Serum Level of Some Trace Elements as Prognostic Factors in Egyptian Patients with HCV-related Liver Disease

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ABSTRACT

Background: A growing body of evidence indicates that many trace elements play an important role in a number of carcinogenic processes that proceed through various mechanisms. Trace elements are involved in chronic liver diseases because these elements may have a direct hepatic toxicity or may be decreased as a consequence of the impaired liver functions. Hepatitis infection may alter serum content of several trace elements e.g. Iron, Copper, Manganese and Zinc. This alteration may play a role on ongoing liver fibrogenesis. Serum metal levels, such as copper (Cu), zinc (Zn), iron (Fe) and manganese (Mn) have been reported to be highly sensitive in the diagnosis of some diseases.

Aim of work: to evaluate the level of some trace metals in HCV and HCC patients, also correlating their levels with HCC tumor size.

Patients and methods: Sixty liver disease patients and fifteen healthy subjects served as a healthy control group. Patients were classified into Group A (n=20) chronic hepatitis C virus infection (compensated), Group B (n=20) patients (uncompensated HCV), and Group C (n=20) patients diagnosed as hepatocellular carcinoma. After verbal consent, all patients detection of subjected to full history taking, clinical examination, and routine laboratory testing, in addition to viral hepatitis markers for the presence of HCV-Ab by linked immune sorbent assay (ELISA), the presence of HCV-RNA by PCR, alpha-fetoprotein, abdominal ultrasonography and estimation of serum minerals (Fe, Cu, Mn, and Zn) using atomic absorption spectrophotometry.

Results: Mn and Cu levels were significant when correlated with the uncompensated HCV patients viral load (r = -0.65 & = 0.51, p < 0.01 & <0.02 respectively). While in HCC patients, only Zn correlated significantly with viral load (P<0.01). No correlation was found between HCV viral load and the studied trace elements in HCV compensated patients. Also, in HCC patients, serum Zn correlated significantly with tumor size greater than 5cm. while Cu serum level was significantly correlated with tumor size less than 5cm. in the uncompensated HCV group, iron serum level showed high significance results when correlated with ALT ( r =0.563 , p <0.01). Regarding correlation of liver function tests with studied trace elements in HCC patients group , the ALK showed high significant correlation with serum Cu level (r = 0.640, P<0.046). Also, T.BIL/D.BIL showed slight correlation with Cu serum level but not yet reach significance (r = 0.583, p < 0.076).

Conclusion: Trace elements were verified to have an essential role in liver disease. Serum copper levels were correlated with the viral load whereas, serum Mn levels showed a promising role in protecting HCV uncompensated patients. Zn might be of importance in regulating viral replication and liver carcinogenesis in HCV patients. While serum Zn levels were correlated with the viral load and liver damage in HCC patients. Further studies have to be performed to confirm the relation between trace elements and HCC development.

INTRODUCTION

Worldwide, HCV is a major etiologic agent of chronic hepatitis: it is a hepatotropic virus causing hepatocellular damage and chronic liver inflammations that progressively can lead to cirrhosis, hepatocellular carcinoma (HCC) and liver failure.

Hepatitis c virus (HCV) is considered the most common etiology of chronic liver disease (CLD) in Egypt (approximately 170 million people is infected with HCV), where prevalence of infected antibodies to HCV (anti HCV) is approximately 10 fold greater than elsewhere worldwide.

Epidemiologically, Hepatocellular carcinoma (HCC) is the commonest primary liver cancer. Incidence is increasing and HCC has risen to become the 5th commonest malignancy worldwide and the third leading cause of cancer-related death. However, the disease is more common in parts of Africa and Asia than in North or South American and Europe. In Egypt, between 1993 and 2002, there was an almost twofold increase in HCC amongst chronic liver patients.
Trace elements are needed for vitamin synthesis, hormone production, enzyme activity, collagen formation, tissue synthesis, oxygen transport, energy production, and physiological processes related to growth, reproduction and health. These elements function as activators of enzyme systems or as constituents of organic compounds and their deficiencies, excesses or malabsorption, contribute to or cause several diseases as cardiac conditions, immune and hormonal dysfunctions, and a host of other maladies.

A growing body of evidence indicates that many trace elements play important role in a number of carcinogenic processes that proceed through various mechanisms. Trace elements are involved in chronic liver diseases because these elements may have a direct hepatic toxicity or may be decreased as consequence of the impaired liver function.

Hepatitis infection may alter serum content of several trace elements e.g. Iron, Cupper, Manganese and Zinc. This alteration may play a role on ongoing liver fibro genesis. Serum metal levels, such as cupper (Cu), zinc (Zn), iron (Fe) and manganese (Mn) have been reported to be highly sensitive in the diagnosis of some diseases.

However, the role of trace elements in chronic liver disease, carcinogenesis and progression of hepatocellular carcinoma (HCC) has not yet been clarified.

The aim of this study was to evaluate the levels of some trace metals in HCV and HCC patients, also to correlate their levels with HCC tumor size.

PATIENTS & METHODS

All the patients included in this study were selected from the National Hepatology and Tropical Medicine Research Institute in Cairo. This study was conducted on 60 patients, (20) patients with chronic hepatitis C virus infection (compensated), (20) patients (un compensated HCV), chosen on the basis of elevated ALT enzyme level, presence of HCV-Ab by linked immune sorbent assay (ELISA), positive result of HCV-RNA by PCR and hepatic histopathology while they were negative for other hepatitis viruses (B and E). The third group was (20) patients diagnosed as hepatocellular carcinoma. Fifteen healthy subjects served as a healthy control group.

Patients with diuretic therapy, zinc supplementation or nutritional support, and those suffering concurrent infections, in addition to those subjected to surgery or chemotherapy had been excluded. After verbal consent, all patients were subjected to full history taking, clinical exam, and routine laboratory testing, in addition to viral hepatitis markers, alpha-fetoprotein, abdominal ultrasonography and estimation of serum minerals (Fe, Cu, Mn, and Zn).

