Particle induced X-ray emission study of blood samples of Indian Kala-azar patients

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Received: 24 June 2015 / Accepted: 8 March 2016 / Published online: 29 March 2016
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Abstract Visceral leishmaniasis (VL) or Kala-azar (KA) is a neglected tropical disease caused by protozoan parasite, *Leishmania* sp. and is fatal, if left untreated. In this study, we measured trace elements (K, Fe, Cu, Zn, Br, Cl, S, Ca, Mn, Cr, Ni, As, Se, Rb and Sr) in the blood of Indian VL patients (32) by particle-induced X-ray emission (PIXE) study. Blood was collected from 36 subjects including healthy controls from Rambagh Kala-azar Hospital, Muzaffarpur, Bihar, India. PIXE experiment was carried out at the Institute of Physics, Bhubaneswar, India and data were analyzed by GUPIXWIN software. We observed first time the association of bromine with the disease. The results showed 48.47 % decrease in Br, 35.16 % decrease in Zn and 29.05 % decrease in Fe in untreated state of the KA patients. In the same group, Cu has been increased by 16.73 %. Cu/Zn ratio has been altered in diseased state. The association of bromine with the disease is reported for the first time and altered levels of trace elements (Br, Cu, Fe and Zn) may come back to normal after completion of the treatment regimen with Amphotericin B.

Keywords Indian Kala-azar · PIXE · Br · Fe · Cu/Zn

Introduction

Leishmaniasis is a spectrum of diseases caused by intracellular kinetoplastid protozoan parasites of the genus *Leishmania* (Santos et al. 2006). The diseases in the human host range from cutaneous leishmaniasis (CL) to diffuse cutaneous leishmaniasis (DCL) to mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) (Reithinger et al. 2007). More than 350 million people are at risk of the infection and the disease causes about 70,000 deaths each year globally (Kaye and Scott 2011). Kala-azar (KA) or VL occurs in India, Bangladesh, Brazil, Iran, Nepal and Sudan (Desjeux 2004). In India, the disease is endemic in Bihar, Jharkhand, West Bengal and eastern Uttar Pradesh and about one hundred fifty million people are living with the risk of being affected by VL (Bora 1999; Cheeran et al. 2010). The clinical manifestations of the disease include prolonged fever, pancytopenia, anemia, emaciation, enlargement of liver and spleen (Singh et al. 2006). Till date there is no vaccine against any form of human Leishmaniasis (Nagill and Kaur 2011). Uses of the current available drugs such as Antimonials, Amphotericin B, Pentamidine, Miltefosine are not satisfactory due to the emergence of resistance, the cost and side effects and teratogenicity respectively (Croft et al. 2006; Rahman et al. 2011). In this background, the assessment of various risk factor/s related to the pathobiology of the disease, should be taken into serious consideration as it might help in formulating the eradication strategies, which is a far cry for Indian KA.

The relation between trace elements and human health has been an area of interest since decades. It has been

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shown by earlier researchers that trace elements like Copper (Cu), Zinc (Zn) and Iron (Fe) have been altered significantly in patients with various diseases including Leishmaniasis (Srinivas et al. 1988; Barollo et al. 2005; Bogden et al. 2007). Zn and Cu are directly involved in metabolic processes critically essential for cell differentiation and replication (Scuderi 1990). These, in turn, control immunological functions of the cells, for example, Zn and Cu are thought to be crucial for the functioning of immunocompetent cells (Matousek de Abel de la Cruz et al. 1993). The defense strategies of organisms depend on the alterations in their levels (Cousins 1985) and they are essential for cell membrane stability (Chvapril 1973), host metabolism (Kocyigit et al. 1998), enzyme activities (Panemangalore and Bebe 1996) and apoptosis (Sprietsma 1997).

Fe is another essential trace element present in almost all cells of the body. Fe is required by the human body for the synthesis of oxygen carrying protein, hemoglobin found in red blood cells and muscles myoglobin. It is essential for the production of proteins crucial for DNA synthesis and cell division (Malakar et al. 2014).

