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EFFECT OF ZINC SUPPLEMENTATION ON NUTRITIONAL IMMUNE DEFICIENCY

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ABSTRACT

In previous studies, during rehabilitation of young children suffering from severe protein-energy malnutrition, we observed, on admission, an increased percentage of immature lymphocytes, and, *in situ* with non-invasive ultrasonography, a thymic mass involution. Normal anthropometric values were recovered after one month of rehabilitation whereas two months were required for immunologic recovery. The aim of the present study was to investigate the effect of a daily zinc supplementation (2 mg per kg) as an immuno-stimulatory treatment to accelerate the immune rehabilitation of these children. A case-historically matched cohort study of 32 malnourished children was planed because the hospital ethical committee refused a comparative study using placebo. Children of both groups underwent nutritional rehabilitation for two months but the test group received a daily zinc supplementation from admission on. Social and psychological aspects were also taken into account. Both groups reached anthropometric recovery in one month. Children with daily zinc supplementation showed faster thymic mass recovery than the control and reached immune recovery in one month while an another month was needed for control children. Zinc supplementation hastened immune recovery that coincided with nutritional recovery and the duration of hospitalization was shortened.

KEY WORDS: Protein-Energy Malnutrition, Children, Thymus, Zinc, Lymphocytes subsets.

INTRODUCTION

Protein-energy malnutrition (PEM) decreases immunocompetence and increases infections in preschool children (1). This immune deficiency secondary to

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malnutrition or nutritionally acquired immune deficiency syndrome (NAIDS) (2), is directly or indirectly responsible for the death of one child every 2 seconds worldwide (3). These children show impaired cellular immunity (4, 5) with thymic involution (6) or nutritional thymectomy (7) and an increase in non T - non B cells or "null cells" (8). This increase in null cells or immature lymphocytes corresponds to "a deficiency of thymic inductive capacity that may result in impaired differentiation and maturation of T lymphocytes"(9).

Concerning Senegalese children who had died of malnutrition, a previous investigation provided direct evidence that nutritional thymic involution is accompanied by an altered thymulin content. Jambon et al. (6) emphasized that "this functional change is probably one of the main causes of the deficiency in cell-mediated immunity." In Bolivian malnourished children, a cross-sectional study showed a high level of immature lymphocytes (CD1a) and an *in vitro* lymphodifferentiative effect of thymulin on peripheral lymphocytes (10). Incubation of immature T lymphocytes (CD1a) with thymulin reduced to half the initial level the percentage of immature cells and the effect was the same at admission as upon discharge (11).

During recovery of severely malnourished children, rapid repletion of weight deficits reduced hospitalization time (12) according to clinical and anthropometrical criteria of discharge (13). Some authors (14) emphasized that weight for height relationship as recovery indicator does not coincide with the recovery of other anthropometric or physiological parameters. For instance, Parent et al. (15) noted that " the return to normal of the immunological indices was more protracted" than biochemical parameters and we observed that anthropometric recovery was reached in 5 weeks while thymic recovery needed 9 weeks (11).

Early discharges based on anthropometric and clinical recovery from hospitalization could explain the frequent relapses of recovered children because they remain immune-depressed. To avoid the relapses and the creation of a "vicious circle" between malnutrition anf infection, it is necessary to cure clinical or nutritional aspects of PEM and also restore the immunological function (15, 16, 17).

We agree with Olusi (16) " to investigate immuno-stimulatory treatment that could be used to rapidly restore their depressed cell-mediated immunity". In PEM, Chandra showed depressed thymulin activity estimated with the Rosettes test (18) and we demonstrated the *in vitro* effect of thymulin incubation on immature lymphocytes (10, 11). Thymulin, like other thymic hormones, could be candidate for such treatments and some authors have used it for other pathologies associated with immune deficiency (19, 20), but the hormonotherapy was excluded for its excessive cost and difficulties to use it in developping countries. Thymulin acquire its biological activity when zinc is added to the "Facteur Thymique Sérique" (FTS) (21), so zinc, as a thymulin cofactor, must be tested before using thymic hormone therapy.

In two recent Chilean studies (22, 23), zinc supplementation in malnourished children increased the cell-mediated immune response to purified protein derivative (PPD) and phytohemagglutinin (PHA) and decreased the morbidity rate. Golden et al. (24) were the first to give a 4-week zinc supplementation treatment and observed restoration of the thymus size, as estimated by X-ray, in severely malnourished children. In the current study, we used two new tools which were not used in

Golden's study : ultrasonography, a non-invasive technique, to quantify the thymic mass and its restoration, along with and MABs (monoclonal antibodies) to evaluate the level of immature lymphocytes (10,11).