Materials and Methods

Fasting blood samples (10 ml) were collected, and left to clot. Serum was separated by centrifugation at 3000 r.p.m for ten minutes and stored at -20°C till time of analysis.

Liver function tests: Using Beckman coulter (synchron Cx9 ALX) Clinical Auto Analyzer,USA

- Determination of Serum alanine transaminase (ALT) by colorimetric method.
- Determination of serum aspartate aminotransferase (AST) using the recommended procedure of IFCC as described by Saris.
- Determination of serum total and direct bilirubin (T.Bil. & D.Bil.) using the modified method of Doumous reference method.
- Determination of serum albumin Alb. is measured using the method of crysol purple for albumin.
- Determination of serum AFP by automated chemiluminescence system (ACS: 180 BAYER GERMANY). ACS: 180 AFP assay. It is a two sandwich immunoassay using direct chemiluminometric technology which uses constant amount of two antibodies. The first antibody, in the lite reagent is an affinity purified polyclonal rabbit anti-AFPantibody labeled with a cridinium ester. The second antibody, in the solid phase is a monoclonal mouse anti AFP-antibody covalently bound to paramagnetic peptides.

Immuno-assay

Enzyme Linked Immunosorbent Assay (ELISA) 4th generation for qualitative determination to hepatitis C virus antibodies in human serum was done. This method is an immunometric assay based on the technique of ELISA as described by Kuo et al. In two steps: First, the wells were coated with polypeptides of structural and nonstructural regions of HCV. Second, antibody to HCV from sample or control allows the enzyme tracer {goat IgG to human conjugated to horseradish
peroxidase (HRP)} to bind to the solid phase. The enzyme activity is therefore proportional to the concentration of HCV-Ab present in samples or controls. Measurement of enzyme activity was performed by adding a colourless chromogen/substrate solution. The enzyme action on chromogen/substrate produces a colour which is measured with photometer at wavelength 450 nm. The reagents of this assay were obtained from Diasorine Co. (Italy). The presence or absence of HCV antibody was determined by comparing the absorbance value of unknown samples to that of the cut-off value. The samples of patients with absorbance values greater than or equal to the cut-off were considered reactive for HCV-Ab.

**Detection of HCV-RNA:** Using the polymerase chain reaction (PCR) as described by\(^{(15)}\).

- Extraction of viral RNA from serum or plasma with the high pure viral RNA kit supplied by QIAGEN (Germany).
- Reverse transcription for viral RNA using strategene RT-PCR kit as described by Lacal and Tronick\(^{(16)}\), reverse transcription occurs to reverse viral mRNA to complementary DNA (C-DNA) than repeatedly amplifying the DNA by using specific primers until the amount of product reaches a level that can be detected by autoradiography ethidium bromide staining.

**Atomic absorption spectrophotometry:**

- Preparation of stock standard solution:
  a) Stock standard of iron solution (1000 mg Fe/litre): 1 mg of iron metal was dissolved in 30 ml of 5 M hydrochloric acid and the volume was completed to one litre in volumetric flask with deionized water.
  b) Stock standard of Copper solution (1000mg Cu/litre): 1 gm of copper metal was dissolved in 50 ml of 5 M nitric acid and the volume was completed to one liter in a volumetric flask with deionized water.
  c) Stock standard of Manganese solution (1000mg Mn/litre): 1 gm of manganese metal was dissolved in 30 ml of 5 M hydrochloric acid and the volume was completed to one liter in a volumetric flask with deionized water.
  d) Stock standard of Zinc solution (1000mg Zn/litre): 1 gm of zinc metal was dissolved in 30 ml of 5 M hydrochloric acid and the volume was completed to one liter in a volumetric flask with deionized water.

**Technique**

The procedure was done according to (5)

1) Serum was diluted (1:1) with deionized water. The atomic absorption spectrophotometer was PYE UNICAM SP 929. It was operated according to the standard instrumental conditions for (Cu, Fe, Mn, and Zn).
2) The blank was aspirated, and the null meter was adjusted, so that a reading of zero was obtained.
3) The working standards for (Cu, Fe, Mn, and Zn) were aspirated, and the absorbance was recorded.

**Calculation:** For serum:

\[
\frac{Ug \times 100 \times \text{dilution}}{100 \times \text{volume of serum}} = \frac{T}{S} \times C
\]

\[T = \text{Absorbance of the sample}
\]
\[S = \text{Absorbance of the standard.}
\]
\[C = \text{Concentration of the standard (ug/100 ml).}
\]

**Statistical Analysis was done using SPSS for Windows version 10.**

Student’s t-test and ANOVA tests were used to assess the difference between groups. The correlation coefficient tests were used for the association between the studied variables. The level of significance was P<0.05.

**RESULTS**

All the patients groups were matched as regards age and sex. All were positive for HCV-RNA but negative for all other viral hepatitis.

The compensated chronic HCV patients groups were all having liver functions with prothrombin in >60%, and platelet count >100x103.

The results of the present study showed a significant difference in the ALT level between the three patients groups (HCV compensated, HCV uncompensated and HCC) (p<0.01), but there was no significant difference between the patients groups in all other parameters of liver function tests (Table1).
Table (1): Anova test for Liver function Tests between Studied Groups

