Low zinc status in guinea pigs and chicks has no effect on reassembly rate of brain microtubules

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Zinc deficiency in guinea pigs and chicks results in peripheral neuropathy. Slowed reassembly of brain microtubules in zinc deficient rats has been reported, and such a defect might explain the neuropathy. To investigate this possibility, growing guinea pigs, chicks, and rats were fed a low zinc diet ad libitum (−Zn:AL), a control diet restricted (+Zn:RF) to the intake of the −Zn:AL group, and the control diet ad libitum (+Zn:AL). When the −Zn:AL guinea pigs and chicks exhibited neuropathy, at approximately 7 wk and 3 wk, respectively, a supernatant fraction of the brain was prepared and microtubule reassembly rates determined. Tubulin concentration and its sulfhydryl content were determined, as was the zinc content of the supernatant fraction. Similar measurements were made on rat brains taken after 3 wk, although the rats did not exhibit neurological signs. The reassembly rates of tubulin from the deficient guinea pigs and chicks were not decreased. That of the −Zn:AL rats was significantly less than that of the +Zn:AL group, but was not different from the +Zn:RF group. The tubulin sulfhydryl concentration was higher in deficient guinea pigs than in controls. There was no correlation between supernatant zinc concentration and the rate of reassembly. Because low zinc status had no effect in the guinea pig and chick, species susceptible to zinc deficiency neuropathy, it was concluded that the observed neuropathy is not the result of deranged properties of tubulin.

Keywords: zinc deficiency; guinea pigs; chicks; rats; tubulin; microtubule reassembly

Introduction

Zinc deficiency in experimental animals adversely affects their nervous system function. The effect on the peripheral system is manifested by abnormal posture and locomotion in chicks,[1,2] guinea pigs,[3,4] and to a lesser extent, in rats, which develop a kangaroo stance.[5] Guinea pigs of low zinc status exhibit signs of hyperalgesia and avoidance of movement,[6] and chicks tend to remain in an immobile and squat position.[7] Zinc deficiency also impairs behavior and cognition in rats,[8,9] suggesting a detrimental effect on the central nervous system.

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Physiological studies show that both guinea pigs[6] and chicks[7] fed a low zinc diet develop peripheral neuropathy characterized by decreased sciatic nerve conduction velocity. This impaired motor nerve function is reversible and returns to normal within 2 weeks after dietary repletion. In the guinea pig, the clinical pathology and decreased conduction velocity are associated with decreased Na,K-ATPase activity in the sciatic nerve. While low sodium pump activity may be causally related to the neuropathy, other possible causes exist. Axonal transport requires functional neurotubules[10] and small concentrations of zinc (5 μmol/L) stimulate rapid axonal transport of proteins in frog nerves.[11] Thus, failure of microtubule formation might be involved in the peripheral neuropathy of zinc deficiency.

There is evidence that the rate of microtubule reassembly, following cold-induced disassembly, is decreased in the supernatant fraction of brain tissue from zinc deficient animals. Hesketh reported a slowed rate of tubulin polymerization in growing rats[12,13] and pigs[13] and Oteiza et al. made similar observations in preg-

nant and non-pregnant adult rats,[14] as well as in fetuses[15] and weanling pups[16] of zinc depleted dams. Addition of 10 μmol/L of zinc to the assay system stimulated reassembly of tubulin in the supernate of zinc deficient rat brains.[12] The effect of zinc deficiency on microtubule reassembly in brain preparations from guinea pigs and chicks has not been investigated.

Microtubules are essential cell structures composed of two similar polypeptide subunits, α- and β-tubulin.[17] They exist in a dynamic equilibrium of assembly-disassembly, a state that is affected by numerous factors. Magnesium and guanosine triphosphate (GTP) promote polymerization while calcium is inhibitory.[18] Zinc ions added in vitro promote aggregation, high concentrations (300–600 μmol/L) leading to abnormal sheet structures[19] and lower concentrations (10–40 μmol/L) giving rise to tubular structures similar to those in cells.[12,20]

The primary objective of this study was to assess the effect of zinc deficiency in three different species on the properties of their brain tubulin. The species, guinea pigs, chicks, and rats, differ in their susceptibility to and the severity of the peripheral neuropathy that results from zinc deprivation. Thus, the possible correlation of clinical neuropathy and tubulin properties was examined. The parameters measured include tubulin concentration in the brain supernate, rate of reassembly, and the sulfydryl content of reassembled tubulin. The concentrations of zinc in plasma and the supernate were measured and related to the rate of polymerization.

