



Estimation of Serum Levels of Heavy Metals in Patients with Chronic Invasive Fungal Rhinosinusitis Before the COVID-19 Era: A Pilot Study

Original Investigation

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Abstract

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Objective: Various metals play role in the survival and pathogenesis of the invasive fungal disease. The objectives of this study were to compare the levels of heavy metals in patients with chronic invasive fungal rhinosinusitis (CIFR) and healthy controls, and to analyze their role in disease outcome.

Methods: Twenty-three patients (15 with invasive mucormycosis and 8 with invasive aspergillosis, Group 1), and 14 healthy controls (Group 2) were recruited. Blood samples were collected from each group into ion-free tubes and analyzed for serum levels of Nickel (Ni), Copper (Cu), Zinc (Zn), Gallium (Ga), Arsenic (As), Selenium (Se), Rubidium (Rb), Strontium (Sr), Cadmium (Cd), and Lead (Pb). The final outcome of the patients during their hospital stay was categorized clinico-radiologically as improved or worsened, or death.

Results: The levels of all metals were higher in Group 1 except for As and Pb. However, the differences in Cu ($p=0.0026$), Ga ($p=0.002$), Cd ($p=0.0027$), and Pb ($p=0.0075$) levels were significant. Higher levels of Zn ($p=0.009$), Se ($p=0.020$), and Rb ($p=0.016$) were seen in the invasive aspergillosis subgroup. Although Zn ($p=0.035$), As ($p=0.022$), and Sr ($p=0.002$) levels were higher in patients with improved outcome, subgroup analysis showed no differences.

Conclusion: The levels of some heavy metals in CIFR significantly differ from those of the general population and also vary with the type of the disease and its outcome. These levels may not have a direct effect on the outcome of the patient, but they do play a role in the pathogenesis of the invading fungus.

Keywords: Chronic invasive fungal rhinosinusitis, invasive mucormycosis, invasive aspergillosis, heavy metals, trace elements

Introduction

Fungal rhinosinusitis is classified as invasive and non-invasive depending on the potential of the fungal hyphae to invade the superficial epithelium. Invasive fungal sinusitis is further divided into acute, chronic, and chronic granulomatous forms (1). Various micronutrients and metals play an important role in the survival and the pathogenesis of the invasive fungal disease. It has been seen in the pathogenesis of fungal microbes that the host hinders microbial growth and virulence by actively restricting essential metals to fungi, a process known as nutritional immunity. As a result of this, fungi have developed various mechanisms and complex regulatory networks to increase the availability of essential metals such as zinc, copper, and nickel for their survival and virulence. The roles of zinc in the growth of *Aspergillus* species and of iron in the growth of *Mucor* species are well-established (2-7). The recent epidemic of invasive mucormycosis in India with the coronavirus disease-2019 (COVID-19) pandemic in the backdrop and the inadvertent use of multivitamins and herbal medicines containing trace or heavy metals (due to the risk to reward ratio in favor of zinc supplementation in COVID-19) may be linked, but to the best of our knowledge, there are no studies available on this topic (8-11). It is also not known whether the levels of these heavy metals are significantly different from those of the general population. We conducted this pilot study in the North Indian population before the COVID-19 era. The primary aim was to compare the levels of various heavy metals between patients diagnosed with chronic invasive fungal rhinosinusitis (CIFR) and healthy controls.

Materials and Methods

A total of 23 adult patients diagnosed with CIFR and 14 healthy controls were included in the study after obtaining ethical clearance from Institute Ethics Committee of All India Institute of Medical Sciences, New Delhi, India (reference number: IEC-436/02.07.2021). Informed and written consent was taken from each of the patients and healthy controls.

The 23 patients included in the study were treatment-naive adult patients (aged >18 years) diagnosed with invasive fungal rhinosinusitis either by microbiological examination (potassium hydroxide wet mount preparation of nasal or palatal crust/swab/ tissue biopsy showing aseptate or septate hyphae) or by histopathological examination of tissue biopsy. The 14 age-matched controls had no pre-existing history of fungal disease. Any patient or control with a history of blood transfusion, prior antifungal treatment, metal intake/poisoning, and not consenting to take part in the study were excluded. Blood samples (10 mL each) were collected from both the patients and the controls into ion-free tubes and sent to the Ecotoxicology laboratory for the estimation of serum

levels of Nickel (Ni), Copper (Cu), Zinc (Zn), Gallium (Ga), Arsenic (As), Selenium (Se), Rubidium (Rb), Strontium (Sr), Cadmium (Cd), and Lead (Pb). The levels of these metals were also compared for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), glycosylated haemoglobin (HbA1c), grade of the disease, and final outcome of the patient during hospital stay.

