters of oxidative damages and inflammatory response after high-intensity eccentric exercise.

Materials and Methods

Subjects: 29 healthy males volunteers and students of UNESC (Universidade do Extremo Sul Catarinense, Criciuma, Santa Catarina state, Brazil) with a mean age of 21.3 ± 4yr, weight of 74.5 ± 7.7 kg and height of 177.2 ± 6.9 cm - were selected. They were nonsmokers, did not take NAC or related supplements, had not participated in resistance training or any other form of structured exercise for at least 6 months, who did not have a history of muscular

lesion or were not carriers of any disease that might compromise the results or aggravated by physical exercise. All the subjects were informed about the purpose of the study and the associated risks, and all of them gave written informed consent. Approval for this study was obtained from Ethics Committee of the UNESC.

Supplementation: Subjects were randomly selected in a single-blind manner to receive either a placebo (capsule containing starch) or NAC supplementation (capsule containing 10mg/kg body mass), without previous control of the diet. Volunteers received one capsule per day for a total of 14 days before the eccentric protocol and for 7 days post-exercise, and were randomly allocated in three groups: placebo (21 days of placebo; n=8), NAC (21 days of NAC; n=9); NAC plus placebo (14 days of NAC+ 7 days of placebo; n=8). During the study, four subjects withdrew from the groups due to personal reasons. Subjects were instructed to maintain their normal diet throughout the duration of the study and to take one capsule daily with their largest meal. Fourteen days after starting supplementation, the subjects performed eccentric exercise.

Eccentric Exercise protocol: Eccentric exercise (EE) of short duration and high intensity was performed 12 hours after the supplementation, with elbow flexion and extension on the Scott bench (equipment used in the muscular activity) at an intensity of 80% of maximum repetition - 1RM (Bompa, 2001). The concentric phase of the exercise was performed with manual assistance from the instructor. The eccentric phase was performed for duration of 6 to 8 seconds. Three sets of the exercises were performed, with 2 minute intervals,

until exhaustion. The 1RM test was performed 24 hours before of the supplementation.

Blood Collection: Blood samples were collected prior to the exercise and on the 2nd, 4th and 7th day after the exercise. Blood (10 mL) was obtained from the cubital vein of the right arm and collected in vacutainer tubes without additives. It was then processed and the serum separated, aliquoted and immediately stored in a freezer at –80°C for later analysis.

Muscle Soreness (MS)**:** MS levels were measured by a visual analogue scale. Subjects marked their subjective rating of MS between 0 (without pain) and 10 (extreme pain) (Revill, Robinson, Rosen, & Hogg, 1976).

Thiobarbituric Acid Reactive Species (TBARS): as indicator of lipid peroxidation, the formation of substances that react to the heating of thiobarbituric acid (malondialdehyde – MDA) measured spectrophotometrically (532nm) and expressed as malondialdehyde equivalents (Draper, & Hadley, 1990).

Protein Carbonylation: oxidative damage in proteins was measured by determining the carbonyl grouping based on the reaction with 2,4-dinitrophenylhydrazine (DNPH). Carbonyl content was determined spectrophotometrically (370nm) using a coefficient of 22.000 M (Levine, Garland, Oliver, Amici, Lenz, Ahn, Shajtiel, & Stadtman, 1990).

Cytokines: Tumor Necrosis Factor-α (TNF-α) and Interleukin 10 (IL-10) were determined by ELISA with commercially available kits (R&D Systems, Minneapolis, MN).

Protein Determination: The quantity of proteins in TBARS and carbonyl assays was measured using the technique of (Lowry, Rosebough, Farr, & Randall, 1951).

Statistical Treatment: Data was expressed in mean and standard error of the mean (SEM), and statistically analyzed using two-way analysis of variance (ANOVA), followed by the SNK post hoc test. The level of significance established for the test was $p<0.05$. SAEG version 9.0 was used.

Results

Muscle Soreness (MS): The results showed a significant increase in MS in all the groups in the second day after EE and a significant decrease in those values in all the groups from the fourth day after EE (Table 1).

Lipoperoxidation: Oxidative damage to membrane lipids was evaluated by the formation of MDA, a sub-product of lipoperoxidation. The results show (figure 1) a significant increase in MDA levels on the 4^{th} and 7^{th} days after the eccentric exercise, in all groups.

Protein Carbonylation (PC): To verify the oxidative damage in proteins, we evaluated the carbonyl groups based on the reaction with dinitrophenylhydrazine. Similarly to lipoperoxidation, the results (figure 2) also show a significant increase in PC on the 4^{th} and 7^{th} days after eccentric exercise in all groups, and NAC supplementation did not alter these results.

Cytokines: to quantify the inflammatory response, we measured the Tumor Necrosis Factor-α (TNF-α) and interleukin 10 (IL-10). All the groups significantly increased the TNF-α on the 2^{nd} day after eccentric exercise. The supplemented groups maintained high levels of TNF-α until the 4^{th} day after EE, decreasing in

the 7th days (figure 3a). Serum concentration of IL-10 increased significantly on the 4th day in all the groups. Only the supplemented groups maintained high levels of IL-10 on the 7th day after EE (figure 3b).

