Differential response of interleukin-2 production to chronic copper supplementation in healthy humans

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ABSTRACT. *Backgound.* **Copper (Cu) is an essential trace element for many biological processes including maintenance of both innate and acquired branches of immunity.** *Objective.* **To measure the effect of copper supplementation on IL-2 and TNF-α production in subjects with lower and higher ceuloplasmin (Cp) values within normal range.** *Design.* **Healthy adults (17 men and 16 women) with normal-low (low Cp) and normal-high Cp (high Cp) values were supplemented with 10 mg Cu/day (as $CuSO_4$) during 2 months.** *Method.* **Before and after supplementation blood mononuclear cells were incubated in the absence or presence of phytohaemagglutinin or lipopolysaccharide for induction of IL-2 and TNF-α, respectively. The secretion of cytokines was measured by ELISA. Cu supplementation did not modify classical biochemical markers of Cu status.** *Results.* **After supplementation, a significant increase in IL-2 production was found only in subjects with normal-low plasma Cp. Before and after Cu supplementation geometric mean and range ± 1 SEM values were 1,566 (1,287-1,905) and 2,514 (2,159-2,927) pg/mL, respectively (two way ANOVA for repeated measures: Cp level $p < 0.001$; time = NS; interaction Cp level and time $p < 0.05$). We did not observe changes in TNF-α production after Cu supplementation.** *Conclusions.* **Cu supplementation increased secretion of IL-2 and not TNF-α, which suggests an activation of proliferative but not inflammatory cytokines. These results support hypothesis that IL-2 may be a good indicator to identify a subgroup of individuals (polymorphism) who differs in Cu metabolism.**

Keywords: copper supplementation, serum copper, ceruloplasmin, IL-2, TNF-α, humans

Copper (Cu) is known to play an important role in the development and maintenance of both innate and acquired branches of the immune system [1, 2]. In animal models and cultured cell lines Cu deficiency results in a decrease of both numbers and function of lymphocytes derived from the thymus [3] and in lower antibody production in response to T-cell-dependent antigens [4]. The interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α) are mainly secreted by activated blood T-lymphocytes and monocyte/macrophage system respectively, where both play a central role in regulating the host response to pathogenic challenges [5, 6]. IL-2 is responsible for progression of T-lymphocytes from the G1 phase to S phase of the cell cycle; TNF-α provides signals for T-B cells interaction [7] and activation of lymphocytes [8]. Inadequate Cu nutrition impairs the production of cytokines in different animal species. Mononuclear cells from lactating diary cows fed low Cu diets produced less interferon gamma when stimulated with concanavalin A [9]; copper deficiency diminished IL-2 synthesis by activated rodent splenocytes [10], mice splenic non-adherent cells [11] and Jurkat T-cell line [12]. Cu deficiency also affected the secretion of inflammatory mediator TNF-α in calve macrophages [13] and U937 monocytic cell line [14]. In humans, impaired secre-

tion of IL-2 receptor (a marker of early T cell activation) by peripheral blood mononuclear cells from healthy subjects who received a low-Cu diet has been described [15].

In recent years, several studies have searched for indicators sensitive to marginal copper deficiency in humans. Studies by Olivares et al. in Chile [16] showed that 25.6% of the adult population in Santiago was below the estimated average copper requirement (0.7 mg/d) proposed by the US Institute of Medicine, suggesting that a proportion of the Chilean population is at risk of being marginally copper deficient. Previous studies done by us have failed to demonstrate copper deficiency by using Cp, serum Cu and Zn-Cu-SOD as indicators of copper status [17], which may be due to lack of copper deficiency in the population or to lack of sensitive indicators of copper status.

The effects of high Cu-intake on proliferative and proinflammatory cytokine production have poorly been understood in humans. We hypothesized those subjects with low ceruloplasmin (Cp) level, within normal range, may respond differently to copper supplementation. We further hypothesized that within the Cp distribution curve that represents the normal population, those representing the extreme tails will show a different response of IL-2 to copper supplementation. Thus, in this study we investigated

the effects of a controlled 2-month copper supplementation on the production of IL-2 and TNF-α in individuals chosen from the ≈ 5% of the upper and lower values of the Cp distribution curve obtained in 800 apparently healthy adults.