The results were classified as improved, worsened, or death as per the following definitions:

1. Improved: Both clinical and radiological improvement.

- Clinical improvement: No clinical evidence of residual disease (necrotic tissue or bone/residual fungal debris/osteomyelitic bone).

- Radiological improvement: No evidence of residual disease on computed tomography (CT) in all cases or magnetic resonance imaging (MRI) in cases involving orbital apex or those with intracranial extension.

2. Worsened: Either clinical or radiological deterioration.

- Clinical deterioration: Progression of disease (involvement of new regions/progression of necrosis) or no clinical improvement despite giving medical therapy and/or possible surgical intervention.

- Radiological deterioration: Progression of disease (involvement of new regions) or persistent residual disease on CT or MRI despite receiving medical therapy and/or possible surgical intervention.

3. Death: Patients who expired during treatment.

Statistical Analysis

Statistical Analysis was performed using the IBM SPSS (IBM SPSS Statistics for Windows, (Version 25.0. Armonk, New York, USA). The levels of heavy metals in both groups were compared using two-tailed t-test considering $p < 0.05$ as significant. The Pearson's correlation coefficient was used to check any correlation between serum levels of heavy metals and ESR, CRP, HbA1c, extent of the disease, and outcome of the patient.

Results and Analysis

There were 23 patients (Group 1) and 14 healthy controls (Group 2). Mean age was 42 ± 5.22 years in Group 1 and 35.6 ± 3.20 years in Group 2. In Group 1, 15 patients were diagnosed with invasive Mucormycosis, and eight patients with invasive Aspergillosis. In both types of invasive fungal disease, unilateral disease was more common than bilateral disease. However, the final outcome was different in the two subgroups. In the invasive Mucormycosis subgroup only 26.6% patients improved, while 26.6% patients worsened, and 46.6% died. In the invasive Aspergillosis subgroup

87.5% of the patients improved and 12.5% worsened, and none died during their hospital stay. The disease extent and final outcome of the patients is given in Table 1.

The levels of all metals analyzed in this study were higher in Group 1 (patients) compared to Group 2 (controls) except for As and Pb; however, statistically significant difference (using two-tailed t-test) was seen only in levels of Cu ($p=0.002$), Ga ($p=0.002$), Cd ($p=0.002$), and Pb ($p=0.007$) between the two groups as shown in Table 2. Levels were found statistically significantly higher for Zn ($p=0.009$), Se ($p=0.020$), and Rb ($p=0.016$) in the invasive Aspergillosis group compared to the invasive Mucormycosis group as shown in Table 3.

Statistically significant differences were also found for Zn ($p=0.035$), As ($p=0.022$), and Sr ($p=0.002$) levels between the improved and the worsened outcome subgroups in all

patients. The levels of these three metals were higher in the improved subgroup as shown in Table 4. On subgroup analysis, there were no significant differences among any of the heavy metal levels in the improved, worsened and death outcome groups of patients with invasive mucormycosis. Subgroup analysis could not be done for comparing heavy metals based on outcome in the invasive aspergillosis group as there were seven patients with improved outcome and only one patient with worsened outcome.

There was statistically significant difference between erythrocyte sedimentation rate (ESR) and CRP in the invasive Mucormycosis and the invasive Aspergillosis groups, but there was no correlation between the serum levels of heavy metals and ESR, CRP, and HbA1c in any of these groups as shown in Tables 4, 5, 6 and 7.

Table 1. Disease extent and outcome of patients

	Extent of disease – number of patients	Outcomes of patients – number of patients
Invasive Mucormycosis	● Unilateral disease – 11/15	Improved – 4
	○ Sino-nasal – 4	Worsened – 4
	○ Sino-orbital – 5	Death – 7
	○ Sino-cranial (extradural) – 1	
	○ Sino-orbito-cranial (extradural) – 1	
	● Bilateral – 4/15	
	○ Sino-nasal – 1	
	○ Sino-cranial (extradural) – 1	
	○ Sino-orbito-cranial – 1 extradural, 1 intradural	
Invasive Aspergillosis	● Unilateral disease – 7/8	Improved – 7
	○ Sino-nasal – 1	Worsened – 1
	○ Sino-orbital – 5	Death – 0
	○ Sino-orbito-cranial (intradural) – 1	
	● Bilateral Sino-cranial (extradural) – 1	

