

# Association between serum zinc and copper levels and antioxidant defense in subjects infected with human T-lymphotropic virus type 1

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**Introduction:** Copper (Cu) and zinc (Zn) are important trace elements that are also structural ions of superoxide dismutase (SOD), which reduce oxidative stress. Zinc deficiency and excess copper have been reported to be associated with inflammation. The human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus, which is believed to cause systemic inflammation. The aim of this study is to measure levels of Zn, Cu, SOD, and prooxidant-antioxidant balance (PAB) in HTLV-1-positive patients and investigate the association between serum Zn and Cu concentrations and levels of oxidative stress in them.

**Methods:** The serum samples of 1,116 subjects who had participated in the “Mashhad Stroke and Heart Atherosclerotic Disorder” study, including 279 HTLV-1-positive and 837 HTLV-1-negative patients, were used. Levels of Zn, Cu, SOD, and PAB were measured.

**Results:** Zinc and SOD levels were lower in the HTLV-1-positive group; however, the difference was statistically significant only for the level of SOD ( $P=0.003$ ). On the other hand, levels of copper and PAB were significantly higher in HTLV-1 positive subjects;  $P=0.004$  and  $P=0.002$ , respectively.

**Conclusion:** In HTLV-1-infected patients, serum Zn concentration is lower and Cu concentration is higher than healthy controls. This altered situation might be either primary or secondary to HTLV-1 infection, which should be investigated in larger studies. We showed that SOD is significantly lower in HTLV-1-infected subjects. As in some other viruses that evolve different mechanisms to potentiate virus replication by changing the physiologic condition of host cells, HTLV-1 too probably decreases the activity of copper-zinc SOD1 by suppressing its gene.

**Keywords:** HTLV-1, trace elements, superoxide dismutase, prooxidant-oxidant balance

## Introduction

The human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that belongs to the Retroviridae family and is classified in the Deltaretrovirus genus.<sup>1</sup> This virus was discovered in 1979 in association with T-cell leukemia.<sup>2,3</sup> Some regions in the world are highly endemic for this virus; including southwestern Japan, sub-Saharan Africa, South America, and the Caribbean area.<sup>4</sup> Northeastern Iran has also been introduced as an endemic region in which the prevalence of HTLV-1 infection was reported to be 2–3%.<sup>5</sup>

Adult T-cell leukemia and an inflammatory condition called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) are the two main diseases caused by this virus.<sup>6</sup> The modes of transmission of the virus are mainly through sexual contact with an infected person; mother-to-child, particularly through breastfeeding; and blood transfusion.<sup>1</sup>

HTLV-1 causes systemic inflammation. The pathogenesis is either autoimmune antigen mimicry, in which the immune system attacks host cells because of antigen

cross reaction with HTLV-1 antigens, or innocent bystander in which the cytokines secreted by HTLV-1-specific cytotoxic cells damage the surrounding tissues.<sup>7</sup>

In patients diagnosed with HAM/TSP, signs of active and acute inflammation were shown to be associated with more severe form of HAM/TSP.<sup>8</sup>

Reactive oxygen species (ROS) are metabolites of oxygen with strong oxidizing capabilities. They are produced by cells that are involved in the host-defense response and can cause endothelial dysfunction by oxidation of cellular signaling proteins.<sup>9</sup> While low concentrations of ROS serve as signaling molecules, chronic or prolonged ROS production is associated with the progression of inflammation.<sup>9</sup> In normal conditions, a balance is maintained between the production and elimination of the ROS. The role of oxidative stress (OS), which is the consequence of an imbalance between ROS and antioxidant factors,<sup>10</sup> has been approved in various inflammatory diseases and chronic infections.<sup>11</sup>

Superoxide dismutase (SOD) is an antioxidant factor that removes the superoxide species.<sup>12</sup> Three forms of SOD are known so far, which include the cytosolic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular superoxide dismutase (SOD3).<sup>13</sup> The catalytic reaction of Cu/ZnSOD is performed by the cyclic reduction and oxidation of the copper ion ( $\text{Cu}^{2+}$ ).<sup>12</sup>

