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ORIGINAL PAPER

Modulatory effects of vitamin C on the relation between physical exercising and oxidative stress at young smokers

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Abstract

In the present paper we were mainly interested in understanding the relevance of smoking and the level of training on the relations that might exist between exercising and oxidative stress. More importantly, considering the increasing biotechnological importance of the vitamin C, we were also interested in observing the effect of vitamin C administration before exercising (40 minutes of cycloergometer aerobic training) on the main oxidative stress markers of the selected untrained young smoking (occasional vs. chronic) subjects. The oxidative stress markers determined were two antioxidant enzymes: superoxide dismutase-SOD and glutathione peroxidase-GPX and a lipid peroxidation marker: malondialdehyde-MDA. Our data demonstrated once again an increased oxidative stress after exercising, as showed by the significant decrease of GPX activity and the increased levels of MDA concentration. Also, the administration of vitamin C resulted in a significant decrease of the oxidative stress (an increase in GPX activity and a decrease of MDA concentration). Thus, considering that oxidative stress is a factor that can be corrected, it seems that various antioxidants such as vitamin C could represent a possible solution for longer physical training or shorter recovery periods. However, future studies are needed to clearly determine which antioxidants, at what dosages and in which combinations will have the greatest positive effect with the lowest risk.

Keywords: vitamin C, smoking, oxidative stress, exercising

1. Introduction

In the last years, an increasing awareness regarding the possible relations that might exist between exercise and the modifications of the oxidative stress was registered. They are many controversies in this area of research, since some researchers demonstrated that oxidative stress could be increased as a result of physical exercise (S.K. POWERS & M.J. JACKSON [1]; S. LEICHTWEIS & al. [2]; M.B. REID & al. [3]), while others reported a decreased oxidative stress and an increased antioxidant status after physical effort (C.T. EVELO & al. [4]; J.D. ROBERTSON & al. [5]). Moreover, some papers showed no significant effect of exercising on the main markers of the oxidative stress (H.M. ALESSIO & A.H. GOLDFARB [6]) (such as the main antioxidant enzymes – superoxide dismutase-SOD and glutathione peroxidase-GPX – on one side and some lipid peroxidation markers such as malondiadehyde-MDA, on the other side). There are several explanations for most of these controversial results: the different methods used for the determination of the enzyme activity – e.g spectrophotometric (A. CIOBICA & al. [7]) vs. chemiluminometric (B.A. STOICA & al. [8]), the different

types of exercise used and the muscles that are involved in that specific type of effort, the differences in the antioxidant enzymes iso-variations, as well as the level of training for the subjects involved in the studies (U.K. SENTÜRK & al. [9]). The results also depend on various co-morbidities such as smoking (W. PARK & al. [10]). In the present report we were mainly interested in understanding the last two factors mentioned above, by studying the relevance of occasional or chronic smoking on the influence of exercising on the oxidative stress status in untrained subjects. Moreover, considering the increasing biotechnological relevance of the vitamin C (F. AGIUS & al. [11]; E. CRUZ-RUS & al. [12]), which is known as a very important and powerful antioxidant (L.L. JI [13]), we were also interested in observing the effect of vitamin C administration after 40 minutes of cycloergometer aerobic exercise on the main oxidative stress markers (two antioxidant enzymes: superoxide dismutase-SOD and glutathione peroxidase-GPX and a lipid peroxidation marker: malondiadehyde-MDA) of the selected untrained young smoking (occasional vs. chronic) subjects.

2. Material and methods

This study included 14 untrained volunteering human subjects, all of them males, and was held at the "Alexandru Ioan Cuza" University of Iasi. The subjects were part of two research groups. The first group included occasional smokers who smoked between 1 and 200 cigarettes per month and the second group consisted of subjects who smoked more than 200 cigarettes per month. The demographical features of the subjects are presented in Table 1.

