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Is there an association between zinc and COVID-19–associated mucormycosis? Results of an experimental and clinical study

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Abstract

Background: The enormous increase in COVID-19–associated mucormycosis (CAM) in India lacks an explanation. Zinc supplementation during COVID-19 management is speculated as a contributor to mucormycosis. We conducted an experimental and clinical study to explore the association of zinc and mucormycosis.

Methods: We inoculated pure isolates of *Rhizopus arrhizus* obtained from subjects with CAM on dichloran rose Bengal chloramphenicol (DRBC) agar enriched with (three different concentrations) and without zinc. At 24 h, we counted the viable colonies and measured the dry weight of colonies at 24, 48 and 72 h. We also compared the clinical features and serum zinc levels in 29 CAM cases and 28 COVID-19 subjects without mucormycosis (controls).

Results: We tested eight isolates of *R arrhizus* and noted a visible increase in growth in zinc-enriched media. A viable count percentage showed a significantly increased growth in four of the eight isolates in zinc-augmented DRBC agar. A time- and concentration-dependent increase in the mean fungal biomass with zinc was observed in all three isolates tested. We enrolled 29 cases of CAM and 28 controls. The mean serum zinc concentration was below the reference range in all the subjects and was not significantly different between the cases and controls.

Conclusions: Half of the *R arrhizus* isolates grew better with zinc enrichment in vitro. However, our study does not conclusively support the hypothesis that zinc supplementation contributed to the pathogenesis of mucormycosis. More data, both in vitro and in vivo, may resolve the role of zinc in the pathogenesis of CAM.

KEYWORDS
Aspergillus, coronavirus disease, Mucorales, zinc, zygomycosis

Valliappan Muthu and Mohan Kumar H contributed equally to the manuscript and are the joint first authors.

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1 | INTRODUCTION

Mucormycosis is a severe infection with a high case-fatality rate and usually occurs in individuals with impaired immunity.[1,2] Coronavirus disease 2019 (COVID-19) has led to an enormous rise of mucormycosis cases in India,[3-5] resulting in several unanswered questions.[6] CAM is also increasingly being reported from Europe and several other countries.[7] During this unprecedented surge of COVID-19–associated mucormycosis (CAM) cases in India, several hypotheses were proposed.[8-10] Most of those assumptions were conjectural except for one study, suggesting an association between CAM and uncontrolled diabetes or inappropriate glucocorticoid therapy.[4] Interestingly, a few patients of COVID-19 managed at home and had not received glucocorticoids also developed CAM.[4,5] Whether unproven therapies, home remedies and over-the-counter medicines contributed to the development of CAM remains unclear. One widely discussed theory in the media and the medical community was that zinc supplementation for COVID-19 management triggered the growth of Mucorales, causing CAM.[11] However, zinc supplementation was also practiced in several other countries without a significant rise in cases of mucormycosis. Zinc is an essential micronutrient for all eukaryotes and is an important factor contributing to the growth and virulence of pathogenic fungi, including *Candida*, *Aspergillus* and others.[12-14] In one study, zinc chelator clioquinol when used with posaconazole (but not amphotericin) was shown to be effective against certain strains of Mucorales.[15]

Herein, we report the results of an in vitro study evaluating the role of zinc on the growth of *Rhizopus arrhizus* isolated from subjects with CAM. We also supplement the experimental data with a case-control study evaluating serum zinc levels in COVID-19 subjects with and without mucormycosis.

2 | METHODS

We performed the current study in two parts, namely experimental and clinical, to evaluate the association of zinc with CAM. The study was performed at the Postgraduate Institute of Medical Education and Research, Chandigarh. The experiment was conducted at the Mycology laboratory (Department of Medical Microbiology), and the serum zinc was estimated at the Department of Biochemistry, as described below. The cases and controls were identified from the emergency services of our hospital. The Institute Ethics Committee approved the study protocol. We obtained written informed consent and used anonymised patient data.

