The effect of astaxanthin and cadmium on rat erythrocyte G6PD, 6PGD, GR, and TrxR enzymes activities in vivo and on rat erythrocyte 6PGD enzyme activity in vitro

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Abstract
In this study, the effects of astaxanthin (AST) that belongs to carotenoid family and cadmium (Cd), which is an important heavy metal, on rat erythrocyte G6PD, 6PGD, GR, and TrxR enzyme activities in vivo and on rat erythrocyte 6PGD enzyme activity in vitro were studied. In in vitro studies, 6PGD enzyme was purified from rat erythrocytes with 2′,5′-ADP Sepharose4B affinity chromatography. Results showed inhibition of enzyme by Cd at IC50: 346.5 μM value and increase of 6PGD enzyme activity by AST. In vivo studies showed an increase in G6PD, 6PGD, and GR enzyme activities (P < 0.05) and no chance in TrxR enzyme activity by AST. Cd ion inhibited G6PD, 6PGD, and GR enzyme activities (P < 0.05) and also decreased TrxR enzyme activity (P > 0.05). AST + Cd group G6PD enzyme activity was statistically low compared with control group (P < 0.05). 6PGD and TrxR enzyme activities decreased without statistical significance (P > 0.05); however, GR enzyme activity increased statistically significantly (P < 0.05).

1 | INTRODUCTION

Cadmium (Cd) is a naturally found heavy metal and is a serious risk factor for human health.1 Cd is used widely in industry and its high retention in biological systems makes it an important environmental pollutant.2 Environmental pollution by Cd is found in soil and in air due to combustion of coal and petrol and also leaks into aquatic systems.3 Human exposure to Cd is by metal industry, manufacturing of Cd–Ni batteries, ingestion of contaminated water and food sources, tobacco smoke and air pollution.4 Following such exposures serious alterations including nervous systems in human and animals, which cause various health hazards.5 Carotenoids form an important pigment group in nature with its diverse effects.6 Carotenoids are found in plants, algae, and microorganisms in high quantities. They are not highly produced in animals and human.7 About 600 carotenoids are identified from natural sources.8 Carotenoids also form the precursor of vitamin A and have important functions in health aspect.9 Consumption of carotenoid-rich fruits and vegetables is shown to reduce different cancer types, cardiovascular diseases, ocular diseases such as cataract, and enhance immune system.10,11 Major groups of carotenoids are lycopene, β-caroten, β-cryptoxanthin, zeaxanthin, and astaxanthin (AST).12 AST is one of the most common carotenoids and found in high amounts in crab, shrimp, and salmon.13 AST has antioxidant,14 anticancer,15 and anti-inflammatory16–18 properties apart from other biological activities.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49; G6PD) catalyzes first step of pentose phosphate pathway. In this reaction, NADPH is produced. This reaction and its byproducts protect red blood cells from oxidative damage.19–21 These reaction byproducts are NADPH and D-ribose 5-phosphate. NADPH produces reduced glutathione therefore protects cells from oxidative agents.22 NADPH is also a coenzyme that contributes to synthesis of some molecules such as fatty acids, steroids, and some amino acids.23 D-ribose 5-phosphate and its derivatives are components of DNA, ATP, CoA, NAD, FAD, and RNA.24

6-Phosphogluconate dehydrogenase (E.C.1.1.1.44; 6PGD) is an important enzyme for biological systems. It catalyzes second oxidative reaction of pentose phosphate pathway. It converts 6-phosphogluconolate to D-ribulose 5-phosphate in the presence of NADP+, and produces NADPH.25 Glutathione reductase enzyme (NADPH; GSSG oxidoreductase, EC 1.6.4.2) catalyzes electron transfer between low- and high-molecular-weight disulfide substrates and reduced pyridine nucleotides.26 Thioredoxin system, which is found
both in prokaryotes and eukaryotes, is composed of thioredoxin, thioredoxin reductase (TrxR, EC 1.6.4.5), and NADPH. Thioredoxin reductases are enzyme belonging to flavoprotein family including lipoamide dehydrogenase, glutathione reductase, and mercury ion reductase.\textsuperscript{[27,28]}

The aim of the present study is to eliminate the inhibitory effect of Cd, which possesses a crucial risk for the living things, on G6PD, 6PGD, GR, and TrxR enzyme activities of two intracellular major antioxidant systems (glutathione and thioredoxin) via AST.

