The effect of zinc deficiency on erythrocyte deformability in the rat

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The influence of zinc (Zn) deficiency on erythrocyte deformability was investigated in the rat. Weanling, male Wistar rats were fed a diet containing <1 mg/kg Zn ad libitum for 3 weeks (−Zn). Controls were fed ad libitum (+ZnAL) or pair-fed (+ZnPF) the same diet containing 100 mg/kg Zn. Weanling rats were included as experimental time zero controls. Erythrocyte deformability was measured at standard shear stress with increasing osmolality in the ektacytometer. Elongation index (the ratio of length to width of the diffraction pattern of the deformed cells) was plotted against osmolality to generate an osmotic deformability profile. Characteristics of this profile were analyzed by a one-factor analysis of variance followed by Tukey's test. Maximum cell deformability (El_{max}) and minimum deformability (El_{min}) were significantly depressed in samples from the +ZnPF group as compared with those from the +ZnAL group ($P < 0.05$). El_{max} and O_{max} (the osmolality at which El_{max} occurs) were increased in the +ZnAL group as compared with the experimental time zero group. There were no differences between the −Zn group and the +ZnPF group when assessed by Tukey's test, but a paired t test indicated that El_{max} was significantly lower in the −Zn group ($P = 0.049$). In summary, zinc depletion itself causes a minor depression in whole cell deformability, while the accompanying decrease in feed intake results in a more pronounced decrease due to diminished membrane flexibility. Erythrocyte deformability is increased in the normal 6-week-old rat compared with that of the 3-week-old weanling rat; this is due to increased membrane deformability.

Keywords: erythrocyte deformability; zinc deficiency; rat; erythrocyte; ektacytometer

Introduction

The proposed physiological role of zinc in the structure and function of biological membranes[1] has been often studied using the single membrane system of the erythrocyte as a model. The stabilizing effect of zinc on the erythrocyte membrane has been demonstrated by the increased susceptibility of red cells from zinc-deficient animals to hemolysis in the presence of hypotonic saline, sodium dodecyl sulfate, sodium dodecyl-N-sarcosine, and mellitin.[2,3] Zinc added in vitro to erythrocytes is also protective against a variety of hemolysins.[4–6]

During zinc deficiency the erythrocyte membrane is sensitive to the low extracellular zinc concentration to which it is exposed. The zinc concentration of the erythrocyte membrane is depressed during dietary zinc deficiency in the rat,[7] and alterations occur in the composition and physical properties of the membrane.[8–11] Many of these membrane alterations could influence the deformability of the erythrocyte, as this physiological property is determined by the flexibility of the cell membrane in addition to internal viscosity and surface-area to cell-volume ratio.[12] Deformability is critical to normal erythrocyte function, as the resting diameter of the erythrocyte exceeds that of the capillaries and endothelial slits that it must traverse.[13] Deformability also influences blood viscosity in large vessels, controls removal of nonfunctional and aged

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erythrocytes by the reticuloendothelial system, and influences release of immature erythrocytes into the circulation.[14] The ektacytometer provides a sensitive tool for the measurement of red cell deformability and differentiation of the contribution of membrane properties relative to the other factors that influence cell deformability.[12]

Low dietary zinc exacerbates the decreased filterability of erythrocytes from vitamin E-deficient rats.[15] Pharmacological levels of zinc administered to patients with sickle cell disease decrease the number of irreversibly sickled cells,[16] and the filterability of erythrocytes from these patients is increased in response to zinc treatment in vitro.[17,18] In the only study that directly assessed the influence of zinc status alone, zinc deficiency had no influence, while the reduced feed intake accompanying the deficiency increased red cell filterability.[10] However, the technique of filterability is not sensitive to altered deformability resulting from membrane damage,[19,20] and the biochemical changes in the red cell membrane in the zinc-deficient state strongly suggest that membrane deformability would be altered. Increased fluidity of the lipid bilayer[8] and increased mobility of spin probes in the protein[9] and cell-surface sialic acid residue[8] domains of the red cell membrane in zinc deficiency have been shown by electron-spin resonance spectroscopy analysis. Because most of the probe used to assess membrane protein mobility binds to the spectrin-actin complex, it has been suggested that spectrin structure and function might be altered in the zinc-deficient state.[9] This idea was further supported by the finding of increased dephosphorylation of spectrin and actin in erythrocyte membranes isolated from zinc-deficient rats.[10] The flexibility of the normal erythrocyte membrane depends on the integrity of skeletal membrane proteins, and abnormalities of these proteins (spectrin in particular) are associated with reduced deformability of the membrane and increased mechanical fragility of the cell.[21] Red cell membranes from zinc-deficient rats also have lower spermidine:spermine ratios although the effect is mainly due to the diminished feed intake of the animals.[11] This change in membrane polyamine composition might also decrease erythrocyte deformability.[22]