2.1 | The role of zinc supplementation in potentiating the in vitro growth of Mucorales

2.1.1 | *Inoculum preparation and quantification*

We used eight randomly selected clinical isolates of *Rhizopus arrhizus* (Table 1) from patients with CAM. The isolates were grown on Sabouraud dextrose agar (SDA) slants supplemented with 0.02% chloramphenicol at 37°C for 3–4 days. The sporangiospores were harvested using sterile bend glass rods and washed in sterile phosphate buffer saline (1x PBS). We used inoculums of 10^3 sporangiospores/mL measured by hemacytometer for further experiments.

TABLE 1 Comparison of fungal biomass of three *Rhizopus arrhizus (R arrhizus)* isolates with and without zinc (Zn) supplementation

Isolate (identification number)	Dry weight, mean value in mg (percentage increment relative to the control)		
	Control (No added zinc)	1×10^{-5} Molar Zn	3×10^{-5} Molar Zn
R arrhizus (P489)			
24 h	10	10.2 (2%)	10.6 (6%)
48 h	11.5	11.9 (3.5%)	12 (4.3%)
72 h	12.8	15.2 (18.8%)	18 (40.6%)
R arrhizus (P503)			
24 h	10	10.4 (4%)	11.6 (16%)
48 h	12.2	12.7 (4.1%)	13.7 (12.3%)
72 h	15.2	15.8 (4%)	20 (31.6%)
R arrhizus (P582)			
24 h	9.8	11 (12.2%)	11.1 (13.3%)
48 h	10.7	12.2 (14%)	12.5 (16.8%)
72 h	15.2	16 (5.3%)	22.3 (46.7%)

2.1.2 | *Viable count estimation*

To determine the effect of three different zinc concentrations on the growth of *R arrhizus* isolates, we enriched the basal culture media, dichloran rose Bengal chloramphenicol (DRBC) agar by adding zinc sulphate heptahydrate ($ZnSO_4$, $7H_2O$). We used zinc sulphate as the source of zinc ions, and the effective zinc concentration in the solution was 22.73% mole fraction of the total 287.6 g moles of $ZnSO_4$, $7H_2O$. A molar equivalent of zinc concentration (0.63 mg/L, 1×10^{-5} M) corresponds to the normal range of serum zinc. We targeted varying concentrations of zinc (1×10^{-5} M, 3×10^{-5} M, and 1×10^{-4} M) to evaluate the growth potentiating effect of zinc on *R arrhizus*. DRBC media without zinc were used as the negative control. A 100 μL of the inoculum containing 10^3 spores/mL of each isolate ($n = 8$) was spread on DRBC agar plates comprising of the test (with $ZnSO_4$) and the negative control groups (without $ZnSO_4$). We repeated the experiment thrice for each isolate. The plates were incubated at 37°C for 24 h, and we manually counted the viable counts (CFU) at three different zinc concentrations and controls.

2.1.3 | *Fungal biomass dry-weight estimation*

Three clinical isolates of *R arrhizus* were used for evaluating the effect of zinc on fungal biomass production. Briefly, we seeded 100 μl of

10^3 sporangiospores/mL into 50 mL asparagine-broth supplemented with two different concentrations of $ZnSO_4.7H_20$ (1×10^{-5} M and 3×10^{-5} M). The experiments were performed in triplicate. After 24, 48 and 72 h of incubation, we filtered the respective growths in the flask using filter paper of known weight, which was subsequently dried. We then measured the dry weight of the fungal biomass of each isolate after 24, 48 and 72 h and compared them with non-supplemented matched controls.

2.2 | Clinical study

We compared the serum zinc levels in subjects of COVID-19 with (cases) and without (controls) mucormycosis. The clinical details and laboratory values of the study participants were obtained from the medical records of the patients attending the emergency services of our institute. Only hospitalised subjects with a confirmed diagnosis (by reverse transcriptase PCR for SARS-CoV-2 in nasopharyngeal or oropharyngeal swabs) of COVID-19 were included. Both the cases and controls were recruited during the same study period. We classified CAM as subjects wherein mucormycosis was diagnosed concurrently or within eight weeks of COVID-19. We defined confirmed mucormycosis (cases) in subjects with a consistent clinical and radiological feature for mucormycosis and microbiology or histopathology evidence of aseptate hyphae (with or without culture reports showing growth of Mucorales) in the tissue or respiratory specimens.[16] The control subjects were hospitalised subjects with COVID-19 without mucormycosis and followed up for at least eight weeks.

