



## THE ROLE OF OXIDATIVE STRESS MARKERS IN B-THALASSEMIC IRAQI PATIENTS INFECTED WITH HEPATITIS C VIRUS DIAGNOSED BY RT-PCR

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### ABSTRACT

Blood transfusion is associated with the risk of infection, especially hepatitis C virus and may lead to peroxidative tissue injury by secondary iron overload in  $\beta$ - thalassemic patients. This study aims to detect HCV-RNA copies in thalassemic patients detected by RT-PCR qualitatively and quantitatively. Also, to measure the role of enzymatic and non-enzymatic anti-oxidants markers in  $\beta$ -thalassemia patients infected with HCV. A total of 200  $\beta$ -thalassemic patients were collected and analyzed for anti-HCV antibodies using Rapid immunochromatographic assay and enzyme-linked Immunosorbent assay methods. Then, the nucleic acids HCV-RNA of positive samples were extracted by modern automated technique. After that, Amplification for HCV-RNA was amplified. The vitamins C and, E in addition to GSH and GGT were also accurately detected. Out of 100 positive ELISA-anti-HCV antibodies, 72(72%) were also positive for RT-PCR considering the assay threshold for the procedure was  $>13$  IU/ ml. The mean viral load in these patients was  $545806 \pm 1009799$  IU/ml ( $1997176 \pm 3802206$ ) Copies/ml. There was an observed difference ( $P \leq 0.005$ ) inactivity of TSB, GGT, Protein, Albumin in sera of HCV patients ( $2.87 \pm 1.41$ ,  $51.1 \pm 60.0$ ,  $6.44 \pm 2.00$ ,  $3.37 \pm 0.71$ ) when compared with healthy subjects ( $0.99 \pm 0.46$ ;  $25.2 \pm 12.0$ ,  $7.09 \pm 0.66$ ,  $3.94 \pm 0.59$ ) respectively while the Globulin detected evinced no significant difference ( $P > 0.005$ ) in HCV patients ( $2.90 \pm 0.80$ ) as compared with healthy subjects ( $3.14 \pm 0.42$ ). Besides there was a significant difference ( $P \leq 0.005$ ) in serum vitamin C, vitamin E and GSH, between HCV patients groups ( $57.2 \pm 46.1$ ,  $34.7 \pm 14.6$ ,  $282.4 \pm 150.5$ ) and healthy subject groups ( $73.0 \pm 18.0$ ,  $44.6 \pm 15.6$ ,  $353.5 \pm 59.37$ ) respectively. The prevalence of HCV infection was much higher among  $\beta$ -thalassemic patients compared with the healthy blood donors. It is recommended to use nucleic acid based-tests for screening blood donors. The current study shows that there is no correlation between ELISA and viral load in hepatitis C virus infection. On the other hand, Real-Time PCR is a confirmatory diagnostic test and is considered as the golden test for the diagnosis and follow up of hepatitis C virus infection. The HCV infection significantly increases ALT, AST, ALP, GGT, and TSB level above the normal range while vitamins C, E, and GSH decreased considerably.

**KEYWORDS:**  *$\beta$ -thalassemia, Hepatitis C virus(HCV), RT-PCR, Anti-oxidants*



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## INTRODUCTION

Hepatitis C virus (HCV) is the substantial causative agent of post-transfusion hepatitis. The distribution of HCV throughout the world is found in varying prevalence rates, affecting about more than 3% of the world's population<sup>1</sup>. HCV infection is one of the principal causes of chronic liver disease worldwide<sup>2</sup>. The long-term natural history of HCV infection is highly variable, and hepatic injury can range from minimal histological changes to extensive fibrosis<sup>3</sup>. HCV infection is a major cause of advanced hepatic fibrosis and cirrhosis, with significantly increased risk for development of hepatocellular carcinoma(HCC)<sup>4</sup>. The hepatitis-C virus belongs to the family Flaviviridae. HCV is classified into seven distinct genotypes (1 to 7) on the basis of their nucleotide sequence<sup>5</sup>. HCV is the pathogen which transmitted parenterally, e.g., sharing of injections and use of contaminated medical equipment<sup>6</sup>. Beta thalassemia is an autosomal recessive hereditary disease that affects the synthesis of beta globin chains in hemoglobin. Beta thalassemia major causes severe anemia, hepatomegaly, splenomegaly, and acromegaly. Regular blood transfusion is a standard treatment for this disease<sup>1</sup>. Although blood transfusion can lead to patient survival in thalassemia, it can also increase the risk of blood-borne infections, such as HCV, hepatitis B virus (HBV) and human immunodeficiency virus (HIV)<sup>7</sup>. Antioxidant systems neutralize the harmful effects of the endogenous Reactive Oxygen Species (ROS) products. Reduced activities of ant-oxidative enzymes manifest oxidative stress present in patients with chronic hepatitis C (CHC). The combined antiviral therapy, which is lately considered to have antioxidant potential, leads to the restoration of antioxidant balance<sup>8</sup>. The occurrence of oxidative stress in HCV infection is explained by chronic inflammation, lipid peroxidation, iron deposition in the liver parenchyma, glutathione (GSH) level alteration, and the effect of non-structural and structural proteins of HCV (core protein, NS3, NS5A) on enzyme activity, mitochondria, and hepatocyte genes<sup>9</sup>. Vitamin E has been recognized as one of the most important antioxidants. Vitamin E inhibits the generation of lipid peroxyl radicals which are induced by free radicals, thereby protecting the cells from the peroxidation of polyunsaturated fatty acids in membrane phospholipids that results in the prevention of red cell hemolysis<sup>10</sup>. Many assays are used to diagnose HCV infected patients, but the measurement of HCV RNA levels has become an