These findings suggested that the ability of erythrocytes from zinc-deficient animals to deform should be reinvestigated with a technique that would more readily detect membrane damage. The objective of this study was to determine the effect of zinc deficiency in the rat on erythrocyte deformability using the ektacytometer. As erythrocyte deformability has not been extensively studied in this species, a secondary objective was to investigate whether developmental changes would occur during the timeframe of the experiment.

Materials and methods

Experimental design

Male, weanling Wistar rats (47–57 g) were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). The animals were aclimatized overnight and assigned to treatment groups the following day. Distilled, deionized water was supplied ad libitum, and the animals were housed individually in stainless-steel cages. The first group of animals was fed an egg white-based diet containing <1 mg/kg zinc ad libitum for 21 days (− Zn; $n=8$). The composition of the basal diet has been previously published.[10] Control rats were either pair-fed (+ ZnPF; $n=8$) or fed ad libitum (+ ZnAL; $n=8$) the basal diet supplemented with 100 mg/kg zinc. Daily feed intake was measured, and the rats were weighed at the beginning and end of the study. A fourth group of weanling (3-week-old) rats representing experimental time zero controls was killed by decapitation on day 1 ($n=9$).

To ensure that blood samples were processed immediately after being obtained, these were collected from the − Zn, + ZnPF, and + ZnAL groups over days 20–22; each group was represented on each day. Blood samples were obtained by cardiac puncture under methoxyflurane (Metofane) anesthesia. There was one exception in which trunk blood was collected from a rat in the + ZnAL group. A blood sample was not obtained from one animal in the + ZnPF group. All blood samples were collected in heparinized polystyrene tubes and placed on ice. Following blood sampling, the animals were killed by decapitation.

A complete blood count was obtained by routine Coulter Counter techniques (Coulter Counter T660, Coulter Electronics Incorporated, Hialeah, FL USA).

Plasma zinc was determined by flame atomic absorption spectrophotometry[23] after diluting the plasma 1:4 with distilled, deionized water. All readings were conducted with the instrument in the absorbance mode. Concentrations were calculated from the absorbance values by use of a linear regression equation, which was obtained with five standard concentrations made in 0.1 mol/L Ultrex grade HNO_3. National Institute of Standards and Technology bovine serum was included in the sample run as a standard reference material. Recovery for this reference material was 105%.

Measurements of erythrocyte deformability by the ektacytometer

Erythrocyte deformability was measured on the day of blood sampling using the ektacytometer (Technicon Instruments Corporation, Tarrytown, NY USA). The principle of the instrument is that erythrocytes suspended in a fluid medium are deformed in a rotational shearing device. The elongated cells diffract a helium-neon laser beam, and the pattern of diffracted light is measured by an image analyzer to give a value for cell elongation (elongation index), which is defined as the ratio of length to width of the diffraction pattern in Equation 1[12]:

$$EI = (L-W)/(L+W)$$

where EI = elongation index, L = length of the diffraction pattern, and W = width of the diffraction pattern.

Whole blood (100 μL) was suspended in a solution containing 3.1% polyvinylpyrrolidone (average molecular weight = 360,000), 6.3 mmol/L Na_2HPO_4, 2 mmol/L NaH_2PO_4, and 0.04% NaN_3 (pH 7.35 ± 0.05) with varying NaCl concentration. The erythrocytes were subjected to constant shear stress (160 dynes/cm^2) in the instrument while the osmolality of the suspending medium was progressively increased from 50 to 500 mOsm/kg. The elongation index of the cells was recorded continuously on a linear X-Y recorder. Parameters of the osmotic deformability profile described previously by Clark et al.[24] were used to characterize cell deformability *(Figure 1)*.