2.2.1 | *Study procedure*

We obtained the following information from the records: (1) age and sex; (2) the presence of diabetes, diabetic ketoacidosis (DKA) or any comorbid illness; (3) the date of diagnosis of COVID-19 and the occurrence of hypoxemia during the disease; (4) the nature and duration of respiratory support; (5) treatment instituted for COVID-19 (dose and duration of glucocorticoids); (6) history of zinc supplementation for COVID-19; (7) the laboratory parameters, including complete blood count, arterial blood gases, liver functions, renal functions, serum zinc levels and others obtained within 24–48 h of hospital admission; and (8) in subjects with CAM, we noted the time lag between COVID-19 and mucormycosis, the site of involvement and the diagnostic test used for confirmation.

2.2.2 | *Measurement of serum zinc levels*

We estimated zinc on archived samples using the atomic absorption spectrophotometer (Agilent AA-200 Analyser). We used a minimum of 600 μL serum sample as per the manufacturer's recommendation. The measuring range was 50–700 μg/dL, and values above the measuring range were retested after the required dilution. The precision (CV) of the instrument was 0.5%–5.8%. Before analysis, the serum sample was briefly diluted with 2% HNO3 in a 1:10 ratio in 'flame mode'. Zinc ions in the serum sample were converted to atomic states using a flame. The atomised element absorbed light from the hollow cathode lamp, elevating it to the excited states. The amount of light energy absorbed was proportional to the number of analyte atoms in the light path. We also tested 10% of the samples in duplicate, and the results were consistent.

2.2.3 | *Statistical analysis*

We used the commercially available statistical software package SPSS 22.0 (IBM SPSS Inc.). Categorical variables are presented as numbers and percentages, and continuous variables as mean (95% confidence interval [CI]). We compared the differences between categorical and continuous variables using the chi-square test and the Mann-Whitney U test, respectively. The viable count (%) was compared between different groups using analysis of variance with the significance level adjusted for multiple comparisons applying Sidak's test. A p-value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | In vitro growth of *Rhizopus arrhizus* in zinc-enriched DRBC agar media

We found the colonies from all the eight isolates on the zinc-supplemented plates to be larger, profluent and more cottony than the control media where zinc was not added (Figure 1). The visual assessment suggested a significant increase in the size of the colonies;

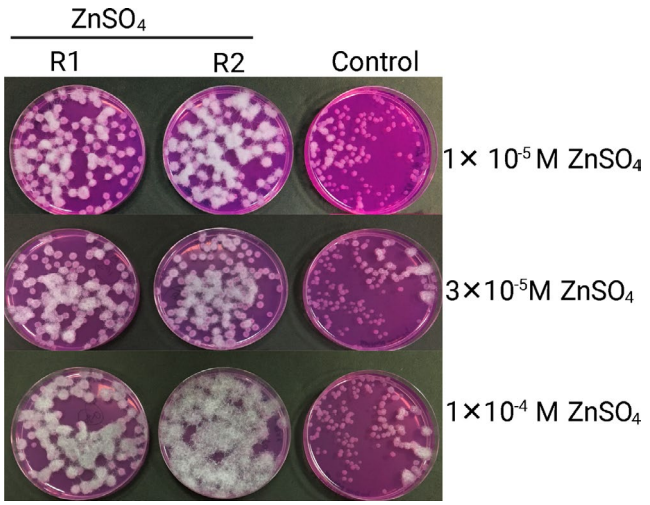

FIGURE 1 Viable counts of *Rhizopus arrhizus* determined at three different concentrations of zinc. R1 and R2 two representative clinical isolates (of the eight tested) of *Rhizopus arrhizus* from CAM patients showing an increased number of colonies and more cottony growth with zinc supplementation

however, this was not quantifiable due to the profuse growth. In addition, we observed a significant increase in growth (viable CFU/mL) of *R arrhizus* in four of the eight isolates incubated in zinc-enriched culture media compared to those without (Figure 2). There was no increase in the viable counts with increasing zinc concentrations (Figure 2).