<table>
<thead>
<tr>
<th>Liver Function Tests</th>
<th>HCV Uncompensated $\bar{\chi} \pm SD$</th>
<th>HCC $\bar{\chi} \pm SD$</th>
<th>$F$</th>
<th>P value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST</td>
<td>46.5±30.4</td>
<td>65.1±43.5</td>
<td>1.175</td>
<td>0.317</td>
<td>NS</td>
</tr>
<tr>
<td>ALT</td>
<td>52 ±32</td>
<td>117±88</td>
<td>4.97</td>
<td>0.010</td>
<td>HS</td>
</tr>
<tr>
<td>ALK</td>
<td>112±73.5</td>
<td>127±61.3</td>
<td>0.296</td>
<td>0.745</td>
<td>NS</td>
</tr>
<tr>
<td>ALb</td>
<td>4.26±0.58</td>
<td>2.37±0.49</td>
<td>64.638</td>
<td>0.277</td>
<td>NS</td>
</tr>
<tr>
<td>AFP</td>
<td>14.2±58.7</td>
<td>472.4±45.3</td>
<td>2.33</td>
<td>0.107</td>
<td>NS</td>
</tr>
<tr>
<td>T Bil</td>
<td>0.89±0.39</td>
<td>1.98±0.82</td>
<td>14.438</td>
<td>0.453</td>
<td>NS</td>
</tr>
</tbody>
</table>

ALT: Alanine transaminase
AST: Aspartate transaminase
ALK: Alkaline phosphatase

Table (2) Correlation between Liver function tests, AFP, HB and White Blood Cells and the Studied Trace elements in HCV Patients Group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Spearman Rank</th>
<th>HCV Uncompensated Patients</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mn</td>
<td>Cu</td>
<td>Zn</td>
</tr>
<tr>
<td>AST IU/ml</td>
<td>r</td>
<td>-0.064</td>
<td>-0.033</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.786</td>
<td>0.887</td>
</tr>
<tr>
<td>ALK mmol/dl</td>
<td>r</td>
<td>-0.146</td>
<td>0.264</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.538</td>
<td>0.259</td>
</tr>
<tr>
<td>ALT IU/ml</td>
<td>r</td>
<td>0.143</td>
<td>-0.124</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.547</td>
<td>0.599</td>
</tr>
<tr>
<td>ALB gm/dl</td>
<td>r</td>
<td>-0.501</td>
<td>0.570</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.024</td>
<td>0.008</td>
</tr>
<tr>
<td>T.BIL/D.BIL mg/dl</td>
<td>r</td>
<td>0.216</td>
<td>-0.274</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.358</td>
<td>0.241</td>
</tr>
<tr>
<td>AFP ng/dl</td>
<td>r</td>
<td>-0.280</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.231</td>
<td>0.189</td>
</tr>
<tr>
<td>HB gm/dl</td>
<td>r</td>
<td>0.155</td>
<td>-0.050</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.513</td>
<td>0.832</td>
</tr>
<tr>
<td>WBC Cmm3</td>
<td>r</td>
<td>0.286</td>
<td>-0.317</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.220</td>
<td>0.173</td>
</tr>
</tbody>
</table>

Liver function tests were not correlated significantly with any of the studied trace elements in HCV compensated group, while in the uncompensated HCV group iron serum level showed high significant value results when correlated with ALT ($r=0.563$, $p<0.01$) (Table 2).
**Table (3):** Correlation between Liver function tests, AFP, HB and White Blood Cells and Studied Trace elements in HCC Patients Group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Spearman Rank</th>
<th>HCC Patients</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mn</td>
<td>Cu</td>
<td>Zn</td>
<td>Fe</td>
</tr>
<tr>
<td>AST IU/ml</td>
<td>r</td>
<td>0.079</td>
<td>0.140</td>
<td>-0.054</td>
<td>0.390</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.828</td>
<td>0.699</td>
<td>0.880</td>
<td>0.264</td>
</tr>
<tr>
<td>ALK mmol/dl</td>
<td>r</td>
<td>-0.097</td>
<td>0.640</td>
<td>0.006</td>
<td>0.439</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.789</td>
<td>0.046</td>
<td>0.986</td>
<td>0.204</td>
</tr>
<tr>
<td>ALT IU/ml</td>
<td>r</td>
<td>-0.333</td>
<td>0.504</td>
<td>-0.054</td>
<td>0.419</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.348</td>
<td>0.136</td>
<td>0.880</td>
<td>0.227</td>
</tr>
<tr>
<td>ALB gm/dl</td>
<td>r</td>
<td>0.445</td>
<td>-0.033</td>
<td>0.278</td>
<td>-0.100</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.197</td>
<td>0.926</td>
<td>0.436</td>
<td>0.781</td>
</tr>
<tr>
<td>T.BIL/D.BIL mg/dl</td>
<td>r</td>
<td>-0.442</td>
<td>0.583</td>
<td>0.085</td>
<td>0.498</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.204</td>
<td>0.076</td>
<td>0.815</td>
<td>0.1425</td>
</tr>
<tr>
<td>AFP ng/dl</td>
<td>r</td>
<td>-0.151</td>
<td>0.455</td>
<td>0.516</td>
<td>-0.273</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.681</td>
<td>0.185</td>
<td>0.126</td>
<td>0.444</td>
</tr>
<tr>
<td>HB gm/dl</td>
<td>r</td>
<td>0.534</td>
<td>0.0426</td>
<td>0.030</td>
<td>-0.048</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.111</td>
<td>0.906</td>
<td>0.933</td>
<td>0.893</td>
</tr>
<tr>
<td>WBC Cmm3</td>
<td>r</td>
<td>0.454</td>
<td>-0.085</td>
<td>-0.194</td>
<td>0.340</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.184</td>
<td>0.815</td>
<td>0.335</td>
<td>0.590</td>
</tr>
</tbody>
</table>

Regarding correlation of liver function tests with studied trace elements in HCC patients group, the ALK showed highly significant correlation with serum Cu level ($r = 0.640$, $P < 0.046$). Also, T.BIL/D.BIL showed slight correlation with higher level of Cu serum but not yet reach significance ($r = 0.583$, $P < 0.076$) (Table 3).