essential part of the management in patients. The most common technique used for detection of HCV is Real-Time PCR assay which allows the detection of PCR amplifiers during the early phase of the reaction and provides a distinct advantage to detect precise PCR products at the end-point of the reaction<sup>11</sup>. Qualitative and quantitative methods for HCV RNA viral load investigations are used to diagnose chronic HCV infection. Further, the patients who need antiviral therapy are identified and the virological responses to antiviral treatment was also monitored<sup>12</sup>. Thus, the study has been undertaken for cut-off diagnosis for the presence or absences of HCV in thalassemic patients by real-time -PCR. Further, the role of vitamins, C and E were detected in addition to Glutathione peroxidases on hepatic damage in thalassemia patient with HCV.

## PATIENTS AND METHODS

A total of 200,  $\beta$ -thalassemia patients (96% male and 104% female) were included in this study. They were referred to the Hereditary Blood Disease Center, Al-Karama Teaching Hospital/ Baghdad. The age of study patients and healthy subjects ranged from 3-44 years with mean  $20 \pm 8.9$  who received regular blood transfusions from March to August 2017. All procedures performed in this study involving human participants were in accordance with the Ethical Approval Committee authentication no.31 on December 6, 2017 - University of Anbar. The study samples were divided into two groups: Group I patients of thalassemia ( $\beta$ -thalassemia patients received regular blood transfusion) composed of 100 patients (49% male and 51% female). All of them were infected with HCV. Group II consisted of patients of thalassemia ( $\beta$ -thalassemia patients received regular blood transfusion) composed of 100 patients (53% male and 47% female) and all of them were not infected with HCV. Group III. Healthy blood donors and randomly selected persons. This group composed of 100 people (50% male, 50% female) whose blood was collected from Iraqi National Center of Blood Transfusion and randomly chosen from the community. All exclusion criteria were applied to confirm their suitability for this group. They are healthy without symptoms of jaundice or chronic liver disease.

### *Serological investigations*

**Biochemical Investigations (Liver Enzymes)** The Celercare® M1 Chemistry Analyzer was used for

detection of liver enzymes which includes Alanine transaminase(ALT), Aspartate aminotransaminase(AST),Alkaline phosphatase(ALP), Total, serum bilirubin(TSB), Gamma-GlutamylTransferase (GGT)and Total protein(TP),(Albumin, Globulin) by using the liver function panel lyophilized kit (MNCHIP).

#### **Rapid immunochromatographic assay**

The detection of anti-HCV antibodies in the samples was performed by one step cassette, CTK, Biotech. Company. The positive or negative results for anti-HCV antibodies in the study specimen was determined by the appearance of specific colored line on the cassette<sup>13</sup>.

#### **Detection of Anti-HCV-Antibody by ELISA**

All serum samples were tested for the presence of antibodies to HCV with a commercial ELISA kit ((Human, Germany). Serum samples were added to these wells. If antibodies specific for HCV are present in the sample, they will form stable complexes with the HCV antigens on the well. A wash step extracted excess sample and a rabbit anti-human IgG conjugated with peroxidase is then added and allowed to incubate. The conjugate will bind to any formed complex of antigen-antibody. After a second wash, a solution of enzyme substrate and Chromogen is added. This solution will turn blue in color if the sample is positive. The blue color changes to yellow after blocking the reaction with sulphuric acid. The intensity of the color is measured by (ELISA reader, Awareness, USA) at 450 nm and it is proportional to anti HCV antibodies concentration in the sample. Wells containing negative samples remain colorless<sup>13</sup>.