Table 1. The demographical features of the experimental groups

Feature	Occasional smokers	Chronic smokers
Age (years)	24.29 ± 1.15	23.00 ± 1.05
Height (cm)	176.10 ± 2.82	176.40 ± 2.16
Weight (kg)	74.56 ± 5.92	75.76 ± 6.08

The study had two phases. In the first one, the volunteers were physically evaluated regarding their aerobic work capacity, in order to establish the type of exercise to which they were subjected. The basic parameters on which the exercise determination was done were the maximal oxygen uptake (VO2max) and the maximal aerobic power (MAP). Before physical assessments, the subjects warmed up taking part in joint gymnastics. During the tests, subjects were provided with water for hydration.

The maximal oxygen uptake (VO2max)

This is considered the maximum rate at which oxygen can be absorbed by the body through the transport from the environment to the active muscles. The assessment of VO2max is generally done by standardized effort tests. The subject pedaled on a Vision Fitness E3200 bicycle at a rate of 80 rotations per minute (RPM) with a load of 143 W (corresponding to the fourth level of resistance of the bike). Blood pressure was measured at the beginning and at the end of the test. This is a progressive effort test on minute levels, the power increasing on each level by 25 W. The subject must carry out the maximum number of levels possible. A pedaling frequency of 80 RPM and a steady breathing rate throughout the whole test must be kept. The effort ceases when the subject can no longer keep up the pace of the previous level he reached. Leaning on the bicycle horns is allowed according to subject's preference, except on forearms. Contact of the foot with the pedal is done on the sole.

Maximal aerobic power (MAP)

This parameter indicates the maximum physical workout scheme of the muscles involved in the effort, being reached simultaneously with VO2max. During a progressive effort test,

VO2max and MAP increase gradually, but there are cases of individuals with similar VO2max and different MAP. The average values of these two parameters are presented in Table 2.

Table 2. The physiological parameters of the experimental group.

Parameter	Occasional smoker	Chronic smokers
VO2max (mL/kg/min)	45.51 ± 2.11	45.10 ± 4.63
Maximal aerobic power (W)	292.00 ± 34.80	283.90 ± 18.59

Also, in order to safely conduct the evaluations, professional medical staff and an intervention team from the Romanian Red Cross (Iasi division) were present in the sports hall. The sports hall in which the effort tests were held has an area of 68.5 m^2, a volume of 230 m^3 with ventilation through a 1 m^2 window. Moreover, during the research, some atmospheric parameters (temperature, humidity and atmospheric pressure) were continuously monitored (Table 3).

Table 3. Atmospheric parameters for conducting the research.

Parameter	Occasional smoker	Chronic smokers
Temperature (°C)	20.81 ± 0.15	20.63 ± 0.19
Atmospheric pressure (mmHg)	751.30 ± 0.71	750.60 ± 0.76
Humidity (%)	36.52 ± 0.31	37.10 ± 0.44

After the physical assessment of the subjects, one week later, the experiment was performed using the Vision Fitness E3200 bicycle between 9 a.m. and 4 p.m. Blood was collected before and immediately after the exercise. Parameters such as the heart rate, blood pressure and the oxygen saturation were monitored before and after the exercise. The exercise required 3 min of cycling at 30% of MAP, followed by 37 min at 50% of MAP. A week later, 12 hours before the experimental effort, the subjects received one 1000 mg tablet of *vitamin C*. The administered tablets contain traces of hydroxypropyl methylcellulose, microcrystalline cellulose, stearic acid, silicon dioxide and magnesium stearate. After the slow assimilation interval (12 h later), the subjects performed the same experimental effort described above for 40 min. Blood was again collected immediately after the effort in 9 ml vacutainers, allowed to clot and centrifuged immediately, aliquoted into Eppendorf tubes and stored at -40°C until measurements. After collecting the biological samples, the subjects remained in the hall to be monitored for at least 15 min. The study was conducted according to provisions of the Helsinki Declaration and all subjects signed an informed consent for participation in this study.

