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Original Article

Effects of dietary vitamin C supplementation on some oxidative status biomarkers in erythrocytes of common carp (*Cyprinus carpio*)

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Abstract: Regarding to the high content of polyunsaturated fatty acids in fish tissues, improving the fish antioxidant status seems to be necessary and may be associated with beneficial effects on fish health. The present study aimed to investigate the effects of dietary vitamin C supplementation (20 mg/kg body weight, 4 weeks) on some oxidative status biomarkers in RBC of common carp (*Cyprinus carpio*). The results showed that the activities of antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) were not changed significantly following dietary vitamin C supplementation in comparison to the control group. Moreover, dietary vitamin C supplementation for 28 days significantly lowered malondialdehyde (MDA) concentration in erythrocyte haemolysate by approximately 26% compared to that of the control group. In conclusion, dietary vitamin C supplementation appears to be able to protect carp erythrocytes against oxidative stress by decreasing lipid peroxidation.

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Introduction

A variety of reactive oxygen species (ROS), derived from reduction of oxygen, ionizing radiations, reactive metals, and other environmental initiators, permanently threatens living organisms (Birben et al., 2012). Oxidative stress is a consequence of an increased generation of free radicals and/or reduced physiological activity of antioxidant defenses against them. Oxidative stress impairs DNA, causes enzymes and membrane disorders, changes the activity of the immune system, and alters the structure of basic biopolymers, which, cause various disorders (Abd Ellah, 2010). In both mammals and fish, insufficient dietary antioxidants have been followed by a decrease in antioxidative defense and increased susceptibility to oxidative stress (Sies et al., 2005; Welker and Congleton, 2009). The antioxidant capacity of fish may be insufficient in captivity (Mohebbi et al., 2011). Regarding to increased environmental pollutants and thus increased risk of oxidative stress, administration of dietary antioxidants would be beneficial for cultured fish. Moreover, in view of the high content of polyunsaturated fatty acids in aquaculture feeds and fish tissues, improving antioxidant capacity of fish seems to be necessary and may be associated with many beneficial effects on fish health (Mohebbi et al., 2011).

Fish tissues are characterized by high concentrations of polyunsaturated fatty acids and may therefore be particularly susceptible to lipid peroxidation. Both oxidative responses and antioxidant potential of fish differ according to species habitat and feeding behavior (Yonar and Sakin, 2011). Dietary antioxidant vitamins may play an important role in protecting fish against oxidant damages. Vitamin C is important for many enzymatic reactions and also acts as a free-radical scavenger. Vitamin C deficiency has been shown to retard growth and impair wound healing in *O. mykiss* and *Oncorhynchus kisutch* (Zhou et al., 2003). Vitamin C supplementation in diet promoted growth in sea bass (*Lates calcarifer*) and common carp (Gouillou-Coustans et al., 1998). On the other hand, it has been documented that vitamin C affects metabolism of lipids and carnitine, which is essential

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for the β-oxidation of long-chain fatty acids (Feller and Rudman, 1988).

Potential functions of vitamin C in RBC include maintenance of plasma ascorbate concentrations by ascorbate or dehydroascorbic acid efflux from red blood cells, transmembrane electron transfer from erythrocyte ascorbate, and antioxidant functions to protect erythrocytes from oxidative damage or to recycle membrane tocopherol (Li et al., 2012). It has been reported that dietary vitamin C supplementation could significantly increase growth performance and antioxidant status in some aquatic animals (Asaikkutti et al., 2016; Gouillou-Coustans et al., 1998).

Common carp (*Cyprinus carpio*) has great commercial importance because it is widely consumed all over the world. Although cyprinids appear to be able to synthesize vitamin C at rates sufficient to meet its physiological needs, dietary supplementation with vitamin C might be beneficial when metabolic demand exceeds endogenous supply (Combs, 1998). Therefore, the present study was conducted to assess the effects of dietary vitamin C supplementation on some oxidative status biomarkers in RBC of common carp.

Materials and Methods

Vitamin C was purchased from Nature Made (California, USA). Commercial enzyme kits for superoxide dismutase (Ransod, RANDOX/SD-125) and glutathione peroxidase (Ransel, RANDOX/RS-505) were obtained from Randox Laboratories (Crumlin, UK). MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Tricaine) and 2-thiobarbituric acid (TBA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rest of the utilized chemicals were of analytical grade and were supplied by Sigma (St Lewis, MO, USA) or Merck (Darmstadt, Germany).