Materials and methods

Animals and diets

Immature rats of Wistar origin (10 per treatment group) and weanling guinea pigs of the Hartley strain (six per group) were obtained from the departmental colonies. One-day-old male Peterson × Arbor Acre broiler chicks (12 per group) were obtained from a local hatchery. At the beginning of an experiment the animals were weighed individually, and assigned randomly to one of three treatment groups. They were housed in stainless-steel wire-mesh cages and the rooms were maintained on a 12-hr light-dark cycle and at a temperature of 22° C. For the baby chicks the battery temperature was thermostatically controlled at 27° C. All animals were supplied deionized water ad libitum and weighed at least weekly.

The basal guinea pig diet (−Zn) was the same as described previously.[4] It was based on EDTA-treated soybean protein and contained 0.9 ± 0.08 mg Zn/kg. The basal rat diet (−Zn) was based on dried egg white and contained 0.3 ± 0.02 mg Zn/kg.[21] The low-zinc chick diet (−Zn) was based on autoclaved egg white and was supplemented with 5 mg Zn/kg (total 5.9) to allow survival for the 3-week period.[22] For each species, one group was fed the low zinc diet ad libitum (−Zn:AL), one group a zinc adequate (basal diet supplemented with 50 mg/kg for chicks and 100 mg/kg for the mammalian species) diet ad libitum (+Zn:AL), and one group the adequate diet restricted (+Zn:RF) to the consumption of the −Zn:AL group.

The guinea pigs were fed the respective diets until the −Zn:AL group exhibited clinical signs of peripheral neuropathy; this occurred on average after consumption of the −Zn diet for 7 wk. At that time, tissues were collected from all groups. Chicks were fed the respective diets for 3 weeks, at which time the −Zn:AL group showed severe signs of deficiency. Tissues were taken from all groups during the next week. A 3-week depletion period was used also for the rats, but they did not exhibit clinical signs of neuropathy during this period.

Tissue preparation and microtubule reassembly

Blood was obtained from chickens by cardiac puncture and from rats and guinea pigs from the trunk. After clot formation, serum was saved for zinc analysis. Immediately upon removal, the brain was placed in cold homogenization buffer, then gently blotted dry and weighed. With slight modifications, the brain tissue was processed as described by Hesketh.[13] After weighing, the tissue was homogenized in 2 vol of cold buffer (10 mmol/L sodium phosphate, pH 7.0, containing 10 mmol/L $MgCl_2$ and 1 mmol/L GTP) using a motor driven teflon/glass tissue grinder. The homogenates were kept on ice for 1 hr to insure complete microtubule disassembly, then centrifuged for 30 min at 100,000*g* at 4° C. The supernatant fluid was decanted and kept on ice until used for reassembly. An aliquot was analyzed for zinc.

For the measurement of reassembly rate, GTP was added to prewarmed aliquots of the supernate to provide 1.5 mmol/L. The rate was determined turbidimetrically by use of a thermostatically controlled model 16 Cary spectrophotometer equipped with a circulating water bath maintained at 37° C and strip chart recorder. The rate of change in absorbance at 350 nm was measured in two aliquots of different protein concentration (approximately 0.5 and 1 mg/mL). After an initial lag phase, the rate became linear and the initial rate, delta A/hr based on 1 mg total supernate protein per mL, was calculated from the mean of the two rates. The results are reported on the basis of tubulin protein concentration, as measured by ^{3}H-colchicine binding; the initial rate is expressed as delta A/cpm.

Colchicine binding

Colchicine binding in the supernatant fluid was determined by the method of Ravindra and Grosvenor.[23] Briefly, aliquots of supernatant fluid were incubated with 100,000 cpm of [ring C-methoxyl-^{3}H]colchicine (4.2 Ci/mmol, Amersham Corp., Arlington Heights, IL) for 50 min. at 37° C in a shaking water bath. The reaction was terminated by addition of 1.2 mg of activated charcoal (Sigma Chemical Co., St. Louis, MO) to adsorb unbound colchicine. The charcoal was pelleted by centrifugation and the radioactivity of aliquots of the supernatant fluid determined in a liquid scintillation counter (Beckman Instruments, Fullerton, CA, model LS-1701) with approximately 30% counting efficiency. The results are expressed as cpm bound per mg of supernatant protein.