#### **Molecular Part of study**

##### **A-Nucleic acids (HCV-RNA) Extraction**

SaMag Viral Nucleic Acids Extraction Kit was designed to be used with damage-12 automatic nucleic acid extraction system for the extraction of Viral RNA (HCV-RNA) from human biological specimens (plasma). The extraction process consisted of steps of lysis, binding, washing, and elution. The purification procedure was optimized for use with 150  $\mu$ l plasma. The frozen sample was thawed at room temperature (15–25°C), and the samples were processed immediately when they have equilibrated to the room temperature. The extraction was performed starting with the SaMag-12 instrument, as follow: cartridges and reaction chambers were inserted. The tip holders, piercing pins, small tip, filtered tips were inserted. Sample

tubes in the sample rack were also inserted, and 1.5 ml Elute tubes were embedded in the sample rack. Under a safe biological cabinet, samples were loaded in sample tubes. After that, the healthy internal subjects were added, and the sample rack was transferred into SaMag instrument. Then SaMag-12 door was closed, and the barcode was used to select Viral Nucleic Acids Extraction kit protocol either SEN-DNA or HCV-RNA virus<sup>14</sup>.

#### **HCV real-time PCR quantification**

A kit for HCV Real-TM Quant is a real-time test for quantitative detection of hepatitis C virus in human plasma. HCV RNA is extracted from plasma, amplified and detected using fluorescent reporter dye probes specific for HCV or HCV IC. Internal control serves as an extraction and amplification control for each individually processed specimen to identify possible inhibition was made . IC is detected in a particular and specific labeled channel other than the HCV RNA. Monitoring the fluorescence intensities during real-time allows the detection and quantification of the accumulating product without the need to open the reaction tube another time after the real-time amplification<sup>15</sup>.

#### **Reagent preparation for amplification**

One set of reagents was thawed and the tubes were vortexed and centrifuged briefly. Reaction tubes were prepared. Reaction Mix preparation was made as follow: 300 $\mu$ l of RT –PCR-mix-1, 200 $\mu$ l of RT –PCR-mix-2,20 $\mu$ l of host start Taq polymerase, and 10 $\mu$ l of M-MLV Reverse transcriptase was added to the tube with DTT. The contents were vortexed thoroughly and centrifuged briefly. In each sample (N) in the new sterile tube, 12.5 N  $\mu$ l of the mix,0.5 N  $\mu$ l of Taq F polymerase and 0.25 N  $\mu$ l of M-MLV were added. 12.5  $\mu$ l of reaction mix was added into each tube. Also, 2.5  $\mu$ l of the extracted RNA sample was added to the appropriate tube with reaction mix and mixed by pipetting all the tubes. Extracted RNA were re-centrifuged for 2 min at maximum speed 16000g and supernatant were taken care. For each run, 6 standards and 1 negative control were prepared as follows: 12.5  $\mu$ l of quantitation standards HCV (QS1 HCV, QS2 HCV, QS3 HCV) were added into 3 labeled tubes; 12.5  $\mu$ l of quantitation standards IC (QS1 IC, QS2 IC, QS3 IC) were added in to 3 labeled tubes. After that, 12.5  $\mu$ l of TE-buffer was added to the tube labeled negative control, and the containers were transferred into the real-time instrument. The program for smart cycler was as follows: Stage 1, 50 °C-1800 sec. (hold); stage 2, 90°C-900 sec;

stage 3 consisted of two temperature cycle (95 °C - 20 sec. Moreover, 60 °C-40 sec. Phase three was repeated for 42 times. The internal control(IC) was detected on the FAM channel and HCV RNA on the CY3 channel. For each control and patient specimen, the concentration of HCV RNA was calculated by using the following formula: HCV RNA copies/specimen(the CY3 channel)/ IC RNA copies/specimen (FAM channel) × coefficient = IU HCV/ml. To obtain the results expressed in copies/ml multiply the IU HCV/ml value by 4) as follow:-

$$\text{IU RNA HCV/mL} \times 4 = \text{copies RNA HCV / ml}^{14-15}.$$

**Serum Level of Vitamin C (Cat.No:YHB3202Hu), E (Cat.NoYHB3208Hu) also, GSH (Cat.No: YHB1369Hu): ELISA Kit (Shanghai, China)**

Enzyme-linked immunosorbent assay based on biotin double antibody sandwich technology to assay Human Vitamin C, E, and GSH. Vitamin C, E, and GSH were added to wells that are pre-coated with Vitamin C, E, and GSH monoclonal antibody and then incubated. After incubation, add anti-VC, VE, GSH antibodies labeled with biotin to unite with streptavidin-HRP, which forms the immune complex. Remove unbound enzymes after incubation and washing and then add substrate A and B. The solution will turn blue and change to yellow with the effect of acid. The shades of the solution and the concentration of Human Vitamins C, E, GSH are positively correlated.

