

Preliminary Studies on Nitric Oxide (NO), Tumor Necrosis Factor Alpha (TNF- α), Intercellular Adhesion Molecule-1 (ICAM-1) and AFP Levels in Serum of Egyptian Farmers Infected with Hepatitis B and C Viruses and Hepatocellular Carcinoma (HCC) Patients

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ABSTRACT

Serum levels of Nitric Oxide (NO), Tumor Necrosis Factor Alpha (TNF- α), Intercellular Adhesion Molecule-1 (ICAM-1) and AFP were evaluated among two groups of Egyptian farmers. Group one was composed of 37 farmers infected with HCV and HBV while group two included 10 HCC patients, hospitalized at NCI. Sera of 10 healthy individuals were used as a control group. Blood samples were collected from 37 farmers resident in El-Sharkia Governorate-Egypt, tested for HBsAg, HCV-antibody by ELISA and HCV by RT-PCR and divided into three subgroups; HBsAg positive group (n=10), HCV-antibody positive and HCV-RNA negative group (n=17) and HCV-antibody and HCV-RNA positive group (n=10). Measurement of serum NO, TNF- α , ICAM-1, AFP, AST, ALT and albumin as well as abdominal ultrasonography examination of the liver were done for all groups. There was a significant increase in NO, TNF- α , ICAM and AFP in HCC positive group, and in NO and ICAM-1 in HBsAg positive group. HCV-antibody seropositive group had a significant increase in TNF- α , ICAM-1 and AFP, while the other HCV-antibody seropositive and HCV-RNA positive group had a significant increase in ICAM-1, AFP, NO and TNF- α . The present results showed significant correlation between NO and ICAM-1 ($r = 0.855$, $p = 0.002$) and between AFP and hepatomegaly percentage ($r = 0.764$, $p = 0.001$) in the group of patients infected with HCV and positive for both antibodies and HCV-RNA. Also this group of patients exhibited high percentage of liver cirrhosis (40%) obtained by ultrasonography examination than the other groups. In Conclusion: The elevation of AFP and its correlation to percentage of the hepatomegaly among group of patients infected with HCV and positive for both antibodies and HCV-RNA as well as the high percentage of cirrhosis observed by ultrasonography examination and the positive correlation levels of NO and ICAM-1 in this group of patients led us to conclude that patients infected with HCV and positive for both antibodies and RT-PCR were strongly associated with HCC among Egyptian farm-

ers compared to others infected with HBV and/or HCV and positive only for antibodies and negative for RT-PCR.

Key Words: *HBV - HCV - HCC - Egypt.*

INTRODUCTION

Viral hepatitis belongs to the most important infectious diseases. Worldwide, more than 300 million chronic hepatitis B surface antigen (HBsAg) and hepatitis C virus carriers exist [27]. In Egypt, viral hepatitis B and C are endemic with high prevalence rate, however the role of virus infection in the pathogenesis of liver diseases is probably much more important than schistosomal infection [8,9].

Hepatitis B, C and D often induce chronic progressive disease including liver cirrhosis with typical complications due to the portal hypertension and with a high rate of association with the development of primary liver cancer [27].

HCC is one of the most common cancers worldwide [17]. Different etiologic factors such as hepatitis viral infection, alcohol, aflatoxin and chemical carcinogens were mentioned in relation to HCC. However, the global distribution of HCC is strongly linked to the prevalence of hepatitis virus infection. The exact pathogenic mechanisms involved in viral-associate HCC are unclear although direct and indirect mechanisms are possible [31].

It is well known that NO, a recently discov-

ered free radical is overproduced in liver cirrhosis and HCC [2,15] and causes DNA damage and mutation in several cell types [16]. NO is an inorganic free-radical gas synthesized from the amino acid L-arginine by a family of isoenzymes called NO synthases (NOS). Two of these are constitutively expressed and a third is inducible by immunological stimuli. NO released by the constitutive enzymes acts as an important signaling molecule in the cardiovascular and nervous systems [14]. NO released by the inducible NO synthase (iNOS) is generated for long periods by cells that have cytostatic/cytotoxic properties for a variety of microorganisms as well as tumor cells [12].

Interestingly, cytokine-mediated death of tumor cells could be achieved through endogenous NO release from tumor cell or as exogenous NO from activated macrophages and endothelial cells [29]. However, NO can induce mRNA for TNF- α in leukemic cells which promotes their morphologic differentiation [28]. TNF- α is an important mediator in the pathogenesis of liver necrosis and failure of microcirculation in HCV and/or HBV infection [32].

