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Measuring the Change in Zinc Ion Concentration in Eye Tear Fluid between Healthy and Parasite Infected Individuals: Relationship between Zinc Ions in Tear Fluid and Parasitic Infection by Soil-Transmitted Helminths

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Abstract

Infection by soil-transmitted helminths has been correlated with lowered concentration of zinc ions in serum. This study investigated the relationship between changes in zinc ion concentration in tear fluid for healthy versus parasitic infected individuals. The study used a serodiagnosis method to determine the presence of parasite infection. Our results conclude that converse to lowered zinc levels in serum, there is a significant increase in the concentration of zinc ions in eye tear fluids for parasite-infected individuals. These results support our continuing efforts to develop a low-cost, point-of-care diagnostic test specific to helminth infection. This will support the on-going global anti-worm treatment efforts.

Keywords: Point-of-care; Parasitic infection; Soil-transmitted helminths; Tear fluid; Zinc

Introduction

Parasitic worms, or helminths, are estimated to affect up to two billion people worldwide [1,2]. There are two major types of phyla of helminths: nematodes, which include all major intestinal worms, and filarial worms that directly impact the lymphatic system. Nematode infections are especially prevalent among school-aged children in the developing world. Pronounced symptoms often include nausea, diarrhoea and high fevers; infection is usually accompanied by further infections or conditions, such as anaemia, growth stunting and malaria, often accompany nematode infections [3].

The high cost of worm infections to education, social, and medical infrastructure has led to a global response from private and public organisations. From tackling the spread of guinea worm eggs with the use of the chemical compound, ABATE©, to public-private collaborations to encourage mass-deworming in schools using the deworming drugs albendazole and mebendazole, deworming strategies have primarily addressed prevention and treatment. The increase in randomised control trial methodology has allowed for great strides to be made to measure the effectiveness of such efforts, through the use of dependent variables such as school absenteeism, serum haemoglobin levels and educational test scores [4].

Diagnosing worm infections, however, is a relatively costly endeavour in infection-prevalent areas, requiring sterile serum, stool samples and laboratory expertise. Measuring and counting the type and presence of eggs in the faeces using microscopy is the standard method for identifying worm infections in South Asia, where the risk of infection from soil-transmitted helminths (STHs) ranges from 12% to 66%, based on the worm type [5].

The aim of this study is to demonstrate the potential the use of eye tears as a serological fluid for diagnosis of nematode infection diagnosis by measuring the increased concentration of zinc ions in eye tear fluid in nematode-infected Indian subjects. We suggest an application of our results towards development of an affordable point-of-care (PoC) diagnostic test, which can be provided as a low-cost diagnosis tool to measure and focus mass deworming efforts and can reorient and refine global deworming strategies. Though correlations between zinc in the blood serum and STH infection have been shown, serodiagnosis of parasitic infection has not been done using metalloprotease ions in eye-tears as an indicator. Our study contributes to the existing scientific literature by significantly correlating the zinc ions in eye tear fluid with parasitic worm infections, thus offering a new method for diagnosis. While there has been some work done on zinc in the blood serum and its relationship with parasitic worm infection, testing for infection using tear fluid has not been previously done.

Tear fluid have been previously used to detect localized eye infections and macular degeneration, such as by identifying the presence of a glutaredoxin-related protein in fungal mycotic keratitis. Tear fluids have also been used to detect systemic biomarker changes, such as with blood sugar, vitamin D and insulin levels in diabetics [6]. For example, the correlation of serum circulatory glucose levels with glucose levels in eye tears has been reported, and is the basis for the development of new non-invasive glucose monitoring devices for diabetics [7].

In this human study, we propose to use eye tears as a diagnostic fluid to possibly detect the sharp increase in serum zinc metalloprotease enzymes that are known to occur during nematode infections. During their infective larval (L3) stage, nematodes secrete large quantities of these enzymes to penetrate host cell walls and aid in establishing a nutritive host environment for them [8]. We hypothesized that just as with many other serum proteins that cross

the blood/eye-tear barrier, these metalloproteins might have elevated concentrations in eye tears. Then, we could indirectly measure their concentration, in normal and infected patients, by liberating the zinc ions present and measuring their concentration by colorimetric analysis [9,10]. Such an assay could be reduced to a simple PoC kit using colorimetric paper analysis, removing the need for sterile conditions, stool sampling and expensive microscope laboratories to determine helminth infections in a susceptible population.