3.2 | Quantification of fungal biomass in the presence of zinc

We tested three isolates. Two isolates were those showing significantly increased CFU counts (P489, P582), while one isolate did not significantly grow with zinc (P503). In all three, we observed a temporal and concentration-dependent increase in the mean fungal biomass. The fungal biomass production in the presence of zinc was higher compared to matched controls; the effect was more pronounced at higher concentrations of zinc and at 72 h (Table 1).

3.3 | Case-control study to compare the serum zinc concentration

During the study period, we identified 31 confirmed cases of CAM, of which two were excluded due to inadequate details. Finally, we enrolled 29 cases and 28 controls (Table 2). Mucormycosis developed after a mean of 11.3 days (range, −5 to 39 days) from the onset of COVID-19. Rhino-orbital (31%) and rhino-orbital-cerebral mucormycosis (51.7%) accounted for the majority of CAM, while pulmonary mucormycosis was seen in 5 of the 29 (17.2%) subjects. The mean age of the cases and controls was not significantly different, and most (71.9%) study participants were men. While hypoxemia

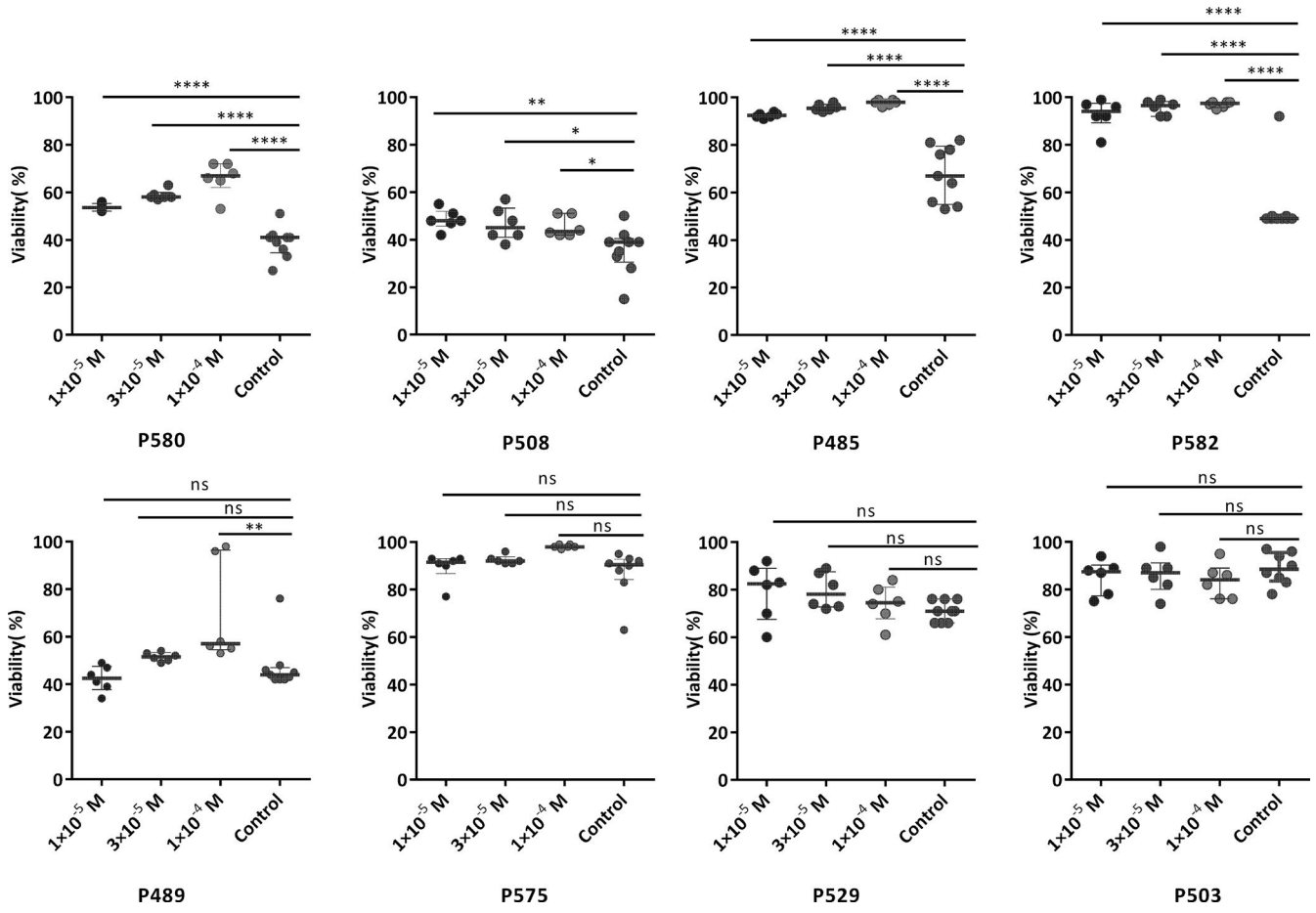

FIGURE 2 Multiple scatter plot showing viable colony count (%) (in the vertical axis) of *Rhizopus arrhizus* isolated from eight different patients with COVID-19–associated mucormycosis at 24 h. The viability count data are presented for the standard culture media without zinc (control) and media enriched with three different zinc concentrations (1×10^{-5} Molar Zn, 3×10^{-5} Molar Zn, and 1×10^{-4} Molar Zn). Each dot represents a replicate for the corresponding patient isolate of *Rhizopus arrhizus*, and the horizontal line (with error bars) represents the median viability percentage (1st to 3rd quartile). The labels P580, P508, P485, P582, P489, P575, P529 and P503 denote the eight different patient isolates. There is a significant increase in the viable count at 24 h with zinc supplementation at all concentrations in four of the eight isolates, while in one significant increase in growth at one of the three concentrations. No