PATIENTS AND METHODS

Design and study group

This study is part of a larger protocol that assessed the response of several biochemical and urinary potential indicators of copper status to 10 mg of copper (as copper sulfate) administered as a single daily dose, for 60 days. Those results will be reported elsewhere. The 800 apparently healthy individuals screened for their serum ceruloplasmin protein were free of acute infectious/inflammatory processes (C-reactive protein in blood samples < 0.8 mg/dL), of chronic illnesses, of anemia (hemoglobin < 120 g/L for women and < 130 g/L for men,) and of chronic multi medication that may interfere with the study. Potential participants received detailed information about the protocol and those who accepted to participate signed an informed consent prior to starting copper supplementation. This consisted of two 5 mg copper gelatin capsules (as copper sulfate), administered in a controlled fashion once per day, with plain tap water, between meals. Blood samples obtained by venous puncture were obtained within 3 days prior to and after copper supplementation.

Fourteen and 19 individuals of both gender, belonging to the lower (low Cp) and higher (high Cp) decile in the Cp distribution curve obtained, respectively, formed the study groups. Participants were 25 to 45 years of age, balanced by sex. The protocol and the consent form were approved by the institutional Review Board on Ethics in Human Research, University of Chile, Santiago, Chile.

Copper status

This was assessed by means of plasma copper, plasma Cp and erytrocyte Zn-Cu-superoxide dismutase (eSOD). Plasma Cu was analyzed by atomic absorption spectrophotometry (Perkin Elmer Model 2280, Norwalk, Conn.), plasma ceruloplasmin protein was determined by nephelometry (Array Protein System, Beckman Instruments Inc., Brea, CA) and eZn-Cu SOD activity was measured using a commercial kit (Bioxytech SOD-525 Assay, OXIS International Inc, Portland OR).

Cell studies

Blood mononuclear cells (BMNC) were isolated on Histopaque gradient (density: 1.119, Sigma Diagnostic, St. Louis, MO), washed twice with phosphate buffer saline (PBS) and suspended at 4.0×10^6 cells/mL in RPMI 1640 culture medium with gentamycin (Elkins-Sinn, Inc. Cherry Hill, NJ).

Induction of IL-2 and TNF-α

BMNC suspension was dispensed in 96-well flat-bottom microtiter plates (1×10^5/well) in the absence or presence of phytohaemagglutinin (PHA: 10 µg/mL) or lipopolysaccharide (LPS: 1.0 µg/mL) for induction of IL-2 and TNF-α, respectively. Cultures in triplicate were incubated for 1 day at 37°C in humidified atmosphere (95% air-95% CO2). Supernatants were collected and kept at -70°C until cytokine assays. Determinations were performed in one batch to avoid inter-assay variations [18]. *In vitro* production of IL-2 and TNF-α were measured by using specific commercial enzyme-linked immunosorbent assay (ELISA: R&D System, Minneapolis, MN). Results were expressed as mean picograms per milliliter (pg/mL) obtained in duplicate experiments.

Statistical analysis

Statistical analyses were performed using Statistica for Windows, release 4.5 (StatSoft Inc., Tulsa, OK) and included two way ANOVA for repeated measures and Pearson correlation. Because the values of IL-2 and TNF-α had a skewed distribution, these values were converted to logarithms before performing mean, SEM and statistical analysis. Results were retransformed into anti-logarithms to recover the original units and expressed as geometric means and range ± 1 SEM range.