ICAM-1 is a useful marker for the determination of the severity of liver disease and hepatic fibrosis, a marker for HCC progression and prognosis and is useful for monitoring the response of disease to treatment [10,23]. It was established that AFP is a relatively specific marker for HCC [33].

Since NO, TNF- α , ICAM-1 were proposed as markers of cell-mediated immunity in addition to AFP, thus we assessed these markers in sera of farmers living in El-Sharkia Governorate and suffering from HBV and HCV infections in addition to HCC patients and control healthy subjects. We compared their results with the liver function tests (AST-ALT and albumin) and ultrasonography findings, to assess the possible relation of HBV and HCV infections to HCC among the Egyptian farmers.

PATIENTS AND METHODS

Fifty seven adult individuals were included in this study. Blood samples were collected from the National Cancer Institute, Cairo University (HCC group) and from the farmers living at Al-Ghazaly and Al-Hesamia regions in El-Sharkia Governorate Egypt (other groups). sera were subjected to the analysis of HBsAg

and anti-HCV antibody by enzyme-linked immunosorbant assay (ELISA) whereas the HCV-RNA was assayed by reverse transcriptase polymerase chain reaction (RT-PCR) and the patients were classified to the following groups:

Group I : Included 10 healthy individuals.

Group II : Included 10 patients with HCC.

Group III: Included 10 patients with HBsAg positivity.

Group IV: Included 10 patients with HCV infection, positive for both antibodies and HCV-RNA by RT-PCR.

Group V: Included 17 patients with HCV infections, positive for antibodies and negative for HCV-RNA by RT-PCR.

All groups were subjected to full clinical assessment, estimation of serum levels of NO, TNF- α , ICAM-1, AFP, AST, ALT and albumin, as well as abdominal ultrasonography.

Methods:

- 1- HCV antibody assay: Determination of hepatitis C (HCV) antibodies was done by the method of Ellner and Neu [7], using kits of Equipar-Italy.
- 2- HBsAg assay: Hepatitis B surface antigen assay was done according to the method of Boniolo et al. [3], using kits of Diasorin-Italy.
- 3- Polymerase chain reaction: HCV-RNA extraction was done according to the QIAamp viral RNA Mini spin protocol (Oncogene Science, USA), amplification of HCV-RNA by RT-PCR was done in 50 μ l final volume using one step RT-PCR system (Gibco) containing: 1x reaction (buffer containing 0.2 mM of each dNTPs and 1.2 mM MgSO₄), superscript II RT-Taq mix, 100 ng sense primer GTG AGG AAC TAC TGT CTT CAC G (nt 47-68), and 100 ng antisense primer ACT GCG AAG CAC CCT ATC CCT ATC AGG (nt 292-312), Ravaggi et al. [19]. The thermocycler was programmed that allow cDNA synthesis followed immediately with RT-PCR amplification automatically. The following cycling conditions were established using a DNA thermal cycler; 50°C for 45 min for one cycle followed by 94°C for 2 min for cDNA synthesis and pre-denaturation then 94°C for 1 min, 55°C for 1

min and 72°C for 2, min for 35 cycles. The PCR product was analyzed on 2% agarose gel [22].

- 4- Nitric oxide measurement: Nitrate concentration in serum as a stable end product of nitric oxide was determined photometrically on microtitre plate by the method of Snyder [25], using kits of Roche Diagnostics-Germany.
- 5- TNF- ∞ measurement: TNF- ∞ was measured by a sandwich enzyme-immunoassay in microtitre plate according to the method of Corti et al. [5], using kits of Immunotech Company, France.
- 6- ICAM-1 measurement: ICAM-1 was measured in serum by a sandwich immunoenzymatic assay in microtitre plate according to the method of Rothlein et al. [21], using kits of Immunotech Company, France.
- 7- AFP: was measured by enzyme-immunoassay in microtitre plate by Abelev method [1], using kits of omega Diagnostics Limited-(UK).
- 8- AST and ALT activities: were carried out according to Reitman and Frankel [20], while serum albumin was estimated according to the method of Drupt [6].
- 9- Ultrasonographic examination: all the sonographic examinations had been carried out using RT-X200, prob 3.5 MHZ convex of General Electric company-USA.
- 10- Statistical analysis: the data obtained were presented in tables as mean \pm standard error. The difference between groups was calculated according to unpaired "t" test [24]. Sensitivity, specificity and cut-off values were calculated according to Sox et al. [26].

