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Low serum zinc levels in an endemic area of visceral leishmaniasis in Bihar, India

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***Background & objectives*: India carries approximately 50 per cent of the global burden of visceral leishmaniasis and majority of patients from the poor, rural communities of Bihar State. Zinc is an essential trace element and its relevance for proper functioning of the entire immune system is already well documented. Though low serum zinc levels have been reported in many parasitic diseases, limited information is available regarding zinc status in human leishmaniasis. We investigated to define the relationship between zinc level in visceral leishmaniasis (VL) patients in endemic and non-endemic regions.**

***Methods*: Venous blood was collected from 88 patients, 16 parasitologically confirmed VL, 35 healthy controls from endemic area (Bihar) and 37 healthy urban controls from non-endemic area, Delhi. In all the three groups, levels of serum albumin, total protein (markers of nutritional status) and zinc were estimated by colorimetric methods.**

***Results:* Serum zinc levels were found to be significantly lower ($P<0.001$) in VL patients than non-endemic controls. The serum zinc levels in VL endemic controls were also significantly lower ($P<0.001$) than non-endemic controls, but these values were not statistically significantly different from VL patients. However, all samples from Bihar (VL patients and controls) had lower serum zinc levels than non-endemic controls from Delhi.**

***Interpretation & conclusion*: Low serum Zn levels, in healthy subjects from Bihar and more significantly in VL patients of this region, are possibly associated with vulnerability and endemicity of visceral leishmaniasis in the region. Further studies need to be done to assess the role of oral zinc supplementation in better management and prevention of VL, particularly in endemic areas.**

Key words Bihar socio-economic status - epidemiology - immune system - malnourishment - visceral leishmaniasis - zinc deficiency

Visceral leishmaniasis (VL) or kala-azar, caused by *Leishmania donovani*, *L. chagasi* or *L. infantum*, is endemic in 62 countries[1]. An estimated 500,000 cases per year and over 90 per cent of cases of VL occur in five countries: India, Bangladesh, Nepal, Sudan, and northeastern Brazil[1,2]. India has about 50 per cent of the global burden of VL, and eastern states Bihar, Jharkhand, East Uttar Pradesh and West Bengal are

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endemic for the disease, though sporadic cases occur across the country[3]. About 90 per cent of these patients are poor and live in the rural areas of Bihar State[3,4]. It is reported that the incidence and prevalence of this diseases remain high in these areas probably due to low socio-economic status of the inhabitants[4,5].

Low socio-economic status has wide repercussions on the societal and personal health of the individuals including the malnutrition, which ultimately leads to compromised host immune status[6,7]. Malnutrition as such is a complex condition involving deficiency of protein and energy with superimposed deficits of other essential trace elements and nutrients. It alters the innate immune response and is reported to be the most frequent cause of immunodeficiency[8]. Epidemiological and experimental studies have documented an increased risk for VL, in the malnourished hosts[6,9]. Although, information about the possible mechanisms for immunity in human VL is limited, it is well documented that for establishment of the infection *L. donovani* evades both the innate and adaptive arms of the immune system. It is also increasingly being recognized that humoral immunity is largely ineffective in containment of the disease and cell mediated immunity is essential[6,10]. A strong correlation has been shown between disease outcome and the nature of the T cell response. In an experimental study *Leishmania* infection in murine model resulted in the development of Th1/Th2 paradigm polarization of immunity[11].

The relevance of zinc in proper functioning of the entire immune system is well documented[12]. Low serum zinc levels have been reported in parasitic diseases in which immune system was affected but cause or effect relationship could not be established. In a murine model Wirth *et al*[13] have reported that short periods of zinc deficiency resulted in rapid atrophy of the thymus, with preferential involution of the cortex, reduced production of thymulin, along with substantial reductions in the total numbers of lymphocytes and phagocytes. The capacity to mount antibody- and cell-mediated immune responses was significantly reduced in zinc deficient mice infected with *Trypanosoma cruzi*[14]. However, with regard to human leishmaniasis, only limited information is available on this aspect. Sprietsma[12] hypothesized that in zinc deficiency, interleukin-1 (IL-1) is incapable of stimulating a strong Th1 response. Therefore, zinc deficiency causes a premature switch from predominantly cellular Thl responses to humoral Th2 responses. Zinc deficiency in humans induces an imbalance in *in vitro* cytokine secretion resulting in decreased secretion of interferon gamma (IFN-γ), tumour necrosis factor α (TNF-α) and IL-2. Thus, severe zinc deficiency impairs host immune response and leads to Th1 deficiency and strong Th2 response. This shift results in susceptibility towards flare up of the infection and better parasite survival inside the host. In this study, we investigated the status of serum Zn concentration in the VL patients and attempted to define the association between serum zinc levels and visceral leishmaniasis. Further, to study whether this nutritional deficiency is specifically limited to the active VL patients or has geographical distribution, we included uninfected individuals from the same VL endemic region of Bihar and non-endemic region of Delhi as controls.

