



# Changes in Liver Tissue Trace Element Concentrations During Hepatitis B Viral Infection Treatment

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## Abstract

Approximately 350–400 million people in the world have Hbs Ag (hepatitis B virus surface antigen) positivity. In the international guidelines, the permanent suppression of replication in chronic hepatitis B virus (HBV) infection therapy is reported as the primary therapeutic goal. Trace elements play a key role in liver diseases. The aim of our study is to determine some trace element concentrations in the liver during HBV treatment periods. The measurement of 11 trace elements (manganese, lead, nickel, chromium, cadmium, iron, copper, zinc, silver, cobalt, and aluminum) was carried out by the method of inductively coupled plasma mass spectrometry in liver biopsy materials (before starting treatment and at the sixth month of the treatment period). There was an increase in zinc and copper concentrations in liver materials at the sixth month of treatment compared to the pre-treatment values (the median zinc value was 48.05 µg/g before treatment and 74.9 µg/g at 6 months after initial treatment,  $p = 0.035$ ; median copper was 2.82 µg/g before treatment and 5.31 µg/g after 6 months,  $p = 0.002$ ). General estimations indicated that zinc ( $p = 0.002$ ), iron ( $p = 0.0244$ ), copper ( $p = 0.0003$ ), and aluminum ( $p = 0.0239$ ) values may be effective in HAI (histological activity index) changes. Only iron levels could be at a very low level effective on the changes caused by fibrosis ( $p = 0.0002$ ). Liver tissue zinc and copper levels increased in parallel with the improvement of inflammation in antiviral-treated HBV patients. In addition, the levels of zinc and copper in the liver tissue can be useful markers for liver tissue damage detection.

**Keywords** Hepatitis B · Trace elements · Liver

It is estimated that approximately 350–400 million people in the world have hepatitis B virus (HBV) infection showing HBV surface antigen (Hbs Ag) positivity [1]. It is accepted that chronic HBV develops in individuals who have been

positive for Hbs Ag for 6 months [2]. Pegylated interferon alpha-2a is a preferred regimen of interferon treatment in terms of efficacy and suitability. Among the nucleoside analogues (NA), entecavir and tenofovir, which are used for HBV

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therapy, are more preferred than other oral nucleoside analogues due to high genetic barriers and potent antiviral properties [1, 3, 4]. It is accepted that viral replication is the main driving force in liver damage. In the international guidelines, the permanent suppression of replication in chronic HBV therapy is reported as the primary therapeutic goal [5–7]. Liver fibrosis is a repair response aimed at restricting the tissue damage that occurs with some mediators [8]. Cirrhosis occurs in response to chronic liver injury and constitutes the final stage of progressive hepatic fibrosis, which is characterized by impaired hepatic architecture and the formation of regenerative nodules [9].

There are a few publications reporting that serum trace element concentrations are highly sensitive for the assessment of several hepatic disorders [10–12]. Trace elements play a key role in liver diseases, especially in degenerative disorders of the liver. Although plasma level of trace elements changes during most of the infections, it is unclear whether this alteration is also present in the infected tissues, such as liver.

There is no literature that has studied the change of trace element levels in the liver tissue with antiviral therapy in chronic HBV patients. The aim of our study is to determine trace element concentrations in liver tissue before and after 6 months of treatment, as well as to determine the difference in concentration between these two states for its diagnostic value. We also aimed to investigate the association of liver trace element levels with changes in histological activity and fibrosis indexes according to the treatment.