Table 2. Mean levels* of heavy metals in patients and controls

No	Heavy metal	Mean level in patients (Group 1)	Mean level in controls (Group2)	p-value
1.	Nickel	1.26±1.09	0.37±0.34	0.472
2.	Copper	1789.73±161.46	1397.96±129.86	0.002
3.	Zinc	1913.37±458.84	1864.52±209.33	0.879
4.	Gallium	1.66±0.24	0.48±0.35	0.002
5.	Arsenic	1.83±0.18	1.92±0.83	0.221
6.	Selenium	118.51±19.80	109.88±13.45	0.553
7.	Rubidium	1183.40±769.46	543.98±150.69	0.229
8.	Strontium	134.38±14.45	122.86±31.19	0.477
9.	Cadmium	0.72±0.15	0.24±0.18	0.002
10.	Lead	4.43±1.45	6.92±2.45	0.007

*Measured as parts per billion (ppb) in 10 mL of serum sample.

Significant p-values are shown in bold.

Table 3. Mean level* of heavy metals in Invasive Mucormycosis vs Aspergillosis

No	Heavy metal	Mean level in patients with Invasive Mucormycosis	Mean level in patients with Invasive Aspergillosis	p-value
1.	Nickel	0.96±0.61	1.82±2.89	0.244
2.	Copper	1762.65±188.57	1840.51±297.61	0.670
3.	Zinc	1481.92±295.68	2722.33±975.20	0.009
4.	Gallium	1.85±0.55	1.30±0.86	0.309
5.	Arsenic	1.75±0.15	1.97±0.44	0.296
6.	Selenium	101.54±14.84	150.33±41.48	0.020
7.	Rubidium	504.64±149.56	2456.08±1903.30	0.016
8.	Strontium	124.17±14.69	153.53±26.41	0.061
9.	Cadmium	0.75±0.21	0.69±0.19	0.739
10.	Lead	4.89±1.80	3.57±2.31	0.419

*Measured as parts per billion (ppb) in 10 mL of serum sample.

Significant p-values are shown in bold.

Table 4. Mean level* of heavy metals in patients with different outcomes

No	Heavy metal	Mean level in patients with improved outcome	Mean level in patients with worsened/expired outcome	p-value
1.	Nickel	1.22±1.10	1.29±0.70	0.951
2.	Copper	1879.74±235.52	1707.23±211.20	0.317
3.	Zinc	2429.01±812.67	1440.69±262.58	0.035
4.	Gallium	1.37±0.64	1.93±0.67	0.276
5.	Arsenic	2.06±0.33	1.62±0.08	0.022
6.	Selenium	133.52±34.90	104.75±17.03	0.169
7.	Rubidium	1953.67±1467.02	477.32±181.39	0.064
8.	Strontium	156.76±21.97	113.86±8.91	0.002
9.	Cadmium	0.78±0.18	0.68±0.24	0.273
10.	Lead	4.40±1.93	4.46±2.14	0.485

*Measured as parts per billion (ppb) in 10 mL of serum sample.

Significant p-values are shown in bold.

Table 5. Mean levels of ESR, CRP and HbA1c in invasive Mucormycosis and invasive Aspergillosis

	Mean level in invasive Mucormycosis group	Mean level in invasive Aspergillosis group	p-value
ESR	52.73	31.8	0.0033
CRP	116.89	8.68	0.00004
HbA1c	9.3	7.2	0.14

ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, HbA1c: Glycosylated haemoglobin.

Significant p-values are shown in bold.

Discussion

Metals play important roles in the pathogenesis of infectious disease as these serve as co-factors in various enzymatic processes. A comprehensive review of the role of various heavy metals in fungal virulence and different

homeostatic mechanisms has been given by Gerwien et al. (2). Maintenance of adequate intracellular concentrations of trace metal ions like zinc, selenium and copper is essential for many biologically important cellular functions and they are often involved in the regulation of bacterial and fungal virulence. The sequestration of these metals by host defense mechanisms (nutritional immunity) results in extremes of environment for infections to use these metals for various cellular processes, including respiration, replication, transcription, translation, signal transduction and cell division. However, pathogens have also developed counter-defense mechanisms to overcome this metal ion limitation (2, 3, 12). Changes in the levels of certain metals have been shown to increase or decrease infection susceptibility, and the levels of these metals also increase or decrease in response to infection (12).