Discussion

The purpose of the study was to verify the effect of NAC supplementation on the development of muscle soreness, parameters of oxidative damages and inflammatory response after high-intensity eccentric exercise.

Initially, we verified the markers of muscular lesion. Several indicators have been used to determine the level of the muscular lesion induced by eccentric exercise as creatine kinase (CK), myoglobin, muscle soreness (MS) (Avery et al., 2003; Beaton et al., 2002; Lee, Goldfarb, Rescino, Hegde, Patrick, & Apperson, 2002). We used MS as markers of cellular damage.

Exercise may induce micro-lesions in the active muscles, especially when exercise is relatively intense, of long duration, and includes eccentric contractions (Beaton et al., 2002). Clinically, this presents itself as muscular discomfort and pain in the stressed muscles, reaching a peak 24 to 48 hours after exercise (Armstrong, Warren, & Warren, 1991; Paschalis, Giakas, Baltzopoulos, Jamurtas, Theoharis, Kotzamanidis, & Koutedakis, 2006). Our results show a significant increase in MS on the second day after the EE in all the groups and that these values decrease in the following days irrespective of NAC supplementation (table 1). Our results are in accordance with other studies that do not suggest a protecting effect of antioxidants against muscle soreness (Childs et al., 2001, Beaton et al., 2002; Shafat, Butter, Jensen, & Donnelly, 2004; Jamurtas Theocharis, Tofas, Tsiokanos, Yfanti, Pachalis, Koutedakis, &

Nosaka, 2005). It is possible that ROS has an important role in the etiology of skeletal muscle damage and MS via oxidation of ion transport systems, leading to disruption of Ca^{+2} -ion homeostasis, impaired mitochondrial respiratory control, distortions in signal transduction pathways, and ultimately, cell dysfunction (Kourie, 1998). Therefore, we believed that quenching of ROS by NAC could protect MS caused by eccentric exercise.

To assess the oxidative damage we measured malondialdehyde (MDA) levels (figure 2) and protein carbonilation (figure 3). Unlike what we believed, the results show significant increase in the level of lipoperoxidation and protein carbonilation on the 4^{th} and 7^{th} days after eccentric exercise in all supplemented groups. It is possible that those results are related with the presence of iron in the serum. The eccentric exercise increased the inflammation and the level of the free iron (Childs et al., 2001). According to Pinho et al. (2005), the use of NAC alone may have limitations and present pro-oxidant effects, due to the facility with which it interacts with iron. This mechanism can lead to the formation of additional ROS and to an increase in oxidative damage.

We have also observed that NAC supplementation did not decrease protein carbonilation after eccentric exercise. These results can be related with phagocytic cells migrating to the tissue after muscular injury (Goldfarb et al., 2005; Stupka, Lowther, Chorneyko, Bourgeois, Hogben, & Tarnopolsky, 2000).

Previous studies show the role of antioxidants in eccentric exercise-induced cytokine production (Petersen, Ostrowski, Ibfelt, Richelle, Offord, Halkjaer, & Pederson, 2001; Toft, Jensen, Bruunsgaard, Ibfelt, Halkjaer, Febbraio, & Pedersen, 2002). These studies show that proinflammatory cytokines increase progressively after eccentric exercise. The cytokine

response to exercise represented a reaction to exercise-induced muscle injury and inflammation (Vassilakopoulos, Karatza, Katsaounou, Kollintza, Zakynthinos, & Roussos, 2003).

TNF-α are the early-response, proinflammatory cytokines that are most likely synthesized by resident macrophages and local post-capillary vascular endothelium, with synthesis occurring rapidly after the onset of injury or infection (Cavaillon, 1994). Changes in serum TNF-α have been reported after a bout of strenuous exercise (Paschalis et al., 2006; Ostrowski, Rohde, Schjerling, & Pedersen, 1999). Here, we demonstrate that TNF-α serum concentrations (figure 3a) increase significantly on the 2nd day after eccentric exercise, decreasing in the following days irrespective of NAC supplementation.

The increase in TNF-α 2nd and 4th days after eccentric exercise in supllemented groups could be related to muscle damage. TNF-α may accumulate in the cell or be released, suggesting a dissociation between intracellular TNF-α production and release (Smith, Anwar, Fragen, Rananto, Johnson, & Holbert, 2000). It is possible that the decreases on the 4th and 7th day after eccentric exercise were due to rapid clearance or an undetectable accumulation at the site of injury (Cavaillon, 1994).

We also observed that serum concentration of IL-10 increases significantly on the 4th day after eccentric exercise in all groups and on the 7th day only in the groups supplemented with NAC (figure 3b).