**Table (4):** Correlation between Albumin, ALT, AFP and Studied Trace elements in HCV Patients Group

<table>
<thead>
<tr>
<th>Variables</th>
<th>HCV Virus Load in IU/ml N=20</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r(Spearman Rank)</td>
<td>P</td>
<td>Significance</td>
</tr>
<tr>
<td>ALB</td>
<td>0.06</td>
<td>0.81</td>
<td>NS</td>
</tr>
<tr>
<td>ALT IU/ml</td>
<td>0.13</td>
<td>0.6</td>
<td>NS</td>
</tr>
<tr>
<td>AFP ng/dl</td>
<td>-0.03</td>
<td>0.95</td>
<td>NS</td>
</tr>
<tr>
<td>Mn</td>
<td>0.29</td>
<td>0.22</td>
<td>NS</td>
</tr>
<tr>
<td>Cu</td>
<td>-0.28</td>
<td>0.23</td>
<td>NS</td>
</tr>
<tr>
<td>Zn</td>
<td>-0.22</td>
<td>0.36</td>
<td>NS</td>
</tr>
<tr>
<td>Fe</td>
<td>-0.08</td>
<td>0.73</td>
<td>NS</td>
</tr>
</tbody>
</table>

No correlation was found between HCV viral load and the studied trace elements in HCV compensated patients group (Table 4).

* No significant

**Table (5):** Correlation between Albumin, ALT, AFP and Studied Trace elements in HCV Uncompensated Patients Group

<table>
<thead>
<tr>
<th>Variables</th>
<th>HCV (uncompensated) Virus Load in IU/ml N=20</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r(Spearman Rank)</td>
<td>P</td>
<td>Significance</td>
</tr>
<tr>
<td>ALB gm/dl</td>
<td>0.12</td>
<td>0.62</td>
<td>NS</td>
</tr>
<tr>
<td>ALT IU/ml</td>
<td>-0.065</td>
<td>0.79</td>
<td>NS</td>
</tr>
<tr>
<td>AFP ng/dl</td>
<td>-0.09</td>
<td>0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Mn</td>
<td>-0.56</td>
<td>0.01</td>
<td>HS</td>
</tr>
<tr>
<td>Cu</td>
<td>0.51</td>
<td>0.02</td>
<td>HS</td>
</tr>
<tr>
<td>Zn</td>
<td>0.18</td>
<td>0.44</td>
<td>NS</td>
</tr>
<tr>
<td>Fe</td>
<td>-0.07</td>
<td>0.78</td>
<td>NS</td>
</tr>
</tbody>
</table>
In this study, only Mn and Cu levels were significant when correlated with the uncompensated HCV patients viral load \( r = -0.65 \) & \( = 0.51, p < 0.01 \) & <0.02 respectively)(Table 5).

* Significant high level of Mn and Cu Uncompensated patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>HCV (uncompensated) Virus Load in IU/ml N=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB gm/dl</td>
<td>0.16</td>
</tr>
<tr>
<td>ALT IU/ml</td>
<td>0.04</td>
</tr>
<tr>
<td>AFP ng/dl</td>
<td>0.09</td>
</tr>
<tr>
<td>Mn</td>
<td>0.41</td>
</tr>
<tr>
<td>Cu</td>
<td>-0.12</td>
</tr>
<tr>
<td>Zn</td>
<td>0.75</td>
</tr>
<tr>
<td>Fe</td>
<td>0.15</td>
</tr>
</tbody>
</table>

While in HCC patients, only Zn correlated significantly with viral load \( P < 0.01 \) (Table 6)

* significant high level of ZN

In the present study, tumor size in HCC patients were categorized as below 5cm and above 5cm. serum Zn was significantly high with tumor size greater than 5cm. while Cu serum level was higher with tumor size less than 5cm (Table 7).

**DISCUSSION**

Measurement of serum trace elements emerge as a recent biochemical studies in patients with chronic liver disease and hepatocellular carcinoma.

The present study revealed that serum Mn and Cu levels are highly significantly correlated with the uncompensated HCV virus load.

This was in line with Suzuki et al.\(^{17}\) who explained this fact by that Cu acts as a cofactor against hepatic fibrosis in chronic hepatitis to liver cirrhosis. In disagreement with our study, Ko et al.\(^{14}\) found that serum Zn only were significantly lower in chronic hepatitis patients than in healthy controls.

This conflict in the finding may be explained by the fact that decreased intake, disturbed intestinal absorption and decreased serum albumin as a carrier of Zn and other trace elements. Also, decreased storage of the liver and increased urinary excretion\(^{18}\).

A conclution of the relation between Zn and HCV is the study of Yuasa et al.\(^{16}\) who stated that Zn has been closely related to the pathogenesis of chronic hepatitis C. And Zn may play an important role as a negative regulator of HCV replication in genome length HCV RNA –replicating cells.

In our study, it was revealed that Zn is statistically highly significant when correlated with HCC \( P<0.01 \). This was in agreement with Jin et al.\(^{19}\) who reported that the level of Zn significantly differed between HCC patients and non-cancerous liver patients.

Kurinchi et al.\(^{20}\) reported that Zn, Cu content is lower in HCC than in the surrounding normal tissue and the cirrhotic control. The same finding were reported by Takashi et al.\(^{14}\) and Nakayama et al.\(^{16}\).

In line with the previous results was Koung et al.\(^{18}\) who found that both the metalloenzyme SOD which need (Cu, Zn) for their activity, in addition to serum Cu /Zn were significantly lower in HCC compared to that seen in surrounding normal liver tissue.

In disagreement with this work, a study done by Wang-Chin et al.\(^{8}\), showed that there was statistically highly significant correlation between serum concentration of both Fe,Cu in HCC .
Furthermore, Arumugam et al.\(^{(21)}\) added that there is a positive correlation between serum Cu and lipid peroxidation products in HCC while there was a negative correlation between serum Cu level and serum antioxidants level in HCC.

Ebara et al.\(^{(25)}\), proved that Cu level in liver parenchyma was significantly higher in HCC than in normal parenchyma (P<0.01) and than in patients with liver fibrosis. And Cu content in liver parenchyma was higher in the presence of HCC than in its absence.

