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The effects of dietary ascorbic acid supplementation on collagen and amino acid concentrations in Japanese quails exposed to heat stress

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Abstract: A considerable body of data exists regarding the role of vitamin C in mammalian physiology; however, there are no data about the effects of dietary ascorbic acid supplementation on collagen concentrations and amino acid levels in animals exposed to heat stress. The present study investigated the effects of supplementary ascorbic acid intake on collagen concentrations in the brain and heart tissue in Japanese quails. In addition, glycine, glutamine, histidine, asparagine, and serine contents in the livers of the same animals were measured. Japanese quails were allocated into 4 groups, each of which was exposed to heat stress (34.8 ± 1.25 °C) for 75 days. Control animals were fed a basal diet, while animals in the experimental groups were fed a basal diet supplemented with 150, 250, or 500 mg of L-ascorbic acid kg^{-1} of diet. Compared to the control group, mean collagen concentration in brain tissue significantly ($P < 0.05$) increased only in Japanese quails given 250 mg of L-ascorbic kg^{-1} of diet. On the other hand, heart tissue collagen content in the quails fed vitamin C did not significantly increase; in fact, the collagen content in the group fed 500 mg of L-ascorbic acid kg^{-1} of diet significantly decreased ($P < 0.01$). Amino acid content in the liver significantly increased in the group fed 150 mg of L-ascorbic acid kg^{-1} of diet ($P < 0.01$ for serine and $P < 0.001$ for the others).

In conclusion, vitamin C had profound effects on collagen synthesis and amino acid metabolism in Japanese quails subjected to heat stress. Results of the present study also indicate that addition of high-dose dietary vitamin C–higher than 250 mg kg^{-1} of diet–may have detrimental effects in quails exposed to heat stress.

Key words: Reactive oxygen radicals, ascorbic acid, quail, collagen, amino acid

Isı stresi oluşturulan Japon bıldırcınlarında oral askorbik asit'in kollajen ve amino asit konsantrasyonları üzerine etkisi

Özet: Vitamin C'nin memeli fizyolojisi üzerindeki etkilerine ilişkin bol miktarda bilgi bulunmaktadır. Ancak ısı stresine maruz kalan canlılarda oral vitamin C verilmesinin kollajen ve amino asit düzeyleri üzerine etkisine ilişkin bir veri

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bulunmamaktadır. Bu çalışmada oral vitamin C ilavesinin sıcak stresine maruz bırakılan Japon bıldırcınlarının beyin ve kalp dokularındaki kollajen konsantrasyonları ile karaciğer dokusundaki glisin, glutamin, histidin, asparajin ve serin amino asitlerinin düzeyleri üzerine etkisi araştırıldı. Bıldırcınlar dört gruba ayrıldı ve gruplardaki bütün hayvanlar 75 gün boyunca ortalama 34,8 ± 1,25 °C olacak şekilde sıcak stresine maruz bırakıldı. Kontrol grubundaki hayvanlar bazal diyet ile beslenirken deney gruplarındaki hayvanların yemlerine sırasıyla 150, 250 ve 500 mg kg^{-1} L-askorbik asit ilave edildi. Kontrol grubu ile karşılaştırıldığında beyin dokusundaki kollajen konsantrasyonu, diyetlerine 250 mg kg^{-1} L-askorbik asit ilave edilen grupta istatistiksel olarak anlamlı ($P < 0,05$) düzeyde arttı, diyetlerine 150 ve 500 mg kg^{-1} L-askorbik asit ilave edilen gruplarda ise değişiklik gözlenmedi. Diğer yandan yemlerine L-askorbik asit ilave edilen bıldırcınların kalp dokusundaki kollajen konsantrasyonunda bir yükselme görülmediği gibi hatta yemlerine 500 mg kg^{-1} L-askorbik asit ilave edilen grubun kalp dokusunun kollajen konsantrasyonunun anlamlı düzeyde ($P < 0,01$) azaldığı saptandı. Karaciğer aminoasit konsantrasyonları yönünden bakıldığında genel olarak ilk deney grubundaki hayvanlarda aminoasit düzeyleri istatistiksel olarak anlamlı derecede ($P < 0,01$ serin için ve $P < 0,001$ diğerleri için) arttı.