Material & Methods

Sample collection: The study was carried out in the department of Laboratory Medicine, All India Institute of Medical Sciences (AIIMS), a tertiary care centre, New Delhi. The study population comprised 88 subjects of whom, 16 were visceral leishmaniasis (VL) patients (group A), 37 healthy controls from non-endemic region (Delhi) (group B) and 35 healthy controls from endemic region (Bihar, India) (group C) as shown in the Table.

VL patients, under investigation were from the State of Bihar, India, where kala-azar is endemic. These

Table. Descriptive statistics of serum zinc levels and markers of nutritional status in VL patients and healthy control groups from endemic and non-endemic regions

	VL patients (group A)	Healthy controls (Non-endemic region) (group B)	Healthy controls (Endemic region) (group C)
Sample size (n)	16	37	35
Mean age (yr)	33.7 ± 9.2	31.6 ± 9.6	30.0 ± 8.6
Male : Female	12 : 4	23 : 14	32 : 3
Mean Zn levels (μM/l)	8.1 ± 2.7*	12 ± 3	8.8 ± 3.1*
Low serum zinc values (<9.95 μmol/l) No. (%)	12 (75)*	13 (35.1)	23 (65.7)
Mean protein levels (mg/dl)	13.4 ± 2.7	13.3 ± 2.1	13.6 ± 1.9
Mean albumin: globulin ratio ± SD	0.5 ± 0.3+	0.8 ± 0.2	0.9 ± 0.1

*P<0.001 compared to group B
+ P<0.001 compared to groups B, C

patients were hospitalized at the medicine, paediatrics and haematology wards in the AIIMS hospital, New Delhi, during September 2005-November 2006 with clinical suspicion of kala-azar. Their blood, bone marrow or splenic aspirates were sent to our laboratory for routine investigations including the kala-azar serology and detection of Leishman-Donovan (LD) bodies. The diagnosis of leishmaniasis was made by antibody detection using recombinant antigens from *L. chagasi*, (Lc-rK39) and another from *L. donovani*, (Ld-rKE-16). The latter is developed and patented by our laboratory[2,3]. Both are commercially available rapid tests and are routinely used for providing confirmatory diagnosis. Further, the diagnosis is confirmed by microscopic demonstration of LD bodies in the bone marrow and splenic aspirates. The samples positive for anti-leishmanial antibodies by both the antigens in qualitative screening tests were further analysed by Ld-rKE-16 quantitative ELISA using a two-fold serial dilution in phosphate-buffered saline to determine the end-point titres.

Since disease is endemic in Bihar, social welfare camps are routinely organized in the endemic areas by us for screening of latent VL infection in healthy individuals[4]. All volunteer asymptomatic family members and villagemates of index case patients from the Samastipur, Vaishali, and Shahibganj districts of Bihar (areas of endemicity) with no enlargement of the spleen or liver constituted the endemic control group. They were not subjected to bone marrow or splenic aspirate examinations for ethical reasons. However, after informed consent their blood samples (5 ml) were collected and serum was first tested on the spot using Ld-rKE-16 rapid spot test (Signal-KA™, Span Diagnostic, India) and then transported to Delhi for further testing. Among these individuals, 35 individuals who were serologically negative for VL were included as endemic control group.

Thirty seven normal healthy individuals were included as non-endemic controls. These individuals were negative for anti-leishmanial antibodies and were clinically healthy. Majority of these individuals were laboratory staff and employees of the Institute, living in Delhi for at least 10 years. Informed consent was obtained for clinical and biochemical study from each patient and healthy control, and study protocol was approved by institutional ethics committee of All India Institute of Medical Sciences, New Delhi.

