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ORIGINAL CONTRIBUTION

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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

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Summary *Background* Intense physical exercise has been associated with an increase of free radical production. When the body's natural defense systems against free radicals are overwhelmed, oxidative stress increases. *Aim of the study* This study examined the effects of a vitamin antioxidant supplement, (composed of 600 mg α-tocopherol, 1000 mg ascorbic acid and 32 mg β-carotene) on oxidative, hormonal, and enzymatic exercise stress markers during habitual training activity over 35 days. *Methods* The plasma concentrations of ascorbic acid, α-tocopherol, β-carotene, testosterone, cortisol and lipid peroxides and the serum activities of lactate dehydrogenase and creatine kinase were measured at four time points: pre-supplementation (PS), pre-training (PT), after training (AT) and 24 h after training (24h-AT) in 13 professional basketball players of the first Spanish Basketball League (ACB). *Results* Antioxidant supplementation led to a significant increase of α-tocopherol and β-carotene from PS to PT. Plasma lipid peroxides decreased about 27.7 % after 35 days of antioxidant treatment. A significant decrease of lactate dehydrogenase serum activity was observed during the 24h recuperation time. During this time the anabolic/catabolic balance increased about 29.8 % in the antioxidant supplemented group, although this increase did not reach statistical significance. *Conclusions* The results of the present study suggest that supplementation with α-tocopherol, β-carotene and ascorbic acid might partially account for the hormonal and enzymatic stress marker profile observed during habitual training activity of professional basketball players.

Key words Antioxidants – Exercise – Peroxidation – Vitamins – Basketball

Introduction

The rise in oxygen consumption concomitant with physical exercise is associated with the production of oxygen free radicals and with the subsequent increase in lipid peroxidation (1–4). A direct relationship between exercise intensity and lipid peroxidation has been reported (2), also associated with skeletal muscle damage (3).

Physical activity also influences changes in serum levels of cortisol and testosterone, depending on the duration and the intensity of the exercise. A decrease in the testosterone/cortisol ratio has been found to be associated with the overtraining syndrome characterised by a decreased sport-specific physical performance (5). This ratio has also now been reported to be an indicator of the actual physiological strain in training (6).

The potential of dietary antioxidants as an endogenous defence to detoxify lipid peroxides produced during exercise has received increasing attention in recent

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years. Results of human studies on the effect of supplementation with antioxidant vitamins on lipid peroxidation or enzyme muscle damage are controversial. Some studies suggested favourable effects of antioxidant vitamin supplementation on these parameters after exercise (7, 8) whereas others failed to demonstrate these effects (9, 10). Vitamin deficiency has shown to limit physical performance (11–14), but vitamin supplementation studies in humans have shown little, if any, effect on athletic performance (15, 16). A couple of studies in humans observed that the increase in the production of free radicals is smaller after the ingestion of antioxidants, regarding the levels achieved in the placebo-control group. Nevertheless, these studies have been performed only with α-tocopherol or ascorbic acid, given together or alone, and few have been performed in elite professional athletes. Most of these studies were carried out under laboratory conditions, which seldom mimic the conditions encountered by athletes during actual competition or training.

The purpose of this study was to examine the effect of a three-compound antioxidant supplement: α-tocopherol, ascorbic acid and β-carotene on oxidative, hormonal and enzymatic exercise stress markers in the routine training activity of professional basketball players.

Subjects and methods

Subjects

Thirteen male professional basketball players participated during the entire study. The participants were informed about procedures and possible risks involved before giving their voluntary consent to participate. The protocol was approved by the institutional review board. Age, weight and height body mass index (BMI) and duration of performed training time were recorded (Table 1). They completed eight to ten training sessions per week and competed once or twice a week in the First Spanish Basketball League (ACB). They were also involved in international competitions. A normal training unit of 90 min consists in a general warm up and stretching (about 10 min), a technical-tactical part (about 30 min), a heavy training load part including training of counterattacks and simulated full or half-court basketball games (about 40 min), and finally a cool down phase (about 10 min).