RESULTS

Participants remained healthy along the study period; daily home visits controlled copper ingestion and appearance of symptoms, revealing that participants remained free of clinically apparent infectious and inflammatory processes. *Table 1* shows the indicators used to define Cu status. As expected, prior to supplementation both serum Cu and serum Cp were significantly different between the study groups (p < 0.001) but eSOD activities were similar. In agreement with C reactive protein, total leukocytes count was normal in all groups at both times of assessment. After

Table 1
Copper status in Cp-low and Cp-high groups before and after copper supplementation

	Before supplementation		After supplementation	
	Low Cp	High Cp	Low Cp	High Cp
Parameter	(n = 14)	(n = 19)	(n = 14)	(n = 19)
Serum copper (µmol/L)*	12.2 ± 4.2	24.5 ± 5.5	13.7 ± 2.2	24.8 ± 5.5
Ceruloplasmin (mg/L)*	255 ± 22	603 ± 119	296 ± 88	526 ± 127
SOD Activity (U/mg Hb)	84.1 ± 18.9	86.7 ± 45.9	78.3 ± 30.2	67.7 ± 28.6
Leukocyte (10^9/L)	7.2 ± 2.0	6.7 ± 1.3	7.5 ± 1.8	7.2 ± 1.7

Values expressed as mean ± 1 SD. SOD = superoxide dismutase. *p < 0.001 between groups before supplementation and after supplementation.
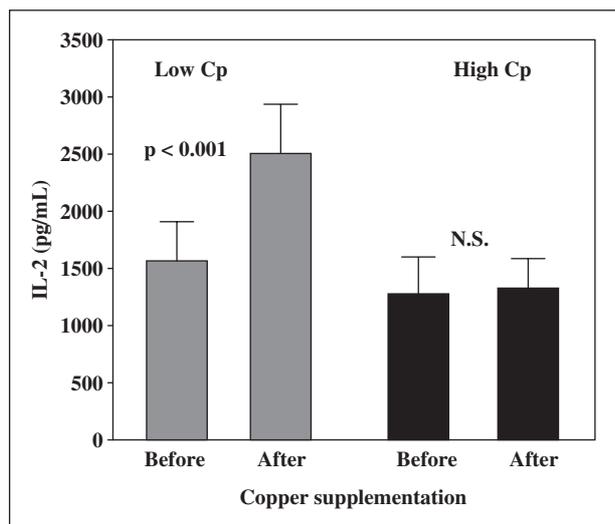
Figure 1
Effect of Cu supplementation on IL-2 production by BMNC. Results are expressed as geometric mean ± SEM (two way ANOVA for repeated measures: Cp value p < 0.001; time = NS; interaction Cp value and time p < 0.05).

supplementation there were no significant changes of serum Cu, Cp protein or eSOD.

IL-2 production by PHA-stimulated BMNC was similar in both study groups prior to copper supplementation (*figure 1*). After supplementation, a significant increase in IL-2 production was found only in the low Cp group (geometric mean and range ± 1SEM in pg/mL = 1,566 (1,287-1,905) to 2,514 (2,159-2,927) before and after Cu supplementation respectively (two way ANOVA for repeated measures: Cp value p < 0.001; time = NS; interaction Cp value and time p < 0.05). No statistical sex-differences on IL-2 production in low Cp subjects were found before and after supplementation. There are not secondary effects on IL-2 production due to zinc and iron status. Number of responders for increased cell output of IL-2 to the Cu supplement were 11/14 and 4/19 in low and

high Cp groups, respectively. In addition, subjects with low plasma Cp showed a higher correlation (r = 0.82, p < 0.001) between IL-2 levels before and after nutritional intervention than those individuals with high Cp (*figure 2*). TNF-α production by LPS-stimulated BMNC in supernatants of cells from both groups were similar between the study groups both prior to and after copper supplementation. Before and after supplementation, mean (range) values were 3825 pg/mL (2516-6689) and 4135 pg/mL (2561-6109) in low Cp volunteers, and 4358 pg/mL (1571-8176) and 4235 pg/mL (1564-9487) in high Cp group.