## STATISTICAL ANALYSIS

Analysis of data was accomplished using the

available statistical package of SPSS-22 (Statistical Packages for Social Sciences-version 22). Data were presented in an essential analytical tool of frequency, percentage, mean, standard deviation, and range(minimum-maximum values). The significance of the difference between different means (quantitative data) was tested using the Student's t-test for the difference between two independent means. The Paired t-test for difference of paired observations or ANOVA test for difference among more than two independent means was achieved. The significance of the difference in different percentages (qualitative data) was tested using the Chi-square test ( $\beta$  -test) with the application of Yate's correction or Fisher Exact test whenever applicable. Statistical significance was considered whenever the P value for the analysis of importance was equal or less than 0.0516-17.

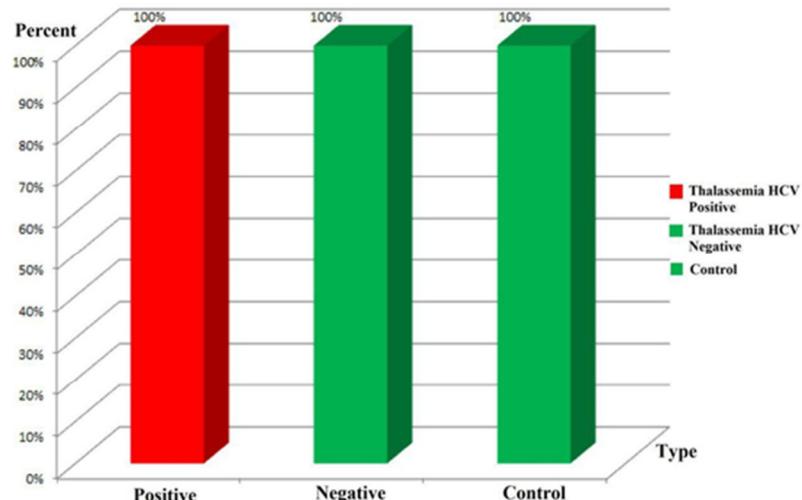
## RESULTS

### Rapid immunochromatographic assay result

One hundred patients with high suspicion of HCV infection revealed positive rapid immunochromatographic assay while the others (100 thalassemic patients and healthy subjects) yielded negative results with a high statistical difference ( $P < 0.0005$ ).

### Enzyme-linked immunosorbent assay result

Regarding the ELISA test, it was found that the results of the rapid immune chromatography assay agreed with those of ELISA in 100 cases. All individuals of Healthy Subjects Group revealed negative ELISA tests results for anti-hepatitis C virus antibody in their sera.



**Figure 1**

**Distribution of patients HCV in thalassemic and healthy subjects according to the ELISA test**

**Molecular RT-PCR result**

Regarding viral load, the quantity of virus, out of 100 positive ELISA-anti-HCV antibodies, 72(72%) was definite for RT- PCR considering the assay threshold for the procedure was  $>13$  IU/ml as shown in

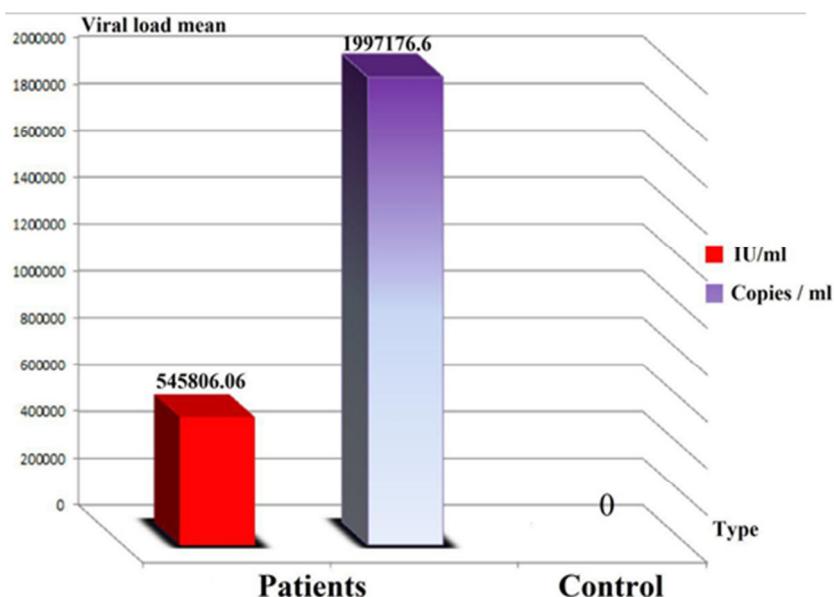
(Table1).RT-PCR versus ELISA results. The mean viral load in these patients was  $545806 \pm 1009799$  IU/ml ( $1997176 \pm 3802206$  Copies/ml) as shown in the following (Figure 2):-