MATERIALS AND METHODS

Severely malnourished children hospitalized in the Materno-Infantil "German Urquidi" Hospital, Cochabamba (Bolivia) were selected. They were treated firstly for respiratory and/or intestinal infections. The children, for which parental consent had been obtained for a 2 month follow-up study, were admitted to the CRIN (Centro de Rehabilitación Inmuno-Nutricional). It is the first Bolivian center able to restore both nutritional and immunological aspects of PEM.

All of them came from poor homes in Cochabamba suburban areas. Their socioeconomic status was characterized by low income, crowded living conditions, lack of sanitation and early weaning.

Kwashiorkor, Marasmus and combined PEM diagnoses were based on weight for height (25), arm per head circumference ratio (26) and clinical findings such as: presence of edema, loss of subcutaneous tissue and diminished muscle mass (27).

To test the effect of zinc supplementation, the hospital ethical committee did not agree on the creation of a placebo group, so all malnourished children admitted to the CRIN received, from admission, 2 mg of element zinc per kg per day. The supplemented children were compared to similarly ill and malnourished children previously treated without zinc. A case-historically match cohort study of 32 malnourished children was designed. Each of the 32 selected children was matched, according to their nutritional pathology, age, sex, and anthropometrical criteria and the same treatment pattern was used (11).

Over a 2-month period, the children received a diet divided into four steps:
1) Initial Phase (1 week): To decrease the risk of transitory lactose intolerance (28), we used a milk-based diet with one-half the lactose concentration, given in 7 feedings per day, supplying 1.5 to 2.5 g of protein and 120 to 150 kcal/kg body weight per day, according to the PEM pattern.
2) Transition Phase (1 week): gradual slow increase of protein and energy.
3) "Caloric-Protein Bombing" Phase (6 weeks): for rapid weight gain with sufficient energy for protein accretion, 5 g of protein and 200 kcal/kg body weight/day were given (29, 30).
4) Discharge Preparation Phase (1 week): gradual decrease of protein and energy.

From CRIN admission, each child was clinically examined daily. Weight, height, arm and head circumferences as well as triceps skinfold thickness were measured weekly according to the standardized methods of Jelliffe (31). Weight for age, height for age and weight for height, were calculated according to the W.H.O. recommendations based on the National Center for Health Statistics distribution (32). Arm-head circumference ratio (Kanawati-MacLaren Index), weight / height ^{2} (Body Mass Index) and upper arm bone-muscle area were calculated too (26, 33).

Thymus size was assessed weekly by mediastinal echographic examination using an echo camera (ALOKA SSD-210 DXII, Tokyo) with a 5 MHz linear pediatric probe. To standardize thymus changes, we determined the longitudinal echographic section area of the left thymus lobe between the second and fourth rib (10, 11).

For identification of lymphocyte subpopulations, 3 to 5 ml of blood were collected by venipuncture with Liquemine (Roche, Paris) as anti-coagulant on days 0, 35 (week 5) and 63 (week 9). After leukocyte separation with Ficoll-Paque (Pharmacia, Uppsala), cell suspension was adjusted to 10^7 cells/ml, divided into 2 aliquots and incubated for 2 h with cell culture medium: RPMI-1640 (Gibco, Gaithersburg, MD) or RPMI-1640 + thymulin (Choay, Paris) (23). Quantification of CD3 and CD1a lymphocytes was performed using OKT3 and OKT6 monoclonal antibodies (Ortho Diagnostic Systems-France, Roissy) with FITC-GAM (fluorescein isothiocyanate conjugate-goat anti mouse) (10, 11). At least 200 cells were counted under a UV fluorescence episcopic microscope.

Differences between groups were compared with a *t* test.

RESULTS

The similarity of the anthropometric parameters on admission confirmed the correct matching of the two groups (Table 1).

TABLE 1
Anthropometric data on admission for the matched groups (mean ± SD)

Parameters	Control Group (32)	Zinc suppl. Group (32)
Age (months)	18.7 ± 7.25	18.84 ± 7.22
Weight (kg)	6.91 ± 1.74	6.80 ± 1.78
Height (cm)	71.78 ± 6.95	71.68 ± 7.09
Head Circumference (cm)	43.87 ± 2.57	43.80 ± 2.38
Arm Circumference (cm)	10.88 ± 1.41	11.03 ± 1.58
Triceps Skinfold T (mm)	4.80 ± 1.89	4.63 ± 1.89
Arm/Head ratio x 10^3	247.38 ± 24.88	251.51 ± 31.16
Upper Arm Muscle Area (cm²)	7.05 ± 1.39	7.41 ± 1.78
Body Mass Index	13.16 ± 1.49	13.02 ± 1.95
HAZ	- 3.2 ± 1.2	- 3.2 ± 1.4
WAZ	- 3.7 ± 1.0	-3.8 ± 1.1
WHZ	-2.4 ± 0.8	-2.5 ± 1.2

HAZ, WAZ, WHZ: z score respectively for Height for age, Weight for age, Weight for height

Anthropometrical and nutritional results.