DISCUSSION

Results show that the 2-month Cu supplementation with 10 mg/day induced an increased of IL-2 but not of TNF-α production in individuals that represented the lower end of the Cp distribution curve obtained in apparently normal population. It calls the attention that this subgroup did not increase plasma levels of Cp after Cu supplementation. We only measured the protein concentration without evaluate the enzymatic activity. It is known that Cu deficiency affects preferentially Cp activity and in lower degree the protein concentration [19]. However, marginal copper deficiency in our volunteers was discarded since no changes in eSOD after Cu supplementation were found. A possible explanation is that individuals with low Cp represent a particular genetic polymorphism for driving Cu metabolism and they would have an increased oxidative stress, after copper supplementation that determines an increased IL-2 production. Recent studies demonstrated that oxidative products like hydrogen peroxide promoted IL-2 production in cultured Jurkat T cells [20]. Future investigation could clarify these alternatives.

It has been difficult to estimate dietary recommendations of copper. Former figures of 1.5-3.0 mg Cu/day [21] have been challenged because a large survey of copper content in the diet showed that up to 30% of the diets measured were below recommendations, however, no apparent copper deficiency was observed in the population [22]. Rec-
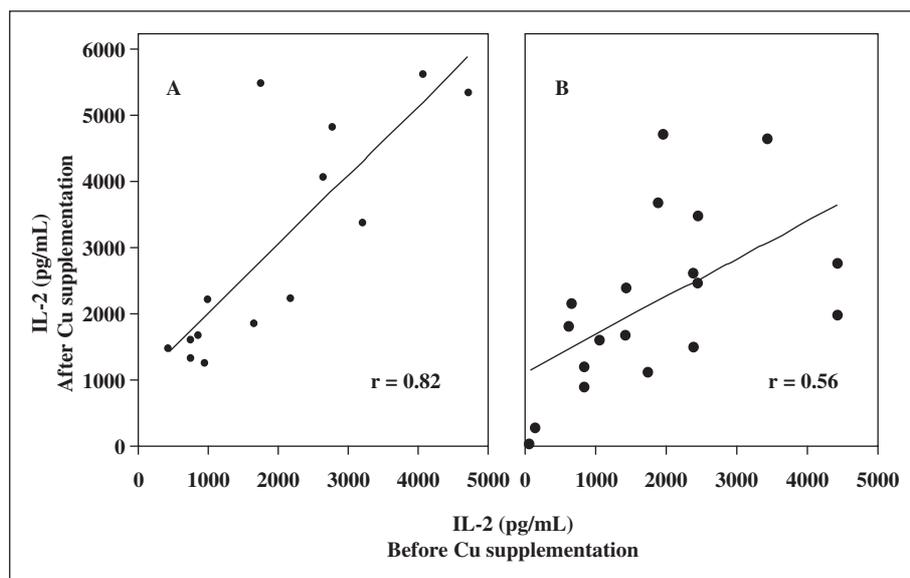

Figure 2
Correlation between IL-2 values obtained prior to and after 2-month controlled copper supplementation. **A)** Subjects with low Cp values. **B)** Subjects with high Cp values.

ommendations published in 2001 lowered the recommended daily dietary allowance for Cu to 0.9 mg/day [23]. With increasing epidemiological evidence indicating a role for suboptimal Cu status in disease, accurate assessment of Cu status in human subjects is becoming a pressing challenge. Unfortunately, it is not clear yet what are the earlier and biologically relevant effects of marginal copper deficiency, and what indicators should be used to detect them. Traditional biochemical markers of Cu status including Cp protein levels (reference values: 200-350 mg/L) and serum copper (reference values: 11-22 umol/L) have proved to be insensitive to marginal decreases in Cu status [24] and unresponsive to Cu intake [25].

IL-2 is the main mediator responsible for activation of T lymphocytes, and its measurement would be a good immunologic biomarker of Cu status [26]. However, effects of high intake of Cu on this cytokine activity in humans are under current investigation. In our study, we observed a significant increase in IL-2 production after PHA stimulation in BMNC among individuals with lower Cp. This change could be related to increase IL-2 mRNA expression, since this correlation has been consistently associated with Cu status in animals and cell lines [12, 27]. Recently, Turnlund and co-workers found diminution of plasma IL-2 receptor (IL-2R) levels after long-term high Cu supplementation [28]. In this study increasing 3 doses for more than 5 months in healthy young men were administered, while we used a unique dose for 2-months in healthy adults from both sex. Taking together, these results suggest that high Cu intake affects differently concentration of IL-2 and density of IL-2R on the cell surface. The significance of these findings on T cell proliferation requires further research.

