Effects of N-acetylcysteine supplementation on cellular damage and oxidative stress indicators in volleyball athletes

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The purpose of this study was to evaluate the effects of N-acetylcysteine (NAC) supplementation on cellular damage and oxidative stress indicators in volleyball athletes. Twenty male volleyball athletes at national level performed a physical training session and were divided into 2 groups, which for 7 days took the placebo substance or NAC. After 7 days the athletes repeated the same training session. In both sessions, blood samples were collected 30 min before and immediately after the training session to measure cellular damage and oxidative stress markers. The main results show that, although higher concentrations of glutathione peroxidase and superoxide dismutase were observed in postsession 1 than those in postsession 2, the other markers showed an increase in antioxidant action after supplementation of NAC, once the effect of experimental conditions (P=0.030) were observed in: time effect (P<0.001) and interaction (P<0.001) for total glutathione; time effect (P<0.001) and interaction (P<0.001) for reduced glutathione; and time effect (P<0.001) for ferric-reducing antioxidant potential. The oxidant action indicated by the protein carbonyl was higher in the placebo group than in the NAC group (P=0.028), but a time effect (P<0.001) for the thiobarbituric acid reactive substances showed lower values in presession 1 than in presession 2. For the cellular damage markers, antagonistic results between markers were found. Based in the results, the supplementation of NAC during a short period was effective in reducing oxidant action and increasing antioxidant action. However, conclusive alterations in the responses of the cellular damage markers were not obtained.

Keywords: Sports, Sports nutritional sciences, Antioxidants

INTRODUCTION

Volleyball is an Olympic modality that is characterized as an intermittent sport, with frequent high-intensity actions, involving explosive actions, short body displacements and numerous jumps (Freitas et al., 2014). In the sports preparation system, athletes are submitted to training with high volume and high intensity, which tend to lead to disturbances in the immune system (Dias et al., 2011), increase the cellular damage markers (Freitas et al., 2014) and increase the oxidative stress levels (Martinović et al., 2011).

Specifically, the increase in oxidative stress becomes a negative factor, because such responses have been associated with a decrease in performance and an increase in fatigue and muscular damage; it even causes states associated with non-functional overreaching and overtraining (Kurkcu et al., 2010; Sahlin et al., 2010). For this reason, some researchers suggest that reduction in oxidative stress can improve exercise tolerance and physical performance...
MATERIALS AND METHODS

Experimental design

Initially, two training sessions were used in order to make an anthropometric evaluation, a progressive maximum treadmill test (ramp protocol) with direct measurement of oxygen consumption, and the submaximal repetitions-to-fatigue test to estimate the one-repetition maximum (1RM). Subsequently, the athletes performed a physical training session and were divided into two groups, and were administered either the placebo substance (maltodextrin) or the antioxidant supplement (N-acetylcysteine) for 7 days. After the 7 days the athletes repeated the same training session. Both sessions had the same design and were held on the same day of the week and at the same time of day. In both sessions, blood samples were collected 30 min before the training session and immediately after the training session to measure cellular damage and oxidative stress markers.

Participants

The study included 20 male volleyball athletes at national level who were divided into the control group (n = 10; age, 15 ± 1 years; body mass, 74.2 ± 8.6 kg; peak oxygen consumption [VO2peak], 50.8 ± 7.0 mL/kg/min) or the experimental group (n = 10; age, 16 ± 1 years; body mass, 78.7 ± 16.5 kg; VO2peak, 46.0 ± 6.1 mL/kg/min).

After being briefed on the procedures and purpose of the study, the participants and legal guardian or a responsible adult signed a written and informed consent document. The study was conducted in accordance with resolution 12/2012 of the Brazilian National Health Council and approved by the Local Ethics Committee and carried out in accordance with the Declaration of Helsinki (approval number: 246/2009).