Sonuç olarak; sıcak stresine maruz bırakılan Japon bıldırcınlarında kollajen sentezi ve aminoasit metabolizması üzerine C vitamininin bariz bir etkisi bulunmaktadır. Bu çalışmadan elde edilen sonuçlar sıcak stresine maruz bırakılan bıldırcınların yemlerine 250 mg kg^{-1}'dan daha fazla oranda vitamin C ilave edilmesinin zararlı etkilerinin olabileceğini göstermiştir.

Anahtar sözcükler: Reaktif oksijen radikalleri, askorbik asit, bıldırcın, kollajen, amino asit

Introduction

Ascorbic acid, also known as vitamin C, is involved in numerous biological processes, including scavenging free radicals (to prevent oxidative damage), acting as a cofactor for such reactions as hydroxylation of proline (necessary for the collagen needed by blood vessels), and wound healing, hormone synthesis, iron absorption, and immune system functions (1-6). Ascorbic acid is required for vascular and connective tissue integrity (7,8). The collagen chain can only be formed if proline and lysine are hydroxylated in ribosomes. In this synthesis, ascorbic acid is used as a cofactor in converting proline residues into hydroxyproline (9). Consequently, ascorbate stimulates procollagen secretion and ascorbic acid deficiency impairs collagen synthesis (9). Under normal conditions a strictly regulated balance is maintained between the amount of collagen synthesized by fibroblasts and the total concentration of this protein in the extracellular matrix.

Despite a considerable body of data regarding the role of vitamin C in mammalian physiology, determination of possible detrimental effects of oxidative stress on cellular metabolism remains an on-going challenge in the biological sciences. Oxidative modification and enhanced degradation of specific proteins occur in intact cells, as well as in pure proteins (10). Amino acids are used for processes other than growth, including responses to stress (11). Glutamine is the most abundant amino acid in blood and in the free amino acid pool of the body (12). Glutamine may exert a protective effect against the damage produced by free radicals (12); however, there are no data about the putative effects of dietary ascorbic acid supplementation on collagen and amino acid concentrations in Japanese quails exposed to heat stress.

The objective of the present study was to investigate the alterations in the collagen concentration and in the levels of some amino acids in Japanese quails subjected to heat stress.

Materials and methods

Animals

The study included 48 laying Japanese quails (11 weeks old) obtained from the Poultry Breeding Unit of Adnan Menderes University Faculty of Veterinary Medicine. The animals were divided into 4 equal groups, kept in cages ($40 \times 40 \times 20$ cm^3), and fed a basal diet (Table 1), with the addition of 0, 150, 250, or 500 mg of L-ascorbic acid kg^{-1} of diet. Vitamin C was obtained from BASF (Aktiengeselschaft, Germany). Water and diets were offered ad libitum. The cages were lit for 16 h per day, and temperature and humidity were measured 3 times a day (0900, 1300, and 2000 h). Mean daily temperature was 34.8 ± 1.25 °C. Average relative humidity in the cages was 43.8 ± 0.53%. The experimental period was 75 days.

Table 1. Ingredients of the basal diet consumed by Japanese quails exposed to heat stress.

