Bull. Environm. Contam. Toxicol. 29, 704–710 (1982)

Ascorbic Acid Enhances the Occurrence of Copper-induced Methemoglobin Formation in Normal Human Erythrocytes *in vitro*

Edward J. Calabrese, Gary S. Moore, and Mark S. McCarthy

Division of Public Health, University of Massachusetts, Amherst, MA 01003

Consumption of large amounts (i.e. up to several grams/day) of ascorbic acid is not uncommon. While daily ingestion of such quantities of ascorbic acid are not thought to be harmful to the general public, there is a growing awareness that certain subgroups within the population may be uniquely sensitive to ascorbic acid. For example, 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 addition, consumption of large amounts of ascorbic acid has been shown to enhance the absorption of inorganic and organic mercury in guinea pigs (BLACKSTONE et al. 1975) as well as cause significant mutagenic action when jointly administered with Cu^{2+} (STICH et al. 1976). The present study attempted to further evaluate the extent of ascorbic acid-copper toxicological interactions by assessing whether ascorbic acid administration affected the occurrence of copper-induced oxidative stress in human erythrocytes in vitro.

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 30% in methemoglobin (METHB). This amount of cupric acetate was administered in 10 µL aliquots per mL 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 h exposure at 37C. 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 six normal humans 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 METHB and levels of GSH. METHB was measured according to the method by BROWN (1973) using potassium ferricyanide and potassium cyanide

0007-4861/82/0029-0704 $01.40

as reagents and measuring changes in optical density at 630 nm. A colorimetric reaction employing 5,5'-dithiobisnitrobenzoic 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 and 2, 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 at the 3 mM level resulted in a 28.36% METHB value (Table 1), as compared to 1.88% METHB for the incubated controls (Table 2). The ascorbic acid additions to the copper treated red cells significantly ($P < 0.001$) 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 52% decrease in the availability of GSH. Ascorbic acid additions either alone or with the copper treatment were not shown to exhibit any statistically significant effect on copper acetate induced decreases in GSH, although there was a non-dose-dependent trend toward somewhat lower GSH values in the ascorbic acid treated groups (Tables 3 and 4).

DISCUSSION

That ascorbic acid may enhance the occurrence of copper acetate induced METHB formation may be explained by its autoxidation in the presence of the copper (II) ion (HALLIWELL & FOYER 1976). Detection of a complex intermediate between copper and ascorbic acid in the oxidation process provide some viability to the above theoretical explanation (HAYAKAWA & HAYASHI 1977). On the practical side, 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. 0.1 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. Finally, it should be noted that erythrocyte copper levels are significantly higher in blood of certain subgroups within the population such as persons with sickle cell trait and disease (SCHAEFFER et al. 1968), and their enhanced peroxidative red cell membrane damage compared to normal

individuals has been attributed to the excess copper (DAS & NAIR 1980). In addition, elevated levels of copper in tissues are not uncommon in areas where the water is corrosive and the households use copper piping (CALABRESE & MOORE 1979). Whether consumption of mega-doses of ascorbic acid would provide an enhanced oxidative/hemolytic risk to such subgroups in the population with enhanced copper levels is unknown, but of public health interest.

Table 1. The Effects of Ascorbic Acid[1] on Copper[2] Induced Methemoglobin[3] in Erythrocytes of Normal Humans

	Control	1 mM	3 mM	5 mM	7 mM	9 mM
Treatment	1	2	3	4	5	6
Sample Size	6	6	6	6	6	6
Mean	28.36	45.80	66.36	61.03	56.54	55.96
Stand. Dev.	3.85	6.89	7.71	8.89	8.22	7.72

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 of normal hemoglobin.