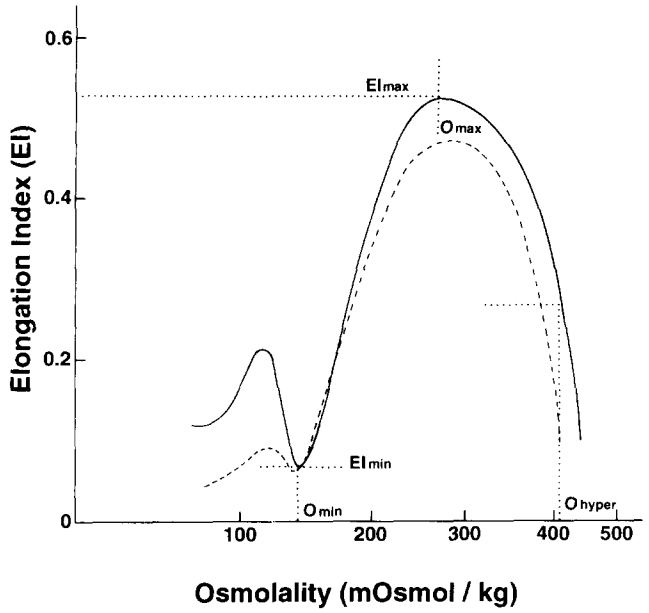

Figure 1 Osmotic deformability profile for normal erythrocytes (- - - -, rat and —, human). Parameters of the curve used to characterize cell deformability are those described by Clark et al.[24]: (1) EI_{max}, the maximum elongation index (EI), (2) O_{max}, the osmolality at which EI_{max} occurs, (3) EI_{min}, the minimum EI obtained under hypotonic conditions, (4) O_{min}, the osmolality at which EI_{min} occurs, and (5) O_{hyper}, the osmolality at which EI is half of EI_{max} on the hypertonic arm.

A freezing point osmometer (model 3MO, Advanced Instrument Inc., Needham Hts., MA USA) was used to measure the osmolality of solutions used in the ektacytometer.

Deionized, distilled water was used for the preparation of all reagents. Glassware was acid-washed in 20% nitric acid, and precautions were taken to prevent trace element contamination from the environment.

Statistical analysis

The data was analyzed using a one-way analysis of variance of a completely randomized design, followed by Tukey's test. The parameters of the osmotic deformability profile were also compared for the −Zn and +ZnPF groups by a paired *t* test.

Source of chemicals

All chemicals were of reagent grade. Bovine serum (standard reference material 1598) was purchased from the U.S. Dept. of Commerce, National Institute of Standards & Technology, Gaithersburg, MD USA. Ultrex grade HNO_3 was obtained from J.T. Baker Inc., Phillipsburg, NJ USA. NaCl, Na_2HPO_4, NaH_2PO_4, and zinc standard (1000 mg/mL) were purchased from BDH Inc., Edmonton, Alta. Canada. Polyvinylpyrrolidone was obtained from Sigma Chemical Company, St. Louis, MO USA. Sodium azide was from Aldrich Chemical Company, St. Louis, MO USA. Methoxyflurane was from MTC Pharmaceuticals, Mississauga, Ont., Canada.

Results

The zinc-deficient diet resulted in a severe zinc deficiency as demonstrated by markedly depressed plasma zinc concentration compared to that of pair-fed or ad libitum-fed control groups. Feed intake was also dramatically decreased, and weight gain was significantly lower than that of the two control groups. The results are shown in *Table 1*.

The hematological profile was similar to that reported previously for dietary zinc deficiency and feed restriction.[25] The mean (± SEM) hematocrit for the zinc-deficient group was 0.411 ± 0.008 as compared with 0.383 ± 0.006 for the pair-fed controls and 0.332 ± 0.007 for the ad libitum-fed control group.

The osmotic deformability profile for erythrocytes from normal (ad libitum-fed zinc adequate) rats is shown in *Figure 1*. The profile for normal human erythrocytes is also shown for comparison, with an explanation of the features of the curve used to characterize cell deformability.