Between group anthropometric recovery.

For both groups, after the first month of hospitalization, weight gain and other parameters such as arm circumference, triceps skinfold (TST), upper arm muscle area (UMA), arm/head ratio and Body Mass Index, were significantly different

from the admission levels (Table 2). Between the first and second month of hospitalization, the difference was not significant except for TST and arm/head ratio. The same was noted with the ponderal indices such as weight for age and weight for height (Table 3).

TABLE 2
Effect of zinc supplementation on anthropometric recovery (mean ± SD).

	Group	week 0	p	week 5	p	week 9
age (months)		n = 32 18.7 - 18.8		n = 32 19.8		n = 26 20.8
Weight (kg)	Control	6.9 ± 1.7	**	8.3 ± 2.2	NS	8.9 ± 2.2
	Zn Sup.	6.8 ± 1.8	***	8.3 ± 1.7	NS	9.0 ± 1.5
Height (cm)	Control	71.8 ± 7.0	NS	72.6 ± 6.9	NS	73.6 ± 6.6
	Zn Sup.	71.7 ± 7.1	NS	72.5 ± 6.8	NS	74.2 ± 5.7
Head C. (cm)	Control	43.9 ± 2.6	NS	44.6 ± 2.6	NS	45.1 ± 2.4
	Zn Sup.	43.8 ± 2.4	NS	44.8 ± 2.2	NS	45.4 ± 1.7
Arm C. (cm)	Control	10.9 ± 1.4	***	12.8 ± 1.6	NS	13.8 ± 1.5
	Zn Sup.	11.0 ± 1.6	***	13.0 ± 1.5	NS	13.9 ± 1.4
Arm/Head	Control	247.4 ± 24.9	***	285.9 ± 26.7	**	304.9 ± 23.8
	Zn Sup.	251.5 ± 31.2	***	291.1 ± 27.7	*	307.0 ± 27.1
TST (mm)	Control	4.8 ± 1.9	***	6.8 ± 2.0	*	7.9 ± 1.7
	Zn Sup.	4.6 ± 1.9	***	7.1 ± 2.0	NS	8.0 ± 2.3
UMA (cm²)	Control	7.1 ± 1.4	***	9.1 ± 2.0	NS	10.3 ± 2.1
	Zn Sup.	7.4 ± 1.8	***	9.4 ± 2.0	*	10.4 ± 1.7
BMI	Control	13.2 ± 1.5	***	15.8 ± 1.8	NS	16.5 ± 1.6
	Zn Sup.	13.0 ± 2.0	***	15.8 ± 1.5	NS	16.3 ± 1.5

Head C : Head circumference, Arm C : Arm Circumference,
Arm/Head : ratio of Arm and Head circumferences x 10^3 (Kanawati-MacLaren Index),
TST : Triceps Skinfold Thickness, UMA : Upper Arm Muscle Area, BMI : Body Mass Index
p: significance level between two points in time (columns) within each group:
   * p< 0.05, ** p<0.01, *** p<0.001; NS: Not significant

TABLE 3
Effect of zinc supplementation on anthropometric recovery (mean ± SD).

	Group	week 0	p	week 5	p	week 9
age (months)		n = 32 18.7 - 18.8		n = 32 19.8		n = 26 20.8
HAZ	Control	-3.2 ± 1.2	NS	-3.2 ± 1.2	NS	-3.2 ± 1.0
	Zn Sup.	-3.2 ± 1.4	NS	-3.2 ± 1.4	NS	-3.0 ± 1.2
WAZ	Control	-3.7 ± 1.0	***	-2.7 ± 1.2	NS	-2.4 ± 1.1
	Zn Sup.	-3.8 ± 1.1	***	-2.6 ± 0.9	NS	-2.3 ± 1.0
WHZ	Control	-2.4 ± 0.8	***	-1.0 ± 1.1	NS	-0.6 ± 1.0
	Zn Sup.	-2.5 ± 1.2	***	-0.9 ± 1.0	NS	-0.6 ± 0.9

HAZ, WAZ, WHZ: z score respectively for Height for age, Weight for age, Weight for height
p: significance level between two points in time (columns) within each group:
 * p< 0.05, ** p<0.01, *** p<0.001; NS: Not significant

Within group anthropometric recovery.

