Effects of Dietary Vitamin C Supplementation on Glutathione, Malondialdehyde and Nitric Oxide Concentrations in brain and heart of Laying Japanese Quails Exposed To Heat Stress (34.8°C)

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SUMMARY

The assessment of a long-term vitamin C supplementation on tissue antioxidant status was investigated using brain and heart tissues obtained from laying Japanese Quails exposed to heat stress (34.8 ± 1.25°C) for 75 days. Forty-eight laying Japanese quail were divided into four groups. Animals in control group were fed with a basal diet, whereas experimental animals were fed with a basal diet supplemented with 150, 250 or 500 mg of L-ascorbic acid/kg of diet. Heart and brain malondialdehyde (MDA) concentrations were the highest in control birds, and decreased in brain of supplemented birds (p<0.01), and in heart of birds receiving the highest vitamin C dosage (500mg/kg) (p<0.05). Compared to controls, the glutathione (GSH) concentrations in heart of birds supplemented by additional vitamin C decreased (p<0.01) whereas the concentrations of this compound remained unaffected by the supplementation of vitamin C. As a consequence, dietary vitamin C supplementation reduces the oxidative stress induced by heat stress on laying Japanese quails. Furthermore, because no evident dose-effect relationship was obtained, a dietary vitamin C supplementation with 150 mg vitamin C/kg diet would be enough to prevent the damaging effects of heat stress on laying Japanese quail.

Keywords : quail - heat stress - vitamin C - glutathione - nitric oxide.

Introduction

Stress induces a cluster of physiological changes [4, 32], including for example, disturbances in metabolism, water, protein, energy and mineral balances, enzymatic reactions, hormonal secretions and blood metabolites and depression of feed intake, efficiency and utilisation [4, 29]. Stress can stimulate numerous pathways leading to increased production of free radicals. It is well known that free radicals generate a peroxidation cascade producing lipid peroxidation, protein oxidation, and contribute to the occurrence of pathological conditions [4, 15, 16]. Mammalian cells are equipped with both enzymatic and non-enzymatic antioxidant defences to minimize the cellular damage caused by interaction between cellular constituents and reactive oxygen species (ROS) [22]. They are generated during normal cellular metabolism; however several conditions are known to disturb the balance between ROS production and cellular defence mechanisms. Among the chain-breaking antioxidants, which prevent propagation of free radical-induced chain reactions, ascorbate and tocopherol are particularly important [27]. Vitamin C, also referred as ascorbic acid or ascorbate, is an important dietary antioxidant which significantly decreases the adverse effects of reactive species such as ROS susceptible to cause...
oxidative damage to macromolecules such as lipids, DNA and proteins [11]. Ascorbate is the only antioxidant that completely protects endogenous lipids from detectable oxidative damage induced by ROS [27]. Ascorbate is able to intercept ROS before they can react with and oxidize lipoprotein lipids. Once ascorbate has been depleted, the remaining antioxidants provide only partial protection from ROS, which may interact with lipoproteins and initiate lipid peroxidation. The end product of lipid peroxidation is malondialdehyde (MDA), which is measured conveniently [2, 19].

Glutathione (GSH) is the most abundant non-protein thiol compound present in mammalian cells and serves many important physiological roles, particularly as cellular antioxidant in peripheral tissue. It acts as an electron donor in the glutathione peroxidase-catalysed reduction of organic and hydrogen peroxides. Subsequently, the oxidized glutathione (GSSG) formed from this reaction is removed from the cell via NADPH-dependent reduction by glutathione reductase [6, 21].

The nitric oxide radical (NO•) plays an important role as a physiological messenger. NO• is formed from L-arginine by NO synthase [25], whose different isoforms exist [7, 28]. Constitutive calcium-dependent isoforms modulate control of vascular tone in endothelial cells or the neurotransmission in neurons, whereas inducible calcium independent isoforms are located in macrophages, chondrocytes, and hepatocytes, and are induced by cytokines and endotoxin. NO• is a very unstable, short half-live gas that breaks down rapidly into the stable products nitrate and nitrite. Ascorbate decreases the levels of superoxide radicals [5, 12], which react with and inactivate NO• [3, 9].