## Materials and Methods

In our study, hepatic tissue trace element levels were measured in microgram per gram before antiviral treatment for HBV and 6 months after treatment was initiated. The liver tissue damage of the patients was assessed by a biopsy, according to Ishak's scoring system [9], before treatment and 6 months after treatment (histological activity index (HAI): histological activity score was scored between 0–18, and fibrosis was scored between 0–6). Patients with HBV DNA above 2000 IU/mL and Hbs Ag positivity for more than 6 months were taken into the study (inclusion criteria). Exclusion criteria were patients with impaired renal function, chronic cardiac disease, and chronic liver diseases diagnosed by other causes than HBV. Written informed consent form was obtained from all patients. Local ethics committee approval was obtained for the patient collection [The study was approved by the Ethics Committee of the University of Mersin, Turkey (decision no: 2014/81, date: 25/04/2014)]. Routine laboratory analysis (platelets, hemoglobin, and liver biochemical tests) values were taken before treatment. In our work, the measurement of 11 trace elements [manganese (Mn), lead (Pb), nickel (Ni), chromium (Cr), cadmium (Cd), iron (Fe), copper (Cu), zinc (Zn), silver (Ag),

cobalt (Co), and aluminum (Al)] was carried out by the method of inductively coupled plasma mass spectrometry (ICP-MS) [12].

Liver samples were obtained with needle (Hepafix Braun 88 mm) biopsies. Liver dry weight tissue heavy metal concentration analyses were performed by an experienced biologist. To avoid external contamination, samples were stored in plastic tubes. The tissue samples used in the metal analysis were held at 105 °C for 72 h and weighed constantly. A mixture of 2 mL of nitric acid (HNO<sub>3</sub>, 65%, p.a.: 1.40, Merck) and 1 mL of perchloric acid (HClO<sub>4</sub>, 60%, p.a.: 1.53, Merck) was added to the tissue samples, which had been transferred to test tubes and were burned at 120 °C for 8 h. After the burning procedure was completed, the tissue samples were transferred into polyethylene tubes. The total volume was prepared for analysis by adding deionized water to reach 10 mL total volume [13]. IAEA-407 was used as reference material in the study. Details of trace element reference material and LOD-LOQ values are given in Table 1.

## Statistical Analysis

All analyses were performed using SPSS version 16.0 for Windows (IBM Inc., Chicago, IL, USA). In the homogeneity evaluation, the parameters with skewness-kurtosis values ranging between +1.5 and -1.5 were considered homogeneous in our study. Differential analysis of pre-treatment and post-treatment trace element values was performed by paired sample *T* test on parametric (homogeneous) data and Wilcoxon test on non-parametric (non-homogeneous) data.

**Table 1** Validation parameters of the analytical method

Trace elements μg/g	LOD (ng g <sup>-1</sup> )	LOQ (ng g <sup>-1</sup> )	R <sup>2</sup>	Certificated values concentration (mg kg <sup>-1</sup> )	Certificated values 95% confidence interval (mg kg <sup>-1</sup> )
Aluminum	1.74	4.18	0.9998	13.8	12.4–15.2
Chromium	1.13	3.78	0.9999	0.73	0.67–0.79
Mangan	0.21	0.75	0.9999	3.52	3.44–3.60
Iron	1.49	5.01	0.9998	146	143–149
Cobalt	0.24	0.69	0.9999	0.10	0.09–0.11
Nikel	0.64	2.23	1	0.60	0.55–0.65
Copper	0.59	2.04	0.9999	3.28	3.20–3.36
Zinc	2.51	8.29	0.9999	67.1	66.3–67.9
Silver	0.13	0.43	0.9999	0.037	0.033–0.041
Cadmium	0.47	1.51	0.9999	0.189	0.185–0.193
Lead	0.36	1.18	0.9999	0.12	0.10–0.14

IAEA-407 was used as reference material

IAEA International Atomic Energy Agency, LOD limit of detection, LOQ limit of quantification

Spearman correlation analysis was performed between HBV DNA and trace element concentrations. We formed generalized estimating equation (GEE) models to evaluate the significance of element concentrations on pre- and post-HAI and fibrosis values (Program SAS University Edition 9.4). We considered HAI as continuous and fibrosis as ordinal and formed GEE models per each trace element which include trace element and time as predictors and HAI or fibrosis as dependent variable. Since HAI has a broader range, we treated it as continuous while fibrosis has less levels. A *p* value of < 0.05 was considered statistically significant.