RESULTS

Serum levels of NO, TNF- ∞ , ICAN-1 and AFP for the five groups under investigation were presented in Table (1). The control healthy subjects group, included 10 individuals with a mean age of 37 \pm 2.2 years (6 males, 4 females). In the control group, the mean levels of NO, TNF- ∞ , ICAM-1 and AFP were 31.3 \pm 2.02 nmol/l, 34.8 \pm 2.14 pg/ml, 264 \pm 4.32 ng/ml and 2.2 \pm 0.91 ng/ml respectively.

As for the HCC group (n=10, mean age 53.1 \pm 1.6 years and included 7 males, 3 fe-

males), the mean levels of NO, TNF- ∞ , ICAM-1 and AFP were 77.1 \pm 8.63 nmol/l, 78.1 \pm 4.31 pg/ml, 324 \pm 12.12 ng/ml and 283 \pm 10.72 ng/ml respectively. These levels were statistically significantly increased compared with control group.

Concerning HBsAg seropositive group (n=10, mean age 36.1 \pm 3.9 years and included 8 males and 2 females), the mean levels of NO, TNF- ∞ , ICAM-1 and AFP were 48.2 \pm 4.73 nmol/l, 39.7 \pm 4.17 pg/ml, 287.6 \pm 3.01 ng/ml and 3.8 \pm 0.45 ng/ml respectively. Statistically, (this group) showed a significant increase in NO and ICAM-1 levels when compared to control group.

Regarding HCV antibodies seropositive cases (n=17) that were negative for HCV-RNA by RT-PCR and included 10 males and 7 females with a mean age of 38.6 \pm 2.4 year. The mean levels of NO, TNF- ∞ , ICAM1 and AFP were 31.8 \pm 1.19 nmol/l, 44.9 \pm 3.33 pg/ml, 312 \pm 3.32 ng/ml and 4.2 \pm 0.29 ng/ml respectively. These results exhibited a significant increase in TNF- ∞ , ICAM-1 and AFP levels when compared to control group, whereas NO level did not show significant change.

HCV antibodies seropositive and HCV-RNA positive group (n=10) obtained in Table (1) and Fig. (1), had a mean age of 38.2 \pm 1.3 years and included 8 males and 2 females. The mean levels of NO, TNF- ∞ , ICAM-1 and AFP were 37 \pm 1.64 nmol/L 42.8 \pm 3.59 pg/ml, 312 \pm 5.78 ng/ml and 14.6 \pm 2.51 ng/ml respectively. Statistically these results exhibited a significant increase in levels of NO, TNF- ∞ , ICAM and AFP.

Concerning liver function tests of all the groups, the results were presented in Table (2). In the control group the mean levels of AST, ALT and albumin were 6.8 \pm 0.57 U/L, 8.0 \pm 0.41 U/L and 4.5 g/dl respectively. In HCC group the mean levels of AST, ALT and albumin were 34.1 \pm 7.0 6U/L, 24.2 \pm 3.33 U/L and 2.9 \pm 0.09 g/dl respectively.

About HBsAg group, the mean levels of AST, ALT and albumin were 16.8 \pm 2.38 U/L, 17.7 \pm 1.69 U/L and 3.2 \pm 0.14 g/dL respectively. In case of HCV-antibody seropositive group that were negative for HCV-RNA by RT-PCR,

the mean levels of AST, ALT and albumin were 12.6 ± 1.69 U/L 13.4 ± 0.86 U/L 3.69 ± 0.07 g/dl respectively. As regards HCV-antibody seropositive and HCV-RNA positive group, the mean levels of AST, ALT and albumin were 13.8 ± 2.11 U/L, 19.2 ± 1.42 U/L and 3.5 ± 0.15 g/dl respectively. Statistically, all the previous groups exhibited significant increase in AST, ALT activity levels and significant decrease in serum albumin compared to control group.