**Table 1**

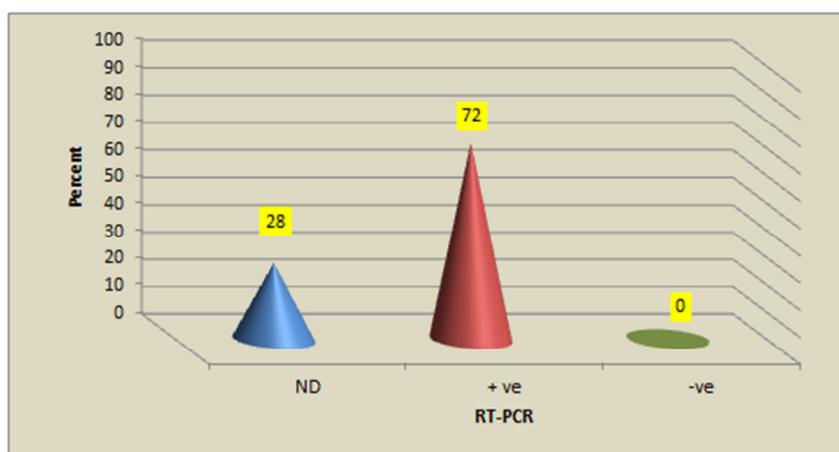
*Amplification results of HCV-genome by RT-PCR in patients with positive anti-HCV antibodies*

| Patients         | ELISA | RT-PCR |          |          | P-value |
|------------------|-------|--------|----------|----------|---------|
|                  |       | ND*    | Positive | Negative |         |
| Healthy subjects | +ve   | 28     | 72       | 0        | 100     |
|                  | -ve   | 0      | 0        | 100      | 100     |
|                  | Total | 28     | 72       | 100      | 200     |
|                  |       | 100.0% | 100.0%   | 100.0%   | 100.0%  |
|                  | ELISA | 0.00   | 0.00     | 100      | 100     |
|                  |       |        |          |          |         |
|                  | Total | 0.00   | 0.00     | 100      | 100     |
|                  |       |        |          |          |         |
|                  |       |        |          | 100.0%   | 100.0%  |

\* ND: Not detectable (equal or less than 13 copy/ml)



**Figure 2**  
*Viral load mean in positive HCV patients*



*Amplification results of HCV-genome by RT-PCR in patients with positive anti-HCV antibodies*

By using the ELISA test, the distribution of HCV +ve patients and healthy subjects groups according to their age was demonstrated. The ages of the patients and control groups were distributed between 1 year to 44 years. The more significant majority of the infected persons with HCV was in

the middle age groups (15-29) years old, while the lowest rate of infected persons was in the age group (30-44) years of old. Gender did not differ significantly among thalassemia patients with or without HCV infection and healthy subjects (Table2).

**Table 2**  
*Distribution of patients with suspicion of HCV infection and healthy subjects individuals among gender according to the result of ELISA*

| Gender           | ELISA    |          | P-value |
|------------------|----------|----------|---------|
|                  | Positive | Negative |         |
| Patients         | Female   | 51       | 104     |
|                  |          | 51.0%    | 52.0%   |
|                  | Male     | 49       | 96      |
|                  |          | 49.0%    | 48.0%   |
|                  | Total    | 100      | 200     |
|                  |          | 100.0%   | 100.0%  |
| Healthy subjects | Female   | 0.00     | 50      |
|                  |          | 0.00     | 50.0%   |
|                  | Male     | 0.00     | 50      |
|                  |          | 0.00     | 50.0%   |
|                  | Total    | 0.00     | 100     |
|                  |          | 0.00     | 100.0%  |

In the present study, there was a significant difference ( $P \leq 0.005$ ) found in the inactivity of ALT, AST, ALK enzyme in the sera of HCV patients ( $59.0 \pm 21.4$ ,  $54.5 \pm 33.4$ ,  $131.3 \pm 60.9$ ) as compared to the healthy subjects ( $25.6 \pm 8.0$ ,  $20.1 \pm 6.9$ ,  $74.1 \pm 27.7$ ). A significant difference ( $P \leq 0.005$ ) in the inactivity of TSB, GGT Protein, Albumin in sera of HCV patients ( $2.87 \pm 1.41$ ,

$51.1 \pm 60.0$ ,  $6.44 \pm 2.00$ ,  $3.37 \pm 0.71$ ) was observed when compared to the healthy subjects ( $0.99 \pm 0.46$ ,  $25.2 \pm 12.0$ ,  $7.09 \pm 0.66$ ,  $3.94 \pm 0.59$ ) while the Globulin detected that there was no significant difference ( $P > 0.005$ ) in HCV patients ( $2.90 \pm 0.80$ ) when compared to healthy subjects ( $3.14 \pm 0.42$ ) as shown in Table 3.