An important halogenated element, Iodine (I) is required for physical development and maintenance of healthy immune system (Malakar et al. 2014). The observation that the crude extract of red algae of the Genus *Asparagopsis* has strong antileishmanial activity (Genovese et al. 2009) and the algae are well known sources of halogenated compounds, gives the clue that these compounds may have some roles in KA disease regression or exacerbation.

Thus increasing attentions have been put forward in analyzing the trace elements in the KA patients' blood in order to perform rapid, sensitive, economical and reliable diagnosis (Rastegar et al. 1984). The present study has been undertaken to evaluate the status of trace element level in the blood of Indian KA patients with the help of particle induced X-ray emission (PIXE), a very sensitive, powerful yet non-destructive multi-elemental analytic technique (Yin et al. 1987; Sudarshan et al. 2000; Ray et al. 2011; Kumar et al. 2010).

Materials and methods

A total of 36 subjects from endemic zones including healthy control were enrolled in the present study with their prior consent. The patients' blood samples were collected at Kala-azar Medical Research Centre (KAMRC), Rambagh, Muzaffarpur, Bihar, India (Table 1). The study groups were divided into following categories: Healthy Control (HC) (mean age = 26.5 years); Untreated (UT) Kala-azar patients group (mean age = 24.4 years) who had not received any treatment with Amphotericin B at the time

Table 1 Patients' age, sex and WBC count (before and after the therapy with Amphotericin B)

Sl. no.	Patients' code name	Age (years)/sex of the patients	WBC count before treatment	WBC count after treatment
1	UT1	22/M	2800	8000
2	UT2	11/M	3400	5800
3	UT3	45/F	5600	9700
4	UT4	30/M	5100	10,400
5	UT5	10/F	6600	7000
6	UT6	25/M	2900	5700
7	UT7	18/M	4900	7900
8	UT8	22/M	4000	3100
9	UT9	60/F	5300	Not done
10	UT10	35/F	5300	6600
11	UT11	12/M	3700	7300
12	UT12	7/F	4200	7500
13	UT13	40/M	2500	6800
14	UT14	7/F	3900	12,700
15	UT15	8/M	4300	6800
16	UT16	8/F	10,100	5400
17	UT17	55/M	2500	6000
18	ICT1	54/M	3900	12,100
19	ICT2	15/M	7400	13,200
20	ICT3	16/M	5900	9800
21	ICT4	12/M	2700	8100
22	ICT5	28/M	5900	10,000
23	ICT6	38/M	3500	4900
24	ICT7	31/M	3300	7400
25	ICT8	30/F	3600	6900
26	ICT9	60/M	3000	6900
27	ICT10	10/F	1000	6500
28	ICT11	55/F	2600	5600
29	CT1	13/M	4300	11,600
30	CT2	20/F	3800	7100
31	CT3	32/M	3400	7300
32	CT4	17/F	2700	3900

point of blood collection; the Complete Treatment (CT) group (mean age = 20.5 years) have completed scheduled drug treatment with Amphotericin B (the normal drug schedule is 15-day therapy with 1 mg/kg body weight). We have another group of patients who have not completed their treatment schedule at the time of blood collection. We grouped them as Incomplete Treatment (ICT) group (mean age = 31.7 years). The purpose of such inclusion was to check any improvements of trace element profiles due to treatment with prevalent drug, have been achieved or not. This study was reviewed and approved by Human subjects Ethical Committee of the KAMRC, Rambagh, Muzaffarpur, Bihar, India.

