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Effect of Ascorbic Acid on Copper-induced Oxidative Changes in Erythrocytes of Individuals with a Glucose-6-phosphate Dehydrogenase Deficiency

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Considerable interest has focused on the potential health implications of consumption of drinking water with elevated levels of copper due to corrosion of copper household pipes. Levels of copper in excess of several ppm in drinking water are not uncommon in communities with corrosive water and where households have copper pipes (CALABRESE & TUTHILL 1978). Human population studies have revealed that consumption of drinking water with copper levels in excess of 1 ppm can lead to elevated tissue levels of copper (SCHROEDER et al. 1966). Several studies (MANSLER & SCHREINER 1976; IVANOVICH et al. 1969) have revealed that elevated copper levels (0.5-0.8 ppm) in dialysis fluid have caused acute hemolysis in those with no known enzymatic deficiencies of red blood cell metabolism. Whether high levels of copper in drinking water presents a potential human health hazard is generally an unexplored question since the prime basis for limiting exposure to copper in drinking water has been for taste rather than health considerations (CALABRESE 1978). However, due to the presence of elevated copper levels in drinking water in many communities, especially in the eastern coastal area of the U.S.A., and the recognition that copper may present a potent oxidant stress, further evaluation of its effects on animal and human systems is warranted.

In addition to enhanced copper exposure via drinking water, many individuals consume ascorbic acid in large amounts (i.e. up to several grams per day). While daily consumption of such amounts of ascorbic acid are not believed to be harmful to most segments of the general public, there is a growing recognition that certain subgroups within the general population may display enhanced sensitivity to ascorbic acid. More specifically, several reports have indicated that persons with an erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) deficiency display enhanced susceptibility to ascorbic acid-induced hemolytic changes (CAMPBELL et al. 1975; UDOMRATN et al. 1977). In fact, as little as 1.5 g/day has been found to produce mild hemolysis in G-6-PD deficient individuals (BEUTLER 1978), while massive amounts have been known to be lethal (UDOMRATN et al. 1977). Investigations in our laboratory have revealed that G-6-PD deficient human erythrocytes display enhanced hemolytic sensitivity to cupric acetate (CALABRESE et al. 1980). More recent studies have revealed that ascorbic acid markedly enhances the occurrence of copper acetate induced pre-hemolytic changes [i.e. increases in methemoglobin (METHB) formation and decreases in reduced glutathione (GSH)] in normal human erythrocytes in vitro (CALABRESE et al. 1982). The

present paper presents the results of an investigation to determine whether ascorbic acid incubation enhances copper acetate-induced pre-hemolytic changes in G-6-PD deficient erythrocytes in vitro as previously reported for normal humans.

METHODS AND MATERIALS

Preliminary testing established a concentration (i.e. 3 mM) of cupric acetate which produced evidence of an oxidant stress such as an approximate 60% decrease in levels of reduced glutathione (GSH) and/or an increase of 25% in methemoglobin (METHB). This amount of cupric acetate was administered in 10 microliter aliquots per milliliter of whole blood. This amount of blood and oxidant was then incubated with increasing levels of ascorbic acid (1.0 - 9.0 mM) for a two hour exposure at 37°C. An incubated control with no ascorbic acid was also employed. Additional controls employing ascorbic acid (1.0 - 9.0 mM) in the absence of cupric acetate were run to assess the potential confounding of the ascorbic acid. Previous experimentation in our laboratory revealed that the acetate ion had no statistically significant ($P > 0.05$) effect on METHB and GSH levels (CALABRESE et al. 1980).

Blood samples from three G-6-PD deficient humans (Mediterranean variant) were collected via venipuncture in heparinized tubes in the morning, held in an ice bath, and used on the day of collection. The hematological parameters measured were selected primarily on the basis of their being widely accepted indicators of oxidative stress. The parameters included: methemoglobin (METHB) and levels of reduced glutathione (GSH). METHB was measured according to the method by BROWN (1973) using potassium ferricyanide and potassium cyanide as reagents and measuring changes in optical density at 630 nm. A colorimetric reaction employing 5,5'-dithiobis(nitrobenzoic acid) (DTNB) was used to measure the amount of GSH in blood according to PRINS & LOOS (1969) at 412 nm.

STATISTICAL ANALYSIS

The data were analyzed via ANOVA to determine the significance of ascorbic acid over the range of concentrations used. If significance was found by ANOVA, a Tukey-Kramer Multiple Comparison of Means was subsequently performed. In the Tukey-Kramer evaluation as seen in Tables 1-4, the solid lines connecting adjacent levels of ascorbic acid indicate a statistical similarity or lack of significant differences between their mean values.

RESULTS

Cupric acetate incubation with no ascorbic acid additions

Table 1. The Effects of Ascorbic Acid[1] on Copper[2]-Induced Methemoglobin[3] in Erythrocytes of G-6-PD Deficient Humans

	Control	1 mM	3 mM	5 mM	7 mM	9 mM
Treatment Group	1	2	3	4	5	6
Sample Size	3	3	3	3	3	3
Mean	24.30	61.62	76.86	74.42	70.20	68.53
Standard Deviation	9.57	17.26	10.23	20.72	17.52	17.50

[1]Ascorbic acid additions made at five levels of increasing concentration.

[2]Copper additions made as 3 mM of cupric acetate.

[3]Methemoglobin measured in relative percent to normal hemoglobin.