Tubulin sulfhydryl determination

Microtubule sulfhydryl content was determined by use of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) as described by Ellman.[24] Aliquots of the brain supernate were allowed to polymerize for 1 hr under the same conditions used for determination of reassembly rate. Microtubules were collected by centrifugation at 100,000*g* for 30 min at 25° C. The pellet was resuspended by homogenization in the phosphate buffer, pH 7.0, using a teflon/glass tissue grinder. Aliquots of the protein suspension were incubated with DTNB in a

3-mL volume. Absorbance at 412 nm was recorded when it became constant. A molar extinction coefficient of 13,600 M/cm was used to calculate sulfhydryl concentration. The results are reported on the basis of μmol per mg of pellet protein.

Supernatant fluid and serum zinc analysis

For zinc analysis, the supernatant fluid used for reassembly was diluted three-fold, and the serum four-fold, with 1.0% HCl. Both were aspirated directly into the flame of an atomic absorption spectrophotometer (Varian SpectrAA-30, Varian Techtron Pty, Mulgrave, Australia).

Protein determination

Protein in the supernatant fluid was determined by the method of Lowry et al.[25]

Statistical analysis

The tubulin data were analyzed using Harvey's Least-Squares Maximum Likelihood Mixed Weighted Computer Program.[26] The least-squares analysis of variance included the main effects of block (day), treatment, the covariate of initial weight, and the initial weight by treatment interaction. The initial weight by treatment interaction was removed from the linear model (pooled with the block × treatment error term) if the interaction was not significant ($P > 0.25$). Least-squares means and standard errors were calculated using the final model. Pre-planned linear comparisons (two-tailed tests) were used to ascertain significant differences between treatment means. Regression coefficients for the initial polymerization rate versus rat brain supernate zinc, adjusted for the model described, were calculated.

Results

Both the guinea pigs and chicks in the −Zn:AL groups showed clinical signs characteristic of zinc deficiency, including evidence of peripheral neuropathy.[6,7] Abnormal posture and locomotion as well as hyperalgesia were always present in the guinea pigs, and an arthritic gait and tendency to squat were prominent signs in the chicks. Although the rats showed loss of appetite, dermatitis, and hair loss, signs of neuropathy were minimal. A slight arching of the back was the only sign observed. All species were severely zinc deficient, as shown by the plasma zinc and body weight gain data shown in *Table 1*. The plasma zinc concentrations were markedly depressed in all species fed the −Zn diets; only in the rats did food restriction affect plasma zinc. Of the three species, guinea pigs had the lowest plasma zinc values whether fed a low or adequate zinc diet.

Data related to tubulin concentration, rate of reassembly, and sulfhydryl content are presented in *Table 2*. The initial rate of polymerization of the tubulin preparation from guinea pig brain was unaffected by zinc status. That of the deficient chick brain was significantly faster than that of controls. These observations were surprising in view of earlier results obtained in the rat[12–16] and prompted us to make similar determinations in the rat under our laboratory conditions.

Table 1 Zinc status of growing rats, guinea pigs, and chicks*

Diet†	Plasma zinc	Weight gain
	μg/mL	g/d
I. Guinea pig (6/group)		
−Zn:AL	0.175 ± .064[a]	0.5 ± 0.2[a]
+Zn:RF	0.647 ± .059[b]	1.9 ± 0.3[b]
+Zn:AL	0.689 ± .062[b]	5.4 ± 0.5[c]
II. Chicken (12/group)		
−Zn:AL	0.245 ± 0.081[a]	5.9 ± 0.6[a]
+Zn:RF	1.28 ± 0.064[b]	6.4 ± 0.4[a]
+Zn:AL	1.31 ± 0.064[b]	32.1 ± 0.7[b]
III. Rat (10/group)		
−Zn:AL	0.229 ± 0.043[a]	0.4 ± 0.1[a]
+Zn:RF	1.03 ± 0.037[b]	0.6 ± 0.3[a]
+Zn:AL	1.23 ± 0.040[c]	4.6 ± 0.4[b]

*Least-squares means ± SEM. Means within a column and for a given species but without a common letter superscript are significantly different ($P < 0.005$).
†−Zn:AL, low zinc diet, ad libitum; +Zn:RF, zinc adequate diet, restricted fed; +ZN:AL, zinc adequate diet, ad libitum.