In an Egyptian study Ismail et al.\(^{(18)}\), found that serum Zn levels were significantly decreased in patients with HCC when compared with patients with chronic hepatitis and liver cirrhosis.

Also, Gur et al.\(^{(9)}\), recorded a significantly progressive decrease in liver tissue content of Zn from patients with viral hepatitis to cirrhosis to HCC parallel to sensitivity of liver damage.

In the present study, both Cu & Zn correlated significantly with HCC tumor size. Cu was significantly increased when tumor size is less than 5 cm while Zn was significantly decreased with tumor size more than 5 cm.

This was in agreement with Jin et al.\(^{(19)}\), who found that in relation to tumor size, an imbalance Cu distribution was observed. The content of Cu present in small HCC (<3.5 cm in average diameter) was greater than that in the surrounding liver parenchyma and when HCC progressed to large one (more than 3.5 cm in average diameter) hepatic Cu was no longer accumulated.

In concordance with our findings, Haratake et al.\(^{(23)}\) concluded that these excessive accumulations of copper and copper-binding proteins might present a helpful finding to distinguish some cases of HCC, especially small HCC. However, in contrast, Takashi et al.\(^{(24)}\) stated that the decrease of Zn and increase Fe in HCC tissue positively correlated with HCC tumor progression.

Ebara et al.\(^{(25)}\) reported that serum Cu levels were significantly higher in malignant hepatic tumors greater than 5 cm in average size in comparison with smaller tumors. Vecchio et al.\(^{(26)}\) study indicates that the storage of copper inside tumor cells is a peculiarity of the fibrolamellar carcinoma (FLC) not associated with cirrhosis, type of HCC with a close relationship to the oncocytic nature of neoplastic hepatocytes.

**Conclusion**

Trace elements were verified to have an essential role in liver disease. Serum cupper levels were correlated with the viral load whereas, serum Mn levels showed a promising role in protecting HCV uncompensated patients. Zn might be of importance in regulating viral replication and liver carcinogenesis in HCV patients. While serum Zn levels were correlated with the viral load and liver damage in HCC patients. Further studies have to be performed to confirm the relation between trace elements and HCC development.

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C- Phycocyanin Effect on IFN-γ Production in Patients with Chronic Hepatitis C Infection: In vitro Study

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ABSTRACT

Hepatitis C virus (HCV) is a serious global health threat and current medical treatment options are limited. Gama interferon (IFN-γ) is an important pro-inflammatory cytokine with antiviral activity however, the mechanism of its action as anti-hepatitis C treatment remains unclear. Many studies suggest that priming with IFN-γ prior to initiation of INF-γ treatment has a beneficial effect in some cases of chronic hepatitis C infected patients through changing the balance of cytokines in favour of a Th-1 type response in the host. C-phycocyanin is a water-soluble biliprotein from the filamentous Cyanobactrium spirulina platensis with a potent antioxidant, anti-inflammatory and anti cancerous properties. In this study we investigated the effect of C-phycocyanin on the production of IFN-γ from the peripheral blood mononuclear cells (PBMC) obtained from patients with chronic hepatitis C with different degrees of liver disease severity. We obtained two groups of blood: the first was from healthy volunteers as a control group and the second was from patients with hepatitis C virus infection. Both groups were subjected to phycocyanin and phytoheamaglutinin as stimulators of the release of interferon gamma from the PBMC. We found that in both groups the level of IFN-γ production without addition of phycocyanin or phytoheamaglutinin was less than after addition of 5ul or 10 ul of phycocyanin. Also the results showed that the mean level of IFN-γ production in the diseased group was higher than that of the control group. Among the diseased group the production was much higher in class B subgroup (Child Biss). The safety of Spirulina and its content C-phycocyanin and its immune stimulatory effects encourage us to recommend its use in vivo study specially in chronic hepatitis C patients who failed to respond to or unfit for the classical interferon therapy.

Key wards: Hepatitis C-virus (HCV), C-phycocyanin (C-Pc), Gama interferon (IFN-γ)

INTRODUCTION

Natural compounds from blue green algae have attracted a great deal of interest as physiologically functional food supplements and several bioactive metabolites have been successfully developed into clinically useful drugs. More than 60 % of anti-cancer and 70 % of anti-infective antibiotics currently in clinical use are natural product or natural product-based. Spirulina has long history of use as food. Its name derives from the spiral or helical nature of its filaments. There are reports that it was used as food in Mexico during Aztec civilization. It is still used as food in the lake Chad area where it is sold as dried bread called dihe. Spirulina has a rich content of protein, vitamins, essential amino acids, minerals and essential fatty acids. Many researches proved that Spirulina and its extracts have many pharmacological benefits as immuno-mulatory function with stimulatory effects on interferon and interleukin I secretion, antioxidant effects, anticancer effects and antiviral effects as it affects the replications of several viruses including Herpes simplex type I, human cytomegalo virus, measles, mumps, influenza A virus and HIV-I virus. Spirulina contains mainly two phycobiliproteins, namely C-phycocyanin (C-PC) and allophycocyanin (A-PC) approximately at a ratio of 10:1. C-phycocyanin comprises a protein and chromophore. The protein moiety consists of α and β subunits of molecular weight in the range of 18.000 and 20.000, respectively and exist as a complex interacting mixture of trimer, hexamer and decamer aggregates. Many reports proved that C-PC has many pharmacological characteristics including anti-inflammatory, anti-oxidant, radical scavenging activities, inhibits cyclooxygenase-2 and has anti-tumor activity. Hepatitis C virus infects an estimated 170 million person's world wide. In most infected
individuals, this remarkable RNA virus evades the immune system and establishes a chronic infection that can lead to cirrhosis, end stage liver disease and hepatocellular carcinoma. While advances have been made in treating HCV, the current therapy, a combination of pegylated interferon IFN-α and ribavirin is poorly tolerated and is effective in only 50% of genotype I HCV- infected patients. Thus, a great need exist for more effective and better tolerated therapies for HCV. Interferon-γ (INF–γ) is a key player in immune response to virus infection and its efficacy for treatment of hepatitis C patients is under clinical trials. INF–γ exerts multiple roles in the regulation of inflammation and immune responses during HCV infection and studies have suggested that it plays a critical role in the control of HCV infection and seems to be well tolerated.