Analysis of Variance

Source of Variation	S.S.	d.f.	M.S.	F
Between Groups	5524.931	5	1104.986	20.21**
Within Groups	1640.180	30	54.673	
Total	6165.11	35		

**Significant at the 0.001 level

Tukey-Kramer Multiple Comparison at the 0.05 level

Control	1 mM	9 mM	7 mM	5 mM	3 mM
28.36	45.80	55.96	56.54	61.03	66.36

Tukey-Kramer Multiple Comparison at the 0.01 level

Control	1 mM	9 mM	7 mM	5 mM	3 mM

Table 2. The Effects of Ascorbic Acid[1] on Methemoglobin[2] in Erythrocytes of Normal Humans

	Control	1 mM	3 mM	5 mM	7 mM	9 mM
Treatment Group	1	2	3	4	5	6
Sample Size	6	6	6	6	6	6
Mean	1.88	1.61	1.71	1.47	1.47	1.61
Stand. Dev.	0.48	0.36	0.23	0.40	0.17	0.43

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	0.725	5	0.145	1.10**
Within Groups	3.950	30	0.132	
Total	4.675	35		

**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
1.88	1.61	1.71	1.47	1.47	1.61

Table 3. The Effects of Ascorbic Acid[1] on Reduced Glutathione[2] of Normal 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	6	6	6	6	6	6
Mean	33.61	25.63	26.18	28.38	26.06	27.63
Stand. Dev.	3.02	5.49	6.51	5.21	7.61	5.38

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	266.798	5	53.360	1.64**
Within Groups	976.388	30	32.546	
Total	1243.186	35		

**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
33.61	25.63	26.18	28.38	26.06	27.63

Table 4. The Effects of Ascorbic Acid[1] on Reduced Glutathione[2] of Normal Human Erythrocytes

	Control	1 mM	3 mM	5 mM	7 mM	9 mM
Treatment Group	1	2	3	4	5	6
Sample Size	6	6	6	6	6	6
Mean	69.08	69.21	62.49	63.44	64.82	66.33
Stand. Dev.	4.01	3.06	1.97	2.60	2.05	2.61

1. All ascorbic acid 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	240.491	5	48.098	6.15**
Within Groups	234.819	30	7.827	
Total	475.310	35		

**Significant at the 0.001 level

Tukey-Kramer Multiple Comparison at the 0.05 level

Control	1 mM	3 mM	5 mM	7 mM	9 mM
69.08	69.21	62.49	63.44	64.82	66.33

REFERENCES

ANGEL, J., B. ALFRED, J. LEICHTER, M. LEE, L. MARCHANT: Internat. J. Vit. Nutr. Res. 45:237 (1975).

BLACKSTONE, S., R.J. HURLEY, R.E. HUGHES: Food Cosmet. Toxicol. 12:511 (1974).

BOULARD, M., K.G. BLUME, E. BEUTLER: J. Clin. Invest. 51:456 (1975).

BROWN, B.P.: Hematology: Principles and Procedures. 1st ed. Lea and Febiger, Phil. (1973).

CALABRESE, E.J., G.S. MOORE: Medical Hypotheses 5:493 (1979).

CALABRESE, E.J., G.S. MOORE, S.L. HO: Environ. Res. 21:366 (1980).

CAMPBELL, G.D. Jr., M.H. STEINBERG, J.D. BOWER: Ann. Intern. Med. 22:810 (1975).

DAS, S.K., R.C. NAIR: Brit. J. Haematol. 44:87 (1980).

HALLIWELL, B., C.H. FOYER: Biochem. J. 155:697 (1976).

HAYAKAWA, K., Y. HAYASHI: J. Nutrit. Sci. Vitaminol. 23:395 (1977).

PRINS, H.K., J.A. LOOS: Glutathione. In: Biochemical Methods in Red Cell Genetics. J.J. Yunis (ed.) Academic Press, N.Y. p. 126. (1968).

SCHAEFFER, K., J.A. LOFTON, S.C. POWELL, H.H. OSBORNE, L.H. FOSTER: Proc. Soc. Exp. Biol. Med. 128:734 (1968).

STICH, H.F., J. KARIM, J. KOROPATRICK, L. LO: Nature 260:727 (1976).

UDOMRATN, T., T.M. STEINBERG, G.D. CAMPBELL, Jr., F.J. OELSHLEGEL, Jr.: Blood 49:471 (1977).

Accepted September 16, 1982