Study design

The study consists of a single-blind, random, clinical trial. The athletes were randomly divided into two groups, supplement and placebo. Participants in the supplement group received 150 mg of α-tocopherol acetate, 250 mg of ascorbic acid and 8 mg of β-carotene four times a day over 35 days, during the competition season 1996/97 of the First Spanish Basketball league (ACB). Evidence indicates that the administration of these (pro) vitamins at the mentioned concentrations, and during a study period of 35 days, seems to be safe and hence, harmful side effects are not expected (17) However, amount and composition of the antioxidants mixture administrated over a long-term period might not be without any harm. Placebo capsules contained hydroxy-methyl-cellulose, glycerol, and lecithin. Artificial food colours were used to obtain the coloured capsules. Capsules, being free of taste, were swallowed by the participants. Antioxidant containing capsules were identical in appearance and matrix with that of placebo. The participants in the placebo group received the same amount of capsules. On days of blood sampling participants continued to take antioxidant supplement or placebo after blood collection. Last blood collection was done after the 24 h recuperation time (24-AT) on day 35. Blood samples (20 ml) were collected in the afternoon, between 17:00 and 18:00, in dark test tubes containing EDTA and refrigerated immediately. Blood was stored overnight in dark sealed boxes in a refrigerator at 4 °C, then centrifuged for 15 min at 2100 x g at 4 °C. Plasma and serum were kept frozen at –40 °C until analysis (after approx. 2 weeks). Butylated-hydroxytoluene (BHT) was added to the samples to prevent further oxidation. For ascorbic acid determination, plasma was stabilised by mixing 1 part of plasma with 2 parts of 10 % solution of metaphosphoric acid. Stability studies have indicated that though vitamin C levels fall after overnight storage they are closely associated with initial values (18). The plasma concentrations of ascorbic acid, α-tocopherol, β-carotene, testosterone, cortisol and lipid peroxides and the serum activities of lactate dehydrogenase and creatine kinase were measured at four time points: pre-supplementation (PS), pre-training (PT), after training (AT) and 24h after training (24h-AT).

Plasma volume reductions have been reported after various types of high-intensity, short-term, moderate and prolonged exercise (19). Because no data on fluid shifts following basketball training or competition are available in the literature, we cannot exclude that the observed differences in biochemical and hormonal parameters (with excepion of the testosterone/cortisol ratio) between the time-points "after training (AT)" and "24 h after training (24-AT) " are caused by plasma volume changes, and not by the effects of the antioxidant supplement. We will specifically address this point in a future work.

Calculations of daily average energy, carbohydrate, protein and fat intake were done by an administrated 24 h recall questionnaire at baseline (pre-supplementation, PS). Subjects recorded food intake of the previous day on a structured questionnaire (work day). This

questionnaire included a food list of 154 items, open items (type of salads consumed etc) and recommendations on how to describe the type, dressing, proportion and quantity of each dish item. Reported information on food intake was converted into nutritional data using the Diet Analysis Nutritionist IV software (N Squared Computing, San Bruno, SA).

Analytical methods

Lipid peroxides were analysed by using assay kits for quantitative determination of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in plasma (LPO–586, OXIS International Inc. Portland, USA). In this assay MDA and 4-HNE were measured by mixing 300 µL of plasma with 225 µL of methanesulfonic acid and 975 µL of 1-methyl–2-phenylindole, incubated for 40 min at 45 °C and absorbance measured at 586 nm (20). The sensitivity of this method is 0.1 γM MDA, and the standard error (SEM) of the measurement is less than 5 % (OXIS International Inc. Portland, USA). α-Tocopherol, and β-carotene were measured by a reversed-phase, high-pressure liquid chromatographic method as described by Arnaud et al. (21). A C18 Nova-Pack column (Millipore, Frankfurt, Germany) and a multi-wavelength UV detector were used to determine α-tocopherol and β-carotene at 295 and 450 nm, respectively. The linear concentration ranged (α-tocopherol 0.18–91.8 µM and β-carotene 0.05–5.75 γM) between run coefficients of variation. Vitamin C was measured by high-pressure liquid chromatography with colourimetric electrochemical detection as described (22). Serum activities of lactate dehydrogenase and creatine kinase were determined by an enzymatic method at 405 nm using commercial kits (Human, Gesellschaft für Biochemie und Diagnostika Taunustein, Germany). Determination of lactatedehydrogenase is linear until δ abs/min of 0.150 (Human, Gesellschaft für Biochemie und Diagnostika Taunustein, Germany) and the linear concentrations of creatine kinase ranges from 0.25–340 nmol (Human, Gesellschaft für Biochemie und Diagnostika Taunustein, Germany). Free testosterone and cortisol were analysed by Radioimmunoassay (Incstar Corporation, Stillwater, Minnesota, USA). Samples were counted in a gamma counter (LKB-Pharmacia 1277, Freiburg, Germany).