Ascorbic acid concentrations were reduced in animals stressed by environmental temperature [20]. Moreover, ambient temperature impairs absorption of vitamin C and increases the dietary requirement of this vitamin [8]. Therefore we intended to study the effects of different dosages dietary vitamin C supplementation on GSH, MDA and NO• concentrations in brain and in heart of Japanese quail exposed to heat stress.

Material and Methods

1. ANIMALS

Forty-eight, 11-week-old laying Japanese quails obtained from the Poultry Breeding Unit of Veterinary Faculty of Adnan Menderes University were used in this study. Animals kept in cages 40 x 40 x 20 cm³ were divided into four groups and fed with basal diet (Table I) eventually supplemented by 0, 150, 250, 500 mg L-ascorbic acid/kg of diet. Each group contained 11, 13, 12, and 12 animals, respectively. Vitamin C was provided by a commercial company (BASF® Aktiengesellschaft, Germany). Water and diets were offered ad libitum. Photoperiod was 16L:8D, and temperature and relative humidity were measured 3 times a day (at 09h.00, 13h.00, and 20h.00). The mean value of the daily temperature was 34.8 ± 1.25°C. Average relative humidity in the house of animals was 43.8 ± 0.53%. The length of the experiment was seventy-five days. On the 75th day the animals were killed by decapitation. Tissue materials were immediately stored frozen at - 80°C after sampling until the analyses are conducted.

2. TISSUE PREPARATION

Tissues were homogenised in 50 mM phosphate buffer (pH 7.4) containing protease inhibitor, 0.2 μM phenylmethylsulfonylfluoride (PMSF) and 1 mM EDTA, at 4°C for 39 s, using a B. Braun homogeniser. Then, homogenate was centrifuged at 1500 g for 5 min at 4°C. The resultant supernatant was used for measurement of the MDA, GSH and NO• concentrations.

3. BIOCHEMICAL ANALYSIS

Total GSH measurements were done by the method of Tietze [31]. Tissue homogenate were deproteinized in a glacial metaphosphoric acid/disodium-EDTA/NaCl solution. In brief, 0.5 ml supernatant or standard (aqueous standard solutions of GSH solution) were diluted with 0.25 mL of sodium phosphate buffer (1M, pH 6.8) and 0.5 mL 5-5’-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.8 g/L in the phosphate buffer), incubated at room temperature for 5 minutes then the absorbances at 412 nm were measured using a Shimadzu UV-160 spectrophotometer. Results were expressed as mg/mg wet tissues.

The MDA production and hence lipid peroxidation were assed in the tissues by the method of Ohkowa [24]. This end product of lipid peroxidation forms a colored complex in the presence of TBA, which is detectable by spectrophotometry at 532 nm. Absorbances were detected with Shimadzu UV-160 spectrophotometer. The 1,1′,3,3′-Tetraethoxypropane was used as a standard and the results were expressed as nmol/g wet tissue.

For the measurement of NO• (nitrite + nitrate), 400 μL of supernatant was denatured by adding 80μL 30% ZnSO₄ solution, stirring and then centrifuging at 10,000xg for at least 20 min at 4°C. First, we activated cadmium (Cd) granules via CuSO₄ solution in glycine-NaOH buffer, thereafter 100(L of deproteinized samples and standards were added. This sample pre-treatment was performed to reduce nitrate to nitrite. NO• was assayed by a modification of cadmium-reduction method as mentioned by Navarro-González and collaborators.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Dry matter (%)</th>
</tr>
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<tbody>
<tr>
<td>Crude protein</td>
<td>21</td>
</tr>
<tr>
<td>Crude cellulose</td>
<td>6</td>
</tr>
<tr>
<td>Crude ash</td>
<td>7</td>
</tr>
<tr>
<td>Limestone</td>
<td>10</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.26</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.45</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.85</td>
</tr>
<tr>
<td>Ca</td>
<td>0.90</td>
</tr>
<tr>
<td>P</td>
<td>0.60</td>
</tr>
<tr>
<td>Na</td>
<td>0.15</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table I. — Ingredients of the basal diet consumed by Japanese quail exposed to heat stress (34.8°C) during 75 days.
ZALVES [23]. The produced nitrite amount was determined by diazotization of sulphanilamide and coupling to naphthylthylene diamine. The samples were read spectrophotometrically using a microplate reader and quantified automatically against a KNO₃ standard curve.

