Pol. J. Food Nutr. Sci., 2014, Vol. 64, No. 4, pp. 277–281
DOI: 10.2478/pjfns-2013-0025
http://journal.pan.olsztyn.pl

Original article
Section: Nutritional Research

Antioxidant Vitamins as Oxidative Stress Markers in Rat Plasma After Physical Exercise – a Short Report

Agata Wawrzyniak[1], Jadwiga Hamułka[1], Małgorzata Drywień[1], Magdalena Górnicka[1], Jolanta Pierzynowska[1], Malwina Wojtaś[1], Małgorzata Gajewska[2], Joanna Frąckiewicz[1], Anna Gronowska–Senger[1]*

[1]*Chair of Nutritional Assessment, Department of Human Nutrition, Warsaw University of Life Sciences (WULS–SGGW), Nowoursynowska 159C, 02–776 Warsaw, Poland*
[2]*Chair of Biochemistry, Department of Physiological Sciences, Warsaw University of Life Sciences (WULS–SGGW), Nowoursynowska 159, 02–776 Warsaw, Poland*

Key words: β–carotene, retinol, vitamin E, vitamin C, exercise, rats

The aim of the study was to verify the hypothesis that β–carotene, vitamin E, vitamin C administered individually or in combination may differently modify their levels in blood plasma being also markers of the oxidative stress. Male Wistar rats were supplemented antioxidants *per os* (α–tocopherol – 2 mg/d, ascorbic acid – 12 mg/d, β–carotene – 1 mg/d), both individually or in combination of 2 or 3, for 14 days. During experiment, half of the animals in each group (n=8) were subjected to treadmill exercise for 15 min at the speed of 20 m/min, to induce oxidative stress. Vitamins in rat plasma were assessed by the high–performance liquid chromatography (HPLC). Results suggest that vitamin E and C supplemented simultaneously may provide some benefit during physical exercise. The significant influence of administered α–tocopherol acetate and physical exercise on the level of α–tocopherol in the plasma was observed. Thus only the concentration of α–tocopherol in blood may be treated as a marker of oxidative stress.

INTRODUCTION

Results of recent research indicated that intense exercise may enhance the need for oxygen, increasing production of its reactive forms [Martins *et al.*, 2011; Powers *et al*., 2011]. The oxidative damage of cell components observed during physical activity and/or during resting may be an indication for supplementing the diet with antioxidants, which may strengthen the organism's antioxidant defence or accelerate a return to prooxidative–antioxidative balance. One of the biomarkers of homeostatic disruption in the organism is the level of low–molecular–weight antioxidants, such as vitamins E and C and β–carotene, in the plasma [Lamprecht *et al.*, 2009; Zeinab *et al.,* 2010; Böhm *et al.*, 2012]. The aforementioned studies indicate mutual interaction of lipophilic compounds and their reciprocal regeneration from free radical forms as well as effective interaction of vitamins C and E, *i.e.* ascorbic acid and α–tocopherol acetate, at the border between the cell's lipo– and hydrophilic phase [Yeum *et al.*, 2004; Traber & Stevens, 2011]. β–Carotene's antioxidant function, as an electron–rich molecule, is stronger with smaller concentrations of oxygen in the cell, but it also complements the antioxidant effect of vitamin E, which works with higher oxygen concentrations. Tocopheryl radicals may be reduced to their original forms by other antioxidants' (vitamin C, coenzyme Q10) effects. Ascorbate has the ability to reduce tocopheryl radicals. This reaction is possible because of the chroman rings in tocopherol molecules, which face the outside of the cell membrane to form ascorbyl radicals [Yeum *et al.*, 2004; Krinsky & Johnson, 2005]. The ascorbic radical, in turn, may return to ascorbate form by reacting with NADPH reductases [Schneider, 2005].

The present study was undertaken because of a lack of unambiguous data regarding the benefits of antioxidant vitamin supplementation in defending against oxidative stress induced physical activity [Lamprecht *et al.*, 2009; Gomez–Cabrera *et al.*, 2012]. The aim of the study was to verify the hypothesis that β–carotene, vitamin E, vitamin C administered individually or in combination may differently modify their levels in blood plasma being also markers of the oxidative stress.

