TRACE ELEMENT ANALYSIS IN HEPATITIS B AFFECTED HUMAN BLOOD SERUM BY INDUCTIVELY COUPLED PLASMA-ATOMIC EMISSION SPECTROSCOPY (ICP-AES)

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Abstract. The objective of this study was to measure the alterations in serum elements, viz., copper (Cu), iron (Fe), selenium (Se) and zinc (Zn) in patients (n = 39) with chronic hepatitis B and to compare them with the results of healthy volunteers (n = 24) by using ICP-AES. Significant low serum levels of Se, Zn and high level of serum Cu were observed in patients with hepatitis B virus, as compared with normal healthy controls. Serum Fe concentrations did not show alterations in hepatitis B patients compared with healthy individuals. The study showed that Cu, Fe, Se and Zn concentrations in serum of hepatitis B patients were 1.499 ± 0.297 ppm, 1.211 ± 0.206 ppm, 0.042 ± 0.014 ppm and 0.550 ± 0.094 ppm respectively. In healthy individuals these concentrations were 1.003 ± 0.159 ppm, 1.272 ± 0.340 ppm, 0.059 ± 0.008 ppm, 0.883 ± 0.070 ppm respectively. The results were statistically compared ($p < 0.05$) with those of healthy individuals. The degrees of involvement of trace elements due to hepatitis B virus in human blood serum were discussed. This study supports the association of trace elements such as Se, Cu and Zn with hepatitis B.

Key words: hepatitis B, serum, copper, iron, selenium, zinc, ICP-AES.

INTRODUCTION

Viral hepatitis is a major human health problem world wide [8, 22]. More than 300 million people throughout the world are affected by hepatitis B virus (HBV) [12, 13]. Around 4% of people have been affected of HBV in India [25]. Serological test is an important tool for the identification of the HBV [4]. Trace elements play an important role in disease caused by virus. The change in trace elements might also be associated with various diseases [20]. The relationship between chronic hepatitis and trace elements has not been understood clearly [16]. Various trace elements are responsible for many biochemical, immunological, and physiological activities. Essential micronutrients are involved in many metabolic pathways in the liver, such as enzymatic functions, protein synthesis, oxidative

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damage and anti-oxidant defense, immunological competence, interferon therapy response regulations and alterations of the virus genomes [27]. Trace element measures in serum/plasma are easy and used commonly. Serum metal level has been reported to be highly sensitive in the diagnosis of liver diseases [10]. The concentration of each trace element varies with different types of liver diseases because these elements may have a direct hepatic toxicity or may be decreased as a consequence of the impaired liver function.

Trace elements such as Zn, Cu, Fe and Se are required for the immune system to function efficiently. The concentration of each trace element varies with different types of liver diseases. The present study has been undertaken to determine the quantitative estimation of trace elements (Cu, Fe, Se and Zn) in hepatitis B affected human serum and compare them with normal human blood serum.

MATERIAL AND METHODS

The randomly selected study group comprised 39 patients with HBV that included 24 males and 15 females (aged 35 ± 11.3 years), ranging between 11 and 63 years. The control group comprised 24 healthy volunteers that included 15 males and 9 females aged between 5 and 61 (mean of 31 ± 8.7) years. Samples were collected from Rajah Muthiah Medical College and Hospital (RMMC&H), Annamalai University. All sera were collected in the morning after fasting 8 hours. Features of the subjects in the study are shown in Table 1.

The healthy volunteers were selected on the basis of no alcoholic, no smoking habits, no history of viral hepatitis and absence of any acute or chronic pathology, clinically evident at the moment of examination, routine clinical check up during the entire period of research, residing in the same geographical region. All people were in the same socio-economic status and similar diet habits. Sera were collected from patients before drug administration. Patients with chronic hepatitis B were diagnosed based on clinical, biochemical, histological and virological evidence that included HBsAg, HBsAb, HBcAb and HBV DNA by PCR technique. Marker enzymes were measured by spectrophotometric method; other parameters were determined by respective commercially available ELISA kit in gastroenterology department, RMMC&H, Annamalai University. HBsAg positive for more than 6 months was considered as chronic hepatitis. Normal range of serum transaminase level was accepted as 40 IU/L. Samples were restricted from other diseases.