The effect of Cu status on pro-inflammatory cytokine production is not well documented and some controversial results have been described. Lukasewycz and associates [29] found that IL-1 production by splenocytes and peritoneal macrophages isolated from mice with sufficient Cu status was lower than in Cu deficient mice. Production of IL-1 and TNF-α by isolated peripheral blood monocytes from Cu-deficient calves was not affected by treatment with a dietary Cu supplement [30]. In the present study, we observed no changes in TNF-α production after controlled Cu exposure suggesting that supplementation did not induce an inflammatory response. Recently, other authors neither found significant changes in normal human plasma IL-6 after long-term high Cu intake [28]. The fact that Scuderi [31] demonstrated an increase of TNF-α production by normal human monocytes that were *in vitro* cultured with increasing concentrations of CuSO$_4$ is probably due to the high-non-physiological concentrations used.

Under the present regimen of Cu supplementation, improvement of IL-2 production may reflect activation and effector activities of host-defense cells. Further studies are needed to evaluate whether low copper doses given during longer periods can influence the TH1/TH2 cytokine pattern in subjects with lower or higher Cp values. Investigations using purified populations of cells, as well as evaluation of other lymphocyte functions like proliferative response are required to confirm and widen our observations. Finally and since Cp influences on the level of expression of T-lymphocytes markers, it would be desirable to know if this plasma protein also contributes to IL-2

secretion and the possible mechanism(s) involved in this process.

REFERENCES

1. Percival SS. Copper and immunity. *Am J Clin Nutr* 1998; 67(suppl): 1064S.

2. Erickson KL, Medina EA, Hubbard NE. Micronutrients and innate immunity. *J Infec Dis* 2000; 182: S5.

3. Bala S, Failla ML, Lunney JK. Alterations in splenic lymphoid subsets and activation antigens in copper deficient rats. *J Nutr* 1991; 121: 745.

4. Prohaska JR, Lukasewycz OA. Biochemical and immunological changes in mice following postweaning copper deficiency. *Biol Trace Elem Res* 1989; 22: 101.

5. Smith KA. Interleukin-2: inception, impact, and implications. *Science* 1988; 240: 1169.

6. Munoz C, Carlet J, Fitting C, Misset B, Bleriot JP, Cavaillon JM. Dysregulation of *in vitro* cytokine production by monocytes during sepsis. *J Clin Invest* 1991; 88: 1747.

7. Aversa G, Punnonen J, de Vries JE. The 26 kD trans-membrane form of tumor necrosis factor-α on activated CD4+ T cell clones provides a costimulatory signal for human B cell activation. *J Exp Med* 1993; 177: 1575.

8. Hackett RJ, Davis LS, Lipsky PE. Comparative effects of tumor necrosis factor-α and IL-1β on mitogenic-induced T cell activation. *J Immunol* 1988; 140: 2639.

9. Torre PM, Harmon RJ, Sordillo LM. Modulation of bovine mononuclear cell proliferation and cytokine production by dietary copper insufficiency. *J Nutr Immunol* 1995; 3: 3.

10. Bala S, Failla ML. Copper deficiency reversibly impairs DNA synthesis in activated T lymphocytes by limiting interleukin-2 activity. *Proc Natl Acad Sci USA* 1992; 89: 6794.

11. Prohaska JR, Lukasewycz OA. Effects of copper deficiency on the immune system. *Adv Exp Med Biol* 1990; 262: 123.

12. Hopkins RG, Failla ML. Copper deficiency reduces interleukin-2 (IL-2) production and IL-2 mRNA in human T-lymphocytes. *J Nutr* 1997; 127: 257.

