Biochimica et Biophysica Acta, 550 (1979) 279—287
© Elsevier/North-Holland Biomedical Press

BBA 78239

THE EFFECT OF COPPER ON ERYTHROCYTE DEFORMABILITY

A POSSIBLE MECHANISM OF HEMOLYSIS IN ACUTE COPPER INTOXICATION

KIRKWOOD F. ADAMS, GEORGE JOHNSON, Jr., KENNETH E. HORNOWSKI and THOMAS H. LINEBERGER

Department of Surgery, Division of General Surgery (Vascular, Trauma, Transplantation), University of North Carolina at Chapel Hill, School of Medicine, Chapel Hill, NC 27514 (U.S.A.)

(Received May 9th, 1978)

Key words: Copper intoxication; Hemolysis; Deformability; (Erythrocyte)

Summary

Although the development of hemolytic anemia as a complication of acute copper intoxication is well documented, the precise mechanism by which copper produces accelerated erythrocyte destruction is unknown. Normal erythrocyte survival depends in part on the ability of the cell to deform and pass through narrow areas of microcirculation in the liver and especially in the spleen. In the present study, it is demonstrated that toxic concentrations of copper rapidly and markedly reduce erythrocyte deformability. This reduction in cell deformability is associated with a marked increase in membrane permeability and osmotic fragility of copper-treated cells. Further, the decrease in deformability occurs despite normal levels of cell ATP and the apparent absence of oxidative damage to the cell. These observations indicate that copper-mediated changes in the erythrocyte membrane may be responsible for reducing the flexibility of the cell. The loss of deformability could act to reduce erythrocyte survival and thus explain the hemolysis associated with copper intoxication in vivo.

Introduction

Although the development of hemolytic anemia as a complication of acute copper intoxication is well-documented, the precise mechanism by which copper produces accelerated erythrocyte destruction is unknown [1—3]. In toxic concentrations, copper has several deleterious effect on the erythrocyte. It acts as a potent oxidant as evidenced by its ability to decrease the cell content of the reducing agent, glutathione [3]. Hemolysates prepared from

cells incubated with copper show a reduction in the activity of several glycolytic enzymes [4]. Finally, excessive amounts of copper increase the permeability of the erythrocyte membrane to potassium [5].

Though the relationship between these toxic effects and the hemolysis caused by copper has not been established, each one could act to reduce cell deformability. Sufficient oxidative challenge could result in hemoglobin denaturation and the formation of rigid cells with Heinz bodies [6]. A reduction in the activity of enzymes involved in the glycolytic cycle could prevent the maintenance of sufficient adenosine triphosphate to preserve membrane flexibility [7]. A change in membrane permeability could produce changes in cell shape which render the cell less deformable [8].

If excess copper significantly reduces erythrocyte deformability, then this would help explain the accelerated cell destruction caused by this ion. A reduction in erythrocyte deformability is a cause of shortened cell survival in a number of hemolytic anemias [9—12]. This study attempts to determine of toxic concentrations of copper do reduce erythrocyte deformability as measured by erythrocyte filterability. The relationship between the known effects of copper on the erythrocyte and changes in deformability was investigated by concurrent study of cell ATP, cell volume control and hemoglobin oxidation.

Methods

Blood was collected in heparinized syringes from a pool of young, healthy human donors. After collection, the blood was immediately centrifuged at $1200 \times g$ for 8 min. Following centrifugation, the plasma and buffy coat were removed from the cells. The cells were then washed three times with a NaCl-Tris buffer (280 osM, pH 7.40) containing 50 mg albumin/l. Following the final wash, the buffer was removed and packed cells were added to an incubation medium containing 145 mM NaCl, 12 mM Tris, pH 7.40. Glucose at a concentration of 6 mM/l and potassium chloride at a concentration of 5 mM/l were also present in the medium. Concentrated Na_2SO_4 or $CuSO_4$ was added to complete the control or experimental media. These cell suspensions were incubated for varying periods of time in an oscillating water bath at 37° C.