Blood samples were taken from all subjects in accordance with standard procedure; six to eight mL of blood was collected from the vein and protected in evacuated tubes without adding any anticoagulant agent. Patients were not administered antiviral treatment previously to this study. Collected blood samples were placed in sterile place and allowed to clot. The blood samples were centrifuged at 300 g for 45 min and the serum was pipetted out and filtered through 0.45 μm membrane filter. The collected sera were stored in plastic vials at –20 ^{o}C until further analysis. This study was approved by the Institutional Ethical Committee.

STATISTICAL ANALYSIS

Statistical analysis has been performed using Statistical package for the social sciences (SPSS, version 11.5) for windows. Continuous variables were expressed as mean ± standard deviation (SD). Data were analyzed using independent sample Student's t test. Significance was assigned for p values (<0.05) with 95% Confident Interval.

TRACE ELEMENT ANALYSIS

Reagent and solutions

Natural element standard solutions (1000 μg/mL) of Cu, Fe, Se and Zn were obtained from Merck, USA. High pure nitric acid was purchased from Fisher Scientific Inc, India. The standard solutions were kept at 4 ^{o}C in dark room. Other chemicals were purchased from Fluka Riedel-de Haen, Scientific Research, India. Standard solutions were prepared freshly from the stocks, with diluted nitric acid (3% v/v). In order to obtain appropriate ICP-AES responses, the experiments were performed using different concentration levels.

Sample digestion

1 mL of serum was transferred to a Teflon beaker and 10 mL of concentrated nitric acid and 2.5 mL concentrate perchloric acid were added. The sample was then brought very slowly to boiling on a hot plate and heated to dryness. If sample blackening occurred during the fuming stage, nitric acid was added dropwise, then the sample was cooled, dissolved again in distilled water and concentrated HCl (10:1) and brought to a volume of 25 mL in a volumetric flask. The solution was analyzed against calibration curves [17].

ICP-AES

An inductively coupled plasma spectrometer has been extensively used in the analysis of major, minor and trace elements in biological material because of its high sensitivity, accuracy, low matrix effect and simpler operation. The ultra-trace elements in such biological fluids could be determined by ICP-AES [9]. A comparative study on the analytical performance of flame atomic absorption, flame atomic emission and inductively coupled plasma (ICP) emission for biological strontium assays, especially in the blood serum, was carried out [17]. The present work was performed using ISA JOBIN YNON – 24.

Operating conditions were:

R.F Generator	: 40.68 MHz, 1000 Watts;
Power required	: 220 ± 10 V, 50/60 Hz, single phase 5 kV;
Flame Temp	: 11000 K;

Plasma : Argon;
Spectra range : 189–800 nm;
Sensitivity : ppb level of detection (±0.002 ppm).

The presence of various elements in the sample was identified by determining the wavelength of the emitted radiation (Cu: 324.754 nm, Se: 196.090 nm, Fe: 238.204 nm, Zn: 213.856 nm) and the concentration was calculated by intensity of the radiation, which might be sufficiently low for certain applications with a simple matrix. Sample and standard were analyzed in triplicate.

RESULTS

All blood serum /plasma samples (39 patients and 24 controls) were analyzed using ICP-AES. The serum Cu concentration was 1.499 ± 0.297 ppm in patients with viral hepatitis B, which was higher than that of controls (1.003 ± 0.159 ppm). Other serum elements (Se: 0.042 ± 0.014 ppm, Zn: 0.550 ± 0.094 ppm) concentrations were found statistically lower compared with the normal (Se: 0.059 ± 0.008 ppm, Zn: 0.883 ± 0.070 ppm). Serum Fe (1.211 ± 0.206) level was not statistically different in normal healthy volunteers (Fe: 1.272 ± 0.340 ppm). Results of this study have been summarized in Figure 1.