Sample preparation for PIXE

Blood from patients and healthy control were collected in tubes with EDTA. The blood samples were then lyophilized using a Vertis Freeze drier at -85°C and made into powder by the mortar pistil. Dry powdered sample were mixed thoroughly with Graphite in the ratio of 7:3 in such a way that the mixture was homogeneous. The pellets of 10 mm diameter were prepared with the help of pelletizer applying 100 kg/cm^2 pressure for PIXE analysis. A multiple target holder carrying 56 pellets were mounted in a scattering chamber maintained at a pressure of 10^{-6} torr. Targets were bombarded by a 2 mm collimated beam of 3 keV protons from a 3MV tandem pelletron at the Institute of Physics, Bhubaneswar, India. The beam current varied between 30 and 50 nA maintaining a count rate of around 200–300 cps. The characteristic X rays from the target were detected by a EG & G Ortec Si(Li) detector placed at 90° to the beam, through a $25\text{ }\mu\text{m}$ mylar window fixed at the detector port of the scattering chamber. For quantitative results, the PIXE spectral analyses were done offline using GUPIXWIN software (Campbell et al. 2010) at UGC-DAE CSR Kolkata Centre, Kolkata, India. Samples were analyzed in triplicate to check reproducibility of the results.

Statistical analysis

Student's *t* test was performed for evaluating statistical significance.

Results

Results of the analysis of the NIST Bovine Liver [SRM 1577c] are in good agreement with those of the certified values (Table 2), establishing thus that the experimental procedure adopted in the present measurements is reliable for analyzing trace elements in blood samples of Indian KA

Table 2 PIXE measurements of NIST standard ‘Trace Elements in Bovine liver’ [SRM 1577c]

Element	Experimental value (ppm)	Certified value (ppm)
S	7378.4 ± 1367.95	7490 ± 340
K	$10,229.4 \pm 233.23$	$10,230 \pm 640$
Ca	148 ± 9.205	131 ± 10
Mn	10.2 ± 1.27	10.46 ± 0.47
Fe	190.7 ± 0.72	197.94 ± 0.65
Cu	284.2 ± 3.04	275.2 ± 4.6
Zn	181.9 ± 2.49	181.1 ± 1.0
Rb	37.5 ± 2.47	35.3 ± 1.1

patients. For Br, no NIST standard value was given and level of Br of the patients groups were compared to that of the control individuals.

Fifteen trace elements (K, Fe, Cu, Zn, Br, Cl, S, Ca, Mn, Cr, Ni, As, Se, Rb and Sr) were detected in the blood of the subjects above by PIXE and significant changes were observed in concentrations for Br, Fe, Cu and Zn in the Indian KA patients. The analyses of our study showed for the first time, the decrease in concentrations of Br (Fig. 1) in patients with KA along with decrease in Fe and Zn and increase of concentration of Cu (Table 3) in the blood samples of the patients with no treatment, UT with respect to the Healthy Control from Endemic zone, HC. We observed significant decrease in Br in untreated group (UT) (6.26 ± 1.89 ppm) as compared to the endemic healthy control group, HC (12.15 ± 2.5 ppm, $P < 0.001$). Other significant decreases were seen for Zn and Fe. For Zn, the value in UT versus HC was 31.81 ± 3.94 versus 49.06 ± 3.58 ppm ($P < 0.001$) and decrease in Fe in blood of untreated versus Healthy Control was 267.96 ± 27.79 versus 377.69 ± 18.86 ppm ($P < 0.01$). The decrease of Br was more prominent than other two elements. In the same situation, we observed an increased Cu concentration in UT with respect to HC (24.98 ± 1.23 vs 21.40 ± 0.098 , $P < 0.01$). Interestingly, patients receiving Amphotericin B as incomplete (ICT) treatment group showed comparatively improved status of Fe ($P < 0.01$) and Zn ($P < 0.001$) concentrations in blood as compared to that for untreated group. However, for Br, the value has not

Fig. 1 Blood Br level has been decreased by nearly 50 % in Indian Kala-azar patient. HC healthy control from endemic zone; UT patient group without any treatment with drug, Amphotericin B at the time of blood collection; ICT patient group whose course of treatment with Amphotericin B have not been completed at the time of blood collection; CT patient group whose treatment with Amphotericin B completed. *** $P < 0.001$; ** $P < 0.01$ [there was no significance in difference at $P < 0.05$ between Healthy Control (HC) and Complete Treatment groups (CT) for Br as well as between Untreated (UT) and Incomplete Treatment (ICT) groups]