## Results

The study group consisted of 16 (80%) male and 4 (20%) female patients. The mean age of 20 patients in our study was  $40.8 \pm 12.1$  years, with the range between 22–62 years of age. Nine of the HBV patients (45%) received lamivudine therapy. The treatments that patients received are shown in Table 2. The biochemical and some hematological parameters before treatment of the study participants are shown in Table 2.

In our study, the median liver HAI score was 7 before treatment, whereas the HAI was 4 after 6 months of treatment. In our study, the HAI value showed statistically significant decrease over the treatment period. Although fibrosis was observed to be lower during the same period, this difference was not statistically significant. The liver fibrosis and HAI values before treatment and at the sixth month of treatment are shown in Table 3.

There was a statistically significant increase in liver zinc concentrations observed at 6 months of treatment compared to pre-treatment values. The median Zn value was  $48.05 \mu\text{g/g}$  before treatment and  $74.9 \mu\text{g/g}$  at the sixth month of treatment (*p* = 0.035). The liver copper concentration increased after 6 months of treatment compared to pre-treatment values (median Cu was  $2.82 \mu\text{g/g}$  before treatment,  $5.31 \mu\text{g/g}$  after

**Table 3** Histopathological changes, according to Ishak's scores, between pre-treatment and the sixth month of HBV treatment

	Before treatment	At the sixth month of the treatment	<i>p</i>
HAI score	7 (3–11)	4 (2–10)	0.013
Fibrosis	2 (0–4)	1 (0–3)	0.16

HAI histological activity index

Median (min-max) were given at irregular intervals

6 months, *p* = 0.002). There were no statistically significant differences in the liver tissue concentration values of Mn, Fe, Ni, Ag, Al, Co, Cd, Pb, or Cr between pre-treatment and at the sixth month. The hepatic trace element levels observed in the treatment of HBV patients are shown in Table 4.

The mean HBV DNA values of the patients who were treated for HBV were  $66.27 \times 10^6$  ( $2110–1.00 \times 10^9$ ) IU/mL before treatment, and all HBV DNA values fell below 20 IU/mL during the sixth month of treatment. The pre-treatment HBV DNA levels correlated positively with liver tissue Cr (*r* = 0.456; *p* = 0.043), Ag (*r* = 0.45; *p* = 0.046), Pb (*r* = 0.55; *p* = 0.013), and Al (*r* = 0.63; *p* = 0.003) levels. There was no correlation between pre-treatment HBV DNA levels and liver tissue trace element values after 6 months of treatment. The correlations of liver trace elements and HBV DNA values (before and at the sixth month of treatment) are shown in Table 5.

In our study, the correlations of biochemical and hematological parameters with the levels of trace elements before and after treatment were examined. There was a correlation between post-treatment platelet level with the value for Ni after 6 months of treatment (*r* = -0.46; *p* = 0.04) and post-treatment liver Cu value (*r* = -0.49; *p* = 0.028). There was also a correlation between the concentrations of liver Fe and bilirubin values after 6 months of treatment (*r* = 0.51; *p* = 0.034).

We used GEE models to find effects of liver trace elements on HAI and fibrosis. We considered HAI as continuous and

**Table 2** Antiviral treatments and hepatic function, age, and bilirubin levels of patients with hepatitis B treatment

Treatment	No. of patient	Age %	AST U/L	ALT U/L	ALP IU/L	GGT U/L	Bilirubin mg/dL	Platelet/ $\mu\text{L}$	Hemoglobin g/dL
Lamivudine	9	$44 \pm 13.5$	45	23.1 (15–32)	$40.2 \pm 5.4$	$79.9 \pm 15.5$	$26.2 \pm 12$	$0.61 \pm 0.27$	$230.000 \pm 37127$
Entecavir	5	$43.4 \pm 13.7$	25	58.4 (13–121)	$99.4 \pm 78.6$	$84.0 \pm 26.6$	$35.2 \pm 20.4$	$0.64 \pm 0.35$	$176600 \pm 50958$
Tenofovir	5	$36.8 \pm 10.3$	25	20.8 (16–25)	$22.2 \pm 6.3$	$75.4 \pm 18.4$	$16.4 \pm 2.9$	$0.74 \pm 0.22$	$177380 \pm 62240$
Telbivudine*	1	38	5	33	152	36	79	0.9	154000
									14