Common carp (total n=60), weighing 60-80 g, were obtained from a local commercial farm. They were held in four glass aquaria, each containing 250 L freshwater. The fish were acclimatized for 7 days (Naeiji et al., 2013) before the commencement of the experiment and were daily fed with commercial fish feed at 3% of total body weight at a fixed time. The aquaria were aerated continuously. Physicochemical characteristics of the water during the experimental period were: dissolved oxygen, 5.5-6 ppm; temperature, 25±1°C; pH, 7±0.5; photoperiod, 12:12 light–dark. The aquaria water was renewed every 48 h (Naeiji et al., 2013). The fish were divided randomly into two groups of 30 each. Group 1 fish were held in two aquaria (each containing 15 fish) and fed with basal diet; served as control. Group 2 fish were held in two aquaria (each containing 15 fish) and fed the basal diet supplemented with vitamin C (20 mg/kg body weight, daily (NRC, 1993)) for 4 weeks. The vitamin-supplemented diet was prepared by spraying vitamin C solution onto the pellet (Treves-Brown, 2000).

At the end of the experimental period, twenty fish were sampled randomly from each aquarium and anesthetized in diluted MS-222. Blood samples were taken by cardiac puncture using heparinized syringes and tubes. After plasma separation by centrifugation at 750×g for 20 min (Nazifi et al., 2010), erythrocyte pellet was washed three times with normal saline solution. The washed centrifuged erythrocytes were hemolyzed by the addition of an equal volume of ice-cold redistilled water (Nazifi et al., 2010) and prepared hemolysate aliquots were stored at -70°C until analysis.

Glutathione peroxidase (GPx) activity was measured using RANDOX-Ransel enzyme kit. In this method, GPx catalyzes the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form, with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured spectrophotometrically, and the results were expressed as units per gram hemoglobin. Hemoglobin (Hb) concentration was measured by cyanmethemoglobin method (Prakash and Banerji, 1972).

Superoxide dismutase (SOD) activity was determined by a modified method of iodophenyl nitrophenol phenyltetrazolium chloride using the RANDOX-Ransod enzyme kit. This method employs xanthine and xanthine oxidase to generate superoxide

Table 1. Mean±SEM of measured oxidative status biomarkers in experimental groups (n= 20 in each group).

Parameter	Control	Vitamin C
Catalase (U/g Hb)	273.41±30.95	310.25±24.74
Superoxide dismutase (U/g Hb)	1573.67±68.53	1557.90±53.67
Glutathione peroxidase (U/g Hb)	744.96±38.37	836.4±38.86
Malondialdehyde (nmol/g Hb)	129.29 ±10.37 [a]	95.25±7.40 [b]

[a, b] Mean±SEM in each row with no common superscript differ significantly (P<0.05).

radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity was then measured by the degree of inhibition of this reaction. One unit of SOD was considered a 50% inhibition of reduction of INT under the condition of the assay. The results were expressed as units per gram hemoglobin.

Catalase (CAT) activity was measured in the RBC hemolysate by the method described by Claiborne (1986) and expressed as units per gram hemoglobin. The decomposition of H_2O_2 can be directly followed by the decrease in absorbance at 240 nm. The difference in absorbance at 240 nm per time unit allows determining the CAT activity. 50 µl of the diluted hemolysate was mixed with 1.9 ml of the phosphate buffer (0.05 M, pH 7) and 1 ml of 30 mM H_2O_2. The decrease in absorption was measured at 240 nm for 1 min. Activity of catalase was calculated using the extinction coefficient of 43.6 $M^{-1}cm^{-1}$. One unit of catalase activity is equal to the amount of enzyme that will decompose 1 µmol H_2O_2 per minute.

Lipid peroxidation was assayed by measurement of malondialdehyde (MDA). Determination of MDA concentration was based on spectrophotometry of the pink-colored product of thiobarbituric acid reactive substances, as described by Latha and Pari (2003). The concentration of MDA was calculated using a molar extinction coefficient value of 156,000 M^{-1} cm^{-1}. The results were expressed as nanomoles of MDA per gram hemoglobin.

Statistical analysis: All experimental values have been represented as mean ±standard error of mean (SEM). The obtained data were analyzed using Student's t-test. The level of significance was set at P<0.05. All calculations were performed using SPSS/PC software, version 18.

Results

The values (mean±SEM) of the measured erythrocyte oxidative status biomarkers in experimental groups are presented in Table 1. The activities of antioxidant enzymes including CAT, GPx and SOD were not changed significantly following dietary vitamin C supplementation in comparison to the control group. As shown in Table 1, dietary vitamin C supplementation for 28 days significantly lowered the MDA concentration in erythrocyte hemolysate by approximately 26% compared to concentration in the control group.