Statistical analysis

Statistical analyses were performed with the SPSS/PC+ software package (SPSS Inc. Chicago). Means and standard deviations of the analytical data were determined for each observation period and for both groups of athletes. An unpaired Student's t test was used to compare mean values of continuous variables between groups. The data within each group were analysed using repeated measures analysis of variance procedure. A paired Student's t test determined if differences at each time point were significant. The normality of each distribution was approved by the test of Kolmogorov and Smirnov in the case of the continuous variables. Statistical confidence interval of 95 % was applicated for analysis.

Results

Tables 1 and 2 show the physical characteristics and intakes of daily energy, macronutrient, vitamin C, α-tocopherol and β-carotene by the participants in the supplement and placebo groups, measured at pre-supplementation time (PS). Age, weight, height, body mass index (BMI) and training time were very similar in both groups. No significant differences in energy, macronutrient and vitamin C, α-tocopherol and β-carotene intakes were observed at pre-supplementation between both groups.

No differences were observed in lipoperoxide (LPO) plasma concentration between groups despite those val-

Table 1 Physical characteristics of the subjects

	Supplement group n=7		Placebo group n=6		Total n=13	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	23.0	3.4	24.7	4.2	23.8	3.8
Height (cm)	199.1	3.9	196.7	3.1	198.0	3.5
Body mass (kg)	96.0	10.1	94.0	9.5	95.1	9.8
Body mass index (BMI)	24.2	0.95	23.9	0.62	24.1	0.80
Training (min/d)	162.1	24.5	157.9	27.5	160.2	25.9

SD Standard deviation; *n* no. of subjects

Table 2 Daily energy, macronutrient, α-tocopherol, β-carotene, vitamin C intake and contribution (%) of macronutrients to energy intake of the subjects

	Supplement group n=7		Placebo group n=6	
	Mean	SD	Mean	SD
Energy (MJ)	16.27	6.76	17.63	10.50
Protein (g)	338	184	206	107
Fat (g)	331	191	208	146
Carbohydrate (g)	504	340	392	248
α-Tocopherol (mg)	11.6	9.4	13,8	12.0
β-Carotene (mg)	1820	850	2590	2240
Vitamin C (mg)	207	213	244	162
Protein (%)	18.7	1.6	20.0	4.3
Fat (%)	37.4	7.0	34.6	10.3
Carbohydrate (%)	43.1	5.0	43.8	10.4

SD Standard deviation; *n* no. of subjects

ues at PT, AT and 24-AT time points were lower in supplement regarding those of the placebo group. A similar pattern of LPO plasma concentrations was observed in both groups, with a trend to increase from PT to AT, and a decrease at 24-AT measured time points. The increase from PT to AT was greater in the supplement (44 %) than in the placebo group (23 %) but without reaching statistical significance (Table 3). The decrease from AT to 24-AT was greater in the placebo group ($p < 0.002$) than in the supplement group. LPO plasma concentrations decreased during the entire treatment (from PS to 24-AT), by about 27.1 % and 10.4 % in the supplement and placebo groups, respectively.

α-Tocopherol, ascorbic acid, and β-carotene plasma concentrations at the pre-supplementation (PS) time point showed no statistically significant differences between the supplement and placebo groups (Table 3). All plasma antioxidant levels were within reference range values (vitamin C: 22–113 μmol/L; α-tocopherol: 11.6–46.4 μmol/L; β-carotene: 150–740 μg/L; reference values from General Lab, Barcelona, Spain). Plasma levels of α-tocopherol and β-carotene increased significantly in the supplement group from PS at all measured points ($p < 0.02$ and $p < 0.04$ respectively). In this group a significant decrease ($p < 0.04$) in α-tocopherol serum concentration from AT to AT–24 was observed. Plasma levels of ascorbic acid in the supplement group remained unchanged from PS to PT and AT, and decreased at 24-AT ($p < 0.02$).