Sample processing: Blood samples were collected, allowed to stand at room temperature for 1 h, centrifuged at 1500 *g* for 5 min and serum was collected and stored in sterile 1.5 ml Nunc tubes. Samples from both LD confirmed patient (group A) and healthy controls (groups B & C) were tested for the presence of antibodies against *L. donovani* using the Ld-rKE-16 Signal-KA™ rapid lateral flow test and quantitative Ld-rKE-16 ELISA[15]. LD cases were diagnosed if they gave suggestive history, had physical findings, and were positive for Ld-rKE-16 and/or Lc-rK39.

Zinc estimation: Serum zinc level was quantified by colorimetry as previously-described[16] using α, β, γ, δ-tetrakis (4-*N*-trimethylaminophenyl) porphine tetratolunesulphonate salt (ttmapp) and ferron catalyst, using a Biomate 3 spectrophotometer (Thermo Electronics, USA), and synthetic quartz glass cuvettes (Hellma, Sigma-Aldrich, USA). This method was previously shown to produce similar results in zinc quantification as atomic absorption spectrophotometry (AAS) with a positive correlation (r = 0.982) and approximately 4 per cent difference in readings[16]. We standardized this protocol for our equipment, resulting in slight differences in methodology. All reagents and buffers were prepared as described and the ratio of working reagent (ferron-ttmapp-acetate buffer solution) to sample was kept fixed (4 parts reagent, 1 part sample). For reagent blank 100 µl distilled water [filtered through 0.2 µm filter (LabPure Analytica, BioAge, India)] instead of serum sample, and absorbance readings calibrated to give absorbance of 0.340 to 0.350. All reagents used in the study were of the analytical grade (Sigma, USA except otherwise mentioned).

Serial dilutions of standard (100 mg/dl) zinc nitrate solution (Merck, Germany) were prepared from 0.00625 to 0.5 mg/dl, using 0.1M HCl, and were incubated in a heating block for 1 h at 37°C followed by colorimetric analysis of each standard and a linear regression of absorbance was prepared. The absorbance values which showed the highest linear correlation to concentration of zinc prepared (r^2 = 0.999, data not shown) were between concentrations of 0.00625 (minimum dilution prepared) and 0.025 mg/dl, producing absorbance values between 0.440 and 0.710, and therefore, serum samples were diluted eight-fold with distilled water in order to successfully achieve absorbance readings in this range. Samples which produced absorbance readings above or below these limits were eliminated from this study. The following equation was used for calculating final serum zinc concentration (µmol/l).

$$[Zn]_s = (1.9125\ \mu mol/l)\ (8)\ ([A_{serum} - A_{serum\ blank}] - A_{reagent\ blank}) / [A_{Zn\ standard} - A_{reagent\ blank}]$$

Where $[Zn]_s$ = serum zinc concentration, A = absorbance, 1.9125 µmol/l is the concentration of zinc prepared in our zinc standard solution (replaced with 0.0125 for representation of zinc values in mg/dl), and 8 is the dilution factor used for dilution of our serum samples. All estimations were repeated 3 times and put in triplicates every time. Average of all 9 readings was taken as final value.

Total protein, albumin, globulin estimation: Total serum protein and albumin were estimated using a Techno 168 Biochemical Analyzer (I.S.E. s.r.l. Group, Italy). Five µl of serum sample was mixed with 500 µl of reagent provided, incubated for 10 min at 37°C, as per protocol, and was further analyzed for total protein and albumin. Globulin level was calculated by subtracting of albumin from total protein.

Statistical analysis: All statistical procedures were performed using SPSS v12.0 (SPSS Inc, USA). All results were expressed as number (percentage) or mean ± standard deviation (SD)/median (range) as appropriate. One way analysis of variance (ANOVA) followed by post-hoc analysis was used to compare the difference in mean values of zinc and protein among the three groups. Since the data were non-normal, the difference in median values of albumin and globulin among groups was compared by using Kruskal-Wallis test with Bonerronni corrections. $P < 0.05$ was considered significant.

Results

All 16 parasitologically confirmed cases were correctly diagnosed by Signal-KA™ test and quantitative ELISA. None of the sample taken from healthy controls (groups B & C) showed reactivity in any of these tests.