Incremental treadmill test

The subjects performed an incremental treadmill test to volitional exhaustion. Each stage lasted 1 min and was increased by 1 km/hr per stage until the subject could no longer continue. The oxygen uptake (VO2) was measured (METALYZER 3B - Cortex, Leipzig, Germany) throughout the test and the average of the final 30 sec was defined as VO2peak. The maximal velocity reached in the test was defined as the Vpeak. When the subject was not able to finish the 1-min stage, the speed was expressed according to the time spent in the final stage: Vpeak = velocity of penultimate stage+ (time, in sec, remained at the last stage/60 sec).

One-repetition maximum test

The load of 1RM was predicted based on a submaximal repetitions-to-fatigue test (1RM = load used = [1.0278–0.0278 × number of repetitions]) (Brzycki, 1993). Multijoint exercises (squat, leg press 45°, bench press, front lat pull-down) and single-joint exercises (leg extension, leg curl, standing calf raise and elbow flexion and extension with dumbbells) were performed. All athletes were familiar with the performance of these exercises.
Supplementation

The experimental group ingested 1,200 mg of oral supplement of NAC (Drozelev, Caçador, Brazil), divided into 2 doses per day: the first at 30 min before lunch (at 11:30 a.m.) and the second at the beginning of volleyball training (at 14:00 p.m.). The control group took the placebo solution (1,200-mg maltodextrin), also divided into 2 doses per day; the first at 30 min before lunch (at 11:30 a.m.) and the second at the beginning of volleyball training (at 14:00 p.m.). Over the 7 days, the researchers carefully monitored the volunteers daily to ensure that they maintained their regular diet and verified whether they had experienced any side effects of the supplementation (no adverse events were reported).

Dietary control

For dietary control, the subjects were submitted to the following instruments: food surveys and a daily food record. The 72-hr food recall consisted of reporting their food intake in the three days prior to blood collection; food consumption was estimated through a daily record held by the volunteer himself, as recommended by the Institute of Medicine, which allows the food and respective portions consumed by participants to be estimated in order to determine the daily energy and of the macronutrient levels after processing in NUTWIN software (São Paulo, Brazil).

Training sessions

Each training session consisted of 60 min of training on the court, comprising flexibility exercises (static and passive stretching), technical part (serve, reception, and attack) and coordination work with play in short games. Later, the athletes performed 30 min of continuous running of relative intensity between an aerobic threshold of 1 and 2. Finally, the athletes performed strength endurance work for upper and lower limbs (5 sets of 20 repetitions at 60% of 1RM for upper limbs, and 6 sets of 20 repetitions at 65% of 1RM for the lower limbs).

Blood samples and biochemical analysis

Blood samples (20 mL) were collected from the antecubital veins of the athletes at predetermined time points (Experimental Design). The collections were performed via vacuum system (BD Vacutainer, São Paulo, Brazil), using one tube containing sodium heparin and one tube without anticoagulants or additives. The serum and plasma from blood samples, obtained immediately by centrifugation of blood (1,000×g, 15 min, 4°C), were stored in Eppendorf tubes and frozen at -80°C until the biochemical analysis.

The activity of the antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD) were quantified in erythrocytes (McCord and Fridovich, 1969). The activity of GPx was determined using Wendel’s method (Wendel, 1981), which uses tert-butyl peroxide as a substrate of the reaction. The activity of SOD was determined based on the inhibition of superoxide radical reaction with adrenaline (Boveris et al., 1983). GSH concentration was assessed using the method proposed by Beutler et al. (1963). Serum antioxidant capacity was measured using the ferric reducing antioxidant potential (FRAP) assay, according to the technique proposed by Benzie and Strain (1996). Plasma lipid peroxidation was determined by detecting the thiobarbituric acid reactive substances (TBARS), particularly malondialdehyde, based on the method described by Esterbauer and Cheeseman (1990). The lipid hydroperoxides (LOOH) present in the serum were quantified by the ferrous oxidation method and complex formation with xylenol orange, as described by Jiang et al. (1992). Protein carbonyl was measured following the method described by Levine et al. (1990). All biochemical tests were made in duplicate.