Table 2 shows the effect of dietary zinc intake on the osmotic deformability profile. Zinc depletion itself had a minor effect on the osmotic deformability profile as can be seen by comparing the deformability parameters for the −Zn group with those from the +ZnPF group. Based on analysis of −Zn and +ZnPF rats by a paired *t* test, EI_{max} was significantly lower in the samples from the −Zn rats ($P = 0.049$). There were no differences between the −Zn and +ZnPF groups when assessed by Tukey's test ($P > 0.05$).

EI_{max} and EI_{min} were both significantly depressed in samples from the +ZnPF group as compared with those from the +ZnAL group as assessed by Tukey's test ($P < 0.05$), indicating a significant effect of decreased feed consumption.

EI_{max} and O_{max} significantly increased between 3 and 6 weeks of age in the growing rat (Tukey's test; $P < 0.05$), indicating a developmental effect. This can be seen by comparing the results from the experimental time zero (weanling) group with those from the +ZnAL group.

Table 1 The effect of dietary zinc level on zinc status

	Experimental group*		
	−Zn†	+ZnPF‡	+ZnAL§
Food intake (g/3 weeks)	129 ± 3[a]	129 ± 3[a]	355 ± 6[b]
Weight gain (g/3 weeks)	6 ± 1[a]	44 ± 2[b]	150 ± 5[c]
Feed efficiency¶	0.04 ± 0.01[a]	0.35 ± 0.01[b]	0.42 ± 0.01[c]
Plasma zinc (μg/mL)	0.23 ± 0.04[a]	0.98 ± 0.09[b]	1.21 ± 0.07[b]

*Results expressed as mean ± SEM. Statistical analysis was a one-factor ANOVA followed by Tukey's test. Means with different superscripts (a, b, and c) are significantly different ($P < 0.05$).
†Weanling rats fed a diet containing <1 mg Zn/kg diet ad libitum for 3 weeks ($n = 8$).
‡Weanling rats pair-fed a diet supplemented with 100 mg Zn/kg diet for 3 weeks ($n = 7$). Plasma Zn was based on $n = 7$ due to insufficient sample collection on one animal.
§Weanling rats fed a diet supplemented with 100 mg Zn/kg diet ad libitum for 3 weeks ($n = 8$).
¶Weight gain/feed intake.

Table 2 The effect of dietary zinc level on the osmotic deformability profile

	Experimental group*			
	Time Zero†	−Zn‡	+ZnPF§	+ZnAL¶
EI_{max}	0.457 ± 0.002[a]	0.444 ± 0.005[a]‖	0.454 ± 0.008[a]‖	0.483 ± 0.006[b]
O_{max}	269 ± 2[a]	269 ± 1[a]	275 ± 3[ab]	279 ± 3[b]
EI_{min}	0.063 ± 0.003[a]	0.052 ± 0.005[ab]	0.043 ± 0.006[b]	0.065 ± 0.003[a]
O_{min}	137 ± 1[a]	137 ± 1[a]	139 ± 2[a]	137 ± 2[a]
O_{hyper}	374 ± 1[a]	380 ± 2[a]	382 ± 3[a]	381 ± 2[a]

*Results expressed as mean ± SEM. Means with different superscripts (a, b, and ab) are significantly different ($P < 0.05$) as assessed by a one-factor ANOVA followed by Tukey's test.
†Weanling (3-week-old) rats at experimental time zero ($n = 9$).
‡Weanling rats fed a diet containing < 1 mg/kg Zn ad libitum for 3 weeks ($n = 8$).
§Weanling rats pair-fed a diet supplemented with 100 mg/kg Zn for 3 weeks ($n = 7$).
¶Weanling rats fed a diet supplemented with 100 mg/kg Zn ad libitum for 3 weeks ($n = 8$).
‖Comparison of the −Zn and +ZnPF groups by a paired t test indicates a significantly lower value for EI_{max} in the zinc-deficient group ($P = 0.049$).