Copper is an essential element for various enzymatic processes. Excess copper is toxic to cells, and therapies have

Table 6. Correlation of ESR, CRP, and HbA1c with levels of heavy metals in patients with invasive Mucormycosis

No	Heavy metal	Pearson's correlation coefficient (r) and p-value (p)		
		ESR	CRP	HbA1c
1.	Nickel	r=-0.090, p=0.749	r=0.238, p=0.392	r=0.122, p=0.663
2.	Copper	r=-0.294, p=0.287	r=-0.326, p=0.234	r=0.250, p=0.367
3.	Zinc	r=0.149, p=0.596	r=0.013, p=0.962	r=-0.273, p=0.323
4.	Gallium	r=-0.276, p=0.319	r=0.435, p=0.105	r=0.16, p=0.568
5.	Arsenic	r=-0.015, p=0.957	r=0.086, p=0.759	r=-0.134, p=0.631
6.	Selenium	r=0.192, p=0.493	r=0.133, p=0.636	r=-0.004, p=0.986
7.	Rubidium	r=-0.132, p=0.639	r=0.096, p=0.731	r=0.346, p=0.205
8.	Strontium	r=0.020, p=0.942	r=0.113, p=0.687	r=0.011, p=0.966
9.	Cadmium	r=0.077, p=0.783	r=-0.151, p=0.588	r=0.532, p=0.409
10.	Lead	r=-0.115, p=0.681	r=0.019, p=0.944	r=-0.086, p=0.759

ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, HbA1c: Glycosylated haemoglobin

Table 7. Correlation of ESR, CRP and HbA1c with levels of heavy metals in patients with invasive Aspergillosis

Serial no.	Heavy metal	Pearson's correlation coefficient (r) and p-value (p)		
		ESR	CRP	HbA1c
1.	Nickel	r=-0.393, p=0.335	r=-0.345, p=0.402	r=-0.247, p=0.555
2.	Copper	r=0.255, p=0.542	r=0.114, p=0.787	r=0.325, p=0.430
3.	Zinc	r=0.425, p=0.293	r=-0.179, p=0.670	r=0.555, p=0.152
4.	Gallium	r=-0.390, p=0.338	r=0.211, p=0.615	r=-0.018, p=0.965
5.	Arsenic	r=0.159, p=0.706	r=0.075, p=0.859	r=-0.086, p=0.839
6.	Selenium	r=-0.100, p=0.812	r=-0.553, p=0.155	r=0.013, p=0.974
7.	Rubidium	r=0.235, p=0.573	r=-0.628, p=0.095	r=0.828, p=0.110
8.	Strontium	r=0.653, p=0.078	r=-0.155, p=0.712	r=0.043, p=0.918
9.	Cadmium	r=0.496, p=0.210	r=-0.434, p=0.281	r=0.261, p=0.531
10.	Lead	r=0.601, p=0.114	r=0.269, p=0.518	r=0.049, p=0.907

ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, HbA1c: Glycosylated haemoglobin

been developed that boost copper delivery to pathogens resulting in toxicity and less virulence (4, 21). The significantly high levels of copper found in Group 1 in our study may be the response of the body to combat the infection. Copper deficiency, though rare, makes humans more susceptible to infection (12).

The roles of cadmium and lead are not well-studied in human invasive fungal disease. Animal models studying the pathogenesis of *Aspergillus fumigatus* demonstrated that cadmium induced the expression of proteins which support the virulence of the fungal pathogen (13). In our study, cadmium levels were significantly higher in Group 1, a finding that may imply its role in maintaining virulence of the fungal pathogen, especially in invasive Aspergillosis. Lead levels were significantly lower in Group 1 in our study. Blood lead levels in humans depend on genetic and ethnic variations and on environmental exposure which further

varies according to geographical areas and local environment (14, 15). Therefore, in our study, neither higher nor lower lead levels could be linked to disease process or pathogenesis, and we did not find any information in the literature for the same.