Erythrocyte filterability was measured using 25-mm diameter polycarbonate membranes (Nuclepore Filtration Products, Pleasanton, California) with a mean pore diameter of 5 μm and a total porosity of $4 \cdot 10^5$ [14]. The membranes were cleaned prior to filtration by sonication for 1—2 min in a Heat Systems Ultrasonic device. Buffer flow rates were determined on each filter prior to use. Those filters having buffer flow rates from 1.5 to 1.8 s/ml were selected for further use. In order to permit repeated use of individual filters, all solutions used in washing and filtering the cells were double-filtered through a 0.22 μm Millipore filter prior to contact with the cells and filters. Samples for the study of erythrocyte deformability were taken directly from the incubation media and diluted to an hematocrit of 0.5% with the double-filtered NaCl-Tris buffer solution. A 10 ml sample of the dilute erythrocyte suspension was filtered at room temperature with no external force other than gravity. The timing was started following the passage of 1 ml of the sample. The time in seconds

required for passage of the cell suspension was determined at volumes of 2, 4, 6, and 8 ml of collected filtrate. Erythrocyte filterability index was quantitated as the slope of the linear plot of time in seconds versus filtered volume recorded in s/ml.

Hemoglobin derivatives were determined using the method of Evelyn and Malloy [17]. Cell samples obtained at different periods of incubation were hemolyzed in M/60 phosphate buffer (pH 6.6) for determination of oxy-, met-, and sulfhemoglobin. The absorbance at 620 nm was measured on a Beckman spectrophotometer, Model 25, to determine sulfhemoglobin. In calculating the amount of sulfhemoglobin present, a correction for the absorbance of oxy- and methemoglobin at 620 nm was employed [18]. Oxyhemoglobin was determined at 540 nm. Erythrocyte ATP was determined by the luciferin-luciferase assay utilizing a liquid scintillation counter [19]. The firefly enzyme was aged overnight in distilled water to reduce background activity. A standard curve was determined prior to running each sample batch. A log-log plot of counts versus concentration of standard was used to give the best fit for the standard curve.

Osmotic fragility was determined by the method of Dacie [20]. The incubated cells were suspended in varying concentrations of buffered NaCl at room temperature for 30 min. The tubes were then centrifuged at $1200 \times g$ for 5 min. The supernatant was removed and its absorbance determined at 545 nm in a Beckman spectrophotometer. The supernatant from the tube containing 0.85 g % of NaCl was used as the blank. The supernatant from the tube containing 0.10 g % NaCl was taken as complete hemolysis. Osmotic fragility curves were constructed by plotting the percent hemolysis versus the sodium chloride concentration. Deoxygenation of cell samples was achieved by gassing the cell suspensions with a humidified 95% nitrogen/5% carbon dioxide mixture (flow rate 0.11/min) during the 3-h incubation.

Ghosts were prepared from washed erythrocytes using a hypotonic lysis method. 35 ml of a hypotonic buffer solution (1 mM Tris in 19 mM NaCl, pH 7.40) were added to 3 ml of packed erythrocytes. The lysed cells were centrifuged at 15 000 rev./min for 20 min at 4°C. After spinning, the buffer was removed and the ghosts were washed again in the hypotonic buffer. The ghosts were then centrifuged as above and the buffer removed. The ghosts were then resuspended in the filtration buffer to an hematocrit of 20. The ghost suspension was divided into three parts and concentrated $CuSO_4$ or Na_2SO_4 was added. After an incubation period of 30 min, each suspension was filtered through an 8 μm Nuclepore membrane. A repeat hematocrit was determined and following resuspension to an hematocrit of 0.5%, a filterability index was determined as described above.

The mean volume of cells obtained following incubation was determined using a Coulter Counter S system.

Erythrocyte sodium and potassium were determined with a flame photometer (Instrument Laboratory, Cambridge, Massachusetts, Model 143.). The cells were washed three times in 6 vols. cold 0.110 M MgCl to remove extracellular sodium and potassium. The results are expressed as mequiv. cation/100 ml erythrocytes. The hematocrits used for calculating these results were not corrected for trapped wash solution.