For markers of cell damage, creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase were measured by the kinetic method. Uric acid, creatinine and urea were measured by the colorimetric method. These analyses were performed using a commercial kit (Labtest, Lagoa Santa, Brazil) and a Bioplus (Model UV-2000, São Paulo, Brazil) spectrophotometer.

Statistical analysis

The data are presented as mean and standard deviation. The comparison of the absolute values was performed using two-way (treatment and time) analysis of variance with repeated measures, followed by a Bonferroni post hoc test. A Mauchly test of sphericity was used to test this assumption and a Greenhouse-Geisser correction was applied when necessary. Additionally, to evaluate the magnitude of the observed differences, the effect size was calculated (eta squared, \( \eta^2 \)) and interpreted as follows: \(< 0.2 \) (small), \(> 0.2 \) and \( > 0.8 \) (large) (Cohen, 1988). The data were analyzed using the Statistica software version 12.0 (Statsoft, Tulsa, OK, USA).

RESULTS

Based on the dietary recalls, it is observed that the total intake (energy intake) and the macronutrient intake (carbohydrate, protein and fat) did not differ \((P > 0.05)\) between session 1 and session 2. Table 1 shows the results of the cellular damage markers after
Table 1. Cellular damage markers after the use of placebo or N-acetylcysteine during training of volleyball athletes

| Variable               | Placebo                  | N-acetylcysteine            |
|------------------------|--------------------------|-----------------------------|
|                        | Session 1 | Session 2 | Session 1 | Session 2 | Session 1 | Session 2 |
|                        | Pre       | Post     | Pre       | Post     | Pre       | Post     |
| CK (U/L)\(^a\)         | 208 ± 56\(^a\)          | 287 ± 74             | 192 ± 13 | 209 ± 70 | 288 ± 113\(^b\) | 393 ± 148 | 193 ± 73\(^c\) | 294 ± 97 |
| AST (U/L)\(^b\)        | 34 ± 11    | 60 ± 25             | 66 ± 66  | 59 ± 35  | 47 ± 22   | 64 ± 45  | 39 ± 18  | 62 ± 48  |
| ALT (U/L)\(^c\)        | 29 ± 8     | 32 ± 11             | 44 ± 30  | 31 ± 7   | 44 ± 13   | 44 ± 18  | 44 ± 23  | 45 ± 12  |
| LDH (U/L)\(^d\)        | 225 ± 17   | 296 ± 22            | 286 ± 60 | 297 ± 64 | 270 ± 45  | 336 ± 53 | 294 ± 54 | 340 ± 67 |
| Creatinine (mg/dL)\(^e\)| 0.8 ± 0.2  | 0.9 ± 0.2            | 0.9 ± 0.2 | 1.0 ± 0.2 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.2 |
| Uric acid (mg/dL)\(^f\)| 4.8 ± 1.0  | 5.4 ± 1.0            | 5.1 ± 0.5\(^g\) | 5.5 ± 0.3 | 4.5 ± 1.0 | 4.4 ± 1.4 | 4.4 ± 1.3 | 5.0 ± 1.0 |
| Urea (mg/dL)           | 15 ± 2     | 14 ± 1              | 16 ± 1   | 17 ± 3   | 15 ± 4   | 16 ± 2   | 17 ± 4   | 14 ± 2   |
| Alkaline phosphatase (U/L) | 121 ± 37  | 127 ± 38            | 120 ± 38 | 121 ± 39 | 101 ± 27 | 106 ± 28 | 105 ± 27 | 106 ± 27 |

Values are presented as mean ± standard deviation.

CK, creatine kinase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.

\(^a\)Time effect (P < 0.001).
\(^b\)Interaction, pre different from the post for same condition (P < 0.001).
\(^c\)Effect of experimental condition, with higher values for placebo condition (P < 0.05).
\(^d\)Effect of experimental condition, with higher values for experimental condition (P < 0.05).
\(^e\)Effect of experimental condition, with higher values for placebo condition (P < 0.05).
\(^f\)Effect of experimental condition, with higher values for experimental condition (P < 0.05).
\(^g\)Effect of experimental condition, with higher values for experimental condition (P < 0.05).