The prooxidant–antioxidant balance (PAB), which can be measured in a single assay,<sup>10</sup> has been reported as a potential cardiovascular risk factor.<sup>14</sup>

Copper (Cu) and zinc (Zn) are important trace elements that act as ion cofactors in proteins, hormones, and receptors and also as cofactors in numerous enzymatic reactions.<sup>15</sup> They are structural ions of SOD<sup>16</sup> and reduce OS by induction of metallothionein synthesis.<sup>17,18</sup> Because of their pivotal role in the redox mechanisms, their imbalanced status may lead to an increased susceptibility to oxidative damage.<sup>19–21</sup>

While acute Zn deficiency causes a decrease in innate and adaptive immunity, chronic deficiency increases inflammation.<sup>22</sup> On the other hand, excess Cu is probably associated with an inflammatory response, although it is not clear whether copper has prooxidant or antioxidant effects. This is because ceruloplasmin, as the main copper-containing protein, has been shown to act both as an antioxidant and prooxidant in different conditions.<sup>23</sup>

Tax, which is an important regulatory protein encoded by the HTLV-1 genome, is essential for the replication of the virus. On the other hand, numerous studies indicate that continuous TAX production is associated with apoptosis, and OS is identified as the mediator of TAX-induced apoptosis.

The oxidative damage induced by Tax is mediated by the transcriptional activation of nuclear factor kappa B (NF- $\kappa$ B) by the Tax. NF- $\kappa$ B is itself a prooxidant nuclear transcription factor.<sup>24</sup>

One of the first proofs of the involvement of prooxidants in the functionality of Tax was the use of antioxidants. A radical scavenger called pyrrolidine dithiocarbamate was shown to strongly suppress the Tax-induced activation of the DNA-binding activity of NF- $\kappa$ B in Jurkat cells.<sup>25</sup>

On the other hand, it has been shown that total antioxidant capacity (TAC) is depleted during HTLV-1 infection.<sup>11</sup>

Considering the fact that both HTLV-1 infection and abnormal serum levels of Zn and Cu could lead to systemic inflammation, and taking into account that OS plays an important role in both of their mechanisms, in the current study, we measured levels of Zn and Cu in HTLV-1-positive patients in a control group. We also assessed the association between serum Zn and Cu concentrations and levels of OS in HTLV-1-infected subjects by measuring SOD-1 and PAB.

## Materials and methods

### Study population

In the current study, the serum samples of 1,116 subjects who had participated in the “Mashhad Stroke and Heart Atherosclerotic Disorder (MASHAD)” study were used, and the participants were selected through cluster-randomized allocation methodology.<sup>26</sup> The study’s protocol was approved by Mashhad University of Medical Sciences Ethics committee and all participants provided informed written consents. This study was conducted in accordance with the Declaration of Helsinki.

The inclusion and exclusion criteria of MASHAD study and the public features of the study’s population including marital status, occupation status, education level, drug use, and biochemical and anthropometry measurements were explained previously.<sup>26</sup> For the current study, 279 HTLV-1-positive patients as well as 837 HTLV-1-negative patients who matched the first group by age and sex were selected.

### Sample collection

Twenty milliliters of blood was taken by venipuncture of an antecubital vein in vacuum tubes. Specimens were centrifuged at room temperature within 30–45 minutes of collection to separate the serum, which were then divided into aliquots and kept frozen at  $-20^{\circ}\text{C}$  for future analysis.

### HTLV-I infection assessment

The serum samples of all participants of MASHAD study were screened for HTLV-1-specific antibodies by ELISA

(Dia.Pro Diagnostic, Italy). Positive cases were assessed for the HTLV-1 genome using PCR for TAX- and LTR-specific primers to confirm the infection. Patients were confirmed to be infected by HTLV-1 if either of the genes were present. A total of 279 HTLV-1-infected patients were identified who were enrolled in the current study.