Compared with the ad libitum-fed control, zinc deficiency in the rat decreased the rate of reassembly slightly ($P < 0.04$, one-tailed test) in our hands, but the −Zn:AL group did not differ from the +Zn:RF group. The decreased rate was associated with a lower concentration of tubulin, as measured by colchicine binding. For this reason the rate of polymerization was corrected for tubulin concentration in all cases.

Because tubulin polymerization is stimulated by the addition of zinc in vitro, one might expect a correlation of the zinc concentration in the supernate with rate of polymerization. There appeared to be an association in the case of the rat supernates, but there was not a significant correlation, as indicated by comparison of treatment group slopes, adjusted for tubulin concentration. The regression coefficients for the −Zn:AL, +Zn:RF, and +Zn:AL groups were −0.024 ± 0.187, −0.183 ± 0.224, and −0.305 ± 635, respectively. The concentration of protein in the supernates of all species was unaffected by zinc status and the sulfhydryl content of the tubulin pellet was affected significantly only in the guinea pig. In this species it was higher in deficient animals than in controls, contrary to observations made in the rat by others.[12,13] In this study, there was a tendency for a higher tubulin sulfhydryl concentration in the zinc deficient rat. It is also of interest that control chick brain tubulin contains a nearly eight-fold higher sulfhydryl concentration than mammalian tubulin.

Discussion

In confirmation of earlier studies,[6,7] low zinc status in the guinea pigs and chicks gave rise to clear clinical signs of periphral neuropathy, but low status produced no or only mild clinical signs in rats. The results of the tubulin reassembly give a definitive answer to the question of possible association of tubulin polymerization with clinical signs of peripheral neuropathy. The spe-

Table 2 Microtubule reassembly rate, tubulin concentration, and tubulin sulfhydryl content in brain supernates from growing guinea pigs, chicks, and rats of different zinc status*

Diet	Initial rate†	Colchicine binding	Supernate zinc	Supernate protein	Sulfhydryls in tubulin pellet
		cpm/mg protein	μg/mL	mg/mL	μmol/mg protein
I. Guinea pig					
−Zn:AL	1.82 ± 0.17	58,389 ± 4401	1.30 ± 0.09^{a,b}	10.8 ± 0.38	0.401 ± 0.025^{a}
+Zn:RF	1.83 ± 0.14	63,618 ± 3816	1.36 ± 0.08^{b}	10.1 ± 0.36	0.317 ± 0.021^{b}
+Zn:AL	2.08 ± 0.15	63,485 ± 4021	1.10 ± 0.08^{a}	10.6 ± 0.37	0.319 ± 0.022^{b}
P value	>0.28	>0.38	<0.05	>0.18	<0.05
II. Chicken					
−Zn:AL	5.48 ± 0.72^{a}	85,477 ± 2489	ND	9.35 ± 0.14	2.66 ± 0.053
+Zn:RF	4.73 ± 0.71^{a,b}	85,594 ± 2476	ND	9.18 ± 0.14	2.78 ± 0.049
+Zn:AL	3.77 ± 0.71^{b}	90,473 ± 2458	ND	9.52 ± 0.14	2.74 ± 0.054
P value	<0.10	>0.17		>0.16	>0.16
III. Rat					
−Zn:AL	0.45 ± 0.05^{a}	95,312 ± 9361^{a}	1.13 ± 0.081^{a}	9.57 ± 0.26	0.627 ± 0.15
+Zn:RF	0.50 ± 0.05^{a,b}	115,609 ± 8126^{b}	1.32 ± 0.070^{b}	9.09 ± 0.25	0.430 ± 0.18
+Zn:AL	0.58 ± 0.05^{b}	121,486 ± 8742^{b}	1.36 ± 0.076^{b}	9.43 ± 0.26	0.361 ± 0.16
P value	<0.08	<0.10	<0.08	>0.2	>0.4

*Least-squares means ± SEM; means within a column and for a given species without a common letter superscript are significantly different; the *P* values relate to the significant differences indicated.
†Initial rate of reassembly is expressed as delta $A \cdot h^{-1} \cdot cpm^{-1} \times 10^5$, the cpm as a measure of the tubulin concentration in the supernate.

cies, guinea pigs and chicks, that had signs of neuropathy showed no change in tubulin concentration and no change, even an increase, in the rate of reassembly. Compared with the ad libitum controls, there was a slight decrease in the polymerization rate in zinc deficient rats, the species most resistant to neuropathy. This was primarily a food intake effect.

