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Clinical studies

Assessing chaperone for Zn, Cu-superoxide dismutase as an indicator of copper deficiency in malnourished children

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ABSTRACT

It is not clear how frequent is copper deficiency in humans. Current copper markers are not sensitive enough to detect early copper deficiency and new markers are needed. CCS is a candidate to become a copper biomarker.

Objective: Measuring CCS mRNA relative expression in malnourished children and compare results (a) with those of the same children after nutritional recovery and (b) with well-nourished children.

Method: On admission to the protocol and after 15 day nutritional treatment, severely (G1 = 18) and moderately (G2 = 10) malnourished children were compared with well-nourished healthy controls (G3 = 15), measuring anthropometric indicators, blood biochemistry, Cu, Fe and Zn serum concentrations, ceruloplasmin, C Reactive protein and mRNA abundance of CCS, SOD and MT2 in peripheral mononuclear cells.

Result: In malnourished groups, mean serum copper concentration was below the cut-off on admission to hospital and increased after 15 days (t-test, $p < 0.01$). On admission to protocol, CCS mRNA abundance in G1 and G2 was higher than in G3 (one way ANOVA, $p < 0.001$). After 15 days, CCS expression decreased as expected (t-test, $p < 0.001$). Initial SOD mRNA relative abundance was higher in study groups than controls and also between G1 and G2 (One way ANOVA, both $p < 0.01$); after 15 days, G1 and G2 were not different (t-test, NS). MT2A abundance of transcripts did not follow a clear change pattern.

Conclusion: CCS mRNA abundance responded as expected, being higher in malnourished children than in controls; nutritional recovery in these latter resulted in decreasing expression of the chaperone, supporting the hypothesis that CCS may be a copper biomarker.

Introduction

Historically, copper deficiency was thought to be rare, but there is increasing evidence indicating that it is present in a variety of situations. Classical cases were described in the Menkes syndrome, a rare genetic disease that often results in death [1,2]. Also, it is reported under a number of pathological conditions, such as anemia [3], gastrectomy and gastric bypass surgery [4], neurologic abnormalities [5], malabsorption syndrome such as celiac disease [5,6] and malnutrition [7]. Cu deficiency has also been reported in association with zinc supplementation [8], preterm infants with low

birth weights [9,10] and elderly patients [11]. Evidence on how frequent is copper deficiency in the general population is scarce. We found that it was highly prevalent in children less than three years of age admitted due to both moderate and severe malnutrition [12]. More recently, we observed that in 23% of men and 30% of women older than 65 years serum copper concentration was below the cut off [13].

One of the difficulties in characterizing milder, acquired forms of copper deficiency and its effects on human health is that classical indicators to assess copper status – mainly serum copper and ceruloplasmin concentrations – are useful to diagnose rather severe changes of copper status, but not sensitive enough to detect milder degrees of copper deficiency.

Novel indicators of copper status have been searched among a series of proteins related to copper metabolism; extensive reviews have analyzed the literature describing that while most of them have failed to respond to variations of copper exposure in a sensitive, reproducible manner [14,15], studies of the chaperone to Zn-Cu-superoxide dismutase (CCS) strongly suggest that this protein

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is a potential copper indicator. Since first descriptions and characterization [16–20], it was described in mammals [21] and in copper deficiency rodent models, its relation to SOD changes and the differences obtained when assessing mRNA CCS and/or CCS protein [22]. More recently, a consistent opposite response (measured as mRNA CCS transcripts) was observed in two experimental conditions assessed in healthy adults [23,24]. Based on this evidence, in this study we tested the hypothesis that CCS mRNA transcripts would be increased in malnourished children as compared to well-nourished children and that they would decrease after nutritional recovery.