Ingredients	Dry matter (%)
Crude protein	21
Crude cellulose	6
Crude ash	7
Limestone	10
Lysine	1.26
Methionine	0.45
Cysteine	0.85
Ca	0.90
P	0.60
Na	0.15
NaCl	0.30

Measurement of total collagen

Samples of heart and brain tissue were stored at −85 °C until analyzed. Total collagen was determined by measuring the concentration of hydroxyproline in each specimen, as described by Reddy and Enwemeka (13). Samples (20-40 mg) homogenized in 2 N NaOH were hydrolyzed by autoclaving at 120 °C for 20 min. Then 450 mL of chloramine-T was added to the hydrolysate and mixed gently, and oxidation was allowed to proceed for 25 min at room temperature. After that 500 mL of Ehrlich's aldehyde reagent was added to each sample and mixed gently, and the chromophore developed by incubating the samples at 65 °C for 20 min. Absorbance of each sample was read at 550 nm using a spectrophotometer. Unknown concentrations of hydroxyproline in each tissue specimen were deduced from a standard calibration curve using L-hydroxyproline. Total collagen content was calculated assuming that 14% of the total amino acids of collagen was hydroxyproline. Collagen content is given as μg of collagen mg^{-1} wet tissue.

Derivatization of amino acids

Liver tissue samples were first homogenized with phosphate buffer and then passed through a 0.45-mm filter. Homogenates were mixed in a 1:1 ratio with the internal standard solution (0.4 mM methionine sulfone in 0.1 M HCl). A volume of 25 mL of standard or sample was dried. The samples were then reconstructed with 20 mL of drying solution [1 M sodium acetate:methanol:triethylamine (TEA), 2:2:1], dried, and dissolved in 20 mL of derivatization solution [methanol:water:TEA:phenylisothiocyanate (PITC), 7:1:1:1]. The derivatization of both primary and secondary amino acids occurred in 20 min at 25 °C, and produced the corresponding phenylthiocarbamoyl derivatives. The samples were then re-dried. Finally, the dried samples were dissolved in 100 mL of PicoTag sample buffer. After sonicating for a few seconds the samples were injected into an HPLC column.

Performance of HPLC

An Alliance 2690 Separation Module (Waters, Milford, MA, USA) HPLC system, which consisted of 2 solvent delivery pumps, 1 auto injector, 1 column heater (46 °C), and 1 Waters 487 UV detector set at 254 nm, was used for reverse phase HPLC analysis. A Waters Millennium 32 chromatography manager system was used to control the system operation, and to collect and process data. All separations were generated on a Waters PicoTag column (30 cm × 3.9 mm) operating at a flow-rate of 1.0 mL min^{-1}. Samples were injected in volumes of 20 mL. The mobile phase consisted of a gradient of 2 eluents (PicoTag eluent A and B). The gradient employed in the separation began with eluent B rising from 3% to 34% in 60 min. After a 10-min washing step with 100% B, the column was re-equilibrated for 20 min with 100% eluent A. A constant flow-rate of 1 mL min^{-1} was maintained. HPLC-grade water was generated with a Millipore MilliQ water purification system (Billerica, MA, USA).

Statistical analysis

Statistical analysis of the differences between groups was performed using ANOVA and the significance of the differences was determined using Duncan's test. Differences were considered statistically significant at $P < 0.05$ against the control group. All values are presented as mean ± SEM

Results

As shown in Table 2, mean collagen content in brain tissue in Japanese quails exposed to heat stress was 2.25 ± 0.32 μg mg^{-1} of wet tissue. In comparison to the control animals (no ascorbate supplementation), mean collagen concentrations in

Table 2. Tissue collagen concentrations in Japanese quails exposed to heat stress. Results are expressed as mean ± standard error ($\bar{X} \pm S_{\bar{X}}$).

