Zinc-induced suicidal erythrocyte death\(^1\)–\(^3\)

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**ABSTRACT**

**Background:** Zn\(^{2+}\) stimulates secretory sphingomyelinase, which in turn produces ceramide, an important trigger of suicidal erythrocyte death or eryptosis. Eryptosis is characterized by exposure of phosphatidylserine (PS) at the erythrocyte surface and by cell shrinkage. As macrophages are equipped with PS receptors, they bind, engulf, and degrade PS-exposing cells.

**Objective:** We examined whether Zn\(^{2+}\) stimulates ceramide formation and PS exposure of erythrocytes and thus may be able to trigger suicidal erythrocyte death.

**Design:** In erythrocytes from healthy volunteers, PS exposure (Annexin V binding), cell volume (forward scatter), cytosolic Ca\(^{2+}\) activity (Fluo3 fluorescence), and ceramide formation (anticeramide antibody) were determined by fluorescence-assisted cell sorting.

**Results:** Exposure to Zn\(^{2+}\) (≥25 μmol/L Zn\(^{2+}\)) significantly increased annexin binding. The effect was paralleled by increase of cytosolic Ca\(^{2+}\) activity (≥25 μmol/L Zn\(^{2+}\)) and by ceramide formation (≥10 μmol/L Zn\(^{2+}\)). Glucose depletion (24 h) similarly increased PS exposure, an effect significantly enhanced in the presence of Zn\(^{2+}\) (≥10 μmol/L Zn\(^{2+}\)).

**Conclusion:** Zn\(^{2+}\) triggers suicidal erythrocyte death, an effect partially due to ceramide formation and an increase of cytosolic Ca\(^{2+}\) activity. Am J Clin Nutr 2008;87:1530–4.

**INTRODUCTION**

Zinc, an essential nutrient, counteracts a variety of infectious diseases (1, 2) including malaria (3), diarrhea (3, 4), and respiratory infections (3, 5, 6). Zinc supplementation is considered particularly important in infants and children (7–10), pregnant women (11), and the elderly (12) and has proven beneficial in particular in infants and children (7–10), pregnant women (11), and the elderly (12) and has proven beneficial. Zinc, an essential nutrient, counteracts a variety of infectious diseases (1, 2) including malaria (3), diarrhea (3, 4), and respiratory infections (3, 5, 6). Zinc supplementation is considered particularly important in infants and children (7–10), pregnant women (11), and the elderly (12) and has proven beneficial in particular in infants and children (7–10), pregnant women (11), and the elderly (12) and has proven beneficial.

Erythrocyte phosphatidylserine exposure is accomplished by cell membrane scrambling (21, 22), which is triggered by an increase of the cytosolic Ca\(^{2+}\) activity (17). The Ca\(^{2+}\) sensitivity of phospholipid scrambling is enhanced by ceramide (15). The increased Ca\(^{2+}\) activity may result from activation of Ca\(^{2+}\)-permeable cation channels, which are activated by osmotic shock, oxidative stress, and energy depletion (23). In addition to cell membrane scrambling, Ca\(^{2+}\) activates Ca\(^{2+}\)-sensitive K\(^{+}\) channels (24, 25), which leads to the exit of KCl with osmotically obliged water and thus to cell shrinkage (26).

Enhanced eryptosis parallels several anemic conditions such as sickle cell disease, thalassemia, glucose-phosphate dehydrogenase deficiency, phosphate depletion, iron deficiency (27), hemolytic uremic syndrome, sepsis, malaria, and Wilson’s disease (16, 27, 28). Beyond that, eryptosis may be triggered by methylglyoxal (29), amyloid (30), listeriolysin (31), paclitaxel (32), chlorpromazine (33), cyclosporine (34), lead (35), and mercury (36). Eryptosis has similarities to, but may be distinct from, erythrocyte senescence (16).

Accelerated eryptosis has been shown to protect against a severe course of malaria (37, 38).

The present experiments were designed to test the hypothesis that Zn\(^{2+}\) ions could stimulate ceramide formation in erythrocytes and that the stimulation of ceramide formation may lead to stimulation of phosphatidylserine exposure. Thus, the present experiments were performed to explore whether exposure to Zn\(^{2+}\) ions triggers phosphatidylserine exposure and to elucidate the underlying mechanisms.

**SUBJECTS AND METHODS**

This study was approved by the local ethics committee of the University of Tübingen (project#: 184/2003V).

