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Energy Dispersive X-Ray Fluorescence (EDXRF) Study of the Blood Samples of Indian Kala-Azar Patients.

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ABSTRACT

Leishmaniasis is a disease spectrum caused by the protozoan parasites of Genus *Leishmania*. In this study, trace elements concentrations (K, Fe, Cu, Zn, Cl, S, Ca, Mn, Rb & Br) of twenty two Kala-azar (KA) patients were measured by Energy Dispersive X-Ray Fluorescence (EDXRF) Spectrophotometer along with six healthy control (HC) (n=28). Significant decrease in the levels of Fe and Zn and increase in level of Cu were found in untreated groups (UT) of KA patients compared to that of HC group (P<0.001, P<0.001 and P<0.01, respectively). Treated group of patients (TR) who received Amphotericin B, showed significant increase in levels of Fe and Zn. Comparison between TR and UT groups for Fe and Zn showed statistically improved status but changes in Cu level was not statistically significant. Meanwhile, no significant difference in level of other trace elements was observed in all experimental groups. Among the ten trace element profiles, Cu, Fe and Zn imbalances were very clear in active KA patients. It corroborated earlier findings including ours. These changes came back to almost normal level after Amphotericin B therapy. Thus drug treatment stabilizes trace elements levels which may act as useful marker for the disease.

Keywords: EDXRF; Copper; Zinc; Iron; Indian KA patients

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INTRODUCTION

Kala-azar (KA) or Visceral Leishmaniasis (VL) is one of the severe forms of Leishmaniasis caused by the parasites belonging to the genus *Leishmania* [1]. Several *Leishmania* species are responsible for a wide range of clinical manifestations, ranging from the mild cutaneous form called Cutaneous Leishmaniasis (CL), the most destructive mucosal inflammation named as Mucocutaneous Leishmaniasis (MCL) and the life-threatening disseminated visceral form, known as Visceral Leishmaniasis (VL) or Kala-azar (KA)[2,3]. Approximate annual incidence of the visceral form is around 500,000 in 62 countries [4] and 90% of the cases are confined to 5 countries namely India, Bangladesh, Nepal, Sudan and Brazil [4]. India accounts for 50 percent of the global burden of VL infections and most of the cases in India are reported from the north eastern states of Bihar, eastern Uttar Pradesh, West Bengal and Jharkhand [5]. Irregular occurrences of VL is also been reported form Gujarat (western India), Tamil Nadu and Kerala (southern India), and sub-Himalayan parts of northern India including Uttar Pradesh, Himachal Pradesh, Jammu, and Kashmir [5-8]. About 90 percent of the VL patients of India are poor and reside in the rural areas of Bihar [9, 10]. Previous reports on VL suggested that the occurrence of the disease remains high in this rural area due to the low socio-economic status of the people [10, 11].

Low socio-economic status effects individuals' diet causing malnutrition and ultimately leads to serious health related problems. Inadequate access to proper nutrient rich food causes trace element deficiency. Trace element deficiency increases susceptibility to infection, decrease immune responsiveness and reduced disease resistance [12] and eventually results in compromised host immune status [13, 14]. Epidemiological reports on VL revealed that there is an enhance risk for VL in the malnourished hosts [13, 15]. Trace element deficiency alters innate immune response and causes immunodeficiency [16].

As stated, trace elements play cardinal roles in many physiological processes, in particular in immunity, metabolism and participate in various bio-chemical reactions [17]. Trace elements are required for proper functioning of a cell at biological, molecular and chemical levels [18]. Some of the trace elements are acts as co-factors for the enzymatic activities. Potassium (K) is the main cation of intracellular fluid that helps to conduct nerve impulses, muscle contraction and also regulates osmotic pressure [19]. Chloride (Cl) is involved in maintaining fluid and electrolytes balance while Calcium (Ca) acts as an essential component of bones and teeth and regulate nerve and muscle function [19]. Manganese (Mn) is one of the co factors of enzymes like hydrolase, decarboxylase, and transferase [20] and also a component of mitochondrial superoxide dismutase involved in glycoprotein and proteoglycan synthesis [19]. Sulphur (S) is present in a very rich amount in connective tissues, skin, hairs, nails and three amino acids like cystine, methionine and cysteine also contain sulphur [19]. The biological role of zinc (Zn), iron (Fe) and copper (Cu) in different pathological condition has been reported in different disease conditions [21]. They are essential components of enzymes, help to attract substrate molecules and facilitate their transition to end products [18]. It is well documented that Zn is a vital trace metal for proper functioning of entire immune system [22]. Cu is required for the development and maintenance of immune system but the direct mechanism of action is yet to be known [23]. Fe is most abundant trace metal in our body and takes part in the synthesis of proteins that are important for DNA synthesis and cell division [17]. It is well established that Zn and Cu are directly involved in metabolic processes that are critical for cell differentiation and replication [24]. Zn and Cu are significant for cell membrane stability [25] and apoptosis [22].