	Dietary vitamin C supplementation				
Tissue Concentrations (μg collagen/mg wet et tissue)	Collagen Control $\bar{X} \pm S_{\bar{X}}$ n = 12	150 mg kg^{-1} $\bar{X} \pm S_{\bar{X}}$ n = 12	250 mg kg^{-1} $\bar{X} \pm S_{\bar{X}}$ n = 12	500 mg kg^{-1} $\bar{X} \pm S_{\bar{X}}$ n = 12	P
Brain	2.25 ± 0.32^{a}	3.21 ± 0.51^{ab}	3.82 ± 0.50^{b}	2.09 ± 0.43^{a}	< 0.05
Heart	1.65 ± 0.13^{a}	1.93 ± 0.15^{a}	1.60 ± 0.15^{a}	1.09 ± 0.86^{b}	< 0.01

[a,b]Different letters in the same line indicate significant differences.

brain tissue in the animals supplemented with vitamin C at 150 mg kg^{-1} increased to 3.21 ± 0.51 μg mg^{-1} of wet tissue. Despite of this apparent rise in collagen content, this alteration was not statistically significant; however, the brain collagen concentration increased from 2.25 ± 0.32 to 3.82 ± 0.50 μg mg^{-1} of wet tissue in quails given 250 mg ascorbic acid kg^{-1} of diet and this increase was statistically significant ($P < 0.05$). The positive effect of ascorbic acid on collagen synthesis in the first 2 experimental groups and on brain collagen concentrations in the third group, which received the highest dose (500 mg kg^{-1}), was lower than in the control group. When compared to the control animals, mean collagen content in heart tissue in quails given 150 mg of ascorbic acid kg^{-1} of diet moderately increased (from 1.65 ± 0.13 to 1.93 ± 0.15). Similar to the brain collagen concentration, mean collagen concentration in heart tissue in the birds supplemented with the highest dose of ascorbic acid (500 mg kg^{-1}) decreased significantly ($P < 0.01$).

Liver amino acid concentrations in all 3 experimental groups were higher than in the control group (Table 3). The amino acids content in the first experimental group (150 mg of ascorbic acid kg^{-1} of diet) was the highest and the increase in amino acids content was significantly higher ($P < 0.01$ for serine and $P < 0.001$ for others) than in the control group and the other experimental groups.

Discussion

Under normal conditions a strictly regulated balance is maintained between the amount of collagen synthesized by fibroblasts and the total concentration of this protein in the extracellular matrix. Heat stress can stimulate numerous pathways that result in

Table 3. Tissue amino acid concentrations in Japanese quails exposed to heat stress. Results are expressed as mean ± standard error ($\bar{X} \pm S_{\bar{X}}$).

	Dietary vitamin C supplementation				
Liver amino acid contents (μmol g^{-1} wet tissue)	Control $\bar{X} \pm S_{\bar{X}}$ n = 10	150 mg $^{-1}$kg $\bar{X} \pm S_{\bar{X}}$ n = 9	250 mg kg^{-1} $\bar{X} \pm S_{\bar{X}}$ n = 11	500 mg kg^{-1} $\bar{X} \pm S_{\bar{X}}$ n = 12	P
Glycine	651.18 ± 50.46^{a}	1745.77 ± 198.32^{c}	1148.00 ± 112.17^{b}	1051.33 ± 121.96^{ab}	< 0.001
Serine	657.54 ± 61.41^{a}	1588.98 ± 237.19^{b}	1023.81 ± 103.06^{a}	886.33 ± 128.62^{a}	< 0.01
Glutamine	542.01 ± 69.25^{a}	2008.11 ± 258.07^{c}	1333.18 ± 243.69^{bc}	1231.16 ± 180.46^{ab}	< 0.001
Histidine	75.58 ± 7.41^{a}	247.08 ± 43.14^{b}	138.04 ± 18.42^{a}	136.50 ± 15.67^{a}	< 0.001
Asparagine	40.07 ± 10.75^{a}	824.67 ± 158.66^{b}	144.27 ± 74.92^{a}	118.65 ± 57.72^{a}	< 0.001

[a,b,c]Different letters in the same line indicate significant differences.