In the placebo group, no changes were observed in α-tocopherol levels between the measured time points. Furthermore, β-carotene levels remained unchanged from PS to PT and AT times, decreasing from AT to 24-AT ($p < 0.03$), while plasma levels of ascorbic acid decreased from PS to the all other time points, reaching statistical significance from PS to PT ($p < 0.01$), and 24-AT ($p < 0.01$). A decrease was also observed from AT to 24AT ($p < 0.02$). β-Carotene plasma concentration remained nearly unchanged during the entire study in this group, with the exception of the significant decrease ($p < 0.01$) from AT to 24-AT. Significant differences in α-tocopherol plasma concentrations at PT ($p < 0.02$), AT and 24-AT ($p < 0.05$), and for β-carotene at PT, AT and 24-AT ($p < 0.001$) were observed between supplement and placebo groups.

The highest serum LDH levels were observed at AT in both groups, being statistically significant in the placebo group ($p < 0.001$). In the supplement group, LDH serum activity increased from PS to AT ($p < 0.01$) and decreased from AT to 24-AT ($p < 0.01$) (Table 4). LDH serum levels increased significantly in the placebo group from PS and PT to AT ($p < 0.02$). Average CK serum activity of the supplement and placebo groups, measured at all time points, were above the normal range (0–190 $U \cdot l^{-1}$) with the only exception being the CK baseline concentration of the placebo group (Table 4). Although there were no significant changes in plasma CK values at the four evaluated time points in either group, we observed highest serum activity of CK at 24-AT. Cortisol plasma concentration reached the highest level at AT in the supplement ($p < 0.02$) and placebo ($p < 0.05$) groups (Table 4). The highest testosterone values were observed at 24-AT in the supplement group ($p < 0.01$ versus PT), and at AT in the placebo group ($p < 0.01$ versus PS). An increase of the free-testosterone/cortisol ratio was observed in the supplement group from AT to 24-AT, just reaching statistical significance ($p < 0.056$).

Table 3 Levels of antioxidants vitamins and lipoperoxides (LPO) in supplement and placebo groups at various measuring times, pre-supplemetation (PS), pre-training (PT), after training (AT) and 24h rest period after training (24h-AT)

Parameter	PS n=13	P <	PT n=13	P <	AT n=13	P <	24-AT n=13
LPO ($\mu mol\ l^{-1}$)							
Supplement (n=7)	2.21±0.68	NS	1.93±0.73	0.052	2.38±0.99	0.059	1.61±0.29
Placebo (n=6)	2.39±0.39	NS	2.16±0.74	NS	3.12±1.08	0.002	2.15±0.84
α-Tocopherol ($\mu mol\ l^{-1}$)							
Supplement (n=7)	23.32±5.56	0.02	30.96±7.75[1]	NS	32.11±6.88*[2]	0.04	28.24±7.40[2]≈
Placebo (n=6)	23.04±3.08	NS	20.51±5.14	NS	23.80±3.36	NS	21.56±1.51
Ascorbic acid ($\mu mol\ l^{-1}$)							
Supplement (n=7)	50.96±22.58	NS	53.31±53.71	NS	54.38±47.00	0.05	16.23±1.70*≅
Placebo (n=6)	48.10±21.09	0.01	12.30±7.85	NS	21.30±18.70	0.02	13.82±10.0#
β-Carotene($\mu g\ l^{-1}$)							
Supplement (n=7)	390±128	0.002	1597±679[3]	NS	1731±859[4]♦	NS	1719.42±859[4]♦
Placebo (n=6)	429±259	NS	459±382	NS	486±310	0.025	398±332

All values are means ± Standard deviation (SD); P means significance of correlative data; *n* no. of subjects.
* $p < 0.02$, ♦ $p < 0.004$, versus PS supplement group, paired t test.
≅ $p < 0.03$, ≈ $p < 0.03$ versus PT supplement group, paired t test.
# $p < 0.01$, versus PS placebo, paired t test.
[1] $p < 0.02$; [2] $p < 0.05$; [3] $p < 0.004$; [4] $p < 0.001$ versus placebo group corresponding, Student t test