significant increase in growth was seen with zinc supplementation in three of the isolates. However, it should be noted that all the three latter isolates had a very high viable colony count (>80%) even at baseline without zinc supplementation (* *p*-value <.05; ** *p*-value <.01; ***p*-value <.001; ****p*-value<.0001; ns—nonsignificant)

TABLE 2 Baseline clinical and laboratory parameters of coronavirus disease (COVID-19) subjects with and without mucormycosis (cases and controls, respectively)

	Controls ($n = 28$)	Cases ($n = 29$)	p value
Age, years	57.2 (52.4–62)	53.0 (47.7–58.4)	.24
Male sex	19/28 (67.9)	22/29 (75.9)	.50
COVID-19 and its management			
Hypoxemia	27/28 (96.4)	16/29 (55.2)	.001
Mechanical ventilation for COVID-19	13/27 (48.1)	7/16 (43.8)	.93
Glucocorticoids	21/26 (80.8)	20/28 (71.4)	.42
Duration of glucocorticoid therapy, days	7.3 (5.7–8.9)	10.5 (7.3–13.7)	.08
Zinc supplementation			
Yes	3/28 (10.7)	6/29 (20.7)	.07
No	17/28 (60.7)	9/29 (31)	
Information not available	8/28 (28.6)	14/29 (48.3)	
Diabetes mellitus	12/28 (42.9)	21/28 (75)	.01
Duration of diabetes mellitus, years	4.9 (1.5–8.2)	5.3 (2.7–7.9)	.85
Diabetic ketoacidosis	4/12 (33.3)	9/21 (42.9)	.72
Glycated haemoglobin in %	9.1 (6.9–11.2)	11.3 (9.4–13.3)	.10
Site of mucormycosis			
Rhino-orbital	-	9/29 (31)	
Rhino-orbito-cerebral	-	15/29 (51.7)	
Pulmonary	-	5/29 (17.2)	
Investigations			
Haemoglobin, g/dL	12.04 (11.19–12.89)	12.54 (11.52–13.57)	.44
Total leukocyte count, cells/μL	15,705 (12,802–18,608)	15,846 (12,990–18,701)	.94
Neutrophil (%)	83.1 (73.1–93.1)	88.5 (84.6–92.4)	.26
Lymphocyte (%)	10.9 (0.65–21.2)	5.8 (2.3–9.2)	.29
Platelet count, $\times 10^3$ cells/μL	216 (178–255)	247 (203–291)	.28
Blood urea, mg/dL	74.2 (48.8–99.6)	65.9 (45.7–86)	.60
Serum creatinine, mg/dL	2.26 (0.95–3.57)	1.48 (0.90–2.07)	.28
Serum bilirubin, mg/dL	0.62 (0.50–0.74)	0.71 (0.58–0.85)	.27
Serum protein, mg/dL	6.60 (6.31–6.89)	6.37 (6.04–6.69)	.28
Serum albumin, mg/dL	3.13 (2.91–3.36)	2.88 (2.67–3.08)	.09
Arterial pH	7.40 (7.36–7.45)	7.39 (7.34–7.44)	.70
Serum bicarbonate, mmol/L	20.1 (17.2–22.9)	17.8 (15.3–20.3)	.21
Serum zinc, μg/dL	58.4 (43.7–73.1)	56.7 (45.4–63.9)	.66