**Erythrocytes and solutions**

Blood was drawn from 9 healthy adult volunteers (not known to the authors) at the Blutspendenzentrale Tübingen (http://www.blutspendenzentrale.de/), and erythrocyte concentrates were prepared with the use of leukocyte depletion filters as described (39). Aliquots of the individual erythrocyte concentrates were either used directly in the independent experiments or stored at 4 °C until usage. Experiments were performed at 37 °C in Ringer solution containing 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl\(_2\), 1 mmol/L CaCl\(_2\), 0.85% glucose, 0.005% bovine serum albumin (BSA), and 1 μmol/L NADH.

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Zn²⁺-INDUCED ERYPTOSIS

Measurement of intracellular Ca²⁺

Intracellular Ca²⁺ measurements were performed as described (23). Briefly, erythrocytes were washed in sodium-chloride Ringer solution and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in 2 mmol/L CaCl₂- and 2 µmol/L Fluo-3/AM-containing sodium-chloride Ringer solution. The cells were incubated at 37 °C for 20 min under shaking condition and washed twice in 2 mmol/L CaCl₂-containing sodium-chloride Ringer solution. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µL Ringer solution in the presence or absence of Zn²⁺. Then, Ca²⁺-dependent fluorescence intensity was measured in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. As an exposure time of 24 h would result in considerable loss of fluorescent dye, exposure time was restricted to 6 h.

Determination of ceramide formation

Cells were stained for 1 h at 4 °C with anticeramide antibody or isotype-matched pure mouse antibody in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) at a dilution of 1:5 as described recently (39). After 3 washes with PBS/1% FCS, cells were stained with polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig-specific antibody.

MgSO₄, 32 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 mmol/L glucose, and 1 mmol/L CaCl₂. Zinc chloride (Sigma, Taufkirchen, Germany) was added to the Ringer solution, was washed in annexin-binding buffer containing 25 µmol/L Zn²⁺ ions (right panel). The numbers indicate the percentage of annexin-binding erythrocytes of the respective cell population as confined by the horizontal line. B) Arithmetic means ± SEM (n = 9) of annexin binding erythrocytes after 24-h treatment with Ringer solution as a function of the Zn²⁺ concentration. *Significant difference (ANOVA using Dunnett’s test as post hoc test, P < 0.05) from absence of Zn²⁺ ions (Ringer solution, white bar).

Fluorescence-assisted cell sorting

Fluorescence-assisted cell sorting (FACS) analysis was performed as described (39). After incubation in the presence or absence of Zn²⁺ ions, cells were washed in annexin-binding buffer containing 125 mmol/L NaCl, 10 mmol/L HEPES/NaOH (pH 7.4), and 5 mmol/L CaCl₂. Erythrocytes were suspended in a solution composed of Annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin-binding buffer (dilution of 1:50). After 10 min of incubation, samples were finally diluted 1:5 in annexin-binding buffer and measured by flow cytometric analysis on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Cells were analyzed by forward scatter, and annexin-fluorescence intensity was measured in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

FIGURE 1. Stimulation of phosphatidylserine exposure at the erythrocyte surface by Zn²⁺. A) Histograms of annexin binding in a representative experiment of erythrocytes incubated for 24 h in Ringer solution (left panel), or in Ringer solution containing 25 µmol/L Zn²⁺ ions (right panel). The numbers indicate the percentage of annexin-binding erythrocytes of the respective cell population as confined by the horizontal line. B) Arithmetic means ± SEM (n = 9) of annexin binding erythrocytes after 24-h treatment with Ringer solution as a function of the Zn²⁺ concentration. *Significant difference (ANOVA using Dunnett’s test as post hoc test, P < 0.05) from absence of Zn²⁺ ions (Ringer solution, white bar).

Measurement of forward scatter of erythrocytes after a 24-h treatment with Ringer solution as a function of the Zn²⁺ concentration. *Significant difference (ANOVA using Dunnett’s test as post hoc test, P < 0.05) from absence of Zn²⁺ ions (Ringer solution, white bar).

FIGURE 2. Erythrocyte shrinkage following exposure to Zn²⁺. A) Histograms of forward scatter in a representative experiment of erythrocytes incubated for 24 h in Ringer solution (left panel), or in Ringer solution containing 25 µmol/L Zn²⁺ ions (right panel). The numbers indicate the GeoMean of the forward scatter of the respective cell population. B) Arithmetic means ± SEM, (n = 9) of forward scatter of erythrocytes after a 24-h treatment with Ringer solution as a function of the Zn²⁺ concentration. *Significant difference (ANOVA using Dunnett’s test as post hoc test, P < 0.05) from absence of Zn²⁺ ions (Ringer solution, white bar).
exposure with subsequent annexin binding in 4.1 ± 0.5% (n = 9) of
the cells. Addition of Zn²⁺ (Zn²⁺ concentrations varying from
1 to 50 µmol/L) to the Ringer solution significantly increased
the percentage of annexin-binding cells in a dose-dependent manner
(Figure 1, A and B).