IL-10 is a primary anti-inflammatory cytokine that acts by inhibiting proinflammatory cytokine production by activated monocytes and macrophages (Ostrowski et al., 1999). Studies have shown significant increase immediately after intense exercise (Smith et al., 2000, Ostrowski et al., 1999, Macintyre,

Sorichter, Mair, Berg, & Mckenzie, 2001) although it is not clear why elevations were observed at different times. However, in the present study, this time period would fit well with the period of resolution of an acute inflammatory response (Smtih et al., 2000), and may, in part, be responsible for the reduction in pro-inflammatory TNF-α that was significantly reduced during those periods.

It is possible that the mechanism by which NAC stimulates the production of anti-inflammatory cytokines 4th and 7th days after eccentric exercise can be related with the inhibition of ROS production with a concomitant decrease in NF-kB activation and expression of cytokine-induced neutrophil chemoattractant (Blackwell, Blackwell, Holden, Christman, & Christman, 1996). In models of pre-existing inflammation, it has been shown that NAC can also modulate phagocytic activity by suppressing oxidative burst, and by potentiating host defense (Villagrasa, Cortijo, Marti-Cabrera, Ortiz, Berto, Esteras, Bruseghini, & Morcillo, 1997; Stolarek, Bialasiewicz, & Nowak, 2002).

In conclusion, the results presented in this study suggest that treatment with NAC don't alter oxidative damage caused by eccentric exercise (lipoperoxidation and protein carbonylation). It seems that NAC has some anti-inflammatory effects and acts on the down-regulation of pro-inflammatory cytokines. However, we suggest further studies in order to clarify the effects of NAC supplementation on pro and anti-inflammatory cytokines.

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Legends

Table 1: Muscle Soreness (MS) in young university students after muscular lesion induced by eccentric exercise. Subjects marked their subjective rating of MS on a 0= without pain; 10=extreme pain. The values are presented as Mean±SD and the significant difference used in relation to pre-EE (^{a}) and in relation to 2 day (^{b}) was from $p<0.05$.

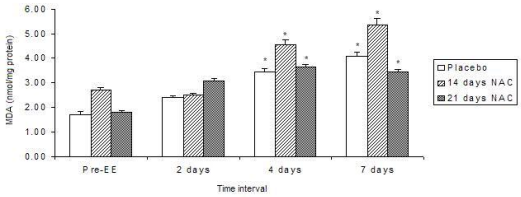
Figure 1: Lipoperoxidation levels in the serum of young university students after muscular lesion induced by eccentric exercise. The values are presented as Mean±SEM and the results expressed in nmol of MDA/mg of proteins. The significant difference used in relation to pre-EE (*) and in relation to placebo ($^{\#}$) was from $p<0.05$.

Figure 2: Carbonylation levels in the serum of young university students after muscular lesion induced by eccentric exercise. The values are presented as Mean±SEM and the results expressed in nmol/mg of proteins. The significant difference used within in relation to pre-EE (*) and in relation to placebo ($^{\#}$) was from $p<0.05$.

Figure 3a: Tumor Necrosis Factor-α (TNF-α) in the serum of young university students after muscular lesion induced by eccentric exercise. The values are presented as Mean±SEM and the results expressed in pg/ml of serum. T he significant difference used within in relation to pre-EE (*) and in relation to placebo ($^{\#}$) was from $p<0.05$.

Figure 3b: Interleukin 10 (IL-10): in the serum of young university students after muscular lesion induced by eccentric exercise. The values are presented as Mean±SEM and the results expressed in pg/ml of serum. he significant difference used within in relation to pre-EE (*) and in relation to placebo ($^{\#}$) was from $p<0.05$.

Groups	Soreness Muscle			
	Pre-EE	2 day	4 day	7 day
placebo	0	5.43±0.8[a]	1.84±0.7[b]	0.87±0.3[b]
14 days NAC	0	5.83±1.0[a]	1.67±0.8[b]	0.00[b]
21 days NAC	0	3.86±0.7[a]	1.29±0.5[b]	0.00[b]



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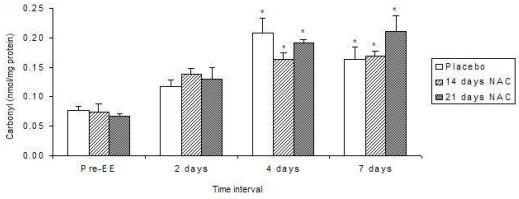

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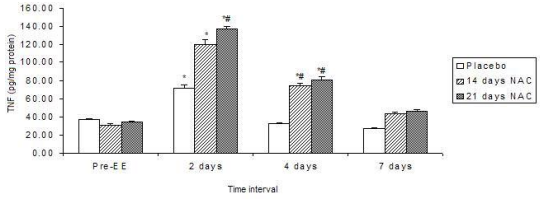

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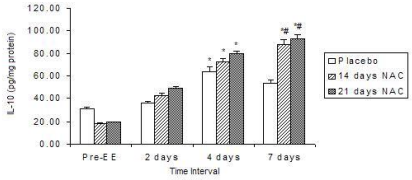

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