Zinc supplementation did not change the rate of anthropometric recovery. This is in accordance with to the observations of Golden and Golden (34) who did not observe an effect of zinc supplementation on anthropometric recovery. They observed an accretion of lean body mass (nitrogen net absorption and nitrogen retention per gram weight gained) measured by mass spectrometry, but our anthropometric estimation of lean body mass did not confirm this observation.

There were no significant differences in height growth during the two months but height gain during the second month was greater in the supplemented group than in the non-supplemented group (1.7 cm vs. 1.0 cm). This agrees with the results of Walker et al. (35) where height recovery occured after weight recovery.

Immunological results.

During the 2 months of hospitalization, variations in mature lymphocytes (T3 or CD3) were similar for the control and zinc supplemented groups of malnourished children (Table 4). The immature lymphocyte (T6 or CD1) levels decreased significantly in both groups. After 5 weeks of hospitalization, the CD1 level in the zinc supplemented group was lower than those of the control group, not only for the same period but also after 9 weeks of hospitalization.

Incubation with thymulin confirmed the results of our previous study (11). In both groups, thymulin incubation increased the level of mature lymphocytes (CD3) and markedly decreased the level of immature lymphocytes (CD1).

TABLE 4
Effect of zinc supplementation on subsets of T-lymphocytes (mean ± SD).

Group	CD	medium	week 0	p	week 5	p	week 9
Control	3	RPMI	n=32 60.9 ± 5.1	***	n=31 65.7 ± 4.8	NS	n=30 65.5 ± 4.0
Control	3	RPMI+Thy	65.7 ± 5.3 (***)	**	69.0 ± 4.2 (**)	NS	69.1 ± 3.8 (**)
Zn Sup.	3	RPMI	n=18 60.6 ± 5.3	*	n=15 65.1 ± 6.9	NS	n=13 67.8 ± 3.5
Zn Sup	3	RPMI+Thy	64.9 ± 3.7 (**)	NS	67.5 ± 7.1	NS	69.6 ± 3.4
Control	1	RPMI	nc=28, nz=28 29.9 ± 5.3	***	nc=26, nz=26 15.5 ± 4.0	**	nc=21, nz=21 11.1 ± 4.9
Zn Sup.	1	RPMI	32.5 ± 7.6	***	-10.1 ± 3.4(***)	*	8.1 ± 2.7 (*)
Control	1	RPMI+Thy	16.0 ± 3.9	***	8.7 ± 2.2	**	6.1 ± 3.6
Zn Sup.	1	RPMI+Thy	17.0 ± 4.1	***	5.9 ± 2.1(***)	NS	5.4 ± 2.1

CD: Cluster of differentiation for T-lymphocytes subsets.
Medium: lymphocytes with basic medium RPMI-1640 or RPMI + thymulin.
nc, nz: number respectively for control and zinc supplemented groups.
Significance between 2 columns : * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; NS: Not significant
    same column, upper row : (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

For the thymus (Table 5), from similar values at admission, the children with zinc supplementation presented faster thymic mass recovery than the control children. The left thymic lobule area (LTLA) reached the previously set threshold of 350 mm² (11, 36) in 5 weeks for the zinc supplemented group, whereas it took 2 months for the control group to attain this level.

TABLE 5

Effect of zinc supplementation on the estimation of thymic mass.

	Group	week 0	p	week 5	p	week 9
Number		32 - 32		26 - 26		23 - 23
LTLA*	Control	70.6 ± 10.0 (NS)	***	229.3 ± 22 (***)	***	387.7 ± 25 (*)
LTLA	Zn Suppl.	81.3 ± 7.4	***	362.2 ± 26	**	453.0 ±17.3

* LTLA : left thymic lobule area between ribs 2-4, (mm²)
mean ± standard deviation; NS: Not significant,
Significance : * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$;

Our results agreed with those of Golden et al. (24). In 4 weeks minimum, with the same zinc supplement, they observed significant enhancement of the radiological shadow of the thymic mass.

Concerning the immunologic parameters used, i.e. the level of immature lymphocytes and the estimation of thymic mass, the children of the supplemented group reached the thresholds previously fixed, 10 percent of immature lymphocytes and 350 mm² of the LTLA (11, 36), in 1 month versus 2 months for the control group (Figure 1).