13. Gengelbach GP, Ward JD, Spears JW, Brown Jr. TT. Effects of copper deficiency and copper deficiency coupled with high dietary iron or molybdenum on phagocytic cell function and response of calves to a respiratory disease challenge. *J Anim Sci* 1997; 75: 1112.

14. Huang ZL, Failla ML. Copper deficiency suppresses effector activities of differentiated U937 cells. *J Nutr* 2000; 130: 1536.

15. Kelley DS, Daudu PA, Taylor PC, Mackey BE, Turnlund JR. Effects of low-copper diets on human immune response. *Am J Clin Nutr* 1995; 62: 412.

16. Olivares M, Pizarro F, de Pablo S, Araya M, Uauy R. Iron, zinc and copper: contents in common Chilean Foods and daily intakes in Santiago, Chile. *Nutrition* 2004; 20: 205.

17. Araya M, Olivares M, Pizarro F, Gonzalez M, Speisky H, Uauy R. Gastrointestinal symptoms and indicators of copper status in apparently healthy adults undergoing controlled copper exposure. *Am J Clin Nutr* 2003; 77: 646.

18. Lopez M, Rios E, Schlesinger L, Olivares M, Nunez MT, Munoz C. Tumour necrosis factor-α transcription in transferrin-stimulated human blood mononuclear cells: is transferrin receptor involved in the signalling mechanism? *Br J Haematol* 2003; 120: 829.

19. Milne DB. Assessment of copper nutritional status. *Clin Chem* 1994; 40: 1479.

20. Nindl L, Peterson NR, Hughes EF, Waite LR, Johnson MT. Effect of hydrogen peroxide on proliferation, apoptosis and interleukin-2 production of Jurkat T cells. *Biomed Sci Instrum* 2004; 40: 123.

21. National Research Council (NRC). *Food and Nutrition Board. Recommended Dietary Allowances, 10th ed.* Washington, DC: National Academy Press, 1989.

22. International Atomic Energy Agency (IAEA). *Human dietary intakes of trace elements: a global literature survey mainly for the period 1970-1991. I. Data list and sources of information*. Vienna: IAEA, 1992.

23. Institute of Medicine Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Molybdenum, Nickel, Silicon, Vanadium and Zinc. *Food and Nutrition Board*. Washington, DC: National Academic Press, 2001.

24. Milne DB. Copper intake and assessment of copper status. *Am J Clin Nutr* 1998; 67: 1041.

25. Kehoe CA, Turley E, Bonham MP, O'Connor JM, McKeown A, Faughnan MS, Coulter JS, Gilmore WS, Howard AN, Strain JJ. Response of putative indices of copper status to copper supplementation in human subjects. *Br J Nutr* 2000; 84: 151.

26. Bonham M, O'Connor JM, Hannigan BM, Strain JJ. The immune system as a physiological indicator of marginal copper status? *Br J Nutr* 2002; 87: 393.

27. Hopkins RG, Failla ML. Transcriptional regulation of interleukin-2 gene expression is impaired by copper deficiency in Jurkat human T lymphocytes. *J Nutr* 1999; 129: 596.

28. Turnlund JR, Jacob RA, Keen CL, Strain JJ, Kelley DS, Domek JM, Keyes WR, Ensunsa JL, Lykkesfeldt J, Coulter J. Long-term high copper intake: effects on indexes of copper status, antioxidant status, and immune function in young men. *Am J Clin Nutr* 2004; 79: 1037.

29. Lukasewycz OA, Prohaska JR. The immune response in copper deficiency. *Ann N Y Acad Sci* 1990; 587: 147.

30. Gengelbach GP, Spears JW. Effects of dietary copper and molybdenum on copper status, cytokine production, and humoral response of calves. *J Dairy Sci* 1998; 81: 3286.

31. Scuderi P. Differential effects of copper and zinc on human peripheral blood monocyte cytokine secretion. *Cell Immunol* 1990; 126: 391.