4. STATISTICAL ANALYSIS

Differences among groups were tested by one-way ANOVA. Duncan test was used to find out the group effects. P<0.05 was set as limit of significance.

Results

As shown in Table II, MDA concentrations in brains and heart from the animals supplemented with vitamin C decreased (p<0.01 for brain, p<0.05 for heart). MDA concentrations in brain were significantly lowered in the 3 ascorbate groups (p<0.01). In heart, MDA concentrations were the highest in control birds (no ascorbate supplementation) and they were significantly decreased in birds receiving the highest dose (500 mg/kg) (p<0.05), whereas, in the other supplemented groups, intermediate values were observed (Table II).

Whereas brain GSH concentrations showed no alteration, heart GSH quantities were significantly lower in the vitamin C supplemented groups compared to the controls (p<0.01). A significant reduction of NO• concentrations in brain (p<0.05) was observed in 500 mg/kg ascorbate supplemented group. The heart NO• concentrations were however similar in all groups.

Discussion

ROS react with intracellular macromolecules, causing irreversible structural and functional damage. Unsaturated lipids of the membrane are the most sensitive to oxidative damage in the cell [14]. Lipid peroxidation caused by ROS results in the disarrangement and, ultimately, disruption of cell membranes, which leads to necrotic death. MDA is the end product of lipid peroxidation. As might be expected, we have found that dietary vitamin C supplementation, particularly the highest dosage, reduced the heart and brain MDA production and improved the resistance of cell membranes to lipid peroxidation induced by high ambient temperature. The high degree of oxidative damage observed in brain and heart from the animals exposed to heat stress (reflected by high MDA concentrations in control birds) is in agreement with previously reported findings [17, 18]. The decreased MDA concentrations in the tissues of animals receiving dietary vitamin C supplementation supports the findings of previous investigators, who reported [27] that ascorbic acid is able to intercept ROS before they can react with and oxidize lipoprotein lipids.

It is somewhat surprising that vitamin C supplementation in Japanese quails exposed to heat stress significantly decreases GSH amounts in heart, but not in brain. A unique physiological relationship exists between glutathione and ascorbate. Ascorbate removes free radicals by donating one or two electrons in redox reactions. The two-electron oxidation of ascorbate results in the generation of dehydroascorbate. Dehydroascorbate can be reduced back to ascorbate. GSH is the substrate for glutaredoxin, an enzyme that reduces dehydroascorbate to ascorbate. Ascorbate is known to compete with superoxide dismutase for removal of superoxide [6, 13]. By the addition of vitamin C, ascorbate levels in tissues reach a competitive range and may eliminate superoxide. The resulting dehydroascorbate would be reduced to ascorbate when GSH is oxidized to GSSG. The regeneration of ascorbate from its oxidised form using GSH could explain the reduction of GSH concentrations in heart of birds supplemented with vitamin C. On the other hand, vitamin C crosses blood-brain barrier with a great efficacy because the vitamin C concentration in brain is 10 fold higher than the blood concentration [1, 30]. But only the vitamin C oxidised form is able to cross the blood-brain barrier [1], and then it is locally reduced to ascorbic acid. This fact suggests that, although the exact mechanisms of the dehydroascorbic acid reduction in brain are not yet identified, they are abundant and very efficient. Because GSH concentrations were not altered in brains of animals dietary supplemented with vitamin C, some glutathione-independent mechanism(s) would be involved, at least in brain. Whatever the vitamin C supplementation dosage, MDA concentrations significantly decreased in brain. In heart, only a significant effect was obtained for the highest supple-