Further researches proved that in addition to the reduction of claudin-1 expression, IFN-γ treatment also led to significant changes in the distribution of claudin-1, CD8, and scavenger receptor class B type I. These receptors are important for virus C to invade the hepatocyte or other susceptible cells. Other effects of IFN-γ include enhancement of Th1 cells and inhibition of Th2 cells, enhancement of immune lysis of HCV infected cells, inhibition of hepatic fibrosis by an effect on TGF-beta and has an effect on HCV induced carcinogenesis.

In this study, we tried to elucidate an immunomodulatory role of natural safe substances as phycoeyanin in patients with chronic viral C hepatitis through stimulation the release of INF–γ from peripheral blood mononuclear cell (PBMC)

SUBJECTS, MATERIALS & METHODS

This study was performed in Internal Medicine, Microbiology and Immunology Departments, Faculty of Medicine, Zagazig University and included 40 subjects. They were divided into two main groups.

Group I: Control group, which included 10 healthy persons (6 males and 4 females their ages ranged from 38 to 62 years) with no history of liver diseases, negative HCV antibodies with normal abdominal ultrasonography.

Group II: Included 30 hepatic patients (18 males and 12 females 38 to 62 years) having chronic HCV infection previously diagnosed by polymerase chain reaction (PCR). This group was further subdivided into 3 subgroups according to Child-Pugh classifications; class A, B and C, each subgroup included 10 samples. All were excluded to be free from renal, cardiac, diabetes mellitus or autoimmune diseases

Methods

1. Liver function test:
   - Serum Alanin amino transferase, Aspartate amino transferase, Alkaline phosphatase using Bio merieux kit.
   - Total direct and indirect bilirubin and serum albumin using Diamond kits.
   - Prothrombin time and INR using Diaplatin kit.

2. Detection of viral markers in serum
   - The presence of Anti-HCV antibodies (IgG) in the serum was determined by qualitative enzyme immunoassay.
   - Viral nucleic acids extraction and isolation. RNA in the serum was extracted, reverse transcribed and amplified using quantitative (PCR). The results were interperated as copies /ml.

3. Detection of auto antibodies:
   Antinuclear antibodies are assayed in all cases of HCV group to exclude auto immune hepatitis by indirect immuno-fluorescence.

4. Mononuclear cells (PBMC) isolation:
   Under sterile safety hood, 5 ml of blood were diluted with equal volume of phosphate buffer solution (Biochrom AG); diluted blood was added over Ficoll-Hypaque solution 1.077 (Biochrom AG) in a ratio of 3:1 for each sample. The tubes were centrifuged at 2000 rpm at room temperature (22°C) for 30 minutes. The buffy coat containing the mononuclear cells was obtained and washed with phosphate buffer at 1:1 volume by centrifugation at 1000 rpm for 10 minutes. The last step was repeated for two extra times but the last time was done with RPMI 1640 media (Furo-lone).

5. Extraction and determination of C-PC:
   C-PC was extracted from blue-green algae according to Bousiba and Richmond as follow: 20 gm of experimental algae was suspended in 200 ml of 0.1 sodium phosphate buffer pH 7.2, containing 100ug/ml lysozome and 10 ml EDTA. The enzymatic disintegration of the cell wall occurred by placing the algae in shaking water path at 30°C for 24 hours. The slurry centrifuged for 1 hour at 10,000 rpm to remove cell debris, yielding a clear supernatant of C-PC. The crude C-PC was centrifuged for 30 hours at 10,000 rpm at 40°C. C-PC was precipitated using ammonium sulphate 75% at pH 7.2 for
6 hours. The precipitated C-PC was dissolved in phosphate buffer and dialyzed over night at 4°C against the same buffer.

6. Culture and stimulation of the peripheral mononuclear cells(5):

200 ml of $1 \times 1000.000/ml$ PBMC were recovered into RPMI 1640 media with 10% (vol/vol) fetal bovine serum (FBs) and 1% (wt/vol) L-glutamine, penicillin (1U/ml) and Streptomyccin (0.1 mg/ml) and incubated in 96 wells of tissue culture plate. 20 ul phytoheamaglutinin (PHA) was added to well. 5 ul of phycocyanin, its concentration was 3.5 Mg/50ml were added to another well, and 10 ul of phycocyanin, its concentration was 3.5 Mg/50ml were added to another well. There was another well without phycocyanin or phytoheamoaglutinin. The culture plates were incubated in the incubator at 37°C, 5% CO$_2$ and optimum humidity. After 24 hours, the supernatant was collected, centrifuged at 1000 rpm for 10 minutes and kept frozen at -20°C. The level of IFN-γ was measured in the cell culture supernatant using ELISA.

7. Interferon gamma assay: (R, D. systems)

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for IFN-γ had been per-coated into a microplate. Standards and samples are pipetted into the wells and any IFN-γ was bounded by the immobilized antibody. After washing away any unbounded substances, an enzyme-linked polyclonal antibody specific to IFN-γ was added. Following a wash to remove any unbounded antibody enzyme reagent, a substrate solution was added and colors developed in proportion to the amount of IFN-γ bound in the initial step. The color development was stopped and the intensity of the color was measured.

8. Calculation of results

Average the duplicate reading for each standard, control, and sample and subtract the average zero standard optical density. ELISA reader [Stat Fox 303, made in Germany] was adjusted at wave length 450 to read the absorbance and four standards were used as calibrator.

RESULTS

Correlation between viral load and other parameters

The relation of viral load and each of the following parameters was determined; age, Hb, AST, ALT, INR, serum albumin, WBCs, PLT, and total bilirubin. There was significant positive correlation between the viral load, age, AST, INR albumin and total bilirubin (Fig. 1, 2, 3, 4, 5).