MATERIAL AND METHODS

Animals and diet

For these experiments, 144 male outbred Wistar rats from certified breeding (Medical University of Białystok, Poland) with an initial body weight of 120–140 g were involved. The animals were fed a diet (Table 1) according to AIN–93M [Reeves, 1997], and had free access to water. Male Wistar rats were supplemented antioxidants *per os* (α–tocopherol – 2 mg/d, ascorbic acid – 12 mg/d, β–carotene – 1 mg/d),

* Corresponding Author: agata_wawrzyniak@sggw.pl (prof. A. Wawrzyniak)

TABLE 1. Composition of experimental diet according to AIN–93M.

Ingredients	Content (%)
Caseine	14.0
Cornstarch	46.5
Dextrose	15.5
Sucrose	9.0
Cellulose	5.0
Soybean oil	4.0
t–Butylhydroquinone	0.0008
Satl mix	3.5
Vitamin mix	1.0
L–Cystine	0.18
Choline bitertrate	0.25
Supplement (#410950)	1.0

both individually or in combination of 2 or 3, for 14 days. During experiment, half of the animals in each group (n=8) were subjected to treadmill exercise for 15 min at the speed of 20 m/min, to induce oxidative stress. Design of experiment was described in Wawrzyniak *et al.* [2013]. The experiment was approved by the III Local Animal Experiment Ethics Committee at the Warsaw University of Life Sciences (Resolution no. 33/2008).

Blood sampling

After the experiment, the rats were anesthetized (intramuscular injection of ketamine and xylazine) at the same time after the end of the training [Wawrzyniak *et al*., 2013] and blood was taken from the left ventricle of the heart. Heparin sodium was used as an anticoagulant. The blood was centrifuged at 4000 rpm for 10 min at 4°C. The plasma was frozen in liquid nitrogen and preserved at –80°C until analysis.

Analysis of vitamins in blood plasma

Vitamins in rat plasma were assessed using the high–performance liquid chromatography (HPLC) (Gilson Company, Middleton, WI, USA) with a UV–VIS detector. The concentrations of retinol and β–carotene were measured according to Gackowski *et al.* [2001]. Hexane solutions of retinol or β–carotene were separated by a C18 RP (4.6 x 250 mm; 5mm) chromatographic column from the Vydac Company (Vydac 201TP54, Hesperia, CA, USA) with a precolumn from the same company. Methanol/acetonitrile (96:4; v/v) was used as a developing mixture for the determination of retinol, and hexane/dichloromethane/methanol (15:5:80; v/v/v) was used as a developing mixture in the determination of β–carotene, each at a flow rate of 1 mL/min. The plasma concentration of α–tocopherol was measured after deproteinising the sample with ethanol and after a two–step extraction of α–tocopherol with hexane following a method modified by Schneider *et al.* [2003], Gronowska–Senger *et al.* [2009]. LiChroCART®250–4 RP–18 (4 x 250 mm; 5mm) with a precolumn (Merc, col. no. 841071, Darmstadt, Germany) was used. Acetonitrile/hexane/isopropanol mixture (65:14:21; v/v/v) was applied as an eluent. The flow rate was 0.8 mL/min. The amount of vitamin C (L-ascorbic acid and L–dehydroascorbic acid) in rat plasma was measured using method modified by Karlsen *et al.* [2005]. Samples were deproteinised with 10% metaphosphoric acid and centrifuged. The overall amount of ascorbic acid in the supernatant was measured after previously reducing the dehydroascorbic acid with hydrochloride tri–carboxyethyl phosphine. The test samples were separated by a Discovery C18 RP (4.6 x 150 mm; 5 μm) column from the Supelco Company (Supelco Analytical cat. no. 504955, Bellafonte, PA, USA) with a precolumn from the same company. A 2% solution of water–phase acetonitrile was applied as eluent, which was formed from a solution of NaH_2PO_4, Na_2EDTA and lautrimonium chloride in the following concentrations: 2.5 mmol/L, 1.25 mmol/L, 2.5 mmol/L. The flow rate was 0.75 mL/min. All of the results were applied to standard curves plotted in accordance with Sigma Company (St. Louis, MO, USA) standards.