Materials and Methods

Study population and design

In this trial, we had two groups of subjects from urban areas in Mumbai, India. The first group from the neighbourhood of Kamathipura, consisted of 48 subjects, with an average age of 10 years. The second population, from the Mazagaon neighbourhood, consisted of 43 subjects, with an average age of 34 years. There were a total of 34 male participants and 47 female participants.

Tear samples

Each participant's age and gender was noted, and in addition, each participant was asked whether they had experienced stomach pain in the last three days. Each participant was made aware of the risks of the study and the requirement that a blood test be taken within three days of the eye tear sample. This study was conducted according to GCP guidelines issued by the ICH and ICMR ethical guidelines in accordance with the laws and regulations of India where the trial was performed. The final approved protocol, informed consent documents and all the study-related documents were reviewed and approved by an Ethics Committee before the start of the study. The procedures and any possible hazards to which the subjects would be exposed were explained and an informed consent statement was read and signed by all participants or their guardians in the case of minors.

Eye tear samples of at least 0.2 mL were taken from each eye of each participant using sterile Schirmer strips. The zinc ion concentration (μg/mL) of each sample was then measured as shown in the general procedure below using colorimetric analysis against a standardized zinc ion concentration curve. All reagents were of AAS or biochemical grade. Aqueous solutions were prepared using metal-free (redistilled demineralized) water [11].

A Tris(hydroxymethyl) aminomethane buffer solution was made up to a 0.4 mol/L concentration, (pH 8.1). A potassium cyanide (KCN) solution was made up to a 0.5 mol/L concentration. A GT solution, pH 8.1, was prepared by dissolving 67 g of guanidine HCl in 90 mL of the tris buffer solution. The pH of the solution was adjusted to 8.1 by a titred amount of 3 mol/L NaOH and/or HCl and diluted to 100 mL with more tris buffer as needed. A GTAC solution was prepared by dissolving 20 mg of sodium L-ascorbate into 10 mL of GT solution plus 0.1 mL of KCN solution and mixed and used immediately upon preparation. The nitro-PAPS (Sigma Aldrich) solution of concentration 0.6 mmol/L was prepared by dissolving 30.2 mg of nitro-PAPS in 90 mL of water. The pH of the solution was adjusted to 8.1 by adding 1 mol/L NaOH and/or HCl and the resultant solution was diluted to 100 mL with water. The chloral hydrate solution was 1 mol/L concentration.

The calibration curve stock standard zinc solution of 50 mg/L was obtained by diluting 5 ml of a 1 mg/mL zinc (zinc nitrate) solution to 100 mL with 0.1 mol/L HCl and the working standard solution was prepared by diluting the stock standard solution to a concentration of 10 mg/L with 0.1 mol/L HCl. The calibration curve was then established with dilutions of the working solution 10 mg/5 mg/2.5 mg/1.25 mg/0.625 mg/0.3125 mg/l (μg/mL).

The dissociation of zinc from protein-bound zinc in both serum and eye tear samples was carried out at pH 8.1 by diluting the serum or eye tear sample 6-fold with GTAC solution and 5 mmol/l of KCN in a shaking microwell plate for 1 h at 25C. The masking effect of cyanide on interfering copper and iron was eliminated by using >0.5 mol/L chloral hydrate as a demasking reagent to dissociate the Zn-cyanide complex without dissociating the Cu- or Fe-cyanide complexes.

Serodiagnosis of helminth infection

Atopic disorders, like helminth infections, show characteristic elevations of serum IgE and IgG4. Studies have supported the claim that both malaria and helminth infection shifts the immune response to Th2 type. Elevated levels of IgE are seen at the serum level when a helminth infection is present. Therefore, for the purposes of this study, serodiagnosis is used to confirm the presence of parasitic infection. Patients with both elevated IgE and IgG levels are believed to be infected with helminthes [12].

IgE Quantitative Test

One monoclonal anti-IgE coated microtiter plate with 96 wells was used. The Abnova assay system uses one monoclonal anti-IgE antibody for solid phase immobilization and goat anti-IgE antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test specimen (serum) was added to the IgE antibody coated microtiter wells and incubated with 100 μL of the Zero Buffer at room temperature for 30 min. The plates were thoroughly mixed for 30 seconds. The incubation mixture was removed by flicking the plate content into a waste container.