\*There was only one patient who received telbivudine treatment, and the value of this patient is included

Mean  $\pm$  SD was given at regular intervals and median (min-max) at irregular intervals

AST and hemoglobin have been expressed as median (min-max) since they do not provide regular intervals in the homogeneity test

ALT alanine aminotransferase, AST aspartate aminotransferase, ALP alkaline phosphatase, GGT gamma glutamyl transpeptidase

**Table 4** The change of liver trace elements before and 6 months of HBV treatment

	Liver concentration ( $\mu\text{g/g}$ )	Before treatment $n=20$	At the sixth month of treatment $n=20$	$p$	
Cr	0.84	(0.09–3.83)	0.66	(0.26–6.60)	0.696
Mn	2.32	(0.22–27.60)	1.88	(0.56–27.20)	0.852
Fe	112.50	(13.20–5985.0)	149.50	(23.30–994.00)	0.940
Co	0.025	(0.002–0.200)	0.024	(0.010–0.191)	0.615
Ni	0.80	(0.09–4.94)	0.65	(0.22–7.85)	0.654
Cu	2.82	(0.01–17.80)	5.31	(0.64–134.00)	0.002
Zn	48.05	(2.40–143.20)	74.90	(29.80–621.70)	0.035
Ag	0.059	(0.009–0.144)	0.048	(0.013–0.506)	0.765
Cd	0.228	(0.009–4.811)	0.395	(0.90–6.135)	0.263
Pb	0.015	(0.001–0.080)	0.100	(0.001–0.270)	0.864
Al	32.00	(5.49–103.00)	22.45	(6.91–381.00)	0.478

Cr chromium, Mn manganese, Fe iron, Co cobalt, Ni nickel, Cu copper, Zn zinc, Cd cadmium, Pb lead, Al aluminum

The results of trace elements are given as median (minimum–maximum) due to skewed distribution

fibrosis as ordinal and formed GEE models per each trace element which include trace element and time as predictors and HAI or fibrosis as dependent variable. Generalized estimation equation models related the effect of trace element changes with Ishak's scores for Zn ( $p=0.002$ ), Fe ( $p=0.0244$ ), Cu ( $p=0.0003$ ), and Al ( $p=0.0239$ ) values that may be effective in predicting HAI changes. Table 6 can be interpreted like a linear regression model is interpreted (namely, a parameter estimate says Zn – 0.0055 means that 1 unit increase in Zn results in 0.0055 unit decrease in HAI, 1 unit increase in Fe results in 0.0002 unit increase in HAI, 1 unit increase in Cu results in – 0.0221 unit decrease in HAI, and 1 unit increase in Al results in 0.0029 unit decrease in HAI). According to the generalized estimation equation models (since we treated fibrosis as ordinal, the resulting parameter estimate in Table 7 from corresponding GEE model was an odds ratio and the interpretation is similar to that of a logistic regression), it was found that only the variation of Fe levels was effective at very low levels to predict the changes caused by fibrosis ( $p=0.0002$ ) (namely, an OR 1.002 found for Fe means that a 1 unit increase in Fe leads to 1.002 times odds of being in a higher fibrosis level). The effects of trace elements

on HAI changes are shown in Table 6, and the effect of trace elements on fibrosis changes are shown in Table 7.

## Discussion

In our study, the changes in liver tissue trace elements of patients receiving antiviral therapy for HBV were analyzed. This is the first study that examines the variation of liver tissue trace element levels before and after treatment. We also analyzed how the trace elements differ according to tissue histopathologic changes. Our results show that Zn and Cu values are affected by antiviral treatment and tissue levels are increased. In our study, no change was observed with treatment at other trace element (Ni, Fe, Mn, Al, Pb, Cd, Ag, Co, and Cr) levels. At 6 months after treatment of HBV patients, the HAI showed a statistically significant decrease. On the other hand, the decrease in fibrosis score was not statistically significant.