The relationship among trace elements and Leishmaniasis have been described by researchers of the field by several techniques like colometric methods [26], Flame Atomic Absorption Spectrometry (FAAS) [27], Atomic Absorption Spectrophotometry (AAS) [28] and Proton Induced X-ray Emission (PIXE) [29] etc. In our present study, we have analysed ten trace elements levels of Indian KA patients before and after the Amphotericin B treatment along with healthy control by Energy Dispersive X-Ray Fluorescence (EDXRF) Spectrophotometer. EDXRF is a very powerful, non destructive multi-elemental analysis technique [30]. The aim of the study was to investigate the changed status, if any, of trace elements in the blood of Indian KA patients in untreated and treated condition by EDXRF which could act as markers. The recovery status of the patients in terms of trace elements levels after drug treatment has been checked.

MATERIALS AND METHODS

The blood samples were collected from Kala-azar Medical Research Centre (KAMRC), Rambagh,

Muzaffarpur, Bihar, India. The study group consists of 22 KA patients along with 6 healthy controls. All individuals gave a written consent that they are agreed with the terms and conditions of the experiments. The study groups were classified into following categories: Healthy Control (HC); Untreated (UT) Kala-azar patients group who had not received any treatment with Amphotericin B at the time point of blood collection; the Treated (TR) group have received scheduled intravenous drug treatment with Amphotericin B. The normal drug schedule is 15-day therapy with 1 mg/kg body weight.

Sample preparation for EDXRF

Blood from studied experimental groups (HC, UT & TR) were collected in heparinised tubes. The blood samples were lyophilized for 36 h and dried blood samples were homogenized using a mortar and pestle and 150 mg sample made into pellets (1 mm thick and 13 mm diameter) using a tabletop pelletizer (Pressure: 100–110 kg/cm^2 for 5 min). Three pellets were made for each blood samples.

Statistical analysis

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

RESULTS AND DISCUSSION

Ten trace elements (K, Fe, Cu, Zn, Cl, S, Ca, Mn, Rb & Br) were detected in the blood of the subjects by EDXRF and significant changes were observed in concentrations levels of Fe, Zn and Cu respectively. The changes in the concentration of rest trace elements in our three studied group were not significant (data not shown). It was observed that Fe and Zn concentrations were significantly lower in UT group compared to that of the HC group. In case of Cu level, significant increase was observed in UT group as compared to the HC group. The EDXRF data showed that in UT group, the concentration of Fe was significantly decreased compared to that of the HC group (1119.71± 187.16 Vs 1554.93± 157.30 ppm, P<0.001) (Figure 1). Zn concentration was also lowered significantly in UT group compared to that of the HC group (20.75±3.37 Vs 32.38±4.67 ppm, P<0.001) (Figure 2). On contrary, we observed an increased Cu concentration in UT group with respect to HC group (5.83±0.74 ppm vs 4.52±0.53ppm, P<0.01) (Figure 3).

Further, KA patients receiving Amphotericin B in TR group showed significantly increased levels of Fe and Zn with respect to UT group. On the other hand, for Cu, the value has been decreased but not significantly between UT and TR group. In TR group, Fe and Zn concentrations were increased significantly with respect to UT group. For Fe, the value in UT vs TR was1119.71 ± 187.16 vs 1441.64 ± 232.35 ppm (P< 0.01) (Figure1) and for Zn, the value in UT vs TR was 20.75±3.37 vs 30.25 ± 5.76 ppm (P< 0.001) (Figure 2). Cu level has been seen decreasing after drug treatment in TR group but change was not significant (Figure 3).

EDXRF data suggested that Amphotericin B drug restored the Fe and Zn levels in treated patients as compared to the untreated group and Cu level also decreased to its normal value of HC group (Table 1). It further corroborated the view that these imbalances in Cu, Fe and Zn in active VL patients could be treated as patho physiological markers [28, 29, 31].