<table>
<thead>
<tr>
<th>Dietary vitamin C supplementation</th>
<th>0 mg/kg</th>
<th>150 mg/kg</th>
<th>250 mg/kg</th>
<th>500 mg/kg</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>(n = 11)</td>
<td>(n = 13)</td>
<td>(n = 12)</td>
<td>(n = 12)</td>
<td></td>
</tr>
<tr>
<td><strong>MDA (nmol/g wet tissue)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Brain</td>
<td>130.64 ± 14.84^a</td>
<td>52.10 ± 16.84^a</td>
<td>62.41 ± 17.03^b</td>
<td>67.70 ± 10.16^b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>1235.42 ± 42.00^b</td>
<td>906.79 ± 38.39^a</td>
<td>873.20 ± 17.40^b</td>
<td>775.49 ± 82.23^b</td>
<td>&lt; 0.05</td>
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<tr>
<td><strong>GSH (mg/mg wet tissue)</strong></td>
<td></td>
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<td>NS</td>
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<tr>
<td>Brain</td>
<td>155.43 ± 10.84^a</td>
<td>170.65 ± 22.56^a</td>
<td>132.14 ± 11.63^a</td>
<td>183.07 ± 16.03^a</td>
<td>NS</td>
</tr>
<tr>
<td>Heart</td>
<td>188.16 ± 44.17^a</td>
<td>61.86 ± 9.74^b</td>
<td>43.49 ± 16.30^b</td>
<td>66.66 ± 8.76^b</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><strong>NO• (µmol/g wet tissue)</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Brain</td>
<td>40.04 ± 2.80^a</td>
<td>39.50 ± 4.07^a</td>
<td>38.70 ± 3.34^a</td>
<td>26.92 ± 3.11^b</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Heart</td>
<td>48.99 ± 6.92^a</td>
<td>37.42 ± 2.04^a</td>
<td>47.09 ± 5.36^a</td>
<td>39.49 ± 2.27^a</td>
<td>NS</td>
</tr>
</tbody>
</table>

^a,b : Different letters indicate statically significant differences in the same line

TABLE II. — Tissue MDA, GSH and NO concentrations in Japanese quail exposed to heat stress (34.8°C) during 75 days eventually supplemented with Vitamin C (150, 250 or 500 mg/kg). Results are expressed as mean ± standard deviations.

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mentation (500 mg/kg), although with intermediate dosages, the MDA concentrations tended to be reduced, but not significantly. Nevertheless, as heart GSH concentrations also similarly lowered in the 3 supplemented groups, it can be assumed that no marked dose-effect relationship was achieved, and that the lowest dose of vitamin C would be sufficient to counteract the adverse effects of heat stress.

It has been shown that NO• could render cells resistant to oxidative stress, by forming complexes with reduced iron, and preventing in this way the formation of strong oxidants [26]. Similarly, kinetically fast reactions between NO• and lipids, and/or organic radicals induce the termination of chain reactions and protect membranes against peroxidation and prevent peroxidative chemistry-induced cell injury [26]. Superoxide radicals react with and inactivate NO• [3, 9]. By decreasing free radical amounts [5, 12], vitamin C would prevent this NO• consumption, but relatively high concentrations of ascorbate (≈10 mol/L) are required to effectively inhibit the thermodynamically favourable reactions between NO• and superoxide [12]. Moreover, ascorbate also increases the synthesis and biological activity of NO• by increasing intracellular tetrahydrobipterin [10]. Contrary to these effect of vitamin C on NO• synthesis, NO• concentrations neither in brain or in heart of animals given 500 mg/kg were enhanced by dietary vitamin C supplementation. Even, NO• concentrations were depressed in brains of birds receiving the highest dose although vitamin C was concentrated in this tissue [1, 30]. Consequently studies are needed to determine the exact mechanism(s) responsible for reduction of the NO• concentrations in brain.

However, the present study clearly shows that dietary vitamin C supplementation reduces the oxidative stress induced by long-term exposure to heat stress in laying Japanese quails, even if, in brain, some antioxidant mechanisms remain to be elucidated.

References