**Table 3**  
*Indices of liver function status (mean  $\pm$  SD) of HCV positive, negative patients, and Healthy subjects*

| Parameters | HCV- positive    | HCV-negative     | p-value | Healthy subject | P-value |
|------------|------------------|------------------|---------|-----------------|---------|
| ALT(U/L)   | $59.0 \pm 21.4$  | $51.5 \pm 22.3$  | 0.419   | $25.6 \pm 8.0$  | 0.000   |
| AST(U/L)   | $54.5 \pm 33.4$  | $47.24 \pm 14.2$ | 0.321   | $20.1 \pm 6.9$  | 0.000   |
| ALP(U/L)   | $131.3 \pm 60.9$ | $118.6 \pm 37.1$ | 0.611   | $74.1 \pm 27.7$ | 0.000   |
| TSB(mg/dl) | $2.87 \pm 1.41$  | $1.9 \pm 1.07$   | 0.001   | $0.99 \pm 0.46$ | 0.001   |
| GGT(U/L)   | $51.1 \pm 60.0$  | $47.5 \pm 60.34$ | 0.099   | $25.2 \pm 12.0$ | 0.000   |
| TP(g/dl)   | $6.44 \pm 2.00$  | $6.66 \pm 0.82$  | 0.303   | $7.09 \pm 0.66$ | 0.000   |
| ALB(g/dl)  | $3.37 \pm 0.71$  | $3.71 \pm 0.74$  | 0.013   | $3.94 \pm 0.59$ | 0.001   |
| GLO(g/dl)  | $2.90 \pm 0.80$  | $2.95 \pm 0.63$  | 0.728   | $3.14 \pm 0.42$ | 0.013   |

*Normal value: ALT <40 U/L; AST <40 U/L; ALP <125 U/L; GGT <50 U/L; TSB <1.46 mg/dl; TP 6-8.3 g/dl; ALB 3.5-5.5 g/dl; GLO 2-4 g/dl.*

In the present study, there is a significant difference ( $P \leq 0.005$ ) in serum vitamin C, vitamin E and GSH, between HCV patients groups ( $57.2$

$\pm 46.1$ ,  $34.7 \pm 14.6$ ,  $282.4 \pm 150.5$ ) and healthy subject groups ( $73.0 \pm 18.0$ ,  $44.6 \pm 15.6$ ,  $353.5 \pm 59.37$ ) respectively.

**Table 4**  
***The mean and standard deviation of VC, VE, and GSH in thalassemia patients and healthy subject's subgroups***

| Parameters | HCV- positive     | HCV- negative    | P-value | Healthy subject   | P-value |
|------------|-------------------|------------------|---------|-------------------|---------|
| V.C        | 57.2 $\pm$ 46.1   | 60.34 $\pm$ 28.4 | 0.564   | 73.0 $\pm$ 18.0   | 0.000   |
| V.E        | 34.7 $\pm$ 14.6   | 30.48 $\pm$ 13.2 | 0.031   | 44.6 $\pm$ 15.6   | 0.000   |
| GSH        | 282.4 $\pm$ 150.5 | 210.5 $\pm$ 74.1 | 0.000   | 353.5 $\pm$ 59.37 | 0.000   |

## DISCUSSION

HCV infection has gained importance, mainly as a significant complication in patients with multiple transfusion during the last decade. This is especially true in countries where HCV is most ubiquitous in the general population and therefore also amongst blood donors. At a global level, between 4.4 % and 85.4 % of the thalassemia patients are positive for anti-HCV antibodies<sup>18</sup>. The distribution of patients according to age were as follow: 73.0% were in the range of (15-29) years; 18.0% in the range (1-14) years and 9% in the range(30-44) years. In our study, the mean age of thalassaemic patients with anti-HCV was 20 $\pm$ 7.0 years. The present results revealed that the mean age groups were significantly higher in patients with positive HCV antibody compared to negative subjects. The higher rate of HCV infection in older patients may reflect more frequency of transfusion and revealed the importance of providing safe blood for reducing the incidence of HCV infection in thalassemic population<sup>19</sup>. There are apparent differences in the distribution of thalassemia patients infected with hepatitis C according to the age groups. The highest percent of incidence in the age group (15–29 years) as it was estimated at 73% while the lowest percentage is in the age groups (30-44). This may be because early detection of the disease gives more significant opportunity for treatment and cure. Also, the existence of infection for a long time leads to compromising passive immunity and deteriorating the liver state. These results come in contrast with the findings of the researcher Abdul-Sada<sup>20</sup> who reported that the incidence of infection increases with older age groups, as the highest percent was 4.29 % for the age group  $\leq$ 49 years while the lowest percentage was 2 % for the age group  $\leq$ 12 years for Hepatitis C patients in An Najaf Al Ashraf governorate. The prevalence rate of HCV RNA in Iraq is 53.2% among Iraqi anti-HCV positive thalassemia patients<sup>21</sup>. Al-Shabany<sup>21</sup> found that out of 69 samples, 25(36%) samples of thalassemic patients were infected with Hepatitis C virus AL-Najaf AL-Ashraf. Out of 100 positive sample by the rapid

