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Original article

Effect of zinc on immune functions in patients with pulmonary tuberculosis

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ABSTRACT

The tuberculosis infection triggers in the host a complex immune response and the involvement of CD4+ lymphocytes and interferon-gamma (INF-γ) in the control processes has been reported. Since nutritional status, e.g. regarding zinc may have potent effects on the immune response, we conducted a zinc supplementation study to gain more knowledge on its effects on immune function in pulmonary tuberculosis. Twenty-one patients with pulmonary tuberculosis completed the 3-month study. Ten of them got 45 mg zinc daily and 11 of them got placebo in addition to drug therapy. Immunoglobulins in plasma and cell proliferation, IFN-γ production and CD markers in peripheral blood mononuclear cells (PBMC) were measured. >The immune system of the patients was activated as reflected by the increased concentration of immunoglobulins in plasma. Still, there was no difference in the ability of the PBMC to proliferate and produce INF-γ in response to concanavalin A between patients and controls. Moreover, there were no significant differences in these variables between the zinc-supplemented and placebo groups after 3 months. In other experiments, the addition of zinc sulphate or iron sulphate in vitro to PBMC tended to decrease the number of CD4+ cells.
Conclusions: The immune system of the tuberculosis patients maintained its activity and in response to pharmacological therapy the immune response seemed to maintain a Th1 orientation. It was not possible to document a role of zinc supplementation for the immune response.

1. Introduction

Active tuberculosis triggers in the host an immune response with the production of crucial cytokines [1]. This can also favour the progression of the infection to chronicity, especially when regulatory T-cells cause the deactivation of cytokines [2,3]. Experimental evidence in murine models showed that lung and spleen CD4+ lymphocytes were more protective than CD8+ lymphocytes in tuberculosis but also indicated the pivotal protective role of interferon-gamma (INF-γ) [4,5]. Moreover in patients with active tuberculosis, the immune response is characterized by a depressed delayed-type hypersensitivity response to tuberculin [3]. There is also evidence of a depression of PBMC proliferation in vitro after stimulation with PPD and a decreased expression of IL-2, IL-4 and INF-γ in these cells and an overexpression of IL-10 and transforming growth factor-beta in monocytes [4,6]. Available data also indicate that in tuberculosis, the pro-inflammatory CD4 Th17 cells are activated, producing IL-17A, IL-17F, tumor necrosis factor, IL-22 and in some cases also INF-γ [3]. In another study, a significantly lower PPD-induced INF-γ production was found together with a higher IL-10 production in vitro in patients with tuberculosis compared to their controls although the absolute number of lymphocytes was similar. Moreover, after 6 months of pharmacological therapy, the in vitro INF-γ production was increased non-significantly and the levels were instead much influenced by gene polymorphisms [7].

Zinc is a potent mediator of host resistance to infection because it can influence the innate and adaptive immune response in many ways [8–10]. It can increase the release of INF-γ and other cytokines by PBMC although at high concentrations [11], and induce the proliferation of CD8+ T-cells in combination with an exposure to IL-2.

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In such studies, the addition of zinc can also affect the proliferation of different cell types in response to various mitogenic stimuli although an excessive supplementation by zinc could also have a deleterious effect on immune functions [8–10].

In the present study, we tried to gain more knowledge on the effects of zinc supplementation on the immune functions of pulmonary tuberculosis patients as well as on the effects of in vitro addition of zinc and iron on the activity of PBMCs from these patients in response to mitogen stimulus.

2. Material and methods

2.1. Patients

The patients were recruited in the districts of Southern Cochabamba city in Bolivia as described elsewhere (Guzman-Rivero et al. submitted). Forty patients having active pulmonary tuberculosis based on the demonstration of acid- and alcohol-resistant *Bacilli* in stained smears of sputum samples were contacted, and 29 of them were selected since they met the additional inclusion criteria of age 15–50 years and no previous tuberculosis episodes. The exclusion criteria were extra-pulmonary tuberculosis, HIV infection, pregnancy, lactation, use of nutritional supplements, and presence of diabetes mellitus, chronic renal failure or liver disease. All patients completed a health questionnaire prior to entering the study and gave their verbal consent for inclusion into the study. Twenty-one patients completed the study and 8 left the study for different reasons (Guzman-Rivero et al. submitted).

2.2. Control subjects

The controls were age- and gender-matched subjects without any clinical signs of previous tuberculosis infection and also residents in the same area as the corresponding patients. Exclusion criteria were pregnancy, lactation, use of nutritional supplements or regular medication, presence of diabetes mellitus, chronic renal failure or liver disease. All controls completed a health questionnaire prior to entering the study and gave their verbal consent for inclusion into the study.