Statistical analysis

Analysis of the variance (ANOVA) was carried out with the SPSS program (version 17; SPSS Inc, Chicago IL, USA). Normality and homogeneity of the data distribution were checked by Shapiro–Wilk test. Data (Table 2) consistent with a normal distribution were subjected to one–way analysis of variance (ANOVA) to assess potential statistical significance for antioxidant vitamins. When significant differences were observed in ANOVA tests, Tukey's post hoc test was applied to locate the source of the significant difference. The interaction between physical exercise and vitamins (T × V) was assessed by 2–way analysis of variance. Pearson's linear correlation coefficient (*r*) was calculated to analyse the strength of the relationship between individual variables. For all analyses, $\alpha=0.05$ was the level of statistical significance. The results are expressed as means and SDs.

RESULTS

No statistically significant differences among all experimental groups were observed in initial (154±14 g) and final body mass (248±22 g) or dietary intake (307±35g) during the study.

After β–carotene was supplemented, this molecule was not detected in rat plasma; therefore, further analysis tested for retinol. Retinol level in nonexercised rat plasma was significantly increased after β–carotene supplementation, while the levels remained unchanged in the exercised groups, compared to the control groups that did not receive this compound (Table 2). Concentration of plasma retinol was significantly decreased in exercised animals supplemented β–carotene (20%), as well as β–carotene and ascorbic acid (15.2%) or all three of the compounds (14.3%). In the experiment, no statistically significant interaction was noted between supplementation of the tested compounds, physical exercise on the concentration of plasma retinol. The results showed a significant influence both due to administered α–tocopherol acetate and physical exercise on the level of α–tocopherol in the plasma. Supplementation of α–tocopherol acetate significantly increased the level of plasma α–tocopherol in exercised and nonexercised animals compared to the corresponding control group. Administering vitamin E together with β–carotene or vitamin C or both significantly increased the level of α–tocopherol only in the exercised groups. Physical exercise, in turn, caused a significant increase in the concentration of α–tocopherol in plasma regardless of whether

TABLE 2. The concentration of vitamins (retinol, vitamin E, vitamin C) in rat plasma in dependence on physical exercise and/or administered vitamins.

Exercise	Groups							P–value
	Retinol (μmol/L)							
	control I	BC	control II	BC + E	BC + C	E + C	BC + E + C	
NT	1.05 ± 0.11^{a}	1.25 ± 0.16^{Ab}	1.02 ± 0.09^{a}	1.05 ± 0.14^{a}	1.12 ± 0.15^{Aab}	–	1.12 ± 0.10^{Aa}	0.01
T	1.01 ± 0.13	1.00 ± 0.12^{B}	0.96 0.11	0.94 ± 0.10	0.95 ± 0.10^{B}	–	0.96 ± 0.07^{B}	0.73
P–value	0.53	0.04	0.30	0.07	0.02	–	0.001	
	Vitamin E (μmol/L)							
	control I	E	control II	BC + E	BC + C	E + C	BC + E + C	
NT	10.2 ± 0.8^{a}	12.7 ± 3.0^{Ac}	10.4 ± 1.0^{ab}	12.1 ± 1.3^{Abc}	–	11.3 ± 1.5^{Aabc}	11.6 ± 1.1^{Aabc}	0.04
T	10.1 ± 1.6^{a}	17.2 ± 0.7^{Bc}	9.6 ± 1.5^{a}	15.2 ± 0.9^{Bb}	–	20.9 ± 1.7^{Bd}	21.1 ± 1.6^{Bd}	< 0.001
P–value	0.92	0.004	0.28	< 0.001	–	< 0.001	< 0.001	
	Vitamin C (μmol/L)							
	control I	C	control II	BC + E	BC + C	E + C	BC + E + C	
NT	23.2 ± 3.8^{a}	24.2 ± 7.8^{a}	21.6 ± 6.6^{a}	–	24.5 ± 4.2^{a}	30.9 ± 4.6^{b}	22.3 ± 3.1^{a}	0.04
T	21.8 ± 2.4^{a}	24.8 ± 3.4^{ab}	21.2 ± 3.7^{a}	–	23.8 ± 4.2^{a}	28.7 ± 4.3^{b}	24.2 ± 5.7^{a}	0.02
P–value	0.40	0.84	0.91	–	0.76	0.39	0.43	