Materials and methods

Design and study groups

This study was conducted in malnourished and well-nourished (control) children receiving medical care at Centro de Nutrición Infantil and patients of Hospital de Pediatría Albina R. de Patiño, in Cochabamba, Bolivia. Stabilization and nutritional recovery was described in detail elsewhere [12]. Briefly, all children admitted for moderate or severe malnutrition constituted potential candidates to this study; they were identified on arrival to hospital, when admitted for an episode of acute infectious diarrhea or upper respiratory infection. Clinical stabilization and feeding followed WHO Guidelines, 1999 [25–27], including milk formulas plus a minerals and vitamins mix (CMV, Nutriset®), providing 285 μg Cu and 2 mg Zn per 100 mL of formula, in addition to the mineral content of powdered cow's milk [27]. Ferrous sulfate (3 mg/kg/d, administered at mid-morning, between meals) was started after infections subsided and administered during the 15 day included in this protocol. After children were free of edema, serum albumin was ≥30 g/L, antibiotic treatment was finishing and clinical signs of infection subsided (approximately 7 days after admission), they were referred to the Nutritional Recovery Center, where anthropometric measures were obtained, children were divided into the study groups following their nutritional status and then participated in this protocol for the following 15 days. Informed consent was signed by the parents or legal guardians prior to incorporation to the study. The protocol was approved by IRB of INTA, University of Chile and the Committees of Research and Ethics, Centro de Nutrición Infantil and of Hospital de Pediatría Albina R. de Patiño, Cochabamba, Bolivia. By suggestion of these committees children were incorporated to this protocol after reaching clinical stabilization and the comparison group was measured only at basal time.

On admission to the protocol at the Nutritional Recovery Center, anthropometric measurements were obtained and children 3 months to 3 years of age were divided into three groups: group 1 (G1) was formed by children with severe acute malnutrition (Z score, median and SD) (Weight/Length [28]: -2.9 ± 1.43 and Length (height)/Age: -3.0 ± 1.15); group 2 (G2), by children admitted with moderate acute malnutrition (Weight/Length between: -2.7 ± 0.20 and Length (height)/Age: -0.8 ± 0.21) [30] and group 3 (G3) (Weight/Length (height): 0.8 ± 0.57 and Length (height)/Age: -0.9 ± 0.35) by apparently healthy and asymptomatic children, who attended a routine medical checkup at the outpatient clinics. Anthropometric measurements were obtained using calibrated equipment and standardized techniques [29], on the day of admission to the Nutritional Recovery Center.

Sampling and procedures

Clinical data, anthropometric measures and blood samples were collected on admission to the protocol (Basal) and after 15 day nutritional recovery (Day 15). Five to eight mL anticubital venous blood was collected at 8–9 AM, after 4 h fasting. Hemoglobin and

hematocrit were performed immediately and the remaining sample was snap frozen to $-40\,^{\circ}$C until processing. CRP was measured by a semi-quantitative technique (Lorne Laboratories, Reading, United Kingdom); cut off (<0.6) was defined following the manufacturer indications; other determinations were hemoglobin (Coulter T540, Hematology Analyzer, Diamond Diagnostics, USA); proteins (DiaLab, Wiener Neudorf, Austria), serum ferritin (ELISA assay; INACG, 1985), serum Fe, Cu and Zn (atomic absorption spectrophotometer, Perkin Elmer Model 2280, Norwalk, Conn.) and ceruloplasmin, by nephelometry (Array Protein System; Beckman Instruments Inc., Brea, CA). A calibration curve of three points for each metal was carried out using a CertiPur solution for Fe (1.19781), Cu (1.9786) and Zn (1.9806) (Merck, Germany). Also, as an internal control, MR-CCHEN-002 (Venus antiqua) and DOlt-2 (Dogfish liver) preparations were used as reference materials to validate the mineral analyses.

Copper status was assessed by copper serum concentration (cut off = ≥80 μg/dL [30]) and ceruloplasmin (cut off = ≥22 μg/dL [31,32]. There are no well-established cut off for serum Zinc concentration for children below 3 years; in this study we defined it at 80 μg/dL, following the International Zinc Nutrition Consultative Group [31] and Hotz et al. [32]. Iron status was assessed by hemoglobin (cut off = ≥12.4 g/dL, as corrected per altitude and age [33,34], ferritin (cut off = ≥10 μg/L) and serum iron (cut off = ≥30 μg/dL [34]. Peripheral mononuclear cells were obtained from the venous samples as described elsewhere [23], and mRNA abundance of CCS, SOD and MT2 were measured [25,26].