![Figure 1](image1.png)  
**Figure (1)** Correlation between viral load and Age

![Figure 2](image2.png)  
**Figure (2)** Correlation between viral load and AST

![Figure 3](image3.png)  
**Figure (3)** Correlation between viral load and INR
Figure (4) Correlation between viral load and Albumin

Figure (5) Correlation between viral load and bilirubin

Correlation between IFN –γ and other parameters
The relation of IFN –γ and each of the same parameters was determined. There was significant positive correlation between the level of IFN–γ and Hb, PLT, ALT, total bilirubin (Fig. 6, 7, 8, 9).

Figure (6) Correlation between interferon –γ production level of and Hb

Figure (7) Correlation between interferon –γ production level of and PLT

Figure (8) Correlation between interferon –γ production level of and ALT

Figure (9) Correlation between interferon –γ production level of and bilirubin

Measurement of γ interferon without addition of phycocyanin or phytohemagglutinin on PBMNC of the two groups: in control group I; it was 67.2 ± 109.2. In group II; subgroup A; it was 116.7 ± 128, subgroup B; it was 400.6 ± 304 and subgroup C; it was 175.2 ± 132.4. There is statistically significant difference among the groups K = 14.9 and P = 0.0018 (Fig. 10).
Figure (10) Measurement of gamma interferon without addition of phycoecyanin or phyto-hemagglutinin of the two groups

Figure (11) Measurement of gamma interferon after addition of 5 µl of phycoecyanin on PBMNC of the two groups

Measurement of gamma interferon after addition of 5 µl of phycoecyanin on PBMNC of the two groups: in control group I; it was 99.87 ± 83.3. In group II: subgroup A; it was 187.6 ± 148.3, subgroup B; it was 739.6 ± 730.7 and subgroup C; it was 363.7 ± 413.3. There was statistically significant difference among the groups (K = 7.65 and P = 0.05) (Fig. 11).

Figure (12) Measurement of gamma interferon after addition of 10 µl of phycoecyanin on PBMNC of the two groups

Measurement of gamma interferon after addition of 10 µl of phycoecyanin on PBMNC of the two groups: in control group I; it was 142.9 ± 101.8. In group II: subgroup A; it was 217.4 ± 172.3, subgroup B; it was 765.1 ± 791.9 and subgroup C; it was 447.8 ± 418.2. There was statistically significant difference among the groups (K = 16.6 and P = 0.001) (Fig. 12).

Figure (13) Measurement of gamma interferon after addition of phytohemagglutinin on PBMNC of the two groups

Measurement of gamma interferon after addition of phyto-hemagglutinin on PBMNC of the two groups: in control group I; it was 854.6 ± 660. In group II: subgroup A; it was 1446.5 ± 521.5, subgroup B; it was 3336 ± 66.4 and subgroup C; it was 2796.2 ± 744. There was statistically significant difference among the groups (K = 31.2 and P = 0.001) (Fig. 13).

DISCUSSION

The overall majority of experimental studies and clinical observations among patients with HCV infection favour immune mediated hepatocellular damage over direct viral cytopathic effect. Recent investigations were done concerning the benefits of gamma interferon in management
of chronic HCV patient especially who failed to respond to classical interferon therapy. It is a unique member of the IFN family. Its receptor, signaling pathway, and cellular effects differ from those of IFN-α and IFN-β. IFN-γ may contribute to HCV clearance in several ways. First, it enhances NK cell activity and induces the expression of inflammatory and potentially antiviral cytokines such as tumor necrosis factor alpha. Second, it facilitates induction and effector function of T cells via upregulation of major histocompatibility complex class I and II proteins and promotes antigen processing via induction of immunoproteasomes. Third, it facilitates T-cell homing from lymph nodes and peripheral blood to the site of infection via induction of T-cell-recruiting chemokines such as IFN-inducible protein 10 (CXCL10), IFN-inducible T-cell chemo-attractant (CXCL11) and monokine induced by IFN-γ (CXCL9). Further results from confocal microscopy and Western blot analysis showed that in addition to the reduction from confocal microscopy and Western blot analysis showed that in addition to the reduction in CLDN1 expression, IFN-γ treatment also led to significant changes in the distribution of CLDN1, CD81, and scavenger receptor class B type I. The main receptors for viral activation. Many recent of researches add an evidence for the immuno-modulatory effects of phycocyanin and phycoerythrin. Phycobiliproteins are a small group of highly conserved chromoproteins that constitute the phycobilisome, a macromolecular protein complex whose main function is to serve as a light harvesting complex for the photosynthetic apparatus of cyanobacteria and eukaryotic groups. The most common classes of phycobiliproteins are allophycocyanin, phycocyanin and phycoerythrin all of which are formed of α and β protein subunits and carry different isomorphic linear tetapyrrrole prosthetic groups. C-phycocyanin (C-PC) is one of the major biliproteins of spirulina platensis, a blue green alga, with anti oxidant and radical scavenging properties. It is also known to exhibit anti inflammatory and anti cancer properties. However the main mechanism of action of C-PC is not clearly under stood. PC is composed of two dissimilar and . protein subunits of 17 000 and 19 500 Da, respectively, with one bilin chromophore attached to the α subunit (α84) and two to the . subunit (β84, β155). The chemical structure of the bilin chromophores in PC is very similar to bilirubin, which is considered to be a physiologically important antioxidant against reactive species. It inhibits oxidative modification of plasma proteins and aromatic amino acid residues.