2–Way ANOVA, P–value	Retinol	Vitamin E	Vitamin C
Exercise (T)	< 0.001	< 0.001	0.73
Vitamins (V)	0.02	< 0.001	< 0.001
T x V	0.12	< 0.001	0.86

Administered vitamins: BC, β–carotene; C, ascorbic acid; E, α–tocopherol; exercise: NT, nonexercised; T, exercised; significantly different means bearing different letters: small letters (a–d) in row, capital letters (A–B) in column ($p<0.05$).

TABLE 3. Correlation (r) between vitamins concentration in plasma.

Pearson correlation	Vitamin E		Vitamin C	
	nonexercised	exercised	nonexercised	exercised
Retinol	0.07	0.02	0.05	0.30
Vitamin E	–	–	0.14	0.64*

*statistically significant correlation ($p<0.001$).

it was supplemented individually or in combination. Plasma α–tocopherol of animals in exercised groups supplemented with vitamins E and C, or with β–carotene and vitamins E and C together, was significantly higher, compared to non––exercised groups, their level of α–tocopherol was 81.9% to 85.0%. We observed a strong interaction between physical exercise and vitamins administration on vitamin E concentration in plasma. The concentration of vitamin C in rat plasma, in turn, remained at similar levels regardless of whether it was administered *per os* or not, and whether the animals were exercised or not. On the other hand, a significantly higher plasma concentration of vitamin C was noted in the animals which were given these vitamins together with vitamin E, regardless of physical exercise.

The tests conducted confirmed a statistically significant correlation between the amounts of vitamins E and C in the plasma ($r=0.64$; $p<0.001$) but only for exercised rats (Table 3). No correlation was found, in contrast, between the concentration of tested vitamins in blood of nonexercised rats.

DISCUSSION

Among the nonexercised rats, a (statistically significant) lower concentration of retinol was observed in plasma of the rats given β–carotene together with α–tocopherol acetate compared to the groups supplemented only with β–carotene. The concentration was comparable to plasma concentration of retinol in the control group when β–carotene was not given. This result is probably due to the fact that carotenoids and vitamin E are transported in the blood in combination with lipoproteins and may compete with each other for binding sides on the lipoproteins [Jenkins *et al.*, 2000]. Thus, vitamin E may have an inhibitory effect on β–carotene metabolism. Meanwhile, no change in plasma retinol concentrations was observed in exercised animals regardless of whether they received vitamins. This may attest to the controlled release of vitamin A from the liver, where it is accumulated [Weber & Grune, 2012]. In fact, vitamin A concentration in plasma of the exercised rats was always lower than that of nonexercised rats (from 3.8% to 20.0%).

Biosynthesis of vitamin C in rat liver meets its daily needs, yet supplemental amounts given orally can improve antioxidant defences in this animal [Djurašević *et al.*, 2008], and influence blood plasma concentrations of the vitamin [Tsao & Young, 1989]. A study by Banhegyi *et al.* [1997] proved that long–term supplementing mice with vitamin C inhibited its biosynthesis in the liver. The results of the Djurašević *et al.* [2008] study showed that supplementing rats with two doses of vitamin C, which differ from each other more than 33–fold caused only a 20% difference in ascorbate plasma concentrations. Analogous dependent relations appeared in human study, with application of 200 mg of vitamin C daily [Levine *et al.*, 1996]. Moreover Schröder *et al.* [2001] did not observe a significant increase of plasma ascorbate concentration after supplementation in basketball players, and explained that by the consumption of ascorbic acid to regeneration of vitamin E and reacting with aqueous peroxyl radical.