Biochemical determinations

Superoxide dismutase (SOD) activity was measured by the percentage of the inhibition rate of the enzyme in reaction with WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5- (2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) substrate (a water-soluble dye) and xanthine oxidase using a SOD Assay Kit (FLUKA, 19160) according to the manufacturer's instructions. The absorbance was monitored at 450 nm (the wavelength for the colored product of WST-1 reaction with superoxide anions), after 20 min of reaction at 37°C. The percent of inhibition was normalized by the protein content and presented as SOD activity units (A. CIOBICA & al. [7]). *Glutathione peroxidase (GPX)* activity was measured using the GPX cellular activity assay kit CGP-1 (SIGMA). This kit uses an indirect method based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled with recycling GSSG back to GSH using glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPX activity (A. CIOBICA & al. [7]). *Malondialdehyde (MDA)* levels were determined uing thiobarbituric acid (TBARs) assay. 200 µL of serum was briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL

of TRIS-HCl (pH 7.4) and 1 mL of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100°C for 20 minutes. Afterwards, samples were centrifuged at 3000 rpm for 10 min and the supernatant read at 532 nm. The signal was read against an MDA standard curve and the results were expressed as nmol/ml (F.P. TROFIN & al. [14]).

Data analysis

The levels of oxidative stress markers (SOD, GPX and MDA) were statistically analyzed using one-way analysis of variance (ANOVA). All results are expressed as mean ± the standard error of the mean (SEM). Post hoc analysis was performed using Tukey's honestly significant difference test in order to compare the groups. F values for which $p<0.05$ were regarded as statistically significant.

3. Results and discussion

Regarding the results we obtained in the case of SOD, the first antioxidant enzyme that decompose the superoxide radicals, we observed that no statistically significant differences were obtained for its specific activity, when we compared the basal group of occasional smokers vs. the SOD activity after exercise ($F(1.12)=4$, $p=0.07$) or vs. SOD activity after exercise + vitamin C ($F(1.12)=4$, $p=0.052$) in this group of occasional smokers (Figure 1). In addition, no significant differences were observed when we compared (using post-hoc analysis) the group of occasional smokers after exercise with the group with the same smoking status after exercise + vitamin C ($F(1.12)=0.2$, $p=0.8$).

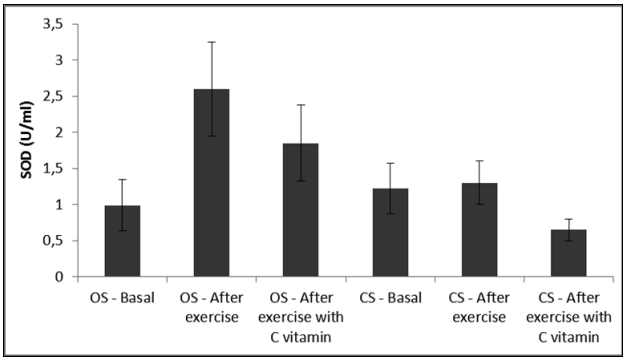

Figure 1. Serum activity of superoxide dismutase (SOD) in the basal, after exercise and after exercise + vitamin C in both occasional smokers (OS) and chronic smokers (CS) groups. The values are mean ± SEM (n=7). Also, post-hoc analysis showed a significant decrease of SOD enzymatic activity in the chronic smokers after exercise and vitamin C vs. exercised alone chronic smokers group ($p=0.01$).

For the group of chronic smokers, we also could not find any significant modifications in the after exercise group ($F(1.12)=3$, $p=0.1$) or after exercise + vitamin C group ($F(1,12)=0.4$, $p=0.5$), as compared to the baseline for the chronic smokers. Still, a significant decrease of the SOD activity was registered with the post-hoc analysis for the chronic smokers after exercise and vitamin C vs. exercised alone chronic smokers group ($F(1.12)=8$, $p=0.01$) (Figure 1). Also, regarding GPX activity, the second antioxidant enzyme which we determined in our study, no significant differences were observed when we compared the basal group of occasional smokers with the GPX activity after exercise ($F(1.12)=0.8$, $p=0.7$) or vs. GPX activity after exercise with vitamin C ($F(1.12)=1$, $p=0.3$) (Figure 2). In addition, no significant differences were observed when we compared using post-hoc analysis the group of occasional smokers