The evaluation of trace element concentrations for the diagnosis of different diseases has been increased in the recent years. Recent researches have focussed mainly on Fe and Zn and Cu [32, 33]. Our result evaluated the levels of important trace metals in three experimental groups: untreated KA patients (UT), patients treated with drug Amphotericin (TR) and healthy control (HC) group by EDXRF. Data suggested that among the ten trace metals, significant changes occurred in Cu, Fe and Zn. Our data has a good agreement with previous reports that Zn and Fe levels are decreased and Cu level is increased in KA patients [26-29]. The connection between Fe, Zn and various pathological conditions is well established and their imbalances in body can cause many disorders [27]. Our immune systems also need Cu for several vital functions [23] and changes in the Cu value in several diseased conditions may help to find the pathophysiological marker. Present study showed that out of ten trace elements, significant changes noticed in the concentration levels of Cu, Fe and Zn which could be restored by drug treatment.

Figure Captions:

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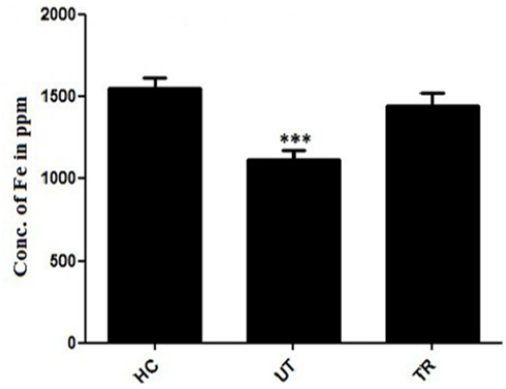
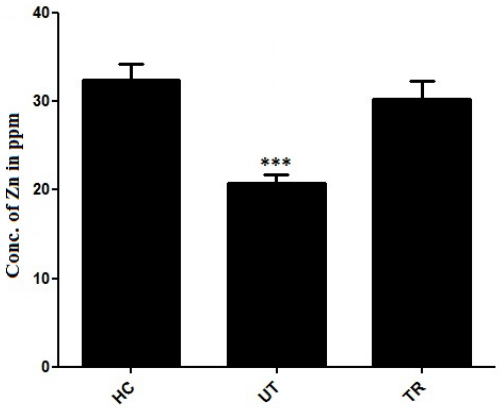
Figure 1: Blood Fe level has been decreased in Indian Kala-azar patient



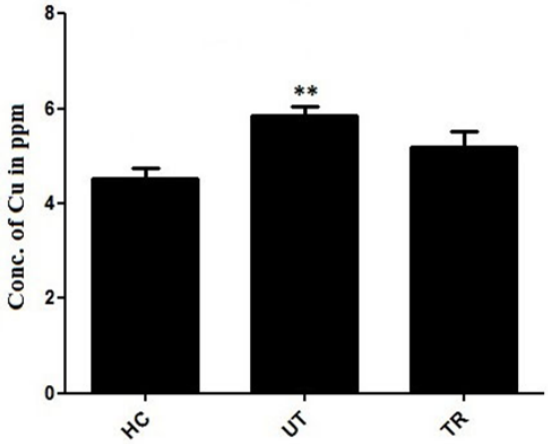
HC = healthy control from endemic zone; UT = patient group without any treatment with drug, Amphotericin B at the time of blood collection; TR= patient group whose treatment with Amphotericin B completed.
***P<0.001 [There was no significance in difference at P<0.05 between Healthy Control (HC) and Treatment groups (TR) for Fe].

Figure 2: Blood Zn level has been decreased 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; TR= patient group whose treatment with Amphotericin B completed.
***P<0.001 [There was no significance in difference at P<0.05 between Healthy Control (HC) and Treatment groups (TR) for Zn].

Figure 3: Blood Cu level has been increased 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; TR= patient group whose treatment with Amphotericin B completed.
**P<0.01 [There was no significance in difference at P<0.05 between Healthy Control (HC) and Treatment groups (TR) for Cu].