## Measurement of serum zinc and copper concentrations

Serum samples were diluted with nitric acid at the ratio of 1:10. Using the flame atomic absorption (Varian AA240FS), the concentrations of Zn and Cu were evaluated. Also, using Zn and Cu standards (Merc and Co. Pharmaceutical Company), the Zn and Cu standard curves were created. The accuracy of the methods for Zn and Cu were  $93\% \pm 4.8\%$  and  $95\% \pm 3.75\%$ , respectively, which were assessed through measuring the confirmed reference material (MercK KGaA 64271 Darmstadt, Germany) comprising known values ( $1000 \pm 2$  mg/L) for Zn and Cu. The intra-assay and inter-assay coefficient of variation for Zn and Cu were also calculated and equal to  $1.5\% \pm 0.2\%$ ,  $2.6\% \pm 0.4\%$ ,  $1.3\% \pm 0.12\%$ , and  $2.11\% \pm 0.32\%$ , respectively.

## PAB measurement

As mentioned before, the PAB test is the only assay capable of checking the balance of oxidants and antioxidants concurrently in a test. PAB values were evaluated in the serum samples through a modified PAB test that has been previously described.<sup>10</sup> Briefly, for making a standard solution, varying portions (0%–100%) of 250  $\mu$ M hydrogen peroxide ( $H_2O_2$ ) were mixed with 3 mM uric acid (in 10 mM NaOH). Sixty milligrams of TMB powder was dissolved in 10 mL DMSO. For making TMB cation, 400  $\mu$ L of the TMB/DMSO was mixed with 20 mL acetate buffer (0.05 M buffer, pH 4.5). Subsequently, 70  $\mu$ L of fresh chloramine T solution (100 mM) was added to this 20 mL of acetate buffer. Next, we mixed the solution and incubated it at the room temperature for 2 hours. Then, 25 U of peroxidase enzyme solution was mixed with TMB cation solution, distributed in 1 mL and stored at  $-20^\circ\text{C}$ . Also, for making TMB solution, 200  $\mu$ L of TMB/DMSO was mixed with 10 mL of acetate buffer (0.05 M buffer, pH 5.8) and the working solution was made by adding 1 mL TMB cation to 10 mL of TMB solution. Afterwards, the solution was incubated for 2 minutes at room temperature and immediately used. After that, 10  $\mu$ L of each of the samples as well as the standard or blank (distilled water) were mixed with 200  $\mu$ L of working solution in each well of a 96-well plate. The plate was incubated for 12 minutes at  $37^\circ\text{C}$  in a dark environment. After incubation, 100  $\mu$ L of 2 N hydrochloric acid (HCL)

was appended in each well and the OD was evaluated with an ELISA reader at 450 nm, with a reference wavelength of 620 or 570 nm. According to the values comparative to the standard samples, a standard curve was drawn. The values of PAB in the arbitrary HK unit are expressed as the percentage of hydrogen peroxide in the standard solution. Then, based on the amounts from the above standard curve, the values of unknown samples were calculated.<sup>14</sup>

## SOD measurement

Primarily, for making Tris–cacodylic acid buffer (0.05 M, pH 8.2) comprising 0.001 M diethylenetriamine pent acetic acid (DTPA), Tris (0.05 M, containing 0.001 M DTPA) was appended to cacodylic acid (0.05 M, containing 0.001 M DTPA) until pH = 8.2 was obtained. Prior to use, the buffer was air-balanced for 1 hour. For pyrogallol preparation, a source of 0.02 M (100 $\times$ ) pyrogallol solution was constructed in water. To remove soluble oxygen, it was flushed with nitrogen for 1 hour, then aliquoted (100  $\mu$ L per aliquot), and finally stored frozen until used. After that, 20  $\mu$ L of each serum and control was added in duplicate wells. Also, using the equilibrated assay buffer, pyrogallol stock solution (0.02 M) was diluted 1:100, and 180  $\mu$ L per well of the solution was added through a multichannel pipettor. The reactions were read on a plate reader at 405 nm at intervals of 5 minutes for 1 hour. The SOD level, which inhibited pyrogallol oxidation by 50% (relative to control), was defined as an SOD activity unit in the described conditions.<sup>27</sup>

## Statistical analyses

In the current study, SPSS Version 18 (SPSS Inc. Chicago, IL, USA) was used for all statistical analyses. The normality of the data was evaluated through the Kolmogorov–Smirnov test. Descriptive statistics containing mean, frequency, and SD were evaluated for all variables. Normally distributed variables were expressed by mean $\pm$ SD, while parameters that were not normally distributed were expressed by median $\pm$ IQR. Chi-square or Fisher's exact assays were applied for the assessment of categorical parameters. To investigate the correlation between PAB, SOD, and HTLV-1, partial correlation and linear regression were utilized. A two-sided *P*-value of  $<0.05$  was considered statistically significant. Figures were drawn using GraphPad Prism 6.