after exercise with the group with the same smoking status after exercise with vitamin C (F(1.12)=1, p=0.29). On the other side, regarding the chronic smokers group, we could actually observe a significant decrease in the enzyme activity in "the after exercise" group, when compared to the chronic smokers basal group (F(1.12)=8, p=0.01). However, no significant differences were observed between the basal chronic smokers group, as compared to the chronic smokers after exercise + vitamin C (F(1.12)=0.6, p=0.4). Still, very importantly, when we performed the post-hoc analysis, we found a very significant increase in the specific activity of GPX in the group of chronic smokers after exercise + vitamin C, when compared with the after exercise alone chronic smokers group (F(1.12)=12, p=0.004) (Figure 2).

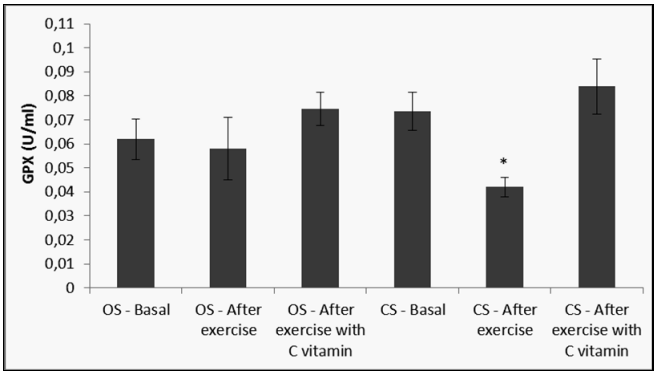

Figure 2. The serum activity of glutathione peroxidase (GPX) in the basal, after exercise and after exercise + vitamin C in both occasional smokers (OS) and chronic smokers (CS) groups. The values are mean ± SEM (n=7). *p < 0.013 vs. CS basal. Also, post-hoc analysis showed a significant increase of GPX enzymatic activity in the chronic smokers after exercise and vitamin C vs. exercised alone chronic smokers group (p=0.004).

In addition, an increased oxidative stress status as a result of exercise performing (as in the case of the aforementioned GPX decrease) was observed when we determined the levels of MDA, as the main lipid peroxidation marker in both our occasional and chronic smokers groups. In the case of the occasional smokers group, we noticed a significant increase of MDA concentration in the "after exercise" group as compared to the basal occasional smokers values (F(1.12)=6, p=0.02). Still, no differences were observed between the basal occasional smokers and the occasional smokers group that performed the 40 minutes exercises and also received the vitamin C (F(1.12)=0.1, p=0.7). However, when we performed the post-hoc analysis we could also observe a significant statistical difference between the occasional smokers group which performed the exercising alone vs. the occasional smokers group that performed the exercises + vitamin C (F(1.12)=7, p=0.02) (Figure 3). Similar aspects were observed in the case of the chronic smokers group, in which case we also initially observed a very significant increase of the MDA levels in the group of chronic smokers that performed the 40 minutes exercising vs. the basal chronic smokers (F(1.12)=14, p=0.002). However, no significant differences were observed between basal chronic smokers and the group that had the same smoking status and performed the exercising + vitamin C (F(1.12)=1, p=0.2). Also, when we performed the post-hoc analysis, we could again observe very significant statistical differences between the group of chronic smokers which performed the exercising only vs. the chronic smokers that were exercising + vitamin C (F(1.12)=18, p=0.001) (Figure 3).