Ultrasonography findings of all groups were presented in Table (3). The data obtained indi-

cated that HCC patients included 10%, 0%, 0%, 10% and 90% for hepatomegaly, splenomegaly, portal fibrosis, cirrhosis and focal lesion, respectively. While HBsAg group, the percentages were 40%, 20%, 0%, 10% and 10% respectively. In the group of patients with HCV-antibody seropositive and negative for HCV-RNA By RT-PCR, the percentages were 35.3%, 11.8%, 17.7%, 0% and 0%, respectively. As for the group of patients with HCV-antibody seropositive and HCV-RNA positive the percentages were 30%, 40%, 10%, 40% and 0% respectively.

Tale (1): Serum levels of NO, TNF- α , ICAM-1 and AFP in HCC and viral hepatitis groups.

Groups	NO nmol/L	TNF- α pg/ml	ICAM-1 ng/ml	AFP ng/ml
Control	31.3 ± 2.02 (100%)	34.8 ± 2.14 (100%)	264.0 ± 4.32 (100%)	2.2 ± 0.91 (100%)
HCC	$77.1 \pm 8.63^*$ (227%)	78.1 ± 4.31 (224%)	$324.0 \pm 12.12^*$ (123%)	$283.0 \pm 10.72^*$ (12863%)
HBsAg	$48.2 \pm 4.73^*$ (154%)	39.7 ± 4.17 (114%)	$287.6 \pm 3.01^*$ (109%)	3.8 ± 0.45 (173%)
HCV-Ab +ve HCV-RNA -ve	31.9 ± 1.19 (102%)	$44.9.0 \pm 3.33^*$ (129%)	$312.0 \pm 3.32^*$ (118%)	$4.2 \pm 0.29^*$ (191%)
HCV-Ab +ve HCV-RNA +ve	$37.0 \pm 1.64^*$ (119%)	$42.8 \pm 3.59^*$ (123%)	$312.0 \pm 5.78^*$ (118%)	$14.6 \pm 2.51^*$ (730%)

() Value in percent taking control as 100%.

* = Significant difference as compared with control ($p < 0.05$).

Tale (2): Liver function tests in HCC and viral hepatitis groups.

Groups	AST U/L	ALT U/L	Albumin g/dl
Control	6.8 ± 0.57 (100%)	8.0 ± 0.41 (100%)	4.5 ± 0.18 (100%)
HCC	$34.1 \pm 7.06^*$ (501%)	$24.2 \pm 3.33^*$ (302%)	$2.9 \pm 0.09^*$ (64%)
HBsAg	$16.8 \pm 2.38^*$ (247%)	$17.7 \pm 1.69^*$ (221%)	$3.2 \pm 0.14^*$ (71%)
HCV-Ab +ve HCV-RNA -ve	$12.6 \pm 1.69^*$ (185%)	$13.4 \pm 0.86^*$ (167%)	$3.69 \pm 0.07^*$ (82%)
HCV-Ab +ve HCV-RNA +ve	$13.8 \pm 2.11^*$ (203%)	$19.2 \pm 14.2^*$ (240%)	$3.5 \pm 0.15^*$ (78%)

() Value in percent taking control as 100%.

* = Significant difference as compared with control ($p < 0.05$).

Tale (3): Ultrasonographic findings of patients with viral hepatitis and HCC.

Group	Hepatomegaly	Splenomegaly	Portal fibrosis	Cirrhosis	Focal lesions
HCC	1 (10%)	0 (0%)	0 (0%)	10 (10%)	9 (90%)
HbsAg	4 (40%)	2 (20%)	0 (0%)	1 (10%)	1 (10%)
HCV-Ab +ve HCV-RT-PCR -ve	6 (35.3%)	2 (11.8%)	3 (17.7%)	0 (0%)	0 (0%)
HCV-Ab +ve HCV-RT-PCR +ve	3 (30%)	4 (40%)	1 (10%)	4 (40%)	0 (0%)

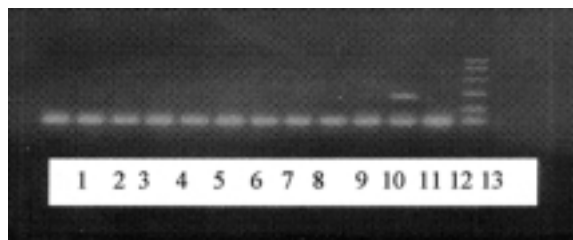


Fig. (1): Agarose gel electrophoresis analysis of HCV-RNA, RT-PCR product lane 13 is PCR marker, lane 11 is positive.