For CK, there was no effect of experimental conditions (P > 0.05). However, there was effect of time (F[3,54] = 25.6, P < 0.001, \(\eta^2 = 0.59\), moderate), with higher values at postsession 1 when compared to the values at preession 1 and postsession 2 (P < 0.001 for all comparisons), as well as higher values at postsession 1 when compared to the values at preession 1 and postsession 2 (P = 0.012). Additionally, higher values post-session 2 were observed when compared to preession 2 values (P < 0.05). In addition, an interaction (F[3,54] = 3.5, P = 0.020, \(\eta^2 = 0.16\), small) was observed. The preession values of the experimental condition and control were lower than those values observed after both sessions of the two conditions (P < 0.001).

For AST, there was no effect of experimental conditions, time effect or interaction (P > 0.05). For ALT, there was only an effect of experimental conditions (F[1,18] = 5.2, P = 0.035, \(\eta^2 = 0.22\), moderate), with higher values for the experimental condition (P = 0.035) when compared to the placebo condition. For LDH, there was an effect of experimental conditions (F[1,18] = 4.7, P = 0.043, \(\eta^2 = 0.21\), moderate), with higher values for the experimental condition (P = 0.043) when compared to the placebo condition; and time effect (F[3,54] = 18.4, P < 0.001, \(\eta^2 = 0.51\), moderate) showed lower values in preession 1 than in postsession 1 (P < 0.001), preession 2 (P = 0.031) and postsession 2 (P < 0.001), respectively. The postsession 2 values were higher than those in preession 2 (P = 0.005).

For creatinine, there was an effect of experimental conditions (F[1,18] = 7.8, P = 0.011, \(\eta^2 = 0.30\), moderate), with higher values for the placebo condition than those in experimental condition (P = 0.011) and time effect (F[3,54] = 4.7, P = 0.005, \(\eta^2 = 0.21\), moderate), with lower values in postsession 1 than in postsession 2 (P = 0.029). For uric acid, there was only a time effect (F[3,54] = 4.8, P = 0.004, \(\eta^2 = 0.21\), moderate), with lower values in postsession 2 than those in postsession 2 (P = 0.024). For urea, there was a time effect (F[3,54] = 3.1, P = 0.034, \(\eta^2 = 0.15\), small); however, the Bonferroni post hoc test did not confirm this difference (P > 0.05). An interaction (F[3,54] = 1.4, P = 0.258, \(\eta^2 = 0.07\), small) was observed, with higher values in postsession 2 of the placebo condition than those values for postsession 1 of the same condition (P = 0.015). For alkaline phosphatase there was no effect of experimental conditions, time effect or interaction (P > 0.05).

Table 2 shows the responses of oxidative stress after the use of placebo or NAC during training volleyball athletes’ training.

For GPx, there was a time effect (F[3,54] = 10.8, P < 0.001, \(\eta^2 = 0.38\), moderate), with lower values in preession 1 than those in postsession 1 (P < 0.001). In addition, the values for postsession 1 were higher than those found in postsession 2 (P < 0.001), respectively. Additionally, an interaction (F[3,54] = 2.9, P = 0.044, \(\eta^2 = 0.14\), small) was observed, in which the values observed pre-session 1 were higher when compared to same condition in the postsession (P < 0.001). In the same way, higher values in postsession 1 than those in postsession 2 of the same condition (placebo) (P < 0.01) were observed. Lower values in preession 2 of the experimental group than those in postsession 2 of the experimental condition (P < 0.05) were observed.