Discussion

Erythrocyte deformability has been measured by various techniques. Filterability has been used most often for clinical purposes, but it is difficult to standardize and does not readily distinguish among the three main cellular determinants of deformability. In particular, filterability is less sensitive to erythrocyte membrane damage.[19,20] Using the ektacytometer the deformability of a population of cells is measured under well-defined fluid shear stress conditions using small quantities of blood.[26] The coefficient of variation for replicate determinations with the ektacytometer is low. Thus, the measurements are particularly sensitive to small changes in the three factors governing cell deformability.[19] The capacity to distinguish which of these factors is responsible for an alteration in deformability[12] was particularly important to our objective of investigating specifically whether a change in membrane structure in zinc deficiency altered the ability of erythrocytes to deform.

Deformability under constant high shear stress was recorded continuously in our study as the osmolality of the suspending medium was progressively increased.[24] The osmotic deformability profile that was generated represents the relationship between the elongation index and osmolality. Red cells from the rat deform in a pattern similar to that of human erythrocytes[27] *(Figure 1)* although EI_{max} (maximum cell deformability) is lower and O_{max} (the osmolality associated with maximum deformability) is slightly higher.* For both normal human and rat cells, maximum deformability occurs near 290 mosmol/kg, the osmolality to which cells are exposed under normal conditions. As osmolality is progressively decreased, the elongation index declines and reaches a minimum (EI_{min}) at an osmolality (O_{min}) at which the cells on average would have attained the maximum possible volume prior to hemolysis. The osmolality associated with minimum elongation coincides with that at which 50% of cells hemolyze in an osmotic fragility assay. Thus, O_{min} provides information about the initial surface area to volume ratio of the cells. The decrease in elongation index under hypertonic conditions results from an increase in intracellular viscosity as a result of osmotic water loss and increasing mean corpuscular hemoglobin concentration.[24]

The osmotic deformability profile can be used to assess the influence on whole cell deformability of internal viscosity, surface-membrane area to cell-volume ratio, and cell membrane flexibility[24]: Reduced cellular surface area to volume ratio causes O_{min} to be increased and EI_{max} to be decreased with no change in the hypertonic arm of the osmotic deformability profile. A change in intracellular viscosity (determined by mean corpuscular hemoglobin concentration) shifts the osmotic deformability profile along the x-axis. Values for O_{min}, O_{max}, and O_{hyper} increase with a decline in mean corpuscular hemoglobin concentration, while EI_{max} is unchanged. When erythrocytes are treated with agents that decrease membrane flexibility, the value for EI_{max} decreases, but there is little shift of the curve along the osmolality axis.

Maximum erythrocyte deformability (EI_{max}) increased between 3 weeks (weaning) and 6 weeks of age in the rat. This finding on its own suggests an increase in the flexibility of the membrane without a change in other factors governing cell deformability. An increase in O_{max} between 3 and 6 weeks of age in the rat is more difficult to explain as a simultaneous increase in EI_{max} and O_{max} was not a pattern described by Clark et al.[24] when they varied the cell determinants of deformability.

Developmental studies have primarily compared the deformability of neonatal erythrocytes with adult red cells. Human neonatal and adult erythrocytes show similar deformability when studied under defined shear stress with the ektacytometer.[28,29] However, neonatal erythrocytes are less filterable[30] than adult cells be-

*Jurgens, V.M., Paterson, P.G., and Card, R.T. (1992). Variability in erythrocyte deformability among species, 35th Annual Meeting of the Canadian Federation of Biological Societies, Victoria, B.C. Canada (abstract)

cause of their larger size. This suggests that the greater minimal diameter of neonatal cells may restrict their passage through the microcirculation, particularly in the spleen. More recently, Engstrom and Ohlsson[31] investigated the morphology and filterability of erythrocytes from the neonatal period to adulthood in the rat. They found neonatal erythrocytes to be less filterable, but the variations in physiological properties between neonatal and adult cells were more pronounced than had been reported in humans. In agreement with our study, they found red cells from 3-week-old rats to be slightly less deformable than those from 6-week-old rats, both in terms of resistance to filtration and filter clogging particles. Between 3 and 6 weeks of age, the hemoglobin and hematocrit of the rapidly growing young rat are gradually increasing toward adult levels following the period of physiological anemia of the newborn.[32] Apparently a rise in maximum cell deformability also occurs during this developmental period.