Discussion

Measurement of circulatory biomarkers of oxidative stress has emerged as a reliable method for screening putative antioxidative agents (Balasenthil et al., 2000). The extent of lipid peroxidation is most frequently measured by estimating MDA levels (Latha and Pari, 2003). Fish erythrocytes have been proposed as a useful model to investigate oxidative stress, since their membranes are rich in long chain n-3 polyunsaturated fatty acids, which are oxidized under oxidative stress conditions (Roche and Boge, 1993; Gabryelak et al., 2000; Nagasaka et al., 2004). Moreover, repeatedly exposure to high concentration of oxygen or presence of iron renders erythrocytes highly susceptible to peroxidative damage (Clemens and Waller, 1987). The effect of vitamin C in decreasing MDA levels in the present study suggest that this vitamin may provide an effective protection against lipid peroxidation. Similar to this finding, it has been reported that the level of thiobarbituric acid reactive-substances (TBARS) in the hepatopancreas and muscle of common carp was decreased following vitamin C supplementation (Hwang and Lin, 2002).

Moreover, Chien and Hwang (2001) indicated that vitamin C can prevent the increase in liver lipid peroxidation due to high water temperature in thorn fish *Terapon jarbua*. Similarly, it has been reported that dietary vitamin C supplementation caused a significant decrease in the levels of tissue lipid peroxides in chicken erythrocytes (Aydemir et al., 2000).

Vitamin C as a strong reducing reagent has a direct reactive oxygen scavenger action. Additionally, depletion of tissue GSH is one of the primary factors that permit lipid peroxidation and vitamin C has been shown to increase intracellular glutathione concentrations (Hwang and Lin, 2002; Johnston, 1993). Another criterion, which might contribute to the lowering effect of vitamin C on peroxidation of hydrophobic regions of the cells may be its ability to reduce the semi-stable β-tocopheroxyl radical (vitamin E radical form after performing the antioxidant role), thus regenerating the metabolically active form of the lipid antioxidant vitamin E (Kontush et al., 1996).

The detoxification of ROS involves the cooperative action of the intracellular antioxidant enzymes SOD, CAT, and GPx. GPx contributes to the oxidative defense of animal tissues by catalyzing the reduction of hydrogen peroxide and lipid peroxides (Harvey, 1997). CAT has an equal importance to in the defense of human erythrocytes against H_2O_2 generating reactions (Harvey, 1997). Superoxide dismutase is also important in the antioxidant defense mechanism and protects against lipid peroxidation (Miller et al., 1993). Animal studies have shown that antioxidant enzymes' levels are variable depending on the availability of antioxidants in food (Meydani, 1993; Puangkaew et al., 2005). It has been reported that SOD and GPx activities increased but CAT activity decreased with an increase of plasma vitamin C levels (Monget et al., 1996). In chicken the activities of erythrocyte SOD and GPx increased following vitamin C supplementation although no significant variations were observed in the CAT activity (Aydemir et al., 2000). Similarly, the present results indicate no significant increment of the measured antioxidant enzymes following vitamin C supplementation in carp erythrocytes.

The results of the present work suggest that dietary supplementation of vitamin C appears to be able to protect erythrocytes of carp against oxidative stress by decreasing lipid peroxidation. However, more investigations are required to elucidate the pharmacokinetic effects of this vitamin and also precise molecular basis of the beneficial effects of vitamin C in fish species.

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چکیده فارسی

تاثیر مکمل غذایی ویتامین C بر برخی بیومارکرهای وضعیت اکسیداتیو در گلبول‌های قرمز کپور معمولی (*Cyprinus carpio*)

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چکیده:
با توجه به مقادیر بالای اسیدهای چرب غیر اشباع در بافت‌های ماهی، بهبود وضعیت آنتی اکسیدانی در ماهی لازم به نظر می‌رسد که می‌تواند اثرات مفیدی در سلامت ماهی داشته باشد. تحقیق حاضر به‌منظور بررسی اثرات مکمل غذایی ویتامین C(۲۰ میلی‌گرم/کیلوگرم وزن بدن، ۴ هفته) بر برخی فراسنجه‌های وضعیت اکسیداتیو در خون ماهی کپور معمولی (*Cyprinus carpio*) انجام شد. نتایج نشان داد که فعالیت آنزیم های آنتی اکسیدان از جمله کاتالاز (CAT)، گلوتاتیون پراکسیداز (GPX) و سوپراکسید دیسموتاز (SOD) در حضور مکمل ویتامین C در مقایسه با گروه شاهد تفاوت معنی‌داری نداشته است. از سوی دیگر، مکمل ویتامین C باعث کاهش معنی‌داری در غلظت مالون دی آلدهید (MDA) گلبول‌های قرمز تا حدود ۲۶ درصد در مقایسه با گروه شاهد گردید. بر اساس نتایج این طرح به‌نظر می‌رسد مکمل غذایی ویتامین C قادر به محافظت از گلبول‌های قرمز ماهی کپور در برابر استرس اکسیداتیو با کاهش پراکسیداسیون چربی می‌باشد.
کلمات کلیدی: ویتامین C، آنزیم های آنتی اکسیدان، پراکسیداسیون چربی، ماهی کپور.