For SOD, there was a time effect (F[3,54] = 17.7, P < 0.001, \(\eta^2 = 0.50\), moderate), with lower values in preession 1 than in postsession 1 (P < 0.001). In addition, the values in postsession 1 were
higher than the values found in postsession 2 ($P < 0.01$), respectively. Additionally, an interaction ($F[3,54]=2.8, P = 0.046, \eta^2 = 0.14$, small) was observed, with the postsession 1 values of the placebo condition being higher than those for presession 1, presession 2 and postsession 2 of the same condition (placebo) ($P < 0.05$).

For total glutathione, there was an effect of experimental conditions ($F[1,18]=5.5, P = 0.030, \eta^2 = 0.23$, moderate), with higher values for the experimental condition than in the placebo condition ($P < 0.05$). In the same way, there was a time effect ($F[3,54]=17.3, P < 0.001, \eta^2 = 0.49$, moderate), with lower values in presession 2 than in other sessions ($P < 0.01$). Additionally, there was an interaction ($F[3,54]=6.6, P = 0.019, \eta^2 = 0.27$, moderate), with higher values in postsession 2 of the experimental condition than those in postsession 2 of the placebo condition ($P < 0.01$). In addition, the values in postsession 2 of the experimental condition were higher than all the values of the same condition ($P < 0.01$).

For GSH, there was a time effect ($F[3,54]=24.8, P < 0.001, \eta^2 = 0.58$, moderate), with lower values in presession 2 when compared to other moments ($P < 0.001$) and an interaction ($F[3,54]=7.1, P < 0.001, \eta^2 = 0.28$, moderate) was observed, with higher values in postsession 2 of the experimental group than those observed in presession 2 and postsession 1 of the same condition (experimental) ($P < 0.001$). In addition, the values from postsession 2 of the experimental group were higher than those in presession 1 and postsession 2 of the placebo condition ($P < 0.05$).

For FRAP, there was only a time effect ($F[3,54]=50.2, P < 0.001, \eta^2 = 0.74$, moderate), with higher values in presession 1 than in postsession 1 ($P < 0.001$) and lower than in presession 2 and postsession 2 ($P < 0.001$). However, there was no interaction ($P = 0.05$). For TBARS, there was only a time effect ($F[3,54]=17.29, P < 0.001, \eta^2 = 0.91$, large), with the values at presession 1 lower than those at presession 2 ($P < 0.001$). For LOOH, there was only an interaction ($F[3,54]=2.9, P = 0.043, \eta^2 = 0.14$, small); however, the Bonferroni post hoc test did not confirm this difference ($P > 0.05$). For protein carbonyl, there was effect of experimental conditions ($F[1,18]=5.7, P = 0.028, \eta^2 = 0.24$, moderate) with higher values for the placebo condition than in the experimental condition ($P < 0.05$) and time effect ($F[3,54]=17.4, P < 0.001, \eta^2 = 0.49$, moderate), with lower values in postsession 1 than in other sessions ($P < 0.01$).

### DISCUSSION

The main results show that, although higher concentrations of GPx and SOD were observed in postsession 1 than those in postsession 2, the other markers showed an increase in antioxidant action after supplementation with NAC, once the effect of experimental conditions, time effect and interaction for total glutathione, time effect and interaction for GSH, and time effect for FRAP were observed. The oxidant action indicated by the protein carbonyl was higher in the placebo group than in the NAC group, but a time effect for the TBARS showed lower values in presession 1 than in presession 2. For the cellular damage markers, antagonistic results between markers were found.
NAC is a donor of thiols that acts as a precursor of intracellular cysteine increasing the production of GSH (Silva et al., 2008). Thus, this supplement would have a potential effect in reducing the hydrogen peroxide levels (H$_2$O$_2$) by increasing the availability and attack of GSH (Zhang et al., 1999). In the present study, it can be considered that there was a higher antioxidant action after the supplementation of NAC, based on the responses of GSH (postsession 2 higher than postsession 1), and especially of FRAP (presession 2 higher than presession 1) and total glutathione (higher concentrations for NAC group).