## Results

### Levels of zinc, copper, SOD, and PAB

Table 1 shows the mean serum concentration of Zn and Cu and also the levels of SOD and PAB in both groups. Zn and

SOD levels were lower in the HTLV-1-positive group; however, the difference was statistically significant only for the level of SOD ( $P=0.003$ ). On the other hand, levels of Cu and PAB were significantly higher in HTLV-1-positive subjects ( $P=0.004$  and  $P=0.002$ , respectively).

The copper-to-zinc ratio was also significantly higher in the HTLV-1-positive group ( $P<0.001$ ).

**Table 1** Mean levels of zinc, copper, SOD, and PAB in HTLV-1-positive and -negative patients

Variable	HTLV-1 negative	HTLV-1 positive	P-value
Zinc, $\mu\text{g/dL}$ (normal range: 65–110 $\mu\text{g/dL}$ )	84.18 $\pm$ 18.26	83.16 $\pm$ 17.56	0.41
Copper, $\mu\text{g/dL}$ (normal range: 80–150 $\mu\text{g/dL}$ )	103.75 $\pm$ 38.56	111.27 $\pm$ 35.77	0.004
Copper/zinc ratio	1.28 $\pm$ 0.82	1.5 $\pm$ 0.64	<0.001
PAB, HK	68.15 $\pm$ 50.67	80.32 $\pm$ 46.59	0.002
SOD, IU	19.94 $\pm$ 14.52	16.79 $\pm$ 12.59	0.003

**Abbreviations:** HTLV-1, human T-lymphotropic virus type I; PAB, prooxidant-antioxidant balance; SOD, superoxide dismutase.

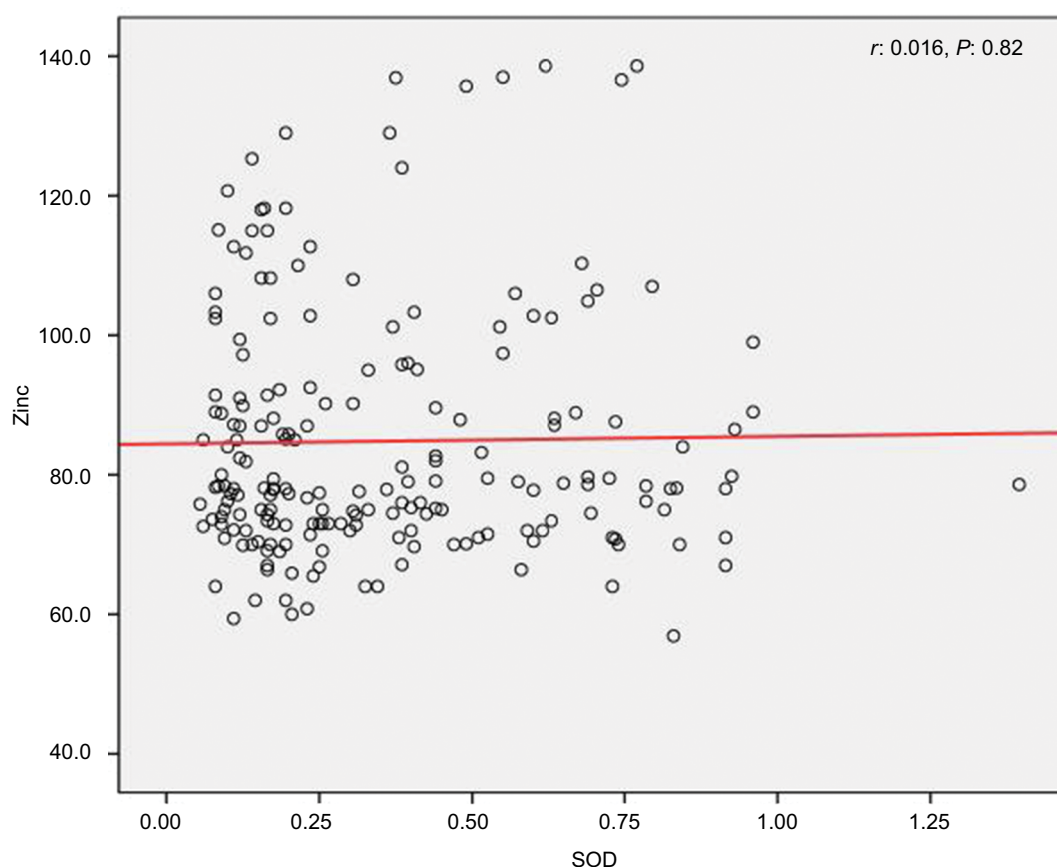
## Correlation between zinc and SOD, SOD and PAB, and copper and SOD