Table 3 Comparison of trace elements (zinc, iron and copper) of healthy subjects and Indian KA patients at different time points of treatment schedule with Amphotericin B

Trace elements/study groups	Untreated group (UT)	Incomplete Treatment group (ICT)	Complete Treatment group (CT)	Healthy Endemic Control (HC)
Zinc (Zn)	31.81 ± 3.94 ^{a,b}	37.89 ± 1.21 ^c	44.37 ± 7.04 ^d	49.06 ± 3.58
Iron (Fe)	267.96 ± 27.79 ^{e,f}	318.56 ± 25.49 ^g	349.09 ± 18.05 ^d	377.69 ± 18.86
Copper (Cu)	24.98 ± 1.23 ^{e,f}	23.14 ± 1.01 ^h	20.95 ± 0.87 ^d	21.40 ± 0.098

Data are mean ± SD, values of *P* were obtained from Unpaired Student's *t* test

^a *P* < 0.001 for Zn level in Untreated subjects versus Incomplete Treated subjects

^b *P* < 0.01 for Zn level in Untreated subjects versus Completely Treated subjects

^c *P* < 0.05 for Zn level in Incomplete Treated subjects versus Completely Treated subjects

^d *P* value non significant at *P* < 0.05 for Zn, Fe and Cu levels in Completely Treated subjects versus Healthy Control

^e *P* < 0.01 for Fe and Cu level in Untreated subjects versus Incomplete Treated subjects

^f *P* < 0.001 for Fe and Cu level in Untreated subjects versus Completely Treated subjects

^g *P* value non significant at *P* < 0.05 for Fe level in Incomplete treated subjects versus Completely Treated subjects

^h *P* < 0.01 for Cu level in Incomplete Treated subjects versus Completely Treated subjects

been increased significantly in ICT group. At the same time in ICT group, decrease in Cu conc. was noticed (Table 3). PIXE analysis of the blood of patients receiving complete course of treatment (CT) with Amphotericin B, showed restoration of normalcy to a great extent with 92, 90, and 76 % recovery of Fe, Zn and Br respectively. On the other hand, the concentration of Cu was reduced to the normal range in healthy control group. The differences of concentration of Br, Fe, Zn and Cu were not significant between HC and CT groups. It was also found that the Cu/Zn ratio of the untreated conditions increased with respect to that in the healthy control and incomplete treatment group respectively and has been restored to near normal value in CT group (Fig. 2).

Discussion and conclusion

Results of our present investigation clearly indicate for the first time the association of Br with the disease, Indian KA. This study further corroborated earlier observations (Al-Samarrai et al. 2008; Van Weyenbergh et al. 2004; Lal et al. 2012) that reported low serum Zn and Fe and high Cu in humans with CL, MCL and VL as well as in dogs. Br as micronutrient has not yet been established but the deficiency of its concentration has been reported in malnourished people (Pasa et al. 2003) and leishmaniasis is a disease where the general health status of the patient becomes poor (Zhavoronkov et al. 1996). Some earlier researchers have reported that the eosinophils kill multi-cellular nematode worms causing filariasis and a bacterium causing tuberculosis and it does so in presence of H₂O₂ with the help of eosinophil peroxidase, a haloperoxidase that preferentially uses bromide over chloride (Thakur

Fig. 2 Cu/Zn may act as patho physiological marker of the disease, Indian Kala-azar. The ratio became almost twofold in KA patients (UT) as compared to the healthy control from endemic zone (HC). HC healthy control from endemic zone; UT patient group without any treatment with drug, Amphotericin B at the time of blood collection; ICT patient group whose course of treatment with Amphotericin B have not been completed at the time of blood collection; CT patient group whose treatment with Amphotericin B completed

2000). Data of our present study gives for the first time, an indication of possible involvement of Br with the etiology of VL entailing the inflammatory processes.