The effect of Zn²⁺ on annexin binding was paralleled by a
decrease of the forward scatter reflecting erythrocyte shrinkage
(Figure 2, A and B). The effect of Zn²⁺ was dependent on the
Zn²⁺ concentration and achieved statistical significance at 25
µmol/L Zn²⁺ (Figure 2B).

As illustrated in Figure 3, exposure to Zn²⁺ for 6 h increased
cytosolic Ca²⁺. The effect was statistically significant at 25
µmol/L Zn²⁺.

As illustrated in Figure 4, exposure of erythrocytes to Zn²⁺
(≥10 µmol/L) led within 24 h to a significant increase of cer-
amide formation. Bacterial sphingomyelinase, utilized as posi-
tive control, similarly increased ceramide formation (Figure 4, A
and B).

Exposure of erythrocytes to glucose depletion resulted in the
expected (40) increase of phosphatidylserine exposure (Figure
5A; compare the upper left histogram with the lower left histo-
gram). The increase of phosphatidylserine exposure after glu-
cose removal was significantly enhanced in the presence of Zn²⁺
(≥10 µmol/L) (Figure 5B). Thus, Zn²⁺ and energy depletion
mutually enhance the susceptibility of erythrocytes to undergo
erptosis.

(Pharmingen, Hamburg, Germany) in PBS/1% FCS at a dilution
of 1:50 for 30 min. Unbound secondary antibody was removed by
washing the cells 2 times with PBS/1% FCS, and samples were
analyzed by flow cytometric analysis on a FACS-Calibur. FITC-
fluorescence intensity was measured in the fluorescence channel
FL-1. As a positive control, erythrocytes were treated for 5 min
with 0.1 unit/mL Streptomyces sp. sphingomyelinase from
Sigma.

Statistics
Because of the large variation in the sensitivity of the individ-
ual erythrocyte concentrates, all data are expressed as arithmetic
means ± SEM, and the number of independent experiments
using blood from different donors is stated. Statistical analysis
was made by ANOVA using Dunnett’s test as post hoc test
(GRAPHPAD INSTAT; GraphPad Software Inc., San Diego,
CA). *P < 0.05 was considered statistically significant.

RESULTS
Incubation of freshly drawn erythrocytes in Ringer solution
for 24 h resulted in low but appreciable phosphatidyserine

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** Increase of cytosolic Ca²⁺ activity in erythrocytes following
exposure to Zn²⁺. A) Histograms of Fluo3 fluorescence in a representative
experiment of erythrocytes exposed for 6 h to Ringer solution without (left
panel) and with (right panel) 50 µmol/L Zn²⁺. The numbers indicate the
mean fluorescence of erythrocytes of the respective cell population. B) Arith-
metic means ± SEM (n = 4) of Fluo3 fluorescence in erythrocytes exposed
for 6 h to Ringer solution without Zn²⁺ (white bar) and with 10 µmol/L Zn²⁺
(light gray bar), 25 µmol/L Zn²⁺ (dark gray bar), or 50 µmol/L Zn²⁺ (black
bar). *Significant difference to value in absence of Zn²⁺ (ANOVA using
Dunnett’s test as post hoc test, P < 0.05).

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Stimulation of ceramide formation in erythrocytes following
exposure to Zn²⁺. A) One-dimensional flow cytometry histograms of anti-
ceramide fluorescein isothiocyanate (FITC)-coupled antibody binding (x
axis) in a representative experiment of erythrocytes incubated for 24 h in
Ringer solution without (control, left panel), or with 25 µmol/L Zn²⁺ (Zn²⁺,
middle panel) or for 5 min in Ringer solution containing 0.1 U/mL sphingo-
myelinase (SMase, right panel). The numbers indicate the GeoMean of an-
other control, similarly increased ceramide formation (Figure 4, A
and B).

Exposure of erythrocytes to glucose depletion resulted in the
expected (40) increase of phosphatidylserine exposure (Figure
5A; compare the upper left histogram with the lower left histo-
gram). The increase of phosphatidylserine exposure after glu-
cose removal was significantly enhanced in the presence of Zn²⁺
(≥10 µmol/L) (Figure 5B). Thus, Zn²⁺ and energy depletion
mutually enhance the susceptibility of erythrocytes to undergo
erptosis.