immunochromatographic assay, 100 (100%) were positive by ELISA and 72(72 %) of them showed positive RT- PCR. In our study, those patients who were considered harmful RT-PCR result which represented in 28(28%) as non-detectable. This may be due to the quantity may be below threshold limit (13 IU/ml). Indicate the resolution of HCV, acute HCV during the period of low-viremia or False positive ELISA for an anti-HCV antibody<sup>23</sup>. However, the results of the two techniques RT-PCR and ELISA should be interpreted with caution because, during infection, when the virus is cleaned up, only the antibody remains positive, and the nucleic acids are generally not detected<sup>24</sup>. Therefore, it appears that initially, a patient may be HCV positive by all tests but clearing up of virus from the serum later and becomes serum PCR negative, yet remains antibody positive and liver PCR positive or these patients may suffer from hepatitis C at the chronic phase of infection, existing antibody without any viruses<sup>25</sup>. The present study agrees with a study mentioned that the false positivity of ELISA had been well documented, Al-Jabali<sup>26</sup> that showed the prevalence of HCV infection in 45 thalassemic patients, 29 positive for RT-PCR tests in Baghdad, Kareem and Salih<sup>27</sup>, in Al-Sulaymaniyah, Al-Mola<sup>28</sup>, and Al-Haris<sup>29</sup> in Al-Najaf. The present study was consistent with another study that showed false-positive ELISA tests for anti HCV which can be seen in patients who have cleared the virus after acute infection by therapy and this might be positive on ELISA which may indicate past infection. Few other authors have also concluded that additional accuracy of antibody screening and confirmatory assays and standardization of molecular testing are necessary to optimize testing and fully characterize the diagnosis of HCV infection<sup>30</sup>. The results of both ELISA and RT-PCR revealed a significant difference between healthy subjects individuals and HCV patients. These findings indicate that both are sufficient for the diagnosis of HCV infection in clinical laboratories in Iraq. In this study, PCR helps to resolve weakly positive or negative ELISA results when clinical signs and/or risk factors are compatible with HCV

infection. Here, the antibody was only detected 1-2 weeks after infection, which reflected the immune response of the host but could not explain the virus replication<sup>31</sup>. In the present study it can be concluded that the RT-PCR was a confirmatory test with higher sensitivity and specificity 100% and 100% respectively, and it was considered a rapid, accurate, and reproducible method indicating that it is not only suitable for clinical diagnosis but also suitable for the screening of HCV to prevent the transmission of this disease. Despite occasional false positive results of ELISA tests, it has many advantages in the diagnostic setting including ease of automation, easy to use, relative cost-effectiveness, and low variability. Additional confirmatory testing is often helpful, and it is better like RT- PCR and should be used in all cases of ELISA positive patient at which the infection either recent or past and before initiating antiviral therapy can be detected. HCV infection clearance/persistence should be evaluated by RT-PCR, as the antibody persists for a longer duration after the virus is cleared<sup>32</sup>. It is suggested that the detection of HCV RNA by RT-PCR has better diagnostic value than the anti-HCV antibody test alone and HCV RNA detection in liver tissue is possible even when it is absent in the serum<sup>33</sup>. There were significant elevations in the liver enzyme in thalassemia patients with an HCV positive as compared with a healthy subject. ALT levels had significantly been affected by HCV infection, and this describes the fact of HCV infection on the liver. The other high results of ALT in thalassemia HCV RNA negative may have resulted from the same cause of iron overload and iron chelating drugs. On the other hand, in the present study, there was a significant increase in the activity of AST enzyme between groups (HCV RNA positive of thalassemia patients and healthy subjects). This was a result of virus multiplication, and other high effects of AST in HCV RNA negative thalassemia patients might result from the chronic blood transfusion and iron overload with iron chelating drugs that the thalassemia patients should have them to manage the iron overload condition<sup>34</sup>. On the other hand, ALP significantly increased in HCV patients ( $131.3 \pm 60.9$ ), and in comparison with healthy subjects group ( $74.1 \pm 27.7$ ). These results were found to have consisted with the study elsewhere that found there is evidence for increases in the activity of alkaline phosphatase in HCV positive patients<sup>35</sup>. In the present study, there was an observed increase in the activity of GGT enzyme in sera of HCV patients as compared to the healthy subjects,  $P < 0.00$ . Gamma-glutamyltransferase