We found a positive relationship between levels of Zn and SOD in HTLV-1-positive patients; however, the relationship was not statistically significant ( $r=0.016$ ,  $P=0.82$ ) (Figure 1). Also, it was shown that there is a significant positive correlation between Cu and SOD ( $r=0.097$ ,  $P<0.001$ ) (Figure 2), as well as a significant reverse correlation between the level of SOD and PAB ( $r=-0.033$ ,  $P=0.016$ ) (Figure 3).

## Discussion

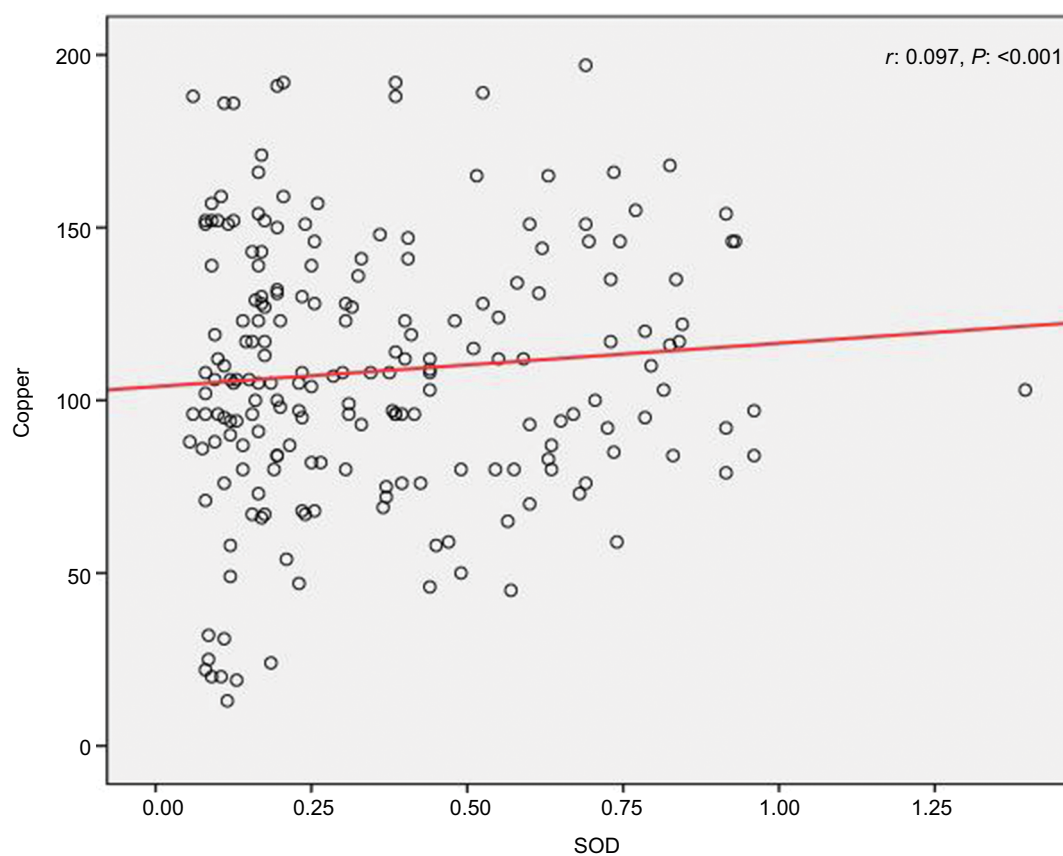
The results of this study show that in HTLV-infected patients, serum Zn concentration is lower and Cu concentration is higher than healthy controls. To the best of our knowledge, no other study has yet assessed the levels of these trace elements in HTLV-1-infected patients.