Light microscopy was performed on samples following incubation by resuspension in native plasma or buffer and observation using a glass slide and cover slip.

All values are reported as mean ± S.D. with n indicating the number of determinations.

Results

To evaluate the effects of copper on erythrocyte deformability, filtration studies were conducted on normal erythrocytes incubated with two concentrations of copper sulfate similar to those found in acute copper intoxication [23, 24]. To rule out the effects of sulfate ion, parallel incubations were performed using sodium sulfate. Within 3 h, copper produced a dramatic reduction in erythrocyte filtration rate. At copper concentrations of 0.24 mM and 0.60 mM the filtration times were 5.4 ± 1.9 s/ml (n = 10) and 42.1 ± 9.6 s/ml (n = 11) respectively. The filtration times differed significantly ($P < 0.001$) from the control filtration time of 2.1 ± 0.3 s/ml (n = 15).

The relationship between erythrocyte filtration and the oxidant action of copper was investigated by two tactics. First, the effect of copper on hemoglobin stability was determined by measuring the amount of oxy-, met-, and sulfhemoglobin present in the cells after varying periods of incubation. After 3 h of incubation, methemoglobin was present in small amounts only at the highest concentration of copper. At this time, there was no sulfhemoglobin present at any copper concentration. After 6 h of incubation, methemoglobin was evident at the lower copper concentration. Sulfhemoglobin had accumulated to a significant degree at both copper concentrations by 24 h of incubation. No significant accumulation of met- or sulfhemoglobin occurred in cells

TABLE I

EFFECT OF COPPER SULFATE AND SODIUM SULFATE ON HEMOGLOBIN STABILITY

	Na_2SO_4 (6.0×10^{-4} M)	$CuSO_4$ (2.4×10^{-4} M)	$CuSO_4$ (6.0×10^{-4} M)
Oxyhemoglogin			
T_0 *	98.3 **	97.3	97.8
T_3	96.9	97.6	90.9
T_6	98.0	86.4	70.7
T_{24}	97.3	76.8	46.3
Methemoglobin			
T_0	0.0	0.4	1.2
T_3	0.0	1.1	6.7
T_6	0.0	12.0	27.0
T_{24}	0.0	16.8	43.8
Sulfhemoglobin			
T_0	1.7	2.3	1.0
T_3	3.1	1.3	2.4
T_6	2.0	1.6	2.3
T_{24}	2.7	6.4	9.9

* T = time of incubation in hours.

** Each hemoglobin derivative expressed as percent of total hemoglobin.

incubated with sodium sulfate even after 24 h. These results are summarized in Table I.

The second tactic used to study the relation between hemoglobin oxidation and reduced cell filtration involved incubations performed after gassing the cell suspension with nitrogen. Despite the absence of oxygen, following these incubations the filtration times at copper concentrations of 0.24 mM and 0.60 mM rose to 5.7 ± 2.6 s/ml (n = 4) and 144.7 ± 37.5 (n = 4), respectively. These filtration times were significantly prolonged ($P < 0.02$) when compared to control filtration time of 3.1 ± 0.9 s/ml (n = 10). The effect of copper on cell volume control was examined by measuring the osmotic fragility of cells incubated with copper or sodium sulfate. Erythrocytes normally have a cell volume which allows for an excess in surface area compared to that required to merely contain the cell volume. The amount of excess surface area may be estimated for normal cells by plotting an osmotic fragility curve. This is obtained by measuring the degree of hemolysis occurring at various concentrations of sodium chloride. Compared to control cells, copper-treated cells have an increase in osmotic fragility and a corresponding shift to the left in the fragility curve (Fig. 1).