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Table 1: Experimental data of ten trace elements level in three experimental groups by Energy Dispersive X-Ray Fluoresence

Sample ID	Mn	P	S	Cl	K	Ca	Fe	Cu	Zn	Br	Rb
HC1	310.23	1540.12	5219.78	4698.17	6478.12	645.32	1475.21	5.14	31.14	5.79	14.32
HC2	289.17	985.14	5178.49	3987.17	7123.78	714.52	1789.78	4.53	38.12	5.89	11.89
HC3	301.33	1125.14	4856.11	4891.76	6894.25	812.42	1342.12	3.72	24.17	7.12	10.25
HC4	209.13	1647.28	5870.13	4896.55	6421.79	698.78	1475.12	4.57	33.12	6.12	6.75
HC5	312.52	852.12	6123.14	4710.12	6871.02	745.00	1647.13	5.04	32.78	8.97	7.10
HC6	254.88	1145.12	4789.12	3654.80	6251.12	789.54	1600.19	4.17	34.96	7.14	7.84
TR1	240.34	1367.53	5261.18	4183.06	6079.56	812.87	1350.43	6.26	28.93	4.003	11.22
TR2	294.86	1470.58	4881.24	4439.05	6108.61	811.05	1389.79	6.13	25.54	5.17	8.89
TR3	308.15	1316.54	5231.7	4135.32	5852.93	782.96	1392.41	5.46	22.90	4.36	6.82
TR4	232.16	1419.05	5265.36	4267.08	5434.75	751.99	1634.69	5.47	37.63	6.38	8.88
TR5	209.98	1795.01	5545.003	4876.643	7839.61	690.77	1829.12	3.45	39.07	3.38	6.60
TR6	245.98	969.97	4759.19	3697.47	5801.49	748.29	1283.91	5.22	27.53	7.73	9.5
TR7	204.59	1275.05	4943.56	4913.49	7307.57	623.17	1577.03	5.18	32.90	7.45	17.82
TR8	223.17	961.49	4611.62	3187.20	5318.75	719.51	1075.77	4.39	27.53	11.3	18.82
UT1	276.47	1424.08	4924.006	3596.36	5714.55	766.07	1165.97	6.00	14.78	3.87	7.83
UT2	258.48	1629.95	5832.34	4908.65	6681.30	886.90	1352.45	5.54	22.59	4.23	13.42
UT3	176.48	1343.86	5343.34	4745.50	6336.04	860.37	1353.50	5.43	22.83	6.96	8.76
UT4	305.16	1376.56	5522.74	4521.66	6395.3	867.79	1344.13	5.09	19.60	3.98	5.006
UT5	348.27	1462.2	5043.53	3845.06	5573.46	740.23	1068.46	5.44	18.98	4.6	6.37
UT6	286.09	1392.34	4841.76	3919.63	5542.35	737.98	1060.05	6.96	20.49	8.12	9.78
UT7	240.36	1456.55	5082.47	4293.39	5874.76	780.67	1248.16	6.22	23.12	6.63	9.37
UT8	275.61	1837.59	4393.01	4340.89	5357.16	719.59	1089.38	5.59	25.37	12.56	10.50
UT9	257.72	1623	5155.98	4999.30	6616.91	877.93	1172.32	6.29	24.84	8.54	10.48
UT10	397.62	1502.38	5373.63	4909.36	6033.35	815.8	1110.25	6.5	23.52	6.34	7.24
UT11	254.98	1389.21	5058.13	5012.28	6127.36	812.74	960.31	4.22	16.23	6.25	11.20
UT12	302.15	721.76	4633.21	1924.21	4133.85	571.58	710.26	5.82	16.68	5.61	9.05
UT13	362.12	1259.26	4750.26	3265.30	5390.34	684.79	920.96	6.79	20.78	3.23	17.40
UT14	241.32	1567.32	4321.8	3789.10	6321.78	819.78	1352.44	6.2	19	11.69	9.1

Table 2: Comparative average value of Iron, Zinc and Copper in three experimental groups (UT, TR & HC) of Indian KA patients

Trace element/Study groups	Untreated group (UT)	Treated group (TR)	Healthy Control group (HC)
Iron (Fe)	1119.71±187.16	1441.64±232.35	1554.93±157.30
Zinc (Zn)	20.75±3.37	30.25±5.76	32.38±4.67
Copper (Cu)	5.83±0.74	5.19±0.091	4.52±0.53

CONCLUSION

In summary, we conclude that during the active KA infection, significant changes are found in Cu, Fe and Zn concentrations which could come back to near normal values after Amphotericin B treatment. It also corroborates earlier findings including ours that Cu, Fe and Zn concentrations may act as patho physiological markers of the disease.

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