Zinc is an essential element for fungal proliferation, and pathogens are still able to thrive in the infected host despite the activities of host nutritional immunity. In animal models, loss of zinc transporters causes *Aspergillus fumigatus* to become avirulent, emphasizing the need for coordinated zinc homeostasis during fungal infection. Fungal growth is known to be inhibited by zinc depletion, and evidence suggests that host cells use zinc sequestration to prevent fungal proliferation. Zinc restriction by host cells is achieved by lowering metal availability via the activity of the host zinc transporters or the expression of zinc-binding proteins which may result in higher levels in serum (2, 3, 5, 16-19). Some

zinc-binding enzymes like superoxide dismutase (SOD) and zinc transporters are also involved in fungal virulence (18-20). *Aspergillus fumigatus* cells lacking SOD1 are more susceptible to reactive oxygen species generated by host defense mechanisms and null mutations in zinc transporters lead to reduced ability of the fungus to grow under zinc deprivation (19). *In vitro* studies on the Mucorales species have also shown that the combination of zinc chelators with antifungal therapy resulted in the synergistic inhibition of the fungus (21). We found higher levels of zinc in Group 1, although not statistically significant perhaps due to small sample size or the habit of taking zinc supplements of healthy adults in the form of multivitamins or alternative medicine without proper consultation even before the emergence of COVID-19. But while comparing the patients in the improved and the worsened subgroups, statistically significant higher levels were found in the improved subgroup, a finding that may reflect that the host defense mechanism had either deprived the infected tissue of zinc or increased the toxicity of zinc. High levels of zinc are also detrimental to fungi and excessive zinc exposure can lead to hyphal growth and change in hyphal morphology. Although fungus develops tolerance and resistance to such toxic levels, it is not well-studied in either *Aspergillus* or *Mucor* (18, 19, 22). We also found statistically significant higher levels of zinc in patients with invasive aspergillosis compared to those with invasive Mucormycosis, again a finding which emphasizes the role of zinc in infection by *Aspergillus* species, but the role of zinc in invasive Mucormycosis is not yet known. The role of other rare metals like nickel, arsenic, selenium, rubidium and strontium in invasive fungal disease is also not fully understood and an area of future research.

Although we found significantly raised levels for some heavy metals, particularly zinc, in patients with improved outcome, we cannot attribute these improved outcomes solely to the higher levels of heavy metal. This is because the outcome of the patient in chronic invasive fungal disease is dependent upon various factors like age, comorbidities, surgical success, residual disease, and tolerance to antifungal treatment. Therefore, the role of heavy metals in deciding the outcome of the patient still remains questionable.

Acute inflammatory markers like ESR and CRP are commonly used parameters for prognostication in invasive fungal sinusitis. In addition to this, poor glycemic control is also one of the risk factors for developing invasive fungal disease (23, 24). We compared the levels of heavy metals based on ESR, CRP, and HbA1c as we expected a positive correlation between these parameters and the levels of those heavy metals which are possibly involved in the pathogenesis of CIFR; however, we did not find any correlation. The statistically significant difference between ESR and CRP between invasive Mucormycosis and invasive Aspergillosis may indicate more severe inflammatory response in

Mucormycosis, but larger studies are required to validate these results.

Our study was limited by small sample size and hospital-based data. Large community-based studies are required to study the roles and effects of these heavy metals on disease process and survival.

Conclusion

Heavy metals play important role in the pathogenesis of invasive fungal disease. Our study was the first of its kind which compared heavy metal levels between patients with chronic invasive fungal disease and healthy controls. We found significant differences in the levels of some essential metals not only between the two groups but also among patients in different disease and outcome subgroups. These levels may not have a direct effect on the outcome of the patient, but they do play a role in the disease process and the virulence of the organism.

Ethics Committee Approval: This study was approved by Ethics Committee, All India Institute of Medical Sciences, New Delhi, India (reference number: IEC-436/02.07.2021).

Informed Consent: Informed and written consent was taken from all patients and healthy controls.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: K.S., A.T., H.V., Concept: K.S., A.T., H.V., Design: K.S., A.T., H.V., Data Collection and/or Processing: J.A.Q., P.V., R.T., S.P., Analysis and/or Interpretation: J.A.Q., S.P., Literature Search: S.K., P.V., R.T., Writing: S.K.

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Main Points

- Heavy metals play important role in the pathogenesis and the virulence of chronic invasive fungal rhinosinusitis (CIFR).
- It is not known whether the levels of these metals differ from those of the general population.
- Our pilot study found significant differences among the levels of some metals, not only between CIFR patients and healthy controls but also between the different disease groups (invasive mucormycosis vs invasive aspergillosis) and the different outcomes (improved vs worsened).

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