An increase in osmotic fragility occurs if the erythrocyte gains cations. An attempt was therefore made to study cell cation changes following copper incubation. Due to the extreme osmotic fragility of cells incubated with copper, there was appreciable lysis of erythrocytes during the washing procedure prior to determination of the cation content. This was reflected by changes in the osmotic fragility and cell volume determinations after wahing cells incubated with 0.60 mM copper sulfate. The mean cell volume declined from 92 to 87 and the osmotic fragility curve shifted toward normal following the washing procedure. Although the lysis of cells prevented an exact determination of cell cation content, cells incubated for 3 h with 0.60 mM copper sulfate NAD 37 ± 4 mequiv./l erythrocyte sodium (n = 4) and 55 ± 7 mequiv./l erythrocyte potassium. This compares with a sodium content of 15 ± 1 mequiv./l erythrocytes (n = 4) and potassium content of 82 ± 2 mequiv./l erythrocytes found in cells incubated with 0.24 mM copper sulfate. These cation concentrations differed markedly from control cells which had a sodium content of 5.9 ± 0.3 mequiv./l erythrocytes (n = 4) and potassium content of 89 ± 3 mequiv./l erythrocytes (n = 4).

Further study of the effect of copper on cell volume control was obtained by measuring the mean cell volume and observing the microscopic appearance of cells following copper incubation. The mean cell volume of cells exposed to 0.24 mM and 0.60 mM copper sulfate were 93 ± 6.9 μm^3 (n = 3) and 95 ± 7.2 μm^3 (n = 3), respectively. These mean cell volumes were increased over a control valve of 89 ± 6.6 μm^3 (n = 3). Results of light microscopic studies of in native plasma showed that 75% of cells incubated at either copper concentration became spheroecchinocytes while control cells maintained a normal biconcave shape. After 3 h of incubation less than 1% cell agglutination was present under filtration conditions at either copper concentration.

Copper has been shown to inhibit several enzymes of the glycolytic cycle in the erythrocyte [4]. The effect of the decreased enzyme activity on cell ATP levels was not determined. Studies of cell ATP concentrations following 3 h of

TABLE II

EFFECT OF COPPER SULFATE AND SODIUM SULFATE ON ERYTHROCYTE ATP

Values reported as mean with standard deviation. n indicates the number of individual tests performed. Samples were obtained after 3 h of incubation. Copper samples did not differ significantly from control.

Additives	ATP (mM/l erythrocytes)	n
Na_2SO_4 (6.0×10^{-4} M)	1.6 ± 0.5	5
$CuSO_4$ (2.4×10^{-4} M)	1.8 ± 0.4	4
$CuSO_4$ (6.0×10^{-4} M)	2.4 ± 0.5	5

incubation revealed no reduction in ATP levels in cells exposed to copper (Table II).

The direct effect of copper on membrane rigidity was examined by measuring the flow characteristics of ghosts prepared with copper or sodium sulfate. These filterability studies indicated that ghosts prepared with sodium sulfate exhibited a filtration time of 3.4 ± 0.5 s/ml (n = 4). Ghosts prepared with 0.24 mM copper sulfate had a somewhat slower filtration time of 4.0 ± 0.9 s/ml (n = 4). When prepared with 0.60 mM copper sulfate the filtration time rose to 7.5 ± 2.3 s/ml (n = 4), which differed significantly from control ($P < 0.0025$).

Discussion

The studies of Burton and Rand [13] demonstrated that the normal erythrocyte is an exquisitely deformable cell that is capable of passage through channels much smaller than its diameter. It was soon appreciated that this extreme deformability was one requirement for normal cell survival. According to current thinking, one mode of erythrocyte destruction involves the selective entrapment of cells with reduced deformability in the liver and especially the spleen. Entrapment occurs due to the inability of the cells to bend and change shape sufficiently to pass through the exacting microcirculation occurring in certain areas of these organs. Cells trapped in this manner are subject to destruction by metabolic depletion, membrane alteration, and phagocytosis. The presence of large numbers of poorly deformable erythrocytes causes increased cell destruction and frequently results in a hemolytic anemia. Evidence for the central role of reduced erythrocyte deformability in the pathogenesis of hemolytic anemia has been obtained from studies of sickle cell disease [9], hereditary spherocytosis, and spur cell anemia of liver disease [12].