Table 4 Levels of hormonal and muscle enzymes in supplement and placebo groups at various measuring times, pre-supplemetation (PS), pre-training (PT), after training (AT) and 24h rest period after training (24h-AT)

Parameter mean ± SD	PS n=13	P <	PT n=13	P <	AT n=13	P <	24-AT n=13
LDH (U l^{-1})							
Supplement (n=7)	387±73	NS	393±69	NS	448±106[1]	0.02	392±71
Placebo (n=6)	332±67	NS	326±47	0.001	415±56[2]	NS	369±50
CK (U l^{-1}) (median range)							
Supplement (n=7)	216 (134–917)	NS	276 (96–1259)	NS	231 (153–724)	NS	410 (174–641)
Placebo (n=6)	146 (65–1440)	NS	230 (96–369)	NS	283 (162–582)	NS	348 (166–859)
Cortisol (µg dl^{-1})							
Supplement (n=7)	9.59±2.99	NS	9.81±2.98	0.02	14.23±4.07[3]	NS	11.74±5.22
Placebo (n=6)	11.72±4.26	NS	8.28±1.57	0.05	14.83±6.32	NS	10.8±2.63[4]
Free testosterone (pg ml–1)							
Supplement (n=7)	19.73±6.32	NS	16.97±4.55	NS	22.72±8.28	NS	25.41±5.11[5]
Placebo (n=6)	20.23±7.32	NS	23.23±5.15	NS	30.86±11.01[6]	NS	24.05±4.09
Free testosterone/cortisol ratio (median range)							
Supplement (n=7)	0.064 (0.041–0.14)	NS	0.06 (0.051–0.098)	NS	0.057 (0.027–0.1)	0.056	0.074 (0.046–0.127)
Placebo (n=6)	0.08 (0.028–0.088)	NS	0.1 (0.049–0.131)	NS	0.073 (0.035–0.234)	NS	0.073 (0.049–0.144)

All values are means ± Standard deviation (SD) or median range; P means significance of correlative data; *n* no. of subjects.
[1] $p < 0.01$ versus PS supplement, paired t test.
[2] $p < 0.02$ versus PS placebo, paired t test.
[3] $p < 0.02$ versus PS supplement, paired t test.
[4] $p < 0.05$ versus PT supplement, paired t test
[5] $p < 0.01$ versus PT supplement, paired t test.
[6] $p < 0.01$ versus PS placebo, paired t test.

Discussion

No significant differences in the plasma concentration of antioxidant vitamins, hormones and muscle enzymes at pre-supplementation were observed in the professional basketball player groups studied.

The high serum activities of creatine kinase and lactate dehydrogenase (Table 4) observed at baseline levels in this study were as high as those found directly after extreme endurance stress (21), or after resistance exercise (22). This could be explained by the high daily degree of physical performance of the professional basketball players during training, the lack of sufficient recovery time, and the specific exercise activities (anaerobic-aerobic) of this sport. The efflux of these muscle enzymes is considered to reflect a change in the normal membrane structure, induced by muscle damage, making it permeable to these molecules. In this sense, increased serum activities of creatine kinase and lactate dehydrogenase are generally accepted as good indicators of muscle damage. The statistically significant increase of LDH at AT in the placebo group, and the contrarily observed significant decrease of this muscle damage marker at 24-AT in the supplement group (see Table 4) might be partially explained by a protective effect of the vitamin antioxidant supplement on exercise-induced damage of muscle cell membranes. Rokitzki and colleagues have shown that supplementation with vitamin E during 151 days of habitual training led to a decrease of the muscle damage marker creatine kinase in professional cyclists (16). We did not observed statistically significant changes of creatine kinase serum activities during the study period in either group (Table 4). High statistical deviation of mean creatinekinase serum activity might explain this fact. However, administration of antioxidants during the study period, might partially account for the creatine kinase pattern observed in the supplemented group.