One important contributor to the lower maximum erythrocyte deformability in rats at 3 weeks of age could be the large number of circulating reticulocytes still present. The mean (± SEM) reticulocyte number of 3-week-old rats was previously reported to be 23.1 ± 3.1% of total circulating red cells as compared to 5.1 ± 1.2 for 6-week-old rats.[25] Membranes prepared from immature reticulocytes are more rigid than those prepared from mature red cells; the lower deformability of reticulocytes is due to reduced membrane deformability and not to differences in cell geometry or cytoplasmic viscosity.[33] Differences in the membrane composition of mature cells from the two age groups could also account for the differences in maximum deformability, but this was not examined in the present study.

The osmotic deformability profiles of the zinc-deficient and pair-fed control animals were similar to that of experimental time zero control animals. Thus, the increase in maximum erythrocyte deformability that normally occurs between 3 and 6 weeks of age in the rat is prevented by dietary zinc deficiency, although the effect is mainly attributable to the associated decrease in feed consumption. Hemoglobin and hematocrit also increased dramatically in the zinc-deficient and feed-restricted animals over the 3 weeks of the study compared with the increase that occurs in ad libitum-fed control rats. This is in agreement with previous studies.[25,34] Erythropoiesis is not increased in these animals, and the mechanism appears to involve their decreased growth.[25]

Acute, severe zinc deficiency per se had a minor effect on the osmotic deformability profile. EI_{max} was significantly lower in samples from zinc-deficient rats when compared with that found in the pair-fed control group. Although this decrease is small, it does indicate that any influence of zinc on erythrocyte deformability is exerted through a stabilizing effect on the membrane. We had, however, predicted a more dramatic effect of dietary zinc deficiency, independent of the level of feed intake, in view of the biochemical and physiological alterations previously reported in the erythrocyte membrane.[2,3,8–11] Given our hypothesis that an alteration in spectrin structure and function might alter deformability during zinc deficiency, the results of a recent study[35] provide some biochemical support for our findings. These investigators reported no effect of dietary zinc deficiency on erythrocyte membrane spectrin content, oligomeric form, or extractibility. The latter suggests that the anchorage of spectrin to the membrane through ankyrin, band 4.1, and other sites is unchanged in zinc deficiency. The only change in the protein composition of the extracted erythrocyte membrane skeleton was a decrease in the percentage of R5, a protein of unknown identity.[35]

The decrease in feed intake that accompanies zinc deficiency had a more dramatic effect on the osmotic deformability profile as shown by significantly lower values for EI_{max} in pair-fed control animals compared with those from ad libitum-fed controls. This alteration alone, without a change in the other parameters of the osmotic deformability profile, is most consistent with decreased membrane deformability due to altered membrane properties.[24] The present findings are in contrast to a previous study using the same animal model in which reduced feed consumption, whether voluntary as in zinc deficiency or imposed by feed restriction, caused an increase in erythrocyte deformability as measured by filterability.[10] This apparent contradiction may be explained by the microcytosis that occurs with reduced feed consumption, as small pore filters are very sensitive to cell size.[28] Using an identical experimental design, one of us[25] previously reported the mean (± SEM) corpuscular volume of erythrocytes from zinc-deficient and pair-fed control rats to be 49.2 ± 1.0 and 49.8 ± 1.4, respectively, as compared with 57.3 ± 1.7 in ad libitum-fed control animals.

Alterations in the erythrocyte membrane previously reported with diminished feed consumption provide possible mechanisms for the decrease in deformability. The elevated cholesterol:phospholipid ratio in the erythrocyte membrane of these animals that has been demonstrated[36] is predictive for a decrease in fluidity and an associated decline in deformability.[37] Other changes that could account for decreased membrane deformability in feed-restricted rats include a decrease in erythrocyte membrane spermidine:spermine ratio[11,22] and a lower red cell ATP concentration.[34,38] A reported decrease in the percentage of bands R4 and adducin in the extracted membrane skeleton of erythrocytes from feed-restricted rats also suggests alterations in the structure of the membrane skeleton.[35]

In summary, dietary zinc deficiency in the rat decreases erythrocyte deformability, and the effect is due to decreased deformability of the membrane. This effect appears to be mainly attributable to the associated reduction in food intake. Our findings also indicate a developmental increase in red cell deformability occurring in the rapidly growing rat between weaning (3 weeks) and 6 weeks of age. This increase in deformability is due to an increase in membrane flexibility.

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