2 \ MATERIALS AND METHODS

2.1 \ Chemicals

2‘, 5’-ADP Sepharose 4B, G6P, 6PGA, Tris, NADP\(^+\), protein assay reagent, NADPH, DTNB, standard serum albumin, electrophoresis chemicals were purchased from Sigma Chemicals. All other chemicals used were analytical grade and obtained from either Merck or Sigma (Germany).

2.2 \ Preparation of the hemolysate

Fresh rat blood samples were transferred into EDTA containing tubes. They were centrifuged for 15 min (2500 \( \times \) g) and plasma as well as leucocytes was removed. Packed red blood cells were washed three times with KCl solution (0.16 M). They were centrifuged in each repeat (2500 \( \times \) g) and supernatants were removed. Obtained erythrocytes were hemolyzed with distilled water (five times the volume of erythrocytes). To remove cell membranes, they were centrifuged at +4°C (10,000 \( \times \) g) for 30 min. Ghost and intact cells were removed and remaining supernatant layer was obtained as hemolysate.\textsuperscript{[29]}

2.3 \ 5’-ADP sepharose 4B affinity chromatography

For 10 mL column volume, two grams of dried 2‘, 5’-ADP Sepharose 4B gel was used. The gel was washed with distilled water (400 mL) to exterminate unwanted ingredients and air was eliminated from the swollen gel. The gel was soaked in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0) and then added into a small column (1 \( \times \) 10 cm) and equilibrated with 50 mL similar buffer. With the equilibration, buffer gel was washed and flow rates for washing and equilibration were adjusted to 50 mL/h with the aid of peristaltic pump. Previously obtained dialyzed sample was loaded on 2‘, 5’-ADP Sepharose 4B affinity column. The flow rate was set to 20 mL/h. Then the column was sequentially washed with 20 mL of 0.1 M K-acetate + 0.1 M K-phosphate (pH: 6.0) until the final absorbance difference became 0.05. Elution step was conducted with 80 mM K-phosphate + 80 mM KCl + 10 mM NADP\(^+\) + 10 mM EDTA (pH 7.5). In final fractions, enzyme activity was measured, and the activity-containing tubes were collected together. All of the experimental procedures were performed at 4°C.\textsuperscript{[30]}

2.4 \ Determination of activity of the enzymes

The activity of G6PD and 6PGD enzymes was measured spectrophotometrically at 340 nm Beutler’s method.\textsuperscript{[31]} The following equation was used in the calculation of activity.

\[
\text{Units (EU)/mL enzyme} = \frac{\left( \Delta A_{340 \text{nm}}/\text{min Test} - A_{340 \text{nm}/\text{min Blank}} \right) \times 100}{(6.22)(0.05)}
\]

Maximum absorbance of NADPH at 340 was used for GR enzyme activity. Following reaction catalyzed by GR enzyme, NADPH decreases. This decrease is determined with spectrophotometry at 340 nm and enzyme activity was determined.\textsuperscript{[32]} The following equation was used in the calculation of activity.

\[
\text{Units (EU)/mL enzyme} = \frac{\left( \Delta A_{340 \text{nm}/\text{min Test}} - A_{340 \text{nm}/\text{min Blank}} \right) \times 100}{(6.22)(0.05)}
\]

Thioredoxin reductase (TrxR) enzyme activity was determined with DTNB method. In this method, TrxR causes a NADPH-dependent reduction in disulfide bonds in DTNB.\textsuperscript{[28]} The following equation was used in the calculation of activity.

\[
\text{Units (EU)/mL enzyme} = \frac{\left( \Delta A_{412 \text{nm}/\text{min Test}} - A_{412 \text{nm}/\text{min Blank}} \right) \times 100}{(13.6)(2)(0.05)}
\]

1. total volume (in milliliters) of assay; df, dilution factor; 6.22, millimolar extinction coefficient of β-NADPH at 340 nm; 0.05, volume (in mL) of enzyme used.