![Figure 5](https://example.com/figure5.png)
The absence of Zn\textsuperscript{2+} erythrocytes after 24-h treatment with glucose-free Ringer solution (0Glc) in panels) of 25 \textmu mol/L Zn\textsuperscript{2+}. The numbers indicate the percentage of annexin-binding erythrocytes of the respective cell population as confined by the horizontal line. B) Arithmetic means \pm SEM (n = 4) of annexin binding of erythrocytes after 24-h treatment with glucose-free Ringer solution (0Glc) in the absence of Zn\textsuperscript{2+} (white bar) or in the presence of 10 \textmu mol/L Zn\textsuperscript{2+} (gray bar) or 25 \textmu mol/L Zn\textsuperscript{2+} (black bar). *Significant difference (ANOVA using Dunnett’s test as post hoc test, *P < 0.05) from the absence of Zn\textsuperscript{2+} ions (glucose-free Ringer solution, white bar).

**DISCUSSION**

The present experiments disclose a novel action of Zn\textsuperscript{2+} on erythrocytes. The trace element triggers erythrocyte cell membrane phospholipid scrambling leading to phosphatidylserine exposure at the cell membrane surface. The effect of Zn\textsuperscript{2+} is paralleled by erythrocyte shrinkage, another typical feature of eryptosis (16). Similarly, cell shrinkage is a hallmark of apoptosis of nucleated cells (41).

The effects are at least partially due to increased cytosolic Ca\textsuperscript{2+} activity, which is known to stimulate phospholipid scrambling of the cell membrane (17, 18) and to activate Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels (24, 25). The K\textsuperscript{+} exit through those channels leads to hyperpolarization of the cell membrane, which drives Cl\textsuperscript{−} toward the extracellular space. The combined exit of K\textsuperscript{+}, Cl\textsuperscript{−}, and osmotically obliged water then leads to cell shrinkage.

The effect is, further, the result of ceramide formation. Previous studies revealed the ability of Zn\textsuperscript{2+} to activate a ceramide-producing secretory sphingomyelinase (14). Ceramide, in turn, sensitizes erythrocytes for the scrambling effect of Ca\textsuperscript{2+} (15).

Exposure to Zn\textsuperscript{2+} further sensitizes the cell for the scrambling effect of energy depletion. Previous studies (40) have shown that energy depletion leads to activation of protein kinase C, which in turn triggers cell membrane scrambling.

The effect of Zn\textsuperscript{2+} requires Zn\textsuperscript{2+} concentrations in the range of those encountered in human plasma (42). Thus, excessive intake of Zn\textsuperscript{2+} could well trigger suicidal death of circulating erythrocytes. Thus, the observed effects of Zn\textsuperscript{2+} could indeed be relevant for in vivo conditions.

Phosphatidylserine-exposing cells are bound to respective receptors on macrophages (19), leading to engulfment and subsequent degradation (20). Accordingly, phosphatidylserine-exposing erythrocytes are rapidly cleared from circulating blood (27). Thus, at least in theory, the stimulation of suicidal death of circulating erythrocytes could cause anemia. Side effects of Zn\textsuperscript{2+} excess indeed include anemia (43, 44), an effect, however, in large part attributed to copper deficiency (45). During malaria, on the other hand, enhanced susceptibility to eryptosis could foster the elimination of infected erythrocytes and thus protect against a severe course of the disease (37, 38). Additional studies are required, however, to test whether the beneficial effect of zinc supplementation in malaria (1–3) is indeed related to accelerated eryptosis.

Zn\textsuperscript{2+} has previously been shown to induce (46–48) and to counteract (49) apoptosis of nucleated cells. The mechanisms effective in erythrocytes may similarly participate in the regulation of nucleated cell death.

In conclusion, exposure to Zn\textsuperscript{2+} leads to phosphatidylserine exposure of erythrocytes. The effect is at least partially due to increased cytosolic Ca\textsuperscript{2+} activity and ceramide formation. In vivo, the effects could lead to accelerated clearance of phosphatidylserine-exposing erythrocytes and thus to development of anemia.

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The authors’ responsibilities were as follows—VK: designed the study, and analyzed and interpreted the data; FL: wrote the manuscript; FL and TW: contributed to the analysis and interpretation of data; TW: contributed to writing the manuscript; and VK, AA and OMN: contributed to the collection of data. All authors reviewed the manuscript. None of the authors had a conflict of interest.

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