Note: The values are presented as mean (95% confidence interval) or numbers (percentage).

was more frequently encountered in controls than cases (92.9% vs 55.2%, $p = .001$), mechanical ventilation for the management of COVID-19 was similar in the two groups (48.1% vs 46.7%, $p = .93$). The proportion of subjects receiving glucocorticoids for COVID-19 was not significantly different between cases (71.4%) and controls (80.8%). Diabetes mellitus was seen in 75% of the cases as opposed to 42.9% of the controls ($p = .01$). However, the duration of diabetes mellitus, the glycated haemoglobin and the proportion of subjects with diabetic ketoacidosis were similar in both groups. The complete blood count, renal and liver function parameters, and arterial pH were not different between the cases and controls.

The information on zinc supplementation was available for 35 subjects (15 cases and 20 controls). Twenty-six of the 35 subjects did not receive zinc supplementation. Six (20.7%) and three (10.7) subjects among the cases and controls, respectively, had received zinc supplementation; the difference was not statistically significant. The mean serum zinc level (56.5 μg/dL) was below the reference range (66–110 μg/dL) provided by the manufacturer and was not significantly different between the cases and controls. Only three subjects had serum zinc levels above the reference range (114, 162 and 162 μg/dL). None of these three subjects had mucormycosis, nor did they receive zinc supplementation. The mean (95% CI) serum

zinc levels in those who received zinc supplementation (59.8 [45.2–74.4] μg/dL; 9 subjects) was significantly higher than those without supplementation (57.1 [42.8–71.5] μg/dL; 26 subjects).

4 | DISCUSSION

We found that half of the *R arrhizus* isolates obtained from CAM patients showed a significant increase in growth in zinc-enriched fungal culture medium. In addition to the increased colony count, we also observed that the colonies were larger and had higher fungal biomass in zinc-supplemented media. However, there was no increase in the colony numbers with increasing zinc concentrations. We did not find a significant difference in serum zinc levels among CAM cases and COVID-19 controls.

Pathogenic fungi (*Aspergillus*, *Candida* and others) grow better in the presence of zinc.[13,17-19] During infections, the human host makes zinc unavailable to the pathogens, causing apparent zinc deficiency.[20] In experimental studies, zinc chelators have shown promising results against *Aspergillus* and several species of Mucorales.[15,21] We found zinc promoted the in vitro growth in half of the *Rhizopus arrhizus* isolates from CAM subjects. The growth did not increase significantly in all others, possibly due to strain-to-strain variation, the availability of other nutritional elements and differences in zinc transporters. Strain-dependent variation was also noted in an experimental study evaluating zinc chelators and antifungal agents.[15] While the effect of zinc on *Rhizopus arrhizus* is not universal, the isolates that responded to zinc enrichment showed significant and unequivocal growth. In an experimental study, investigators found that zinc acted as a catalyst in promoting the in vitro growth of *Rhizopus nigricans* (now *Rhizopus stolonifer*).[22] The authors found more efficient energy utilisation by the fungi on adding zinc. Zinc reduced the economic coefficient of the fungus (weight of the sugar or carbohydrate consumed/growth of the fungus) and led to an abundant and profuse growth of the fungi. Additionally, other micronutrients (copper, manganese and molybdenum) promoted fungal growth only in the presence of zinc.[22] To our knowledge, the current study is the first to evaluate the effect of zinc enrichment on the growth of Mucorales isolated from CAM patients.