Sample size and analysis of results

The group studied represents all children younger than 3 years of age admitted to the Nutritional Recovery Center with moderate and severe malnutrition, who were previously treated at hospital and reached clinical stabilization in a week. Sample size was calculated using serum Cu concentration as dependent variable [7], with an alpha value of 5% and beta 90%. Analyses were performed using STATISTICA 6.1 for t-test for unpaired samples and Tukey's Multiple Comparison Test as post hoc test. Lethality in malnourish children maybe high; also, at the hospital where this study was conducted, parents of malnourished often demand early discharge; in view of this, potential drop off was potentially high and therefore all candidates available during the studied period were invited to participate. Calculated sample size was 10 children per group. Analysis of results included descriptive statistics, t-test for unpaired samples and multivariate analysis for repeated measures (Two-way ANOVA).

Results

Fifty malnourished children consecutively admitted to hospital with acute diarrhea and/or upper respiratory infections were referred to the Nutritional Recovery Center after being clinically stable. Forty-five of these accepted to participate in this protocol, signed the consent and provided complete data and were analyzed, divided into three groups: G1 was formed by 18 children (with severe malnutrition); G2 by 10 children (with moderate malnutrition, 12 were incorporated to the protocol, 2 died shortly after admission) and G3 by 15 age balanced asymptomatic children that attended the well-baby clinic. Some relevant general characteristics on admission and after 15 day nutritional recovery appear in Table 1. On admission to hospital, five of those with severe malnutrition suffered marasmic malnutrition and fifteen presented edematous malnutrition; at incorporation to this protocol all were free of edema and their serum albumin was ≥30 g/L. After Basal assessment, children progressed as expected without secondary

Table 1
General characteristic of studied children on admission (Basal) and after 15 day nutritional recovery (Day 15).

n	Group 1 18	Group 2 10	Group 3 15
Age (months)	15.7 (7.6–27.7)	16.4 (10.5–25.9)	15.9 (9.1–29.2)
Girls/boys	13/7	5/5	9/6
Basal			
Hemoglobin (g/dL)	10.9 (8.6–13.7)	10.5 (9.3–12.0)	11.9 (9.6–14.1)
Hematocrit (%)	33.5 (25.7–40.1)	33.0 (29.2–37.1)	38.3 (33.7–42.1)
Total proteins (g/L)	59.1 (32.2–75.2)	71.2 (66.9–78.2)	70.1 (65.2–73.5)
Albumin (g/dL)	30.7 (15.2–47.0)	40.8 (37.4–46.0)	41.1 (37.4–47.0)
Globulins (g/dL)	28.5 (15.6–37.0)	30.2 (25.8–36.6)	28.9 (23.6–34.0)
Day 15			
Hemoglobin (g/dL)	10.5 (8.4–13.0)	10.2 (9.1–11.7)	
Hematocrit (%)	31.9 (24.3–38.9)	31.0 (27.5–33.4)	
Total Proteins (g/L)	59.4 (39.0–76.2)	70.1 (65.7–74.0)	
Albumin (g/dL)	31.1 (19.6–47.3)	40.3 (38.8–42.6)	
Globulins (g/dL)	28.3 (19–36.6)	29.7 (26.0–33.3)	

Values are expressed as mean and (range).
Differences between groups at Basal and Day 15 = NS.
Differences in Group 1 and in Group 2 between Basal and Day 15 = NS.

infections, food intolerances or other complications. Four of the children with edema on admission lost weight after the 15 day protocol whereas the remaining ones gained a mean of 48 g/kg/day.

At basal time, serum ferritin, Fe, Cu and Zn were different between groups (one way ANOVA, $p < 0.019$; $p < 0.038$; $p < 0.0001$ and $p < 0.005$, respectively). After 15 day treatment CRP did not decrease, but serum ferritin diminished in both malnourished groups to values comparable those observed in G3. Also, serum copper increased; comparison between G1 and G2 were not significant (paired *t* test, NS) and values did not reach those observed in group 3; however, differences within groups (comparing basal time against day 15 (Table 2) were significant in G1 and in G2 (both $p < 0.01$, *t*-test).