2.3. Study design

Patients were randomly allocated to receive zinc or placebo coded capsules for 3 months as described elsewhere (Guzman-Rivero et al. submitted). Each zinc capsule contained 315 mg of zinc gluconate (corresponding to 45 mg zinc) and each placebo capsule contained 315 mg of cornstarch, both specially prepared for our study by a company (Farmacia Artesanal, Cochabamba, Bolivia). All patients received simultaneously the standardized treatment regime for active tuberculosis of adults proposed by PHAO/WHO [12,13] (two months of daily doses of isoniazid, rifampicin, pyrazinamide and ethambutol followed by 4 months of daily doses of isoniazid and rifampicin, dosed in mg/kg body weight) together with one capsule per day (zinc or placebo) taken in the fasting state early in the morning. Controls were not given any zinc or placebo capsules. No dietary advice was given to the subjects in the study.

2.4. Collection of blood samples

For patients blood was sampled twice, at time zero (T0) before the start of the treatment and after 3 months (end of zinc supplementation, T1), and for controls, blood was sampled at time zero only. Blood was collected by venipuncture (after 12 h of fasting and 30 min of relaxing) into heparinized tubes. Plasma was obtained by centrifugation, aliquoted and stored at −80 °C until processing. The cell pellet was used for isolation of mononuclear cells in the IIBISMED laboratory, Cochabamba, Bolivia.

2.5. Immunoglobulin and C-reactive protein measurements

The measurement of plasma immunoglobulins (IgA, IgG and IgM) and C-reactive protein (CRP) concentration was performed in the laboratory of Skåne University Hospital, Lund, Sweden. The samples were transported on dry ice from Bolivia to the laboratory. The analytical methods involved the addition of antibodies directed to the different Igs and the resulting agglutinates were measured by turbidimetry on a Cobas instrument using accredited methods.

2.6. Proliferation of peripheral blood mononuclear cells (PBMC)

This was performed as described [14]. Briefly, blood samples were collected into heparinized tubes and plasma was removed after centrifugation (5000 *g* for 10 min) and replaced by twice the volume of phosphate-buffered saline (PBS). The diluted blood was layered onto Histopaque (density 1.077) and centrifuged for 30 min at 3000 *g*. The PBMCs were washed twice with RPMI-1640 containing penicillin/streptomycin (1:100) and finally re-suspended in the same medium. The suspension was adjusted to a final concentration of 1×10^6 cells/mL after performing a manual cell counting (dilution 1:100 with Türk solution). The viability was determined in a Neubauer hemocytometer after dilution 1 + 1 with Trypan blue solution and the mean (SD) cell viability was 84 (9) %. PBMC were cultured in RPMI-1640 medium, fetal bovine serum (FBS) and antibiotics. For stimulation of cells, concanavalin A (Con A) was added at a final concentration of 5 μg/mL, and in other wells mixtures of Con A plus zinc (1 μmol/L) or iron (1 μmol/L) (both as sulphate) were added. The final volume of the culture was 300 μL/well and the cultures were performed in triplicate for 6 days. Proliferation was measured at the end of culture period as total DNA content by propidium iodide staining [15]. Briefly, the cells were permeabilized for 30 min with 500 μL of 100% ethanol and then incubated in the dark for 30 min with 500 μL of propidium iodide at 10 μg/mL in PBS. Fluorescence (excitation, 535 nm; emission, 612 nm) was measured in a fluorometer (Fluoroskan Ascent fl, cat. no 5210450, Labsystems Thermo Scientific, Helsinki, Finland). The fluorescence values for the triplicate cultures were averaged and expressed as relative fluorescence units (RFU).

2.7. Measurement of production of interferon-gamma (INF–γ) and of CD4+/CD8+ lymphocyte subpopulations in cultured peripheral blood mononuclear cells

Cells were stimulated by the addition of Con A and trace elements during culture as described above but with an incubation time of 3 days. For measurement of INF-γ, the cultured samples were centrifuged, and the supernatant was stored at −80 °C. The cell pellet was re-suspended in one volume (500 μL) of FBS and one volume of 20% DMSO in RPMI and stored in liquid nitrogen until cell phenotype analysis. Supernatants of cell culture were later thawed and an ELISA Quantikine® test kit was used for measurement of the concentration of INF-γ. The detection limit was 8.0 pg/mL (data supplied by the manufacturer) and the coefficient of variation was 6% for our assays.

Cells preserved in liquid nitrogen were thawed by keeping the vials at 37 °C for 2 min and then suspended in PBS solution, centrifuged at 3000 *g* for 10 min and re-suspended in 1% FBS in PBS. The CD cell markers BD FACSCount™ kit was used for quantification of CD4+ and CD8+ following the manufacturer's instructions. Briefly, the cells were fixed in formaldehyde solution, and monoclonal antibodies fluorescence-labelled against CD4+/8+ markers were added and the samples were measured in a FACSCount™ instrument