Previous studies reported the effectiveness of the ingestion of NAC (1,200 mg × 8 days) as a strategy to improve the pro-oxidant status in healthy subjects (Zembron-Lacny et al., 2010) and to increase GSH and muscle cysteine in well-trained individuals (Medved et al., 2004). However, in a study by Leelarungrayub et al. (2011) the supplementation of NAC for 7 days (1,200 mg × day) did not alter the total antioxidant capacity. In contrast, the study of Trewn et al. (2013) did not find increases in total glutathione levels after 7 days of supplementation with NAC in trained cyclists, but a decrease in lipid peroxidation after the supplementation was observed.

Analyzing the oxidant action, it was observed that the concentrations of protein carbonyl were lower in the NAC group than in the placebo group. However, the TBARS were lower in pre-session 1 than in the presession 2 moment. In this sense, besides the increase in the antioxidant action, some previous studies have reported a decrease in the pro-oxidant activity. Zembron-Lacny et al. (2010) using NAC (1,200 mg × 8 days) pointed to the effectiveness of the use of NAC in the reduction of protein carbonyl. Slattery et al. (2014) reported that, after a supplementation with NAC (1,200 mg × 9 days), lowers TBARS concentrations after sprint exercise involving well-trained cyclists were observed, concomitantly with higher total antioxidant activity and a reduction of inflammatory parameters after the exercise.

Although there was an increase in antioxidant activity and a decrease in oxidative action, based on the results of cellular damage markers it is not possible to say that the supplementation with NAC generated a protective effect. In fact, antagonistic results between the damage markers were observed. In this regard, a previous study also demonstrated that the supplementation of NAC for 7 days (1,200 mg × day) did not modify the concentration of a cellular damage marker (CK) or the concentrations of a pro-inflammatory cytokine (tumor necrosis factor-α) (Leelarungrayub et al., 2011).

In this sense, it must be considered that the increase in cellular damage markers in the bloodstream is not caused only by injury in the sarcolemma, but also by an increase in the porosity of the sarcolemma due to the intensity of exercise. Thus, the importance of the joint analysis of different markers of cellular damage is evident (Bessa et al., 2008; Branco et al., 2016). Thus, based on the antagonistic results of the joint analysis, no protective effect can be accredited to the supplementation of NAC.

Regarding the performance, divergent results are described in the literature. In well-trained individuals it was observed that a supplementation of NAC increased performance in submaximal activity (Medved et al., 2004). In sedentary individuals, a supplementation of NAC for 7 days (1,200 mg × day) decreased muscle fatigue and increased the VO$_{2max}$ (Leelarungrayub et al., 2011). In a study by Zembron-Lacny et al. (2010) the authors described that although a supplementation of NAC increased the pro-oxidant status, an improvement in the performance was not observed. However, the type of exercise seems to have an influence, since Trewn et al. (2013) suggest that a supplementation of NAC alters the metabolism of the substrate (increases the fat oxidation) and changes the type of muscle fiber recruited (greater involvement of oxidative fibers), factors that generated decreases in performance (mean power) during high-intensity interval exercise. However, Slattery et al. (2014) reported an improvement in performance involving a sprint in a cycling race simulation after intake of NAC (1,200 mg × 9 days).

Some of the divergences in the results found in the literature can be explained, in part, by the difference in the characteristic of the subjects (e.g., sedentary, physically active, athletes), type of exercise (endurance × power) and doses of NAC used. Thus, future studies considering these characteristics should be conducted.

In summary, the present study showed that the supplementation of NAC (1,200 mg/day) during a short period (7 days) was effective in reducing oxidant action and increasing antioxidant action. However, conclusive alterations in the responses of the cellular damage markers were not obtained. New experiments with different times of supplementation need to be formulated in order to evaluate the time-course of the NAC. In addition, evaluations of the performance and perceived exertion of the athletes should be included, since one of the limitations of the present study was that these parameters were not assessed.

**CONFLICT OF INTEREST**

No potential conflict of interest relevant to this article was reported.
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