ANALYSIS OF VARIANCE

Source of Variation	S.S.	d.f.	M.S.	F
Between Groups	5712.830	5	1142.566	4.46**
Within Groups	3073.611	12	256.134	
Total	8786.441	17		

**Significant at the 0.025 level

Tukey-Kramer Multiple Comparison at the 0.05 level

Control	1 mM	3 mM	5 mM	7 mM	9 mM
24.30	61.62	76.86	74.42	70.20	68.53

Table 2. The Effects of Ascorbic Acid[1] on Methemoglobin[2] in Erythrocytes of G-6-PD Deficient Humans

	Control	1 mM	3 mM	5 mM	7 mM	9 mM
Treatment Group	1	2	3	4	5	6
Sample Size	3	3	3	3	3	3
Mean	4.86	4.70	4.69	4.57	4.56	5.18
Standard Deviation	1.25	1.44	1.59	1.70	1.49	1.67

[1]All levels incubated in absence of oxidant stressor.

[2]Methemoglobin measured in relative percent to normal hemoglobin.

ANALYSIS OF VARIANCE

Source of Variation	S.S.	d.f.	M.S.	F
Between Groups	0.815	5	0.163	0.07**
Within Groups	28.111	12	2.343	
Total	28.926	17		

**Not significant at the 0.025 level

Tukey-Kramer Multiple Comparison at the 0.05 level

Control	1 mM	3 mM	5 mM	7 mM	9 mM
4.86	4.70	4.69	4.57	4.56	5.18

Table 3. The Effects of Ascorbic Acid[1] on Reduced Glutathione[2] of G-6-PD Deficient Human Erythrocytes Incubated with Copper[3]

	Control	1 mM	3 mM	5 mM	7 mM	9 mM
Treatment Group	1	2	3	4	5	6
Sample Size	3	3	3	3	3	3
Mean	21.32	8.39	8.98	10.16	14.02	15.29
Standard Deviation	5.51	2.63	1.30	0.16	4.24	4.02

[1]Ascorbic acid additions made at five levels of increasing concentration.

[2]Reduced glutathione measured in mg per 100 mL RBC.

[3]Copper additions made as 3 mM of cupric acetate.

ANALYSIS OF VARIANCE

Source of Variation	S.S.	d.f.	M.S.	F
Between Groups	362.894	5	72.579	5.95**
Within Groups	146.284	12	12.190	
Total	509.178	17		

**Significant at the 0.01 level

Tukey-Kramer Multiple Comparison at the 0.05 level

Control	1 mM	3 mM	5 mM	7 mM	9 mM
21.32	8.39	8.98	10.16	14.02	15.29

Table 4. The Effects of Ascorbic Acid[1] on Reduced Glutathione[2] of G-6-PD Deficient Human Erythrocytes

	Control	1 mM	3 mM	5 mM	7 mM	9 mM
Treatment Group	1	2	3	4	5	6
Sample Size	3	3	3	3	3	3
Mean	53.49	49.50	43.33	41.38	38.75	36.75
Standard Deviation	8.42	10.35	14.16	16.54	18.75	20.40

[1]All levels incubated in the absence of an oxidant stressor.

[2]Reduced glutathione measured in mg per 100 mL RBC.

ANALYSIS OF VARIANCE

Source of Variation	S.S.	d.f.	M.S.	F
Between Groups	623.099	5	124.620	0.53**
Within Groups	2839.985	12	236.665	
Total	3463.084	17		

**Not significant at the 0.05 level

Tukey-Kramer Multiple Comparison at the 0.05 level

Control	1 mM	3 mM	5 mM	7 mM	9 mM
53.49	49.50	43.33	41.38	38.75	36.75

at the 3 mM level resulted in a 24.30% METHB value (Table 1), as compared to 4.86% METHB for the incubated controls (Table 2). The ascorbic acid additions to the copper treated red cells significantly ($P < 0.025$) increased the formation of METHB, with 3 mM being the most effective concentration (Table 1). However, by themselves, these ascorbic acid additions did not affect METHB formation (Table 2). Incubation with cupric acetate alone resulted in a 60% decrease in availability of GSH. Ascorbic acid additions either alone or with the copper treatment induced a non-dose-dependent trend toward somewhat lower GSH values (Tables 3, 4). These trends were statistically insignificant ($P > 0.05$) in the Tukey-Kramer comparison.

DISCUSSION

The ascorbic acid incubation enhanced the occurrence of copper acetate induced METHB formation and decreases in GSH in G-6-PD deficient erythrocytes. These findings are consistent wtih previous studies of CALABRESE et al. (1982) with normal human red cells. However, the G-6-PD deficient red cells responded in a more sensitive manner, especially at the lower concentrations of ascorbic acid employed. Research with animal models revealed that ascorbic acid incubation also markedly enhanced copper acetate induced hemolytic changes in Dorset sheep, an animal model with an erythrocyte G-6-PD deficiency, but not in Sprague-Dawley rats (CALABRESE et al. 1982a).

It should be emphasized that the level of copper employed here (i.e. 3 mM) is considerably greater than normally present in plasma being on the order of 15-30 times greater than normal (BOULARD et al. 1975; CALABRESE et al. 1980), while the lowest level of ascorbic acid employed (i.e. 1.0 mM) is about 10 times greater than normal, and five times greater than in persons consuming three to five grams of ascorbic acid per day (ANGEL et al. 1975). Subsequent studies should be designed to assess the demonstrated association of ascorbic acid and copper at more realistic levels of exposure.

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