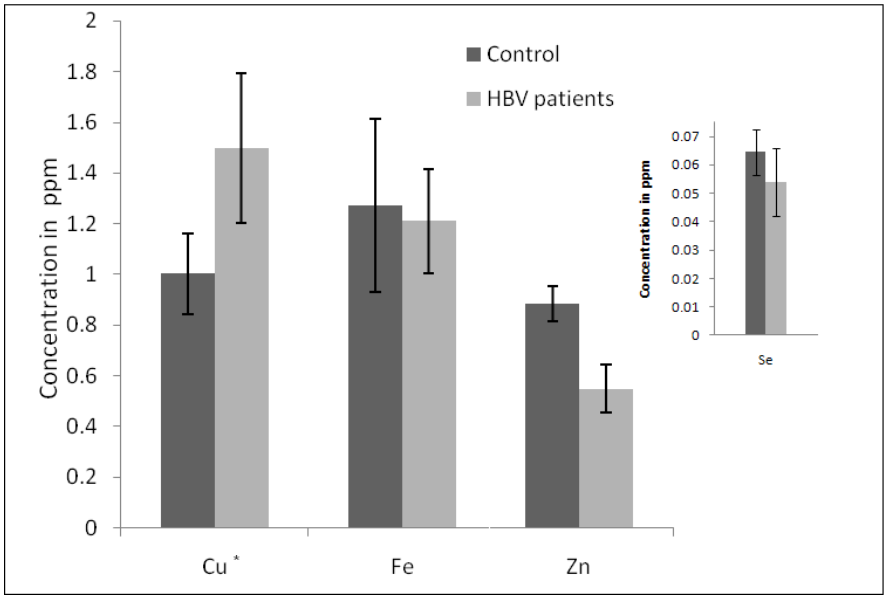

Fig. 1. Serum Cu, Fe, Se, Zn concentrations (ppm) in the control (n = 24) and the HBV patients (n = 39) shown as mean ±SD (*$p < 0.05$).

Table 1

Age and gender distribution of control and HBV patients group

Group	Age (years)			Gender
	n	Mean ± SD	Range of years	M/F
Control	24	31 ± 8.7	5–61	15/9
Patients (HBV)	39	35 ± 11.3	11–63	24/15

SD – Standard Deviation

DISCUSSION AND CONCLUSION

Trace elements are used as a diagnosing tool during disease; it is important to know whether the balance is changed in free or bound elements. Chronic hepatitis is insidious, in that the primary clinical signs are not readily evident until a well-established pathogenic process has developed. Differences in trace elements have been associated with various diseases.

The above results show that serum Cu concentrations of HBV patients are higher than normal individual serum concentrations. These elevated serum Cu levels indicate an alteration of Cu metabolism during the acute phase of uncomplicated hepatitis [7]. It may be resulted from defense strategies of organism and induced by hormone like substances [3]. As the disease progresses from chronic hepatitis to liver cirrhosis, serum calcium, magnesium, phosphorus and zinc concentrations decrease while copper concentration increases [18]. It may be explained by the release of copper from damaged necrotic hepatocytes [26].

Decrease in serum Se might indicate the development and progression of HBV. It also links to the disease progress of some viral agents in relation to the biosynthesis of selenoproteins [1] and decrease in serum Se significantly increases the risk of cancer mortality [21]. Declining serum Se might involve reduced biosynthesis of the hepatically derived Se transport protein selenoprotein P (SePP) for its human promoter is negatively regulated by proinflammatory cytokines in vitro [5]. Four-year animal studies showed that dietary supplement of Se reduced the HBV infection by 77.2% [29].

Zn is an essential element that is found in all cells. It stimulates the activity of approximately 100 enzymes [23] and binds to several viruses. The reduced zinc concentration indicates the severity of liver damage [22]. Kalkan *et al.* [10] have reported Zn concentration associated with viral hepatitis decrease with the development of hepatitis patients; it reveals that Cu concentration increases statistically while Zn, Se level decreases. Decrease in serum Zn is due to poor

appetite, during the infection with the help of leukocyte endogen mediator (LEM) and uptake of more Zn to synthesize nucleic acid, protein and enzymes by liver cells. With progression of the liver damage, due to poor appetite, impaired function of intestines and stomach and high pressure of the portal vein, the zinc intake and absorption decreases and also the low content of serum albumin results in less combination with zinc and because of the diffusion characteristic of blood zinc, it is easily lost through urine and sweat [3, 28]. Fota Markowska *et al.* [15] studied the serum Zn level dynamics in patients with acute hepatitis B and the early recovery periods. They observed significantly decreased serum Zn levels during hospitalization and the supplementation of Zn to HBV patients resulted in early recovery. Loguercio *et al*. reported that cirrhotic patients had a significant decrease of serum Zn and Fe level [14]. In the present study the slight variation of iron concentration may be due to the accumulation of iron in liver parenchyma [24].