Erythrocyte deformability may be determined by several methods. In this study, deformability was assayed with a Nuclepore filtration system [14]. Similar filtration systems have demonstrated abnormal cell flexibility in a number of different hemolytic states. Investigators using Nuclepore filters have been able to correlate reduced cell filtration rates with rapid cell destruction and selective entrapment of rigid cells of the spleen [12]. Using a Nuclepore filtration system, this study establishes that toxic concentrations of copper reduce erythrocyte deformability. It seems likely that the ability of copper to

reduce cell deformability plays a significant role in accelerating erythrocyte destruction following copper intoxication.

Normal erythrocyte deformability depends on the existence of hemoglobin in a soluble form within the cell interior. Earlier studies of erythrocytes incubated for prolonged periods with toxic concentrations of copper demonstrated oxidative damage and denaturation of hemoglobin [25]. Subsequent studies have shown that copper acts as an oxidant stress which in sufficient concentration simply overwhelms the reducing capacity of the erythrocyte [3]. This allows the oxidation and precipitation of hemoglobin to occur. On the basis of these studies, it was proposed that oxidative damage and precipitation of hemoglobin led to rigid erythrocytes and the severe hemolysis associated with copper intoxication.

This study was initiated because of the demonstration that heavy metals interfere with normal erythrocyte membrane properties [7]. Since copper is rapidly accumulated by the erythrocyte [27], this suggested that damage to the cell membrane and deformability might occur before oxidative damage to hemoglobin developed.

In order to test this possibility, deformability was measured after brief incubation of erythrocytes with toxic concentrations of copper. These determinations revealed a profound reduction in deformability despite only short exposure to copper. Examination of hemoglobin after this period of incubation revealed no evidence of oxidative damage. In addition, similar incubations in a nitrogen/carbon dioxide atmosphere still resulted in a highly significant decrease in cell deformability. The results of these experiments point to major toxic effects of copper independent of its oxidant properties. The exact mechanism by which copper disrupts normal cell deformability was not determined by this study. Measurement of ATP, a key metabolite required for normal membrane flexibility demonstrated no deficit in copper-treated cells.

Examination of two other erythrocyte properties did give clues to the mechanisms by which copper caused a reduction in cell deformability. Normal erythrocytes enjoy an excess of surface area in relation to the volume of the cell. This allows extreme cell flexibility by permitting changes in shape or cell deformation without an increase in cell volume. This property is reflected by a resistance to osmotic lysis which allows some increase in cell volume without cell disruption. Copper alters this structural relationship of the erythrocyte as indicated by an increase in osmotic fragility following copper incubation. Copper-treated cells become less able to change their shape and have a reduced cell deformability. Such cells having reduced deformability have difficulty passing through narrow vascular channels and are subject to entrapment and destruction in the liver and spleen.

The precise biochemical abnormality responsible for the increased osmotic fragility of erythrocytes exposed to copper was not determined. Enhanced osmotic fragility occurs as a consequence of a relative increase in cell water or loss of cell membrane. An increase in cell water occurs when the normal permeability of the membrane is disrupted so that a net accumulation of cations occurs [26]. These studies indicate that copper does alter membrane permeability to cations, but due to technical difficulties, no definite gain in cations was demonstrated. Although no definite gain in cations was demonstrated, further studies

of the mean cell volume and light microscopy of cells incubated with copper did demonstrate that some spherocytic change had occurred. Further studies of cation fluxes, erythrocyte water, and critical hemolytic volume will be required to determine whether this spherocytic change results from a gain cell water alone or also depends on a loss of membrane surface area.

The membrane properties of erythrocyte ghosts exposed to toxic concentrations of copper reveal a second aspect of the cell injury caused by this ion. High copper concentrations cause the ghosts to lose their intrinsic filterability. This effect of copper could occur by several mechanisms. The method of ghost preparation used in this study does cause the retention of 1—2% hemoglobin within the ghost [21], but is seems unlikely that damage to this small amount of hemoglobin can accont for the changes observed. It is possible that copper affects the physical and structural properties of the proteins of the membrane. It is well known that another heavy metal, calcium, has profound effects on cell deformability, presumably secondary to its alteration of membrane protein relationships [7]. It is also possible that difference in ghost size, shape, and aggregation independent of changes in membrane rigidity might account for the differences observed.