The tubulin concentration in brain supernate was quantified by ^{3}H-colchicine binding and expressed as cpm per mg of total protein. When so expressed, the rat preparation showed a difference in concentration. But when expressed in terms of total cpm (data not shown), the tubulin content did not correlate with treatment in either the rat or the other species tested. To account for any individual preparation differences in tubulin concentrations, we expressed the initial rate of microtubule formation in terms of slope per unit of tubulin (cpm of bound colchicine).

The rat data reported here are in partial agreement with the results of others[12–16] who have reported that the slowed microtubule reassembly can be attributed largely to lower zinc concentrations in brain supernates of zinc deficient animals. Our data substantiate earlier observations that the initial rate of microtubule formation is lower in rats of low zinc status than in ad libitum-fed controls. However, the reassembly rate of restricted-fed controls was not different from that of either the +Zn:AL or −Zn:AL groups, suggesting that the reduction in reassembly in the rat is largely attributable to depressed food intake. In earlier studies only Oteiza et al.[16] included a restricted-fed group, and their work suggests that part of zinc status effect on microtubule reassembly is secondary to the associated depression in food intake.

Consistent with the results of Hesketh[13] and Oteiza et al,[14,16] supernate zinc was depressed in the rats as a consequence of zinc deficiency, that of the −Zn:AL group being 85% or less of the controls. In the guinea pig brain supernate, the zinc concentration was highest in the zinc deficient group. The reason for this difference is not obvious, but it should be noted that, even in severe zinc deficiency, there is little or no change in whole brain zinc concentration in most species studied.[4,15] There was not a significant correlation of supernate zinc concentration and reassembly rate in the rats studied here. One variable source of the supernate zinc in all studies is the blood plasma left in the brain tissue, because none of the brains were perfused. The plasma of the deficient animals contained less zinc than controls and would contribute less zinc. Any soluble zinc metalloprotein in the brain would likely appear in the supernate, but zinc would not be in a free ionic form and probably would not differ in concentration in any case. Redistribution of brain zinc pools has been associated with zinc deficiency.[27,28] Essentially nothing is known about the zinc concentration in extracellular and cerebrospinal fluids, so it is difficult to make an inference about intracellular free zinc, in vivo, of animals with different zinc status.

Microtubules are formed by polymerization of tubulin subunits in association with microtubule-associated proteins (MAPs).[17] The different effect of zinc deficiency on rate of reassembly in brain supernates of guinea pigs and chickens as compared with rats may be related to microtubule or MAP characteristics. Brain tissue contains different types of microtubules,[29] and cold-labile microtubules contain a higher proportion of MAP 2 than do cold-stable microtubules.[30] Such diversities are of particular interest considering the possibility that the effect of zinc on microtubule reassembly may be related to an interaction of zinc with MAPs. The weak affinity with which zinc binds tubulin and the low intracellular free zinc concentration indicate that the reported effects of zinc on microtubule reassembly is related to its effect on MAPs.[15,31]

It has been proposed[12,13] that the decreased reassem-

bly rate associated with zinc deficiency is related to a reduction in tubulin free sulfhydryl groups. The data presented here do not support such a concept. In rats, the zinc deficient group had the slowest rate of microtubule reassembly and the highest concentration of tubulin sulfhydryls. Similarly, microtubules collected from extracts of zinc deficient guinea pigs were found to have a higher sulfhydryl content than corresponding control groups.

Oteiza et al.[16] suggested that impaired microtubule assembly is one of the underlying biochemical lesions responsible for the teratogenicity associated with zinc deficiency. It seems quite clear that defective microtubule assembly is not responsible for the peripheral neuropathy observed in guinea pigs and chicks.[6,7] The biochemical and physiological bases for the different responses of zinc deficient guinea pigs and chickens as compared with rats, as regards tubulin properties, are not yet apparent. However, the effect of food restriction appears to play a role. The need for multi-species comparisons in nutrition is reinforced by the data presented.

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