Assessment of the disorders of trace element metabolism should be based not only on the dietary intake, but also on various factors such as absorption, transportation, storage, excretion, and metalloproteinase and metallo-enzymes synthesis which are impaired when liver cell is damaged [2, 19].

In conclusion, the trace elements such as Cu, Zn and Se are found to be statistically significant ($p < 0.05$); this study confirms the variation of their concentration in HBV affected patients compared with healthy volunteers. Change in trace elements associated with some biological reaction takes place in the liver. Further research, both basic and applied, is needed to assess properly the possible role of malnutrition in contributing to the emergence of novel viral diseases.

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REFERENCES

1. BANARES, F.F., E. CABRE, M. ESTEVE, Serum Selenium and risk of large size colorectal adenomas in a geographical area with a low selenium status, *Am. J. Gastroenterol.*, 2002, **97**, 2103–8.
2. BIANCHI, G.P., G. MARCHESINI, M. BRIZI, B. ROSSI, G. FORLANI, G. LEONARDI, Nutritional effects of oral zinc supplementation in cirrhosis, *Nutr. Res.*, 2000, **20**, 1079–89.
3. CESUR, S., S.A. CEBECI, G.O. KAVAS, S. AKSARAY, D. TEZEREN, Serum copper and zinc concentrations in patients with chronic hepatitis B. *Journal of Infection*, 2005, **51**, 38–40.
4. CHRISTOPHER, R., W. EDWARDS, A.D. BOUCHIER, C. HASLETT, E. CHIVERS, *Davision's Priciple and Practice of Medicine*, 2nd Edition, ELPS, 1995.