In our work we found that the mean level of IFN-γ in the diseased sub groups (A, B and C) was higher than control group in resting condition. Also, the production of IFN-γ by PBMCs was higher in hepatitis patients than control group after addition of C-PC and phytohemagglutinin .This was in agreement with the results of Missale et al. and Tsai et al. who reported higher concentration of INF gamma in the serum of patients with chronic viral C hepatitis. In contrast to this result Jirillo et al. reported that a significant decrease of INF-γ was reported in patients with viral hepatitis C. These conflicting results may be due to changes in Th1 & Th2 responses among patients with chronic viral hepatitis C. Also it might be explained by the difference in study size. Also, this work showed that INF-γ production was higher in hepatic patient than in control group after addition of 5 µl and 10 µl of C-PC. This adds an evidence for the immunomodulatory effects of phycocyanin and supported by a group of researches from University of California-Davis who reported that Spirulina and its extracts stimulated the secretion of interleukin-1, LL-4 and interferon IFN-γ to nearly 2.0, 3.3, and 13.6 times basal levels respectively. Also they reported that induction of IFN-γ by Spirulina was found to be comparable to that seen after phytohemagglutinin (PHA) stimulation. In spite it was different in our results where induction of IFN-γ by phytohemag-glutinin was higher than that induced by phycocyanin. It may be corrected by increasing the dose of phycocyanin added to PBMC. Also in paper presented at the 30th annual meeting of the Japanese Society for immunology, Saeki et al. presented the results of a human study using spirulina extract and reported that IFN-γ secretion activity was enhanced significantly after two weeks of spirulina extract administration. Also he added that, surprisingly, IFN-γ and NK cell activities continued up to 6 months after administration of extract was discontinued.

In the present work , the mean level of IFN-γ production was increased in the diseased group after addition of 5µl and 10µl of phycocyanin but the increase was most obvious in child B followed by C & lastly A.

Although many human studies showed low level of IFN-γ in chronic HCV infection, our-
vitro study showed that PBMCNCS were still able to produce IFN-γ, even in advanced stage of liver disease, in resting state and after stimulation by Phycocyanin with different doses. This means that the body does not make enough natural interferon to effectively fight infection and the addition of exogenous help (Phycocyanin that stimulation PBMCNs to produce IFN-γ) may aid in fighting off the infection. The safety of spirulina and the high stimulatory effect of its constituent C-PC on IFN-γ production, encourage for further in-vivo study and on a large scale of people.

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تأثر الفيروسات على انتاج الأنتيرفرون جاما في مرضى
الالتهاب الكبدى الفيروسي المعظم (س) : دراسة خارج الجسم

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***قسم النبات كلية العلوم، جامعة الزقازيق.

يعد الآن أكثر من 10% من الأدوية المعضاة للسرطان والمضادات الحيوية منتجات طبيعية أو مشتقة من مصادر
طبيعية.

الفيروسات هي مركب طبيعي يحترف من الطحالب الزرقاء وقد أتاح له تأثيرات مختلفة كمضاد للالتهاب ومضاد
للأشكال. وحيدا قد ظهر الفيروسات نشط ضد الأورام.
الفيروسات يعتبر من مكونات سيبلونية باطنيز والتي تسمى الآن أورتوسيبا والتي طالما استخدمت كغذاء.

الهدف من الدراسة:
دراسة تأثير الفيروسات على انتاج الأنتيرفرون جاما من خلايا أحادية اللدوى في مرضى الالتهاب الكبدى الفيروسي
المزمن (س) دراسة خارج الجسم.

المتطلبات والملاحظات:
- شملت الدراسة على 40 شخصا من غير المصابين بأمراض الكلى والقلب وداء السكري ومرض ذاتية المنااعه أو
- يتناولون أي دواء يؤثر على مناعة الجسم.

وتم تضمينهم إلى:

مجموعة (أ) وشملت على 10 أشخاص من الأصحاء كمجموعة ضابطة.
- وظائف الكبد وشملت (أنتنزيمات الألبان، أمينوتراسفيراز، الفوسفاتيز القولوي) واسترات أمينوتراسفيراز – البيلوبين
- بالكامل – (ألبومين، وزن البروتين).
- الأعماق التكيفية على البطن بالإضافة إلى مقاس نسبة الانتيرفرون جاما قبل وبعد إضافة الفيروسات المنفرز من خلايا
- أحادية اللدوى تنذر علاج.

أوصيت نتائج الدراسة ما يلي:

1- محتوى الدم كانت
2- وظائف الكبد وشملت (أنتنزيمات الألبان، أمينوتراسفيراز – الفوسفاتيز القولوي، واسترات أمينوتراسفيراز – البيلوبين
- بالكامل، والألبومين، وزن البروتين).
3- الأعماق التكيفية على البطن بالإضافة إلى مقاس نسبة الانتيرفرون جاما قبل وبعد إضافة الفيروسات المنفرز من خلايا
- أحادية اللدوى تنذر علاج.

وأوضح نتائج الدراسة ما يلي:
1- في المجموعة الطبيعية وفQuiz: What is the main topic of the document? The document is about the effects of viruses on the production of interferon gamma in patients with alcoholic hepatitis. The study was conducted outside the body.

What is the main topic of the document? The document is about the effects of viruses on the production of interferon gamma in patients with alcoholic hepatitis. The study was conducted outside the body. The study was conducted outside the body.
في ضوء الدراسة الحالية نستخلص التوصيات الآتية:

1. الخلايا أحادية النوع لها القدرة على إنتاج الإنترفيرون جاما خارج الجسم ونحن ننصح بدراسات على الإنسان باعطاء الفيروسات تم قياس الإنترفيرون جاما في دم مرضى الإلتهاب الكبدي الفيروسي المزمن. 
2. إجراء محاولات لعطاء الفيروسات مع الإنترفيرون ألفا في مرضى الإلتهاب الكبدي المزمن أو للمرضى الغير مناسبين لإعطاء الإنترفيرون ألفا.
3. ولأن الفيروسات للقدرة على إنتاج الإنترفيرون جاما لذلك ممكن استخدامها كغذاء للمرضى والأصحاء وهو يعتبر من إحدى مكونات سيرولينا التي تتميز بدرجة من الأمان.