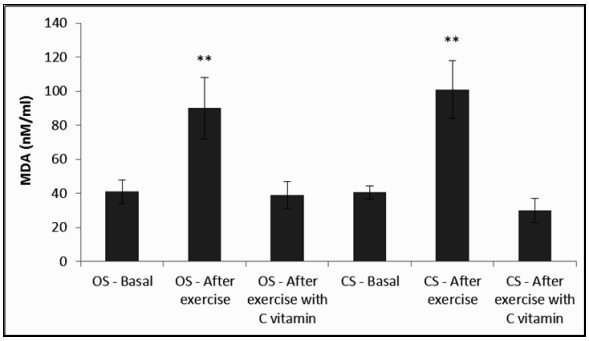

Figure 3. The serum concentration of malondiahdehyde (MDA) in the basal, after exercise and after exercise + vitamin C in both occasional smokers (OS) and chronic smokers (CS) groups. The values are mean ± SEM (n=7). **p < 0.02 vs. basal (both OS and CS). Also, post-hoc analysis showed a significant MDA decrease in both the occasional and chronic smokers groups after exercise and vitamin C vs. exercised alone groups (p < 0.02).

In the present paper we were mainly interested in understanding the relevance for the level of training and smoking status (occasional vs. chronic) in the relations that might exist between exercising and oxidative stress status (as seen through the values of its main markers such as SOD, GPX and MDA). Moreover, considering the increasing biotechnological relevance of the vitamin C (F. AGIUS & al. [11]; E. CRUZ-RUS & al. [12]), we were also interested in observing the effect of vitamin C administration before exercise on the main oxidative stress markers (two antioxidant enzymes: SOD and GPX, and a lipid peroxidation marker-MDA) at the selected untrained young smokers (occasional vs. chronic). Our data demonstrated once again an increased oxidative stress status after 40 minutes of cycloergometer aerobic exercise, as showed by the significant decrease of GPX activity and increased levels of MDA concentration. Importantly, the administration of vitamin C resulted in a significant decrease of the aforementioned oxidative stress, manifested both in a significant increase of the enzymatic activity of GPX, as well as in a reduction of the concentration of the main lipid peroxidation marker MDA. Also, the smoking status seems to be relevant in this context, since we could observe a significant decrease of SOD enzymatic activity in the chronic smokers after exercise and vitamin C vs. exercised alone chronic smokers group, which was not observed in the case of the occasional smokers. In addition, post-hoc analysis showed a significant increase of GPX in the chronic smokers after exercise and vitamin C vs. exercised alone chronic smokers group that was also not observed in the occasional smokers group. Still, no changes between occasional and smokers groups were noticed in the case of MDA. The significant increase that we could observe in the case of SOD's activity and the decrease of GPX activity after exercising, could be explained by a defensive mechanism of the cell, as a result of the increased oxidative stress status (L.L. JI [13]; M. PADURARIU & al. [15]) generated though the 40 minutes exercising. These compensatory changes could also represent the reason for the differences observed in the modifications of SOD (increased) and GPX (decreased enzymatic activity), when we compared the results of the occasional vs. chronic smoking effects on the oxidative stress status. As mentioned, previous results in this area of research are controversial, with a multitude of reports stating opposite findings, as for example in the case of SOD activity, which was reported to be either decreased (P.M. TIIDUS & al. [16]) or increased (H. OHNO & al. [17]; S.K. POWERS & al. [18]). Moreover, other authors such as H.M. ALESSIO & A.H. GOLDFARB [6] stated no significant modifications in the specific enzyme activity of