5. DREHER, I., T.C. JAKOBS, J. KOHRLE, Cloning and characterization of the human selenoprotein P promoter. Response of selenoprotein P expression to cytokines in liver cells, *J Biol Chem*., 1997, **272(**46**)**, 29364–71.
6. GUR, G., Y. BAYRAKTAR, D. OZER, M. OZDOGAN, B. KAYHAN, Determination of hepatic zinc content in chronic liver disease due to hepatits B virus, *Hepatogastroenterology*, 1998, **45,** 472–476.
7. HATANO, R, Accumulation of copper in liver and hepatic injury in chronic hepatitis C, *J. Gastroenterol. Hepatol.*, 2000, **15**, 786–779.
8. JAFRI, W., N. JAFRI, J. YAKOOB, M. ISLAM, S. FARHAN, A. TIRMIZI, T. JAFAR, S. AKTHAR, S. HAMID, H.A. SHAH, S.Q. NIZAMI, Hepatitis B and C: prevalence and risk factors associated with seropositivity among children in Karachi, Pakistan, *BMC Infectious Diseases*, 2006, **6,** 101–105.
9. KAHN, A.M., H.L. HELWEG, A.G. REDEKER, T.B. REYNOLDS, Urine and serum zinc abnormalities in disease of liver, *Amer. J. Clin. Pathol.*, 1965, **44**, 426–429.
10. KALKAN, A., V. BULUT, S. AVCI, I. CELIK, N. KEMAL BINGOL, Trace elements in viral hepatitis, *J. Trace Elem. Med. Biol.*, 2002, **16**, 227–230.
11. KANG, Y.J., Z. ZHOU, Zinc prevention and treatment of alcoholic liver disease, *Mol. Aspect Med.*, 2005, **26**, 391–404.
12. KAO, J.H., N.H. WU, P.J. CHEN, M.Y. LAI, D.S. CHEN, Hepatitis B genotypes and the response to interferon therapy, *J. Hepatol.*, 2000, **33(**6**)**, 998–1002.
13. LEE, W.M., Hepatitis B virus infection. *N. Engl. J. Med*., 1997, **337**, 1733–45.
14. LOGUERCIO, C., V. DE GIROLAMO, A. FEDERICO, S.L. FENG, E. CRAFA, V. CATALDI, Relationship of blood trace elements to liver damage, nutritional status, and oxidative stress in chronic nonalcoholic liver disease, *Biol. Trace Elem. Res.*, 2001, **81**, 245–254.
15. MARKOWSKA, H.F., A. PRZYBYLA, I. BOROWICZ, R. MODRZEWSKA, Serum zinc (Zn) level dynamics in blood serum of patients with acute viral hepatitis B and early recovery period, *Ann. Univ. Marie Curie-Sklodowska Med.*, 2002, **57**, 201–209.
16. MERAN, L., F. SIRMATEL, S. AHI, M. TARAKCIOGLU, Plasma copper and zinc levels in chronic viral hepatitis, *Saudi Med. J.*, 2004, **25(**8**)**, 1066–9.
17. NAKAYAMA, A., H. FUKUDA, M. EBARA, H. HAMASAKI, K. NAKAJIMA, H. SKURAI, A new diagnostic method for chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma based on serum metallothionein copper and zinc levels, *Biol. Phar. Bull*., 2002, **25(**4**)**, 426–431.
18. PRAMOOLSINSAP, C., N. PROMVANIT, S. KURATHONGS, Serum trace metal levels in patients with acute hepatitis B, *Southeast Asian J. Trop. Med. Public Health*, 1996, **27**, 476–480.
19. PRAMOOLSINSAP, C., N. PROMVANIT, S. KOMINDR, P. LERDVERASIRIKUL, S. SRIANUJATA, Serum trace metals in chronic viral hepatitis and hepatocellular carcinoma in Thailand, *J. Gastroenterol*., 1994, **29**, 610–615.
20. RAGHUNATH, R., R.M. TRIPATHI, K. VINOD, A.P. SATHE, R.N. KHANDEKAR, K.S.V. NAMBI, Assessment of Pb, Cd, Cu and Zn exposures of 6–19 year old children in Mumbai, *Environ. Res.*, 1991, **80**, 215–222.
21. SANDSTEAD, H.H., Understanding zinc: recent observations and interpretations, *J. Lab. Clin. Med.*, 1994, **124**, 322–327.
22. SANKARYA, S., S. ONCU, B. OZTURK, Effect of prevalence applications on prevalence of hepatitis B virus and hepatitis C virus infections in west Turkey, *Saudi Med. J*., 2004, **25(**8**),** 1070–82.
23. SHANKAR, A.H., A.S. PRASAD, Zinc and immune function: the biological basis of altered resistance to infection, *Am. J. Clin. Nutr*., 1998, **68**, 447–463S.

24. SOMI, M.H., A. OSTAD RAHIMI, B. MOSHREFI, P. REZAEIFAR, G.J. MAGHAMI, Nutritional status and blood trace elements in cirrhotic patients, *Hepatitis Monthly*, 2007, **7(**1**)**, 27–32.
25. TANDON, B.N., S.K. ACHARYA, A tandem epidemiology of hepatitis B virus infection in India, *Gut*, 1996, **38** (suppl. 2), S56–S59.
26. TAYLOR, E.W., R.G. NADIMPALLI, C.S. RAMANATHAN, Genomic structures of viral agents in relation to biosynthesis of selenoproteins, *Biol. Trace Element Res.*, 1997, **56**, 63–91.
27. VANHOE, H., C. VANDECASTEELE, J. VERIECK, R. DAMS, Determination of trace elements in a human serum reference material by inductively coupled plasma mass spectrometry, *J. Microchimica Acta*, 1989, **99**, 373–379.
28. YASUYUKI, A., M. MISUHIKO, A. YASUO, Liver cirrhosis and metabolism (sugar, protein, fat and trace elements), *Hepatol*. *Res*., 2004, **30**, 46–58.
29. YU, S.Y., Y.J. ZHU, W.G. LI, Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong, *Biol. Trace Elem. Res.*, 1997, **56(**1**)**, 117–124.