The plasma concentrations of ascorbic acid and α-tocopherol at baseline (Table 3) were comparable with those found in marathon runners (16) but lower than in German second league basketball players (25) or Argentine soccer players (26). As expected, there was a significant elevation of α-tocopherol and β-carotene plasma concentration levels in the supplement group after the 32 days of supplementation. In contrast, we did not observed a significant increase of plasma ascorbate concentration after supplementation. This fact, besides the observed decrease of ascorbic acid levels in the placebo group from pre-supplemented values at all evaluated times, might be explained by the consumption of ascorbic acid by scavenging aqueous peroxyl radicals and regenerating vitamin E (27, 28). There is strong evidence of such an interaction of these two vitamins in vitro studies (27, 28). In contrast, only results of a few in vivo studies were supportive of an in vivo interaction between Vitamin C and E (29, 30), However, as in the present study neither urine concentration of vitamin C nor dehydroascorbic acid and 2,3 diketo-L-gulonic acid

were measured, this assumption must be take with caution.

α-Tocopherol is recognised as the most important liposoluble antioxidant while, as commented above, ascorbic acid acts as a vitamin E regenerator. This regenerative effect might explain the more pronounced decrease in the plasma concentration of ascorbic acid concentration in both groups at 24-AT compared to that of α-tocopherol at the same time. However, its role in supplementation in "in vivo" studies remains controversial (31).

Lipid peroxide plasma concentration decreased (Table 3) although not statistically significant ($p < 0.09$), after 35 days (from PS to 24-AT) in the supplement group (27.7 %). One might speculate that this would lead to a reduction of muscle damage during habitual training activity. The measurement of lipid peroxide plasma concentration was performed by the analysis of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) plasma concentrations, molecules formed after lipid peroxidation (32). Nevertheless, since only certain lipid peroxidation products generate MDH (33), the assay used in this study only measured free MDH and 4-HNE in the samples and do not provide for liberation of MDA bound to proteins via Schiff base (OXIS, unpublished data). Thus, results of changes in LPO plasma concentration must be carefully interpreted.

Plasma cortisol and testosterone responses to exercise are related to intensity and duration of exercise (34–36), and to the training status of the subjects (35). In our study, cortisol plasma levels showed a similar pattern of change in both groups, with an increase after a period of training, and a decrease at 24 h post-training. This pattern has been previously described (36, 37). In our study, variation patterns of testosterone were different in both groups. In the placebo group, the highest testosterone plasma concentration was reached immediately after exercise, as previously described by other authors (36, 37). However, in the supplement group this concentration was reached at the end of the 24 h rest period after training. As a consequence, we observed a borderline, statistically significant increase ($p < 0.056$) in the testosterone/cortisol ratio in the supplement group during the 24 h recuperation period after training. The testosterone pattern observed in the supplement group has also been described in runners supplemented with branched chain amino acids after 2 h of running exercise (38), and linked to a reduction of the exercise-induced endogenous amino acid oxidation, subsequent skeletal muscle protein degradation, and prevention of testosterone muscle clearance (38–40). In fact, exercise-induced free radical generation appears to lead to an increase in protein oxidation in skeletal muscle, and antioxidant supplementation would reduce this process (41). The protection of protein structures by antioxidants might lead to the observed effect on the anabolic/catabolic ratio in the supplement group.

The main value of the present study is that it has been performed in elite basketball players during a regular competition season reflecting the habitual conditions under which the administration of antioxidant vitamin supplements could be useful for these athletes. A limitation of the study is that blood extractions before and after an exercise session were not performed in the same training unit but in two training sessions separated by two days. To achieve the collaboration of professional basket players in a scientific study is, in our experience, difficult, being nearly impossible to extract, twice within the same day, about 40 ml of blood from the athlete. However, the data obtained agree with other studies that obtained data before and after the same training session.

The results of this study provide evidence that supplementation with α-tocopherol, β-carotene and ascorbic acid could be useful in reducing lipid peroxides, serum peak LDH levels and testosterone clearance after the training period in habitual training activity of professional basketball players. The supplementation with α-tocopherol, β-carotene and ascorbic acid might partially account for the hormonal and enzymatic stress marker profile observed during habitual training activity of professional basketball players.

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