SOD, even if both acute and chronic exercising was performed. Even more, there are opinions that it could be very unlikely for the increased capacity of the enzymatic scavenging of superoxide radical to be a major protective adaptation against free radical damage in exercise-trained muscle (M. HIGUCHI & al. [19]). As also demonstrated in the present paper, it seems that the level of training could be an important explanation for this variety of results (U.K. SENTÜRK & al. [9]). It was previously showed that endurance training can actually result in a reduction of some lipid peroxidation markers such as MDA during moderate-intensity exercise, while the activation of antioxidant enzymes such as catalase increases and could actually modulate the MDA levels after training (H.M. ALESSIO & A.H. GOLDFARB [6]). Another important factor that supports the aforementioned controversial results could be represented by the smoking status. Thus, as demonstrated in this study, it seems that there is a very strong and complex correlation between the smoking status and the oxidative stress markers modifications. They are also controversies regarding the effects of smoking and nicotine on oxidative stress status (A. CIOBICA & al. [7]; L. HRITCU & al. [20]). While some reports stated that nicotine administration may result in oxidative stress by inducing the generation of reactive oxygen species in the periphery and central nervous system (D. QIAO & al. [21]), some authors suggested that nicotine may have antioxidant properties in the central nervous system, which are mainly intracellular through the activation of the nicotinic receptors or even extracellular by acting as a radical scavenger that binds to iron (M.B. NEWMAN & al. [22]). In fact, it is believed that low concentrations of nicotine may act as an antioxidant and even play a neuroprotective role, while an increased dosage of nicotine may induce neurotoxicity and stimulate oxidative stress (Z.Z. GUAN & al. [23]). Regarding the use of vitamin C, it is generally considered that although some recent papers demonstrated that exercise-induced oxidative stress could be prevented by antioxidants (M. ARAÚJO & al. [24]; L. SUN & al. [25]), there is still insufficient knowledge about the possible protective effects of antioxidants against exercise-induced oxidative damage (F.P. TROFIN & al. [26]). As mentioned before, various controversies are also characterizing this area of research, since it was showed for example that vitamin C is able to increase the running capacity until exhaustion (L. PACKER & al. [27]), while other authors demonstrated that actually vitamin C cannot prevent training-induced oxidative stress (K. GOHIL & al. [28]). Also, there are other reports demonstrating that both vitamin C and E did not affect in any way the performance of the subjects (S.K. POWERS & M.J. JACKSON [1]; S.K. POWERS & al. [29]), while Ji group stated that vitamin C administration could decrease muscle fatigue (L.L. JI [13]). Also, for vitamin E it is showed that it could delay the onset of muscular fatigue (G.P. NOVELLI & al. [30]) and also reducing some specific tissue-damage during exercise (M.J. JACKSON & al. [31]). Moreover, similar aspects were reported for other antioxidants such as N-acetylcysteine (C. SHINDOH & al. [32]). Thus, considering that oxidative stress is a factor that can be corrected, it seems that various antioxidants could represent a possible solution for longer physical training or shorter recovery periods. However, although this seems to be a promising research field, future studies (including the ones that our group is performing) will need to clearly identify the relation between exercising and oxidative stress status. In addition, this also suggests the possible importance of nutrient and antioxidant supplementation to support the enzymatic defense system (A. CIOBICA & al. [33]). Moreover, despite the fact that the use of agents which modulate oxidative stress represents an exciting opportunity in this area of research, future studies will need to clearly determine which antioxidants, at what dosages and in what combinations will have the greatest positive effect with the lowest risk. It must be considered the importance of free radicals in many biological reactions, as it is known for example that overdosing with this specific vitamin could affect the heart,

especially in the case of acute prolonged exercise, mainly through a pro-oxidant reaction with transitional metal ions (F.P. TROFIN & al. [26]).

4. Conclusions

Our data demonstrated once again an increased oxidative stress status after 40 minutes of cycloergometer aerobic exercise, as the significant decrease of GPX activity and the increased levels of MDA concentration showed. More importantly, the administration of vitamin C resulted in a significant decrease of the oxidative stress, manifested both in a significant increase in the enzymatic activity of GPX, as well as in a reduction of the concentration of the main lipid peroxidation marker MDA. Also, the smoking status seems to be relevant in this context, since we could observe a significant decrease of SOD enzymatic activity in the chronic smokers after exercise and vitamin C supplementation vs. exercised alone chronic smokers group, which was not observed in the case of the occasional smokers. In addition, we showed a significant increase of GPX in the chronic smokers after exercise and vitamin C supplementation vs. exercised alone chronic smokers group that was also not observed in the occasional smokers group. Thus, considering that oxidative stress is a factor that can be corrected, it seems that various antioxidants could represent a possible solution for longer physical training or shorter recovery periods. However, future studies will need to clearly determine which antioxidants, at what dosages and in what combinations will have the greatest positive effect with the lowest risk.

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