Although serum levels of both Zn and Cu are in normal ranges for the Iranian population in both cases and control groups,<sup>28–30</sup> further studies are required to reveal whether the different serum Zn and Cu levels in HTLV-1 patients has



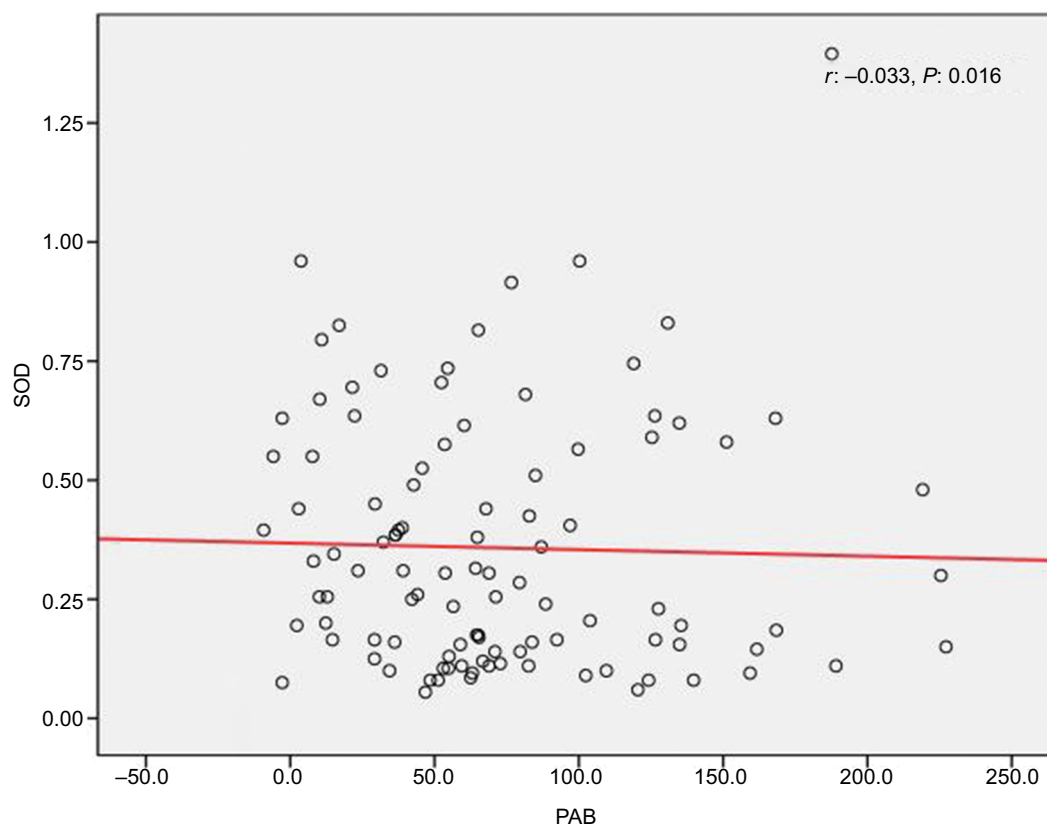
**Figure 1** Correlation between levels of zinc and SOD in human T-lymphotropic virus type I-positive patients.  
**Abbreviation:** SOD, superoxide dismutase.





**Figure 2** Correlation between copper and SOD.

**Abbreviation:** SOD, superoxide dismutase.



**Figure 3** Correlation between SOD and PAB.

**Abbreviations:** PAB, prooxidant-antioxidant balance; SOD, superoxide dismutase.

led to the increased susceptibility to infection in them or the infection itself has led to different Zn and Cu levels.