(GGT) has been widely used as an index of liver dysfunction and marker of high alcohol consumption, obstructive liver diseases, and use of enzyme-inducing drugs, lead to increased free radical production and the threat of glutathione depletion. Also, GGT has been known as one of the markers of oxidative stress because it is an enzyme playing an essential role in the extracellular catabolism of GSH as an indicator of intracellular antioxidant<sup>36</sup>. Serum GGT might also be associated with the development of HCC more generally, as well as individually, in patients with chronic hepatitis C. In the present study, the mean level vitamins C and E in addition to Glutathione peroxidase was significantly decreased in thalassemia group among cases with HCV patients compared to the healthy subject. Our patients, especially those with HCV infection, did have a significant depletion of vitamins E and C, indicating that iron overload and HCV infection were more influential than supplementing these nutritional factors in transfusion-dependent thalassemia major patients. HCV infection is an important cause of liver damage especially in the thalassemic patient with multi-blood transfusion; the HCV-related liver damage is characterized by increased iron storage (possibly induced by the virus), which elicits free-radical-mediated peroxidation<sup>37</sup>. A considerable decrease of arachidonic acid and other polyunsaturated fatty acids of the red blood cell membrane, and a decreased level of plasma vitamin E in cirrhotic patients with HCV infection suggest that stimulated polyunsaturated fatty acids oxidation may be associated with the progression of HCV infection in chronic liver disease<sup>38</sup>. At the same time, liver iron storage may be necessary for determining the prognosis of HCV-related chronic hepatitis<sup>39</sup>. In our study, levels of vitamins E and C were significantly lower in the significant thalassemia patients with HCV infection. Sousa and associates<sup>40</sup> reported in study evaluated the involvement of polymorphisms of the antioxidant enzymes Catalase (CAT) and glutathione peroxidase (GSH) in liver fibrosis and hepatocellular carcinoma (HCC) in patients with HCV. It was observed that the GSH polymorphism, alone or combined with the CAT, was associated with a high risk of fibrosis severity and HCC. Also, GSH polymorphism was associated with advanced stages of HCC. In patients with HCV, a marked depression was noted in the GSH activity, the critical enzyme in the process of utilization of the active forms of oxygen and the products of peroxidation, which play a pivotal part in defense of the cell membrane structures<sup>41</sup>. In their study on

Egyptian thalassemia major(TM) patients, Tolba and associates<sup>42</sup> 2015 found decreased antioxidants level in the serum along with hypertriglyceridemia, hypercholesterolemia, low HDL levels, and also increased malondialdehyde level so that all these factors contributing to the development of atherosclerosis in TM patients. Choudhary and associates<sup>43</sup> reported a deficiency in levels of reduced glutathione in thalassemia of western Rajasthan, India which was lower than in healthy subjects. These results go hand in hand with the previous study, which suggested that glutathione (GSH) is a principal intracellular reducing agent, which is very sensitive to oxidative pressure and has several essential functions such as protection against oxidative stress, gene expression regulation, induction of apoptosis activation and proliferation in T lymphocytes. According to some authors, during the replication of HCV, the amounts of MN, SOD, hemeoxygenase, CAT and GSH are increase as an adaptive response to non-structural proteins of HCV. In contrast, some researchers claim that intracellular GSH status does not change during replication and that the level of ROS is not high enough to initiate an adaptive increase in GSH. However, oxidative damage is characterized by reduced levels of GSH, and up regulation of antioxidative enzymes such as CAT, GPx and SOD, according to most authors<sup>44</sup>.

## CONCLUSIONS

The study suggested that the prevalence of HCV infection was much higher among  $\beta$ -thalassemic patients compared to the healthy blood donors. Seropositivity. Routine Screening of donated blood for HCV is highly recommended, and screening programs for blood donors in blood transfusion

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centers can be useful, it is recommended to use nucleic acid tests for screening blood donors. Also, the current study concluded that there is no correlation between ELISA and viral load in hepatitis C virus infection ( $P<0.05$ ). It also indicated that seropositivity does not reveal the presence of active HCV infection. On the other hand, Real-Time PCR is a confirmatory diagnostic test and is considered as the golden test for the diagnosis and followup of hepatitis C virus infection. HCV infection significantly increases ALT, AST, ALP, GGT, and TSB level above the normal range while vitamin C, E, and GSH showed decreased considerably.

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## AUTHORS CONTRIBUTION STATEMENT

Sahar K. Al-Ani achieved biochemical analysis, experimental data, and all other analysis. Prof. Dr. Mushtak T.S. Al-Ouqaili performed research plan and design in addition to his great effort in molecular part of the research and supervised on this work. Also, Dr. Muthanna M. Awad contribute and help in the physiological part.

## CONFLICT OF INTEREST

Conflict of interest declared none.

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