FIG 1. Decrease in immature lymphocytes and recovery of thymic mass during 2 months hospitalization (The horizontal line represents the cut-off point for immature lymphocytes and left thymic lobule area respectively).

a) decrease of immatures cells (%T6)   b) recovery of thymic mass (mm²)
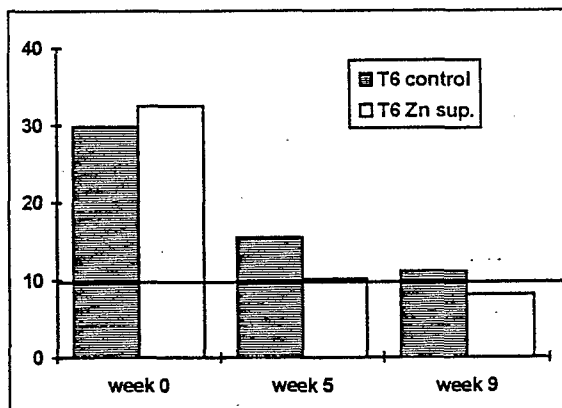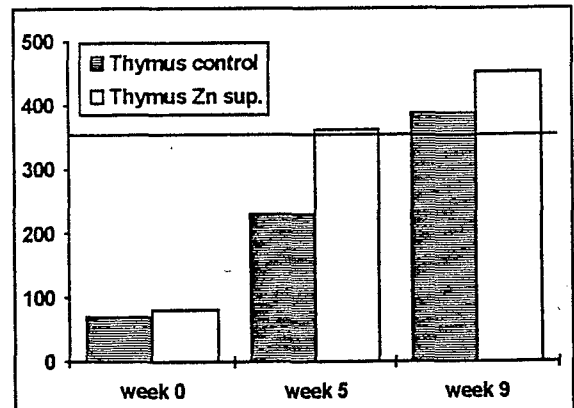
Castillo-Duran et al. (21) and Schlesinger et al. (22) gave the same supplement but used other immunologic parameters (skintest with PHA). After 3 months recovery, Schlesinger et al. (23) observed an increase in the positive responsiveness and Castillo-Duran et al. (22) noted a decrease in the incidence of infections.

DISCUSSION

In a previous study, we demonstrated (11) that nutritional recovery was faster than immune recovery. Discharge of severely malnourished children when they reached anthropometric and clinic recovery ('apparent health') but were still immune depressed, could explain the frequent relapses of these recovered children.

Our study confirmed that anthropometric recovery occurred during the first month of CRIN hospitalization. Some anthropometric parameters such as WAZ or WHZ, used to fix the discharge date, presented a lower rate of variation than other parameters such as UMA or TST during the recovery period(124, 122, 172 and 145 % of the admission levels, for WAZ, WHZ, UMA and TST respectively). Upper arm muscle area (UMA) and triceps skinfold thickness (TST) indirectly estimate muscle mass and fat body mass (37, 38, 39). They can be used to monitor anthropometric recovery while thymic echography is used to monitor immunologic recovery (11).

The thymic echography is a non-invasive technique never used to immune diagnosis in PEM. Previous studies of colleagues in Senegal (6) and our current studies in Bolivia (11, 36) are lonely studies. The cutoff fixed derived from apparently healthy bolivian children of the same range of age. For children with more than 90% of reference weight for height and 90% of reference height for age, the average of the standardized area of the left thymic lobule was 350 mm² (36). Results from other countries are needed. On the day, only a colleague in a pediatric hospital in Cuba has obtained similar results and confirmed our observations (personal communication, unpublished yet).

Physiological doses of zinc supplement (2 mg of elemental zinc per kg of body weight per day) during the 2 months of the CRIN hospitalization did not enhance anthropometrical recovery but significatively reduced the immune recovery time, compared to similarly ill and malnourished children previously treated without zinc. Zinc supplementation from admission acted as an immune stimulating factor and immunologic recovery could coincide with anthropometric recovery.

From a nutritional and immunological viewpoint, we could consider these children to be sufficiently well to return to their home environment, and these malnourished-recovered children could be discharged after only 1 month of hospitalization. Obviously, this reintegration was even better when human aspects, such as sociologic and psychologic were considered (40).

Pharmaceutical zinc salt was obtained from foreign country. The supply of zinc for daily supplement represented an additional cost of one US dollar per month of hospitalization and per infant. Compared to the dietetical cost of inpatients (30 $US per month per child)), zinc supplement during hospitalization was one of the

cheapest ways to reduce the gap between nutritional and immune recoveries, to hasten the discharge and to save one other month of hospitalisation.

Zinc was used with a pharmaceutical purpose: immunostimulation. In spite of mild doses used, zinc supplementation must be reduced to hospitals or specialized centers such as the CRIN. Supplementation after discharge could be justified only when local zinc deficiency is underlined.

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