It is apparent from this study that the erythrocyte lesions induced by toxic concentrations of copper form a complex pattern. Following brief exposure to copper, the deformability of the cells is dramatically reduced. This reduction in deformability is accompanied by a loss of the proper surface area to cell volume ratio and less ghost flexibility. Continued exposure to copper is marked by the accumulation of poorly soluble hemoglobin that probably further decreases the deformability of the cell. These diverse types of cell injury converge to produce an erythrocyte which is poorly deformable and doomed to premature destruction during the course of natural circulating through the body.

References

1 Chittani, H.K., Gupta, P.S., Gulati, S. and Gupta, D.N. (1965) Am. J. Med. 39, 849—854
2 Manzler, A.D. and Schreiner, A.W. (1970) Annu. Intern. Med. 73, 409—412
3 Mentz, E.N. and Sagone, A.L. (1972) J. Lab. Clin. Med. 80, 405—413
4 Boulard, M., Blume, K.G. and Seutler, E. (1972) J. Clin. Invest. 51, 459—461
5 Vincent, P.C. and Blackburn, C.R.B. (1958) Aust. J. Exp. Biol. 36, 471—478
6 Lubin, A. and Desforges, J.F. (1972) Blood 39, 658—665
7 Weed, R.I., LaCelle, P.L. and Merril, E.W. (1969) J. Clin. Invest. 48, 795—809
8 Glader, B.E., Fortier, N., Albala, M.M. and Nathan, D.G. (1974) N. Engl. J. Med. 291, 491—496
9 Jandl, J.H., Simmons, R.L. and Castle, W.B. (1961) Blood 18, 133—148
10 LaCelle, P.L. (1970) Semin. Hematol. 7, 355—371
11 LaCelle, P.L. and Weed, R.I. (1969) J. Clin. Invest. 48, 48a
12 Copper, R.A., Kimball, D.B. and Durocher, J.R. (1974) N. Engl. J. Med. 290, 1279—1284
13 Rand, R.P. and Burton, A.C. (1963) Biophys. J. 4, 115—135
14 Chien, S., Luse, S.A. and Bryant, C.A. (1971) Microvascular Res. 3, 183—203
15 Chien, S., Usami, S., Dellenback, R.J. and Gregersen, M.I. (1967) Science, 157, 827—829
16 Jandl, J.H., Engle, L.K. and Allen D.W. (1960) J. Clin. Invest. 39, 1818—1836
17 Evelyn, K.A. and Malloy, H.T. (1938) J. Biol. Chem. 126, 655—662
18 Henry, R.J. (1974) Clinical Chemistry, Principles and Techniques, pp. 1144—1154, Harper and Row, New York
19 Stanley, P.E. and Williams, S.G. (1969) Anal. Biochem. 29, 381—391
20 Dacie, J.V. and Lewis, S.M. (1963) Practical Hematology, pp. 135—141, Grune and Stratton, New York
21 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
22 Mentzer, W.C., Bertram, H.L. and Emmons, S. (1976) N. Engl. J. Med. 294, 1200—1204

23 Chugh, K.S., Singhal, P.C. and Shamara, B.L. (1975) Ann. Int. Med. 82, 226—227
24 Klein, W.J., Metz, E.N. and Price, A.R. (1972) Arch. Int. Med. 129, 578—582
25 Deiss, A., Lee, G.R. and Cartwright, G.E. (1970) Ann. Int. Med. 73, 413—418
26 Osui, F.A., Naiman, J.L. and Blum, S.F. (1969) N. Engl. J. Med. 280, 909—916
27 Shields, G.S., Markowitz, H., Klassen, W.H., Cartwright, G.E. and Wintrobe, M.M. (1961) J. Clin. Invest. 40, 2007—2015
28 Gubler, C.J., Lahey, M.E., Cartwright, G.E. and Wintrobe, M.M. (1953) J. Clin. Invest. 32, 405—414
29 Jandl, J.H. and Simmons, R.L. (1967) Br. J. Hematol. 3, 19—31