increased production of free radicals (14). As such, heat stress reduces antioxidative capacity in such tissues as heart and brain. It is reported that collagen synthesis is decreased by free radicals (14); however, the mechanism of this process remains largely unknown (15). On the other hand, vitamin C is an aqueous-phase, non-enzymatic antioxidant that prevents radical formation (16), and is essential for the production and maintenance of collagen. It modulates collagen mRNA transcription (3,17), plays an essential role both in the formation of the collagen triple helix and in its stabilization (17), and is involved in the formation of strengthening collagen cross-links (17,18). Accordingly, there is direct evidence that a lack of vitamin C alters collagen synthesis, but there are no data demonstrating that additional dietary intake of ascorbic acid above the minimum requirement stimulates collagen synthesis. In the present study the increase in the mean collagen concentration in brain tissue in animals given 250 mg of ascorbic acid kg^{-1} of diet was significantly higher than in the control animals. This might be attributed to the modulatory effects of vitamin C on collagen mRNA transcription or to a decrease in the degradation rate of procollagen mRNAs by downregulation of matrix metalloproteinase mRNA expression, which is responsible for collagen degradation. Nonetheless, mean collagen concentration in brain tissue in animals given 500 mg of ascorbic acid kg^{-1} of diet was significantly ($P < 0.05$) lower that in the control birds. Ascorbic acid is known to have the potential to increase production of hydroxyl radicals for hydrogen peroxide via the Fenton reaction, and depending on circumstances, can either protect against or potentiate damage (12,19). As collagen synthesis was decreased by free radicals, the apparent reduction in collagen levels in brain tissue in the animals given 500 mg of ascorbic acid kg^{-1} of diet may have been due to the production of hydroxyl radicals.

The mean collagen concentration in heart tissue in the first experimental group (150 mg of ascorbic acid kg^{-1} of diet) increased slightly, but not significantly. Regarding tissue collagen concentrations, there were no significant differences between the control and first 2 experimental groups (150 and 250 mg of ascorbic acid kg^{-1} of diet, respectively). The third experimental group (500 mg of ascorbic acid kg^{-1} of diet), however, had significantly ($P < 0.01$) lower levels of collagen in the heart. Based on these results, ascorbic acid seems to have acted differentially on collagen synthesis in brain and heart tissue. In other words, supplementation of dietary ascorbic acid at 250 mg of ascorbic acid kg^{-1} of diet had a stimulatory effect on collagen synthesis in brain tissue, while it decreased collagen synthesis in heart tissue. As there is a more than 10-fold gradient between the concentrations of ascorbic acid in the brain and serum (7,20), the contrasting results in the 2 organs may be attributed to the high levels of vitamin C in the brain, which is accustomed to high levels of ascorbic acid.

The present study shows for the first time that supplementation with dietary ascorbic acid had a profound effect on amino acid metabolism in the brain and heart tissue in Japanese quails subjected to heat stress. Animals in all 3 experimental groups had higher amino acid levels than the control group. In particular, the amino acid concentrations in the first experimental group (150 of ascorbic acid kg^{-1} of diet) were consistently the highest and the increase in amino acid contents was significantly higher than that in the other groups. Glutamine is the most abundant amino acid in the free amino acid pool of the body (12,21) and may have a protective effect against the damage produced by free radicals (12). Furthermore, glutamine is 1 of the 3 amino acids of glutathione, an important tripeptide involved in antioxidant defense. Like glutamine, glycine is also a constituent of glutathione. It is well known that an excess amount of ascorbic acid has a pro-oxidant effect; therefore, the decline in glutamine and glycine concentrations in the animals given an excess amount of ascorbic acid may have been due to supporting glutathione synthesis. Concerning the increases in the levels of the other amino acids in the experimental animals, they may have been related to energy production in order to maintain the repair processes impaired by heat stress; however, the mechanisms underlying the increase in the synthesis of amino acids remain to be elucidated.

In conclusion, redundancy of ascorbic acid may be harmful to quails exposed to heat stress, in terms of both collagen synthesis and amino acid production. In addition, to determine the possible relationships between ascorbic acid and amino acid metabolism further research is needed.

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