A thorough literature review does not retrieve a sufficient number of studies to examine changes in tissue trace element concentrations of patients receiving HBV therapies. Trace element studies in HBV patients are mostly performed in serum

**Table 5** The correlations between liver trace elements and HBV DNA values (before and at the sixth month of treatment)

HBV DNA (IU/mL)	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ag	Cd	Pb	Al
Before treatment											
<i>r</i>	0.456	0.141	0.112	0.023	0.090	-0.216	0.282	0.451	-0.125	0.546	0.627
<i>p</i>	0.043	0.553	0.639	0.924	0.706	0.361	0.228	0.046	0.600	0.013	0.003
At the sixth month of treatment											
<i>r</i>	0.015	0.334	-0.197	0.237	0.023	0.339	0.263	-0.018	-0.312	0.258	0.053
<i>p</i>	0.950	0.150	0.405	0.314	0.925	0.143	0.262	0.940	0.180	0.272	0.826

Cr chromium, Mn manganese, Fe iron, Co cobalt, Ni nickel, Cu copper, Zn zinc, Cd cadmium, Pb lead, Al aluminum

**Table 6** The effect of liver tissue trace element levels on HAI changes (in generalized estimation equation models, in which the effects of elements on pre- and post-treatment values are investigated)

Trace element	Estimate	Standard error	<i>p</i>
Zn	-0.0055	0.0018	0.002
Cr	-0.0326	0.2715	0.9044
Mn	-0.0629	0.0465	0.1762
Fe	0.0002	0.0001	0.0244
Co	-6.0009	6.8638	0.382
Ni	0.1129	0.2420	0.6408
Cu	-0.0221	0.0061	0.0003
Ag	-1.7478	3.6155	0.6288
Cd	-0.1196	0.2682	0.6556
Pb	-0.5191	5.4986	0.9248
Al	-0.0065	0.0029	0.0239

*Cr* chromium, *Mn* manganese, *Fe* iron, *Co* cobalt, *Ni* nickel, *Cu* copper, *Zn* zinc, *Cd* cadmium, *Pb* lead, *Al* aluminum

samples [14]. In a study conducted by Balamtekin et al. [15], Mn, Mo, Se, and Zn values in a pediatric patient group were found to be higher in acute HBV patients than a healthy control group. In the same study, it was stated that these trace elements do not have any effect on patients' interferon- $\alpha$  response. In our study, it was found that there was an increase in Zn and Cu tissue levels with no difference in the Mn concentration of liver tissue.

In the analysis performed by Rahelic et al. [16], serum trace element levels were measured in liver cirrhosis patients with HBV. In this study, serum Zn levels in cirrhotic patients were found lower than non-cirrhotic patients, and serum Cu and Mn levels of cirrhotic patients were found higher than non-cirrhotic patients. In the same study, Zn levels were found to

be lower in encephalopathy patients [16]. Most patients in our study were early-stage chronic HBV patients. Even in the case of early-stage liver disease, we found that there was an increase in liver Zn levels after the sixth month of treatment.

In the study of Papanikolopoulos et al. [17], there were no differences among the serum trace elements in patients with chronic liver disease due to different etiologies (viral, drug, autoimmune, and cryptogenic hepatitis). In the same study, serum Zn values in acute hepatitis patients were found to be higher than the control group. In this study, a correlation was found between prothrombin and Cu serum levels in patients with severe hepatitis. There was also a correlation between AST and serum Zn levels in severe hepatitis group, but no such correlation was found in the mild hepatitis group [17]. Conversely, the post-treatment Zn tissue concentration was found higher in our study. In our analysis, AST were measured before treatment, which indicated no correlation with tissue trace elements; however, there was a correlation between platelet and Ni as well as between Fe and serum bilirubin levels. In the study of Abedienkenari et al. [18], blood Zn and Cu values were found to be lower in the group with high AST and ALT values than in the group with normal transaminase values [18]. There was no correlation between tissue concentrations and transaminase levels in our study.