The well was then washed with distilled water to remove any residual test specimen, and IgE antibody labelled with horseradish peroxidase (conjugate) was added. 150 μL of Enzyme Conjugate Reagent was added into each well and gently mixed for 10 seconds. The conjugate binds immunologically to the IgE on the well, resulting in the IgE molecules being sandwiched between the solid phase and the enzyme-linked antibodies. After incubation at room temperature for 30 min, the wells were washed with water to remove unbound-labelled antibody. 100 μL of TMB Substrate Reagent (1-step) solution of was added and incubated at room temperature, in the dark for 20 min, resulting in the development of a blue colour. The colour development was stopped with the addition of 100 μL of the Stop Solution. The colour was changed to yellow and measured spectrophotometrically at 450 nm. The concentration of IgE is directly proportional to the colour intensity of the test sample.

IgG Human ELISA Test

Abcam's IgG Human Enzyme-Linked Immunosorbent Assay (ELISA) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Human IgG in serum and plasma. This assay employs an antibody specific for Human IgG coated on a 96-well plate. All reagents were equilibrated to room temperature (18-25°C) prior to use. Standards and samples were pipetted into the wells and IgG present in a sample was bound to the wells by the immobilized antibody. The wells were washed and the 100 μL biotinylated anti-Human IgG antibody is added. After washing away unbound

biotinylated antibody, 100 µL of the HRP-conjugated Streptavidin was pipetted to the wells. The wells were again washed, a 100 µL of the TMB substrate solution was added to the wells and they were incubated for 20 min in the dark to allow the colour to develop in proportion to the amount of IgG bound. The Stop Solution changed the colour from blue to yellow, and the intensity of the colour was measured at 450 nm immediately.

For information on reagent preparation, see Abnova KA0216 Human (IgE) ELISA Assay kit and Abcam: ab100547 – IgG Human ELISA Kit.

Results

The data from this study was analysed in two stages. First, we performed a two-sided t-test to test the null hypothesis that there was no significant relationship between the zinc concentrations in eye tears of the infected versus the not-infected population. In this stage of the analysis, those with elevated IgE antibody levels were assumed to be infected, while those with normal levels were assumed to be not infected. With a p-value of less than 0.001, the null hypothesis was disproved. The results show that the infected population was likely to have a tear zinc concentration between 3.66 µg/mL and 4.4 µg/mL at the 95% confidence interval.

The same analysis was conducted for the levels of zinc ions in the blood serum. Using a two-sided t-test for the same population, the null-hypothesis was again disproved; this indicated a significant relationship between parasite infection and the concentration of zinc ions in the serum at the 95% confidence interval. These results showed that the concentration of zinc of the infected population was between 0.82 µg/mL and 0.88 µg/mL. The results of the test were significant at $p<0.001$. The results derived here corroborate a number of studies exploring the relationship between levels of zinc in blood serum and (a) the increased risk of parasite infection at low concentrations of zinc in the serum and (b) the use of zinc supplements and measurements to assess the effects of treatment [13-15] (Table 1).

	Infected population	Not-infected population
Number of subjects	23	58
Average concentration of Zn+ in tear fluid	4.78 µg/mL	2.42µg/mL
Range of concentration of Zn+ in tear fluid	2.7 µg/mL–6.7 µg/mL	0.7 µg/mL–5.7 µg/mL

Table 1: Summary of data sample.

However, as mentioned earlier in this paper, while IgE assays are used to establish the presence of a broad range of parasites, IgG assays provide a more accurate measure of the presence of soil-transmitted helminths and other ascariasis causing parasites. Of our sample size of 81 subjects, 40 did not demonstrate elevated antibody levels at all, and so were deemed not infected. Of the remaining 41 subjects, 23 exhibited both elevated IgE levels and IgG levels, while the remaining 18 subjects exhibited only elevated IgE levels. We assumed that these 23 with elevated IgE and IgG levels were likely to be infected with soil-transmitted helminthes, and studied this population in the second part of our analysis. We performed a two-sided t-test to test the independence of infected versus not infected populations in tear zinc and blood serum zinc levels.

The first t-test disproves the null hypothesis, showing that those infected with STH are likely have elevated zinc concentrations in their eye tears between 4.39 µg/mL and 5.17 µg/mL while those without STH infection had ranges of zinc concentration in the tears between 1.87 µg/mL and 2.34 µg/mL at 95% confidence interval. The concentration of zinc ions in the serum was lower for the infected population than for the not-infected population, with the infected population sample exhibiting serum zinc levels between 0.76 µg/mL and 0.87 µg/mL, while the healthy sample exhibited levels between 0.99 µg/mL and 1.05 µg/mL. All results were statistically significant at $p<0.001$.