At basal time, CCS mRNA relative abundance was higher both in G1 and G2 in comparison to G3 (one way ANOVA, $p < 0.001$); differences were also significant between G1 and G2 (*t*-test, $p < 0.01$). After 15 day treatment, CCS expression decreased as expected in G1 and G2, and was different between G1 and G2 (*t*-test, $p < 0.001$) (Fig. 1).

Table 2
Biochemical characteristics of studied children on admission (Basal) and after 15 day nutritional recovery (Day 15).

	G1	G2	G3
Basal			
Ceruloplasmin (μg/dL)	40.1 ± 18.7	45.7 ± 3.9	41.0 ± 9.6
CRP (mg/L)	14.0 (7.6–25.6)	7.1 (3.7–13.6)	10.1 (4.8–21.4)
Ferritin (μg/L)	13.8 (5.6–34.5)	14.6 (5.1–41.7)	5.8 (2.8–12.2)[a]
Iron (μg/dL)	93.0 ± 33.6	60.5 ± 26.3	80.4 ± 26.3[a]
Copper (μg/dL)	66.2 ± 16.6	84.3 ± 14.3	131.5 ± 17.9[a]
Zinc (μg/dL)	63.2 ± 13.0	56.4 ± 15.2	74.7 ± 11.7[a]
Day 15			
Ceruloplasmin (μg/dL)	39.3 ± 13.0	47.6 ± 10.9[b]	
CRP (mg/L)	14.3 (6.0–34.0)	10.5 (3.0–37.4)	
Ferritin (μg/L)	8.1 (2.6–24.7)	6.0 (2.4–14.8)	
Iron (μg/dL)	78.9 ± 36.9	61.3 ± 25.5	
Copper (μg/dL)	109.7 ± 29.7	110.5 ± 17.7	
Zinc (μg/dL)	65.6 ± 14.9	66.4 ± 10.3	

Comparison G1/G2.
[a] Basal time: One way ANOVA, $p < 0.001$. (Post hoc test: Tukey's Multiple Comparison Test.)
[b] After 15 days: *t*-test, $p < 0.001$.
Comparison basal/15 days:
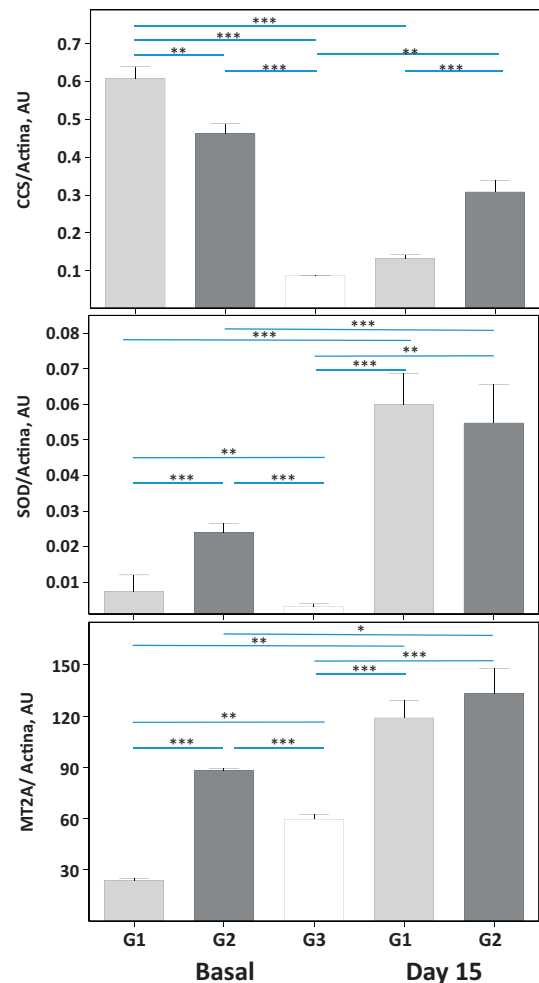Cu G1: <0.01, *t*-test.
Cu G2: <0.01, *t*-test.