Zinc supplementation is widely believed to be safe and offers benefit against a variety of infections, including SARS-CoV-2.[23] However, a randomised controlled trial failed to show the benefit of zinc supplementation in COVID-19.[24] While most patients had normal serum zinc concentrations, the mean values were significantly higher (2 μg/dl) in those with zinc supplementation than without, both in cases and in controls. However, the clinical significance of this finding is not known. We found no difference in serum zinc levels in CAM cases and controls. While serum zinc levels may be useful in evaluating for toxicity or deficiency, it is not known whether they reflect zinc availability at the cellular level. Further, we collected serum samples after the development of mucormycosis, and zinc levels may differ during incubation of the pathogenic fungi in CAM. Moreover, most of our patients were either not on zinc supplementation, or this information was not available.

What does our study add to the existing knowledge on the pathogenesis of CAM? Our preliminary experiment suggests that zinc promotes the in vitro growth of some isolates of *Rhizopus arrhizus*. A previous study has shown that the activity of polymorphonuclear cells against various Mucorales and *Aspergillus* may be overwhelmed due to increased fungal burden.[25] Theoretically, the increase in fungal biomass with zinc may result in mucormycosis despite a normal immune function. However, our study was an in vitro experiment and whether zinc increases the growth of *Rhizopus arrhizus* in vivo remains to be seen. Also, we tested only a few isolates of *Rhizopus arrhizus*. Whether zinc will have a different effect on other pathogenic Mucorales is unknown.

In summary, our experiment suggests that zinc may potentiate the in vitro growth of half the isolates of *Rhizopus arrhizus* obtained from patients with CAM. We did not find a difference in serum zinc levels in COVID-19 patients with and without mucormycosis. Conclusive proof of zinc in the pathogenesis of invasive mucormycosis requires further research.

CONFLICT OF INTEREST

VM, MKH, RAP, DZ, HC, SMR, NKP, AKP, NS, SS, AC and RA have no conflict of interest.

AUTHOR CONTRIBUTION

Valliappan Muthu: Conceptualization (supporting); Data curation (lead); Formal analysis (lead); Methodology (lead); Project administration (supporting); Writing-original draft (lead); Writing-review & editing (lead). **Mohan Kumar:** Data curation (equal); Investigation (equal); Resources (equal); Writing-review & editing (equal). **Raees Paul:** Data curation (supporting); Investigation (supporting); Methodology (supporting); Writing-review & editing (supporting). **Deepy Zohmangaihi:** Data curation (supporting); Investigation (supporting); Methodology (supporting); Writing-review & editing (supporting). **Hansraj Choudhary:** Data curation (supporting); Investigation (supporting); Methodology (supporting); Writing-review & editing (supporting). **Shivaprakash M Rudramurthy:** Data curation (supporting); Investigation (supporting); Methodology (lead); Resources (supporting); Writing-review & editing (supporting). **N. K. Panda:** Data curation (supporting); Project administration (supporting); Resources (supporting); Writing-review & editing (supporting). **Ashok Pannu:** Data curation (supporting); Investigation (supporting); Project administration (supporting); Resources (supporting); Writing-review & editing (supporting). **Navneet Sharma:** Data curation (supporting); Investigation (supporting); Methodology (supporting); Writing-review & editing (supporting). **Sadhna Sharma:** Investigation (supporting); Project administration (supporting); Writing-review & editing (supporting). **Arunaloke Chakrabarti :** Conceptualization (supporting); Data curation (supporting); Resources (lead); Writing-review & editing (supporting). **Ritesh Agarwal:** Conceptualization (lead); Data curation (supporting); Formal analysis (supporting);

Investigation (supporting); Methodology (supporting); Visualization (lead); Writing-original draft (supporting); Writing-review & editing (supporting).

DATA AVAILABILITY STATEMENT

Data are available on request with the corresponding author.

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