A significant decrease in serum zinc levels was observed in VL patients (group A) as compared to non-endemic controls (group B) (8.1 ± 2.7 vs. 12 ± 3 µmol/l, P=0.001). However, regional controls from Bihar (group C) also showed levels of serum zinc similar to that of group A (Table). While comparing the results of two control groups (A vs B) the serum zinc values were significantly different (12 ± 3 vs. 8.8 ± 3.1 µmol/l), (P<0.001), implying that the average resident of Bihar will have lower zinc levels than those who live in Delhi. Zinc deficiency in humans is considered when the serum zinc levels are < 9.95 µmol /l. Using this criterion, zinc deficiency was found in 75 per cent of VL patients and 65.7 per cent of healthy controls from Bihar, while only 35.1 per cent Delhi controls had defined zinc deficiency (Table). This difference between groups A and B was significant (P<0.001).

To understand whether the low serum zinc levels were either due to protein energy malnutrition or zinc intake, we measured total protein levels in all three groups (A, B & C). Total serum protein levels were similar in the three groups but the albumin/globulin ratio was significantly lower (P<0.001) in VL patients as compared to both healthy control groups (B & C) (Table). The difference of mean albumin/globulin ratio between the two regional controls was statistically insignificant indicating that there was no generalized protein energy malnutrition in the population studied.

Discussion

Leishmaniasis has remained endemic in eastern parts of India including Bengal and Bihar, since its description in the literature for more than 200 years ago[17]. The Bihar state is frequently afflicted with flood and soil has become deficient in zinc[18]. Our results also showed low serum zinc levels in both VL patients as well as healthy controls from endemic area of Bihar. It has been debated that why kala-azar (VL) is endemic in this region even though we have the suitable vector, temperature and other conditions suitable for kala-azar transmission in other parts of India. Our findings are in consonance with nutritional data of Bihar[5] that more than 50 per cent children of 9 regions of Bihar had at least Grade I documented malnourishment based on weight for age and the level of nutritional deficiencies are related to social background of the population[19]. Several studies have earlier documented that kala-azar is a disease of socially and economically backward classes of the state[19]. Besides kala-azar patients, majority of controls from Bihar displayed overt zinc deficiency. However, the fact that serum albumin and total protein levels from the regional controls from Bihar were within normal limits and comparable to urban controls of Delhi indicates that these patients were specifically zinc-deficient at baseline and the deficiency was not due to general malnutrition. Our findings support similar observations made in a study done in a kala-azar endemic population in Bangladesh, where more than 50 per cent of healthy community members showed zinc deficiency[20].

It is well established that zinc has an important role in immune system and subjects deficient in zinc, iron or retinol have increased susceptibility to a variety of infectious agents, specially the visceral leishmaniasis[20]. Our observed difference in the serum

zinc level in patients infected with *L. donovani* is clinically significant supporting the previous reports of lower serum zinc levels associated with infection by intracellular pathogens[21,22] seen in *in vitro* condition[23], in leishmania infected dogs[24] and humans with cutaneous leishmaniasis[25]. However, our study also indicates an association between human visceral leishmaniasis endemicity in Bihar and nutritional deficiency of zinc. Low serum zinc and high serum globulin levels are reported to be associated with decreased Th1 and increased Th2 response. This shifting deteriorates the protective host immune response against the intracellular pathogens such as *Leishmania*[21,22]. To produce an adequate $CD4^+$ TH type 1 immune response adequate zinc stores are required. Inability to do so is thought to be responsible for further dissemination of the organism and progression to visceral leishmaniasis[23,26]. An increased Th2 response with immunoglobulin production in cutaneous leishmaniasis has been previously shown[27] and the significantly increased serum globulin in VL patients, but not in other groups, is consistent with this and supports the hypothesis that patients who progress to visceral leishmaniasis were not able to produce an adequate Th1 response. The controls from Bihar with low serum zinc, however, did not show an increased serum globulin, indicating that zinc deficiency alone does not initiate an increased Th2 response and antibody production. Serum zinc has been hypothesized to act by upregulating gene expression of IL-2 and IFN-γ of the Th1 pathway and downregulating IL-1β, IL-8, TNF-α, and other inflammatory cytokines of the Th2 pathway. Zinc is also believed to play a role in macrophage activation during the Th1 immune response[23], responsible for eliminating the intracellular amastigotes. Since zinc deficiency could play a major role in the pathogenesis of other intracellular infectious diseases, zinc supplementation in these patients such as human papilloma virus (HPV)[28] and cutaneous leishmaniasis[29], showed increased cure rates.

Based on the present study, we postulate that oral zinc supplementation may have an additive effect in the chemotherapy and prevention of visceral leishmaniasis, in the kala-azar endemic regions of Bihar.

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