In conclusion, when examining a helminth infected population, our study showed that those infected exhibited higher levels of zinc ions concentration in tear fluid than those with no parasite infection, and those with only IgE elevated antibody levels. The concentrations of zinc ions in the tear fluid were 55.95% higher for the infected population than for the not-infected population. Figure 1 shows a cluster of infected patients exhibiting higher levels of zinc ions in the tears than uninfected patients.

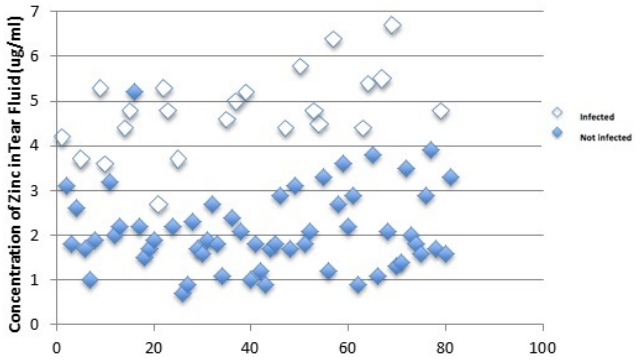

Figure 1: Relationship between Infected Population and Concentration of Zinc Ions in Tear Fluid.

Discussion

This study continued the work of our earlier studies first examining zinc ion concentrations in the eye tears of normal healthy individuals that established the result that zinc ion concentration in healthy eye tears falls in a fairly narrow range of 1.0 to 4.0 µg/mL followed by a small study that showed that a severe parasite infection (stool analysis) leads to an elevation in zinc eye tear levels. This new study made use of ELISA serum assays to determine helminth infections in a much larger and diverse pool of subjects and showed a statistically significant

relationship between elevated zinc ion concentration in eye tears and helminth infections.

These results are our first step towards creating a point-of-care, non-sterile test to measure helminth infections. It is key to note that diagnosis is not a part of the standard recommendations to governments made by international health IGOs and NGOs. Most governments are advised to subsidize periodic mass deworming: the WHO recommends mass treatment once or twice a year in regions where worm prevalence is above 20% and 50%, respectively [16]. This is to be expected, given the current very high costs of testing. Knopp et al. estimate that the cost per child of testing is 1.88 USD, but Ahuja et al. demonstrate the combined cost of testing and treatment is realistically at least six times higher. Though the predominantly used Kato-Katz test is highly accurate, it is both costly and inconvenient. Many cost-benefit analyses on the benefits of mass deworming have been conducted without the benefit of accurate infection diagnoses and epidemiological data, only highlighting the positive effects of mass deworming using secondary measures such as education attendance and active labour days [17].

These are sound recommendations, given the status quo. However, the absence of a cost-effective, epidemiologically effective and easy diagnostic test could change this status quo. This study hopes to lay the foundations for the development of such a test, and believes that there are positive public health, fiscal and philosophical externalities to be gained from it.

Conclusion

Based on our discussion above, it is apparent that developing a low-cost, point-of-care, diagnostic for parasite worm infections is a promising pursuit. Several efforts have been made to develop tools for accurate and rapid ion-sensing, suitable for low-cost PoC and in-field testing applications [17,18]. These results are the first steps for developing a similar product. Mass deworming efforts have come under recent scrutiny with the Cochrane Review's publication disputing evidence for mass deworming [19]. The development of a low-cost PoC diagnostic would fit into the ecosystem of global worm infection prevention, diagnosis and cure, as another component of major global public health interventions.

Limitations

This study looks at helminth infection as a binary data system, though the severity of worm infections often depends on the parasites larval stage in the gut and the number of identifiable eggs. Also, in addition to patients with parasitic infection, patients with atopic allergic diseases such as atopic asthma, atopic dermatitis, and hay fever have also been shown to exhibit increased total IgE levels in blood, limiting our diagnoses. Therefore, future research should both measure zinc concentration in the blood serum and selectively examine stool samples to correlate the presence of helminth infection with these ELISA results in a given population. This would also give the opportunity to better analyse how the range of zinc ion concentration in eye tear fluid might correlate with the severity of infection. Furthermore, while using serodiagnosis is effective, this study could have been improved using an assay specific to human IgG4, which better correlates with STH infection [20].

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