Among the tested vitamins only the plasma α–tocopherol significantly increased after exercise and supplementation. Exercise is considered as a cause of enhanced production of free radicals and intensified oxidative stress. α–Tocopherol can react with free radicals and protect cells and their structure from oxidation [Traber & Atkinson, 2007]. It was observed that the concentration of plasma α–tocopherol increased after exercise in supplemented groups, that may be due to rapid redistribution from tissues as a result of the enhanced production of free radical [Aguilo *et al*., 2005; Gronowska–Senger *et al*., 2009]. As mentioned above, vitamin C acts as vitamin E regenerator and vitamin E may inhibit β–carotene metabolism, that might explain the more pronounced decrease of their plasma concentration compared to α–tocopherol. It indicates that α–tocopherol, acting as an antioxidant, may be used as a marker of oxidative stress.

In the present study, no statistically significant correlation was found between vitamin C dose and its plasma concentration. Nonetheless, a statistically significant increase in concentration of vitamins C and E was observed in exercised and nonexercised groups supplemented these vitamins together, which seems to confirm the beneficial effect connected with their supplementation. Taking these results into consideration, it might be assumed that the increased utilization of vitamin E as an antioxidant factor in condition of physical exercise–induced stress initiates vitamin C synthesis in the liver as a tocopheryl radical regenerating compound, which, together with vitamin C supplementation, increases its concentration in blood plasma.

CONCLUSIONS

Our study suggests that only the concentration of α–tocopherol in blood may be treated as a marker of oxidative stress induced by physical exercises. On the basis of the current study we suggest the need of simultaneous vitamin E and C supplementation during physical exercise in humans. Future study should consider supplementation of different doses of these vitamins for different types of physical exercises and their duration.

ACKNOWLEDGEMENTS

The authors acknowledge financial support from the Ministry of Science and Higher Education (grant N N312 410037).

REFERENCES

1. Aguilo A., Tauler P., Funtespina E., Tur J.A., Córdova A., Pons A., Antioxidant response to oxidative stress induced by exhaustive exercise. Physiology & Behavior, 2005, 84, 1–7.
2. Banhegyi D., Braun L., Csala M., Puskas F., Mandl J., Ascorbate metabolism and its regulation in animals. Free Radic. Biol. Med., 1997, 23, 793–803.
3. Böhm F., Edge R., Truscott G., Interactions of dietary carotenoids with activated singlet oxygen and free radicals: Potential effects for human health. Mol. Nutr. Food Res., 2012, 56, SI, 205–216.
4. Djurašević S.F., Djordjević J., Drenca T., Jasnić N., Cvijić G., Influence of vitamin C supplementation on the oxidative status of rat liver. Arch. Biol. Sci., 2008, 60, 169–173.
5. Gackowski D., Kruszewski M., Jawien A., Ciecierski M., Olinski R., Further evidence that oxidative stress may be a risk factor responsible for the development of atherosclerosis. Free Radic. Biol. Med., 2001, 31, 542–547.
6. Gomez–Cabrera M.C., Ristow M., Viña J., Antioxidant supplements in exercise: worse than useless? Am. J. Physiol. Endocrinol. Metab., 2012, 302, E476–E477.
7. Gronowska–Senger A., Górnicka M., Kołodziejska K., Tocopherol acetate vs oxidative stress induced by physical exercise in rats. Pol. J. Food Nutr. Sci., 2009, 59, 263–269.
8. Jenkins M.Y., Mitchell G.V., Grundel E., Natural tocopherols in a dietary supplement of lutein affect tissue distribution of tocopherols in young rats. Nutr. Cancer, 2000, 37, 207–214.
9. Karlsen A., Blomhoff R., Gundersen T.E., High–throughput analysis of vitamin C in human plasma with the use of HPLC with monolithic column and UV–detection. J. Chromatogr. B, 2005, 824, 132–138.
10. Krinsky N.I., Johnson E.J., Carotenoid actions and their relation to health and disease. Mol. Asp. Med., 2005, 26, 459–516.
11. Lamprecht M., Hofmann P., Greilberger J.F., Schwaberger G., Increased lipid peroxidation in trained men after 2 weeks of antioxidant supplementation. Int. J. Sport Nutr. Exerc. Metab., 2009, 19, 385–399.
12. Levine M., Conry–Cantilena C., Wang Y., Welch R.W., Washko P.W., Dhariwal K.R., Park J.B., Lazarev A., Graumlich J.F., King J., Cantilena L.R., Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. Proc. Natl. Acad. Sci. USA, 1996, 93, 3704–3709.
13. Martins R.R., de Oliveira Macedo U.B., Leite L.D., Rezende A.A., Brandão–Neto J., Almeida M.G., Lipoic acid and moderate swimming improves the estrous cycle and oxidative stress in Wistar rats. Appl. Physiol. Nutr. Metab., 2011, 36, 693–697.
14. Powers S.K., Nelson W.B., Hudson M.B., Exercise–induced oxidative stress in humans: cause and consequences. Free Radic. Biol. Med., 2011, 51, SI, 942–950.
15. Reeves P.G., Components of the AIN–93 diets as improvements in the AIN–76A diet. J. Nutr., 1997, 127(5 suppl), 838S–841S.