Both Zn and Cu are claimed to protect organisms against infectious diseases and to regulate innate immune response.<sup>31</sup> However, the etiology of altered plasma Zn and Cu concentrations associated with proinflammatory conditions is yet to be determined.<sup>32</sup>

The increased serum copper-to-zinc ratio is shown to be associated with an altered homeostatic status after a destabilizing event and is also associated with an inflammatory response.<sup>32</sup> Also, the combination of low serum Zn and high serum Cu is contributed to an increased risk of cardiovascular diseases.<sup>33</sup> Our results show a significant increase in the copper-to-zinc ratio in the HTLV-1-infected subjects in comparison to controls. This finding could be an explanation for the systemic inflammation seen in HTLV-1 infection, both in asymptomatic carriers and symptomatic patients.

We showed that SOD1 is significantly lower in HTLV-1-infected subjects. It is well known that viruses evolve different mechanisms to potentiate virus replication, by changing the physiologic condition of host cells. For example, it has been shown that influenza virus A increases superoxide anion level in human alveolar cells, mainly by suppressing the copper–zinc SOD1 gene.<sup>34</sup> In another example, the expression of the regulatory Tat protein from the human immunodeficiency virus type 1 (HIV-1) was shown to suppress the expression of cellular Mn-containing superoxide dismutase (Mn-SOD).<sup>35</sup> This might be the case for what is happening in HTLV-1 infection as well.

Also, the important regulatory protein Tax from HTLV-1 is shown to resemble other viral and cellular oncogenes like adenovirus E1A, simian virus 40 large tumor antigen, and the human papilloma virus E7 proteins, which aside from their transforming properties have the ability to induce apoptosis.<sup>24</sup> On the other hand, it has been demonstrated that the induction of OS was a prompt effect of TAX function and this OS is a mediator of TAX-induced apoptosis.<sup>24</sup> This effect is commonly observed in other viral proteins that cause apoptosis, such as HIV-1 Tat protein.<sup>35</sup> As HTLV-1 and HIV-1 share similar genomic organization and tropism for immune cells, in particular CD4<sup>+</sup> and CD8<sup>+</sup> T cells, their role in OS could be similar.

In a study that evaluated the serum level of trace elements Zn, Cu, Mg, and Se, and assessed the level of OS in children with type 1 diabetes, levels of all four elements and also the OS parameters were significantly lower in diabetic patients than in the controls.<sup>36</sup>

We found a positive correlation between SOD and Cu and a negative correlation between SOD and PAB. We, therefore,

assume that there might be a negative correlation between Cu and PAB. This assumption is in favor of what has been reported by Alamdari et al, which is a negative correlation between PAB and ceruloplasmin.<sup>14</sup> They have discussed that ceruloplasmin behaves as an antioxidant.

Regarding the association between HTLV-1 infection and antioxidant defense, in 2014, Shomali et al have investigated the TAC in the serum of HTLV-1-infected patients. They have reported that TAC is depleted during HTLV-1 infection. This reduction was seen in both asymptomatic carriers of HTLV-1 and those who had symptoms of HAM/TSP.<sup>11</sup> This finding is relevant with the lower SOD levels in HTLV-1-infected subjects, which is shown in the present study.

Revealing the exact mechanisms by which ROS are involved in the regulation of T-cell functions is important to achieve a good insight of the immune response, and to develop new treatments for the control of immune-mediated diseases.<sup>37</sup>

## Limitations

In this study, assessment of the relation between SOD and zinc and copper levels was not separately done in asymptomatic HTLV-1 carriers and those with symptoms. As symptomatic patients usually have higher viral loads, it is necessary to distinguish the two groups when studying the effects of the virus on different biologic mechanisms.

## Conclusion

The decreased serum Zn and increased serum Cu in HTLV-1-infected subjects along with an increased Cu/Zn ratio confirms the fact that infection with this virus is associated with general inflammation. On the other hand, because the antioxidant capacity is reduced in these patients, supplying them with antioxidants could help postpone the incidence of symptoms.

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## Disclosure

The authors report no conflicts of interest in this work.

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