Fig. 1. Relative abundance of CCS (A); SOD (B) and MT2A (C) in peripheral mononuclear cells children on admission (Basal) and after 15 day nutritional recovery (Day15). At basal time: One way ANOVA, *$p < 0.001$; **$p < 0.01$. After 15 days of treatment: *t*-test, $p < 0.001$.

SOD relative abundance was increased in G1 and G2 as compared to G3 and also differed when comparing G1 and G2 (One way ANOVA, both $p < 0.01$). After treatment, SOD expression increased but differences between G1 and G2 did not reach statistical significance in both malnourished groups (G1 and G2, *t*-test NS). The relative abundance of MT transcripts showed a different pattern, G1 was lower and G2 was higher than G3 (One way ANOVA, both $p < 0.001$). After treatment, MT2A expression increased in both G1 and G2 (*t*-test, NS).

Discussion

Characteristics of the malnourished and well-nourished groups support previous studies that confirms that copper deficiency is frequent among both severely and moderately malnourished children [12], making these groups attractive to assess copper related proteins that may be indicative of changes of copper status, in humans and in real life conditions. Results support the hypothesis that CCS expression changes during copper deficiency and also that this protein responds to copper repletion associated with nutritional recovery. As expected and following the mean group serum copper concentrations, CCS mRNA abundance of transcripts in malnourished children were increased both in cases suffering moderate and severe malnutrition when compared to asymptomatic, healthy age balanced controls. This is interesting because moderate

malnutrition represents a less intense detrimental situation in comparison with severe malnutrition; therefore, results suggest that CCS mRNA abundance of transcripts already respond in this milder condition and changes are detectable. The subsequent decrease of CCS mRNA transcripts after treatment with copper is also in agreement with evidence obtained measuring CCS protein in cell lines tested in conditions of initial Cu deficiency and subsequently in copper supplemented media [18,20,21], and preliminary results described in humans [23,24]. Obtaining similar responses in humans suffering non-experimental copper deficiency helps further advancing in the assessment of CCS as potential copper indicator.

Although in this study the assessment of malnourished individuals has given the opportunity to evaluate copper proteins in humans and in a real life situation, authors are aware of the limitation that this poses. Ethical constraints did not permit assessing contemporary blood samples after 15 day treatment in the healthy comparison group. However, considering the short time elapsed between the initial and final times of assessment (15 days), it seems reasonable to expect that the single basal determination may serve for comparison purposes. Another ethical constraint was given by the need to administer full treatment to patients since admission to hospital, making impossible to isolate copper and zinc and iron as study variables. Nevertheless, it is of interest that in these conditions changes of mRNA CCS transcripts were detected, as expected. A third point for discussion is how infection might influence the results obtained. After 15 day nutritional treatment children were clinically recovered from infections, but CRP values were still high; the fact that CRP was also high in healthy children (Group 3) suggests that all these children had persistent or chronic infections. Detailed analysis of these aspects are beyond this study, but it is indeed relevant that in the presence of all these confounding variables, changes of mRNA CCS transcripts were still detectable and significant.

Assessment of SOD has given inconclusive data [15,35]. In this study, results of SOD expression are as expected, since on admission both groups of malnourished children expressed more SOD transcripts than G3; however, results of this study do not permit explaining why G2 expressed more SOD than G1. It is interesting that after copper repletion, both malnourished groups increased their SOD expression well above the levels observed in G3 at basal time. To what extent this represents copper repletion or the response to the inflammatory state associated with malnutrition cannot be answered with results of this protocol. In summary, this study provides one more piece of information to the body of evidence necessary to define whether CCS is a sensitive copper indicator. Future studies should assess whether and how changes of mRNA transcripts concur to those described for CCS protein; sensitivity, specificity and predictive value of the proposed markers should be calculated and whether mRNA CCS transcripts or CCS protein is the best economic option to develop as a test applicable to massive groups.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgement

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