Many common trace elements in human body like Cu, Fe and Zn etc. have been seen to influence the immune system immensely (Thomas et al. 1995; Dardenne 2002; Percival 1998). The macrophage, the primary sentinel of the body against parasite infection, is reported to be adversely affected by zinc deficiency (Sen et al. 2008). This reduces its abilities of phagocytosis and cytokine

production. Zinc is not only crucial for nonspecific immunity elicited by neutrophils, natural killer cells and macrophages but also important in development of acquired immunity. It controls T lymphocytes activation, Th1 cytokine production and B lymphocyte development and antibody production (Sen et al. 2008). Interestingly, workers in the fields (Shankar and Prasad 1998; Singh et al. 2008) suggested the cause of endemicity of Leishmaniasis in eastern parts of India including Bengal and Bihar may be due to Zn deficiency in the flood afflicted soil.

In diseased condition, Cu has been seen in increased state. The Cu concentration in the brain, heart and spleen was found significantly higher in thalassemic patients than non thalassemic persons and lower serum Zn and magnesium levels and higher Cu and Potassium levels in thalassemic major patients were scored compared to that of the control (Al-Samarrai et al. 2008). In case of cutaneous leishmaniasis, the positive correlation between plasma Cu and parasite-specific IgG in the patient groups strongly suggests that this trace element might interfere in antileishmanial immune response which may lead to a non-protective Th2/humoral immune response and this in turn, may help in progress of the disease (Van Weyenbergh et al. 2004). The increased serum Cu in KA patients by PIXE was in congruence with the observations made by other groups (Mukhopadhyay et al. 2000; Van Weyenbergh et al. 2004; Lal et al. 2012).

Our results showing depletion in Fe concentration in the blood samples of VL patients is in perfect tune with the observations that serum Fe concentrations were significantly lower in patients with Leishmaniasis (Van Weyenbergh et al. 2004). It was reported by earlier researchers that Br deficiency has some bearing with inflammatory disease associated with Fe deficiency (Chuwa et al. 1996).

Evaluation of Zn and Cu has revealed correlation between various disease states as well as the healthy or cured condition (Ray et al. 2011; Reza et al. 2011). We have also seen that the status of these micronutrients have been changed towards normalcy along with the treatment with Amphotericin B. Increased plasma Cu is not always associated with all kinds of Leishmaniasis. In Mucocutaneous Leishmaniasis which is characterized by chronic inflammatory conditions, serum Cu is not increased (Ribeiro-de-Jesus et al. 1998) and thus only increased Cu level in blood may not be considered as pathological marker rather it is Cu/Zn ratio which holds true for indicating the disease status. The Cu and Zn have pro-oxidant and antioxidant properties and their imbalance may be expected to reflect the status of oxidative stress (Mezzetti et al. 1998). In our study, calculations of Cu/Zn ratio from UT (>80 % increment with respect to HC) and ICT groups (>38 % increment with respect to HC) of study groups

corroborates the view that increase in Cu/Zn ratio occurs in Leishmaniasis (Van Weyenbergh et al. 2004). Our study further supports the view that complete restoration of Cu/Zn ratio has been observed after treatment (Van Weyenbergh et al. 2004). The CT group showed about 94 % recovery as compared to that of HC group. So, Cu/Zn ratio may be an indicative marker for Indian KA also.

By employing PIXE technique, this study revealed for the first time, the association of Br with Indian Kala-azar and further supported the observations that there are decrease of trace elements like Zn, Fe and increase of Cu in the blood of VL patients. This study also showed that the level of these trace elements can be restored to near normal in the blood of the patients at the end of the treatment regimen with Amphotericin B. The improvement of the WBC counts (Table 1) of the KA patients after the completion of treatment has also been noticed. The study further corroborated that Cu/Zn ratio may be a pathophysiological marker of the disease.

Acknowledgments The work described here is under the University Grant Commission-Department of Atomic Energy (UGC DAE) Collaborative Research Scheme. Ms. Sangita Lahiry was the project fellow of the scheme. For PIXE experiments, authors are thankful to the Director, Institute of Physics, Bhubaneswar, India. The co operations of Director of Public Instructions, Govt. of West Bengal, India and the Principal, Barasat Govt. College, Kolkata respectively, are duly acknowledged.

Compliance with ethical standards

Conflict of interest None.

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