2.5 \ Determination of protein

Quantitative protein determination was performed at 595 nm according to Bradford method. Bovine serum albumin solution was used as standard.\textsuperscript{[33]}

2.6 \ In vitro effect of AST and Cd

To assess the effect of AST and Cd on enzyme activity of 6PGD, five different AST concentrations (0.08, 0.16, 0.24, 0.32, and 0.48 mM) and five different Cd concentrations (50, 100, 200, 300, 500, and 750 μM) were added to purified enzyme containing tubes separately. IC\textsubscript{50} values (inhibitor concentration that reduces the total enzyme activity by half) were evaluated via drawing % Activity – [I] plots.

2.7 \ In vivo effect of AST and Cd

Thirty-two (250–300 g) male Wistar albino rat was obtained from Bingol University Experimental Research Facility. Animals were divided into four groups as; Control (i.p. isotonic saline), Cd (40 mg/L day), AST (20 mg/kg/day), Cd + AST (40 mg/L day + 20 mg/kg/day). Animals were kept in a room with constant temperature (20–22°C) and controlled light/dark cycle (12 h light/12 h dark). Water and food were provided ad libitum. Rats were kept in this setup for 1 week and following adaptation to these conditions experiments started. Twelve hours prior to experiments, food was ceased. At the end of 30th day, blood samples were obtained and prepared for experimental analysis.\textsuperscript{[34,35]}
Study was performed after acceptance of protocols by BUHADYEK (Date:21.02.2018/2018/2, Decision:02/05).

### 2.8 Analysis of kinetic data

For in vitro analysis, Microsoft Office Excel 2010 was used and results are given with standard error. For in vivo analysis, SPSS statistics 20 program was used. Results were analyzed with one-way ANOVA and post hoc Least Significant Difference test. $P < 0.05$ was accepted as statistically significant.

### 3 RESULTS

In this study, 6PGD enzyme was obtained from rat erythrocytes and purified with 2',5'-ADP Sepharose 4B affinity chromatography at 1.37 EU/mL specific activity, with 61.5% yield and 244.7 times purification. In vitro effects of AST and Cd on purified rat erythrocyte 6PGD enzyme activity were investigated. Obtained results show that Cd ion inhibits enzyme at IC$_{50}$; 346.5 μM value (Figure 1). On the other hand, AST increases 6PGD enzyme activity (Figure 2).

In vivo studies were conducted on rat erythrocyte G6PD, 6PGD, GR, and TrxR enzyme activities and effects of AST and Cd ions on their activities. Results show that AST increases G6PD, 6PGD, and GR enzyme activities; however, this increase did not exceed statistical significance ($P > 0.05$). No chance was observed in TrxR enzyme activity. On the other hand, Cd ion inhibited G6PD, 6PGD, and GR enzyme activities very significantly ($P < 0.05$) and also decreased TrxR enzyme activity ($P > 0.05$). Finally, when AST + Cd group was compared with control group for enzyme activity change, G6PD enzyme activity was significantly decreased ($P < 0.05$), 6PGD and TrxR enzyme activities were decreased without significance ($P > 0.05$), whereas GR enzyme activity was significantly increased ($P < 0.05$) (Figures 3–5).

### 4 DISCUSSION

AST is an antioxidant found in algae, fish, crustaceans, and birds.$^{[36]}$ AST has potent antioxidant activity shown by scavenging reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, singlet oxygen, and also inhibit in vitro lipid peroxidation.$^{[37]}$ Cd is toxic and has low excretion rate from body therefore is an important toxic metal.$^{[38]}$ One of the most important effects of heavy metals such as Cd is oxidative stress.$^{[39]}$ Oxidative stress is caused by excessive ROS production exceeding cellular antioxidant defense.$^{[40,41]}$
\textbf{CONCLUSION}

Our study shows an inhibitory effect of Cd on G6PD, 6PGD, GR, and TrxR enzyme activities and AST, which has an antioxidant nature, was effective on reversing this inhibitory effect.

\section*{CONFLICTS OF INTEREST}

The authors declare that there is no conflict of interest.

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\section*{REFERENCES}