In an autopsy study after natural deaths, the mean liver Zn level was 53.38  $\mu\text{g/g}$ , while the Cu level was 4.91  $\mu\text{g/g}$  [19]. In the autopsy study performed by Satarug et al. [20], the mean wet liver tissue cadmium level was found to be 0.95  $\mu\text{g/g}$ , while the median Cd value in our study with dry liver weight was found to be 0.228  $\mu\text{g/g}$  before treatment and 0.395  $\mu\text{g/g}$  after treatment. Gur et al. [21] conducted a study of liver tissue Zn concentrations in HBV patients and found that liver Zn concentrations decreased as the disease progressed. In this study, the mean dry liver zinc level was 3.83  $\mu\text{mol/g}$  in

**Table 7** The effects of liver tissue trace element levels on fibrosis change (in generalized estimation equation models, in which the effects of elements on pre-treatment and post-treatment values are investigated)

Trace element	Odds ratio estimate	Standard error	95% confidence limits (lower)	95% confidence limits (upper)	<i>p</i>
Cr	1.162	0.1909	0.79947501	1.68961383	0.4308
Mn	0.968	0.0275	0.91887976	1.02347121	0.2642
Fe	1.002	0.0004	1.00070025	1.00230265	0.0002
Co	0.343	5.8594	3.5325E-06	33,356.2353	0.8552
Ni	1.138	0.173	0.81098964	1.59791555	0.4537
Cu	0.999	0.0064	0.98668938	1.01166754	0.8886
Zn	0.999	0.0014	0.99630684	1.00190181	0.5401
Ag	8.143	3.0733	0.01971255	3363.03796	0.495
Cd	1.073	0.1428	0.81115185	1.41963529	0.6211
Pb	0.005	4.3198	98.09	22.1646793	0.214
Al	0.999	0.0029	0.99431621	1.00561571	0.9796

*Cr* chromium, *Mn* manganese, *Fe* iron, *Co* cobalt, *Ni* nickel, *Cu* copper, *Zn* zinc, *Cd* cadmium, *Pb* lead, *Al* aluminum

healthy controls and 1.1 μmol/g in cirrhotic patients. The improvement in liver Zn concentrations in HBV patients with treatment in our study suggests that the reduction in disease severity may lead to elevated Zn levels in the liver, which supports the work of Gur et al [21].

In the study conducted by Versieck et al [22], it was reported that the mean serum Cu concentration increased in patients with acute hepatitis. The authors suggested that increased Cu concentration may be due to hepatic necrosis or impaired hepatic Cu excretion. Hatono et al [23] reported that the amount of hepatic Cu increased as fibrosis progressed [23]. During our treatment, liver Cu concentrations were evaluated before and after 6 months of treatment. However, in our study, the level of Cu in the recovery phase was found to be statistically higher in the liver.

The limitation of our work is the small number of patients who were biopsied. Another limitation is that most patients with HBV that had biopsies had early-stage HBV. For this reason, evaluation of liver trace element accumulation in cirrhotic patients may require further autopsy studies. In our study, however, a biopsy of each patient prior to treatment and after the sixth month of treatment may provide premise data for the literature.

In conclusion, in our study, liver tissue Zn and Cu levels increased in parallel with the improvement of inflammation in HBV patients who received antiviral treatment. Our study is a premise study for examining the change of trace elements with biopsy repetition; however, it will be necessary to evaluate the trace element changes that occur during HBV treatment with greater number of patients and longer treatment periods for reaching a strict conclusion.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflicts of interest.

**Ethics** This study was approved by the Ethics Committee of Mersin University, Turkey (Committee Decision no: 2014/81, date: 25/04/2014). All the patients signed their informed consent form before participation in the study. The written informed consent from the biologist was included in the study. The study was carried out in accordance with the principles of the ethical Declaration of Helsinki.

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