16. Schneider C., Chemistry and biology of vitamin E. Mol. Nutr. Food Res., 2005, 49, 7–30.
17. Schneider M., Niess A.M., Rozario F., Angres C., Tschositsch K., Golly I., Battenfeld N., Schäffer M., Northoff H., Dickhuth H.H., Fehrenbach E., Trommer W.E., Biesalski H.K., Vitamin E supplementation does not increase the vitamin C radical concentration at rest and after exhaustive exercise in healthy male subject. Eur. J. Nutr., 2003, 42, 195–200.
18. Schröder H., Navarro E., Mora J., Galiano D., Tramullas A., Effects of α–tocopherol, β–carotene and ascorbic acid on oxidative, hormonal and enzymatic exercise stress markers in habitual training activity of professional basketball players. Eur. J. Nutr., 2001, 40, 178–184.
19. Traber M.G., Atkinson J., Vitamin E, antioxidant and nothing more. Free Rad. Biol. Med., 2007, 43, 4–15.
20. Traber M.G., Stevens J.F., Vitamins C and E: beneficial effects from a mechanistic perspective. Free Rad. Biol. Med., 2011, 51, SI, 1000–1013.
21. Tsao C.S., Young M., Effect of exogenous ascorbic acid intake on biosynthesis of ascorbic acid in mice. Life Sci., 1989, 45, 1553–1557.
22. Wawrzyniak A., Górnicka M., Hamułka J., Gajewska M., Drywień M., Pierzynowska J., Gronowska Senger A., α–Tocopherol, ascorbic acid and β–carotene protect against oxidative stress but reveal no direct influence on p53 expression in rats subjected to stress. Nutr. Res., 2013, 33, 868–875.
23. Weber D., Grune T., The contribution of β–carotene to vitamin A supply of human. Mol. Nutr. Food Res., 2012, 56, SI, 251–258.
24. Yeum K.J., Russell R.M., Krinsky N.I., Aldini G., Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma. Arch. Biochem. Biophys., 2004, 430, 97–103.
25. Zeinab S.A., Khadiga S.I., Abdel–Razik H.F., Eman E.S., Effect of carrot and wheat germ oil supplementation on antioxidant status of rats exposed to benzene. Pol. J. Food Nutr. Sci., 2010, 60, 175–181.

Submitted: 14 April 2014. Revised: 14 July 2014, 17 September 2014, 29 September 2014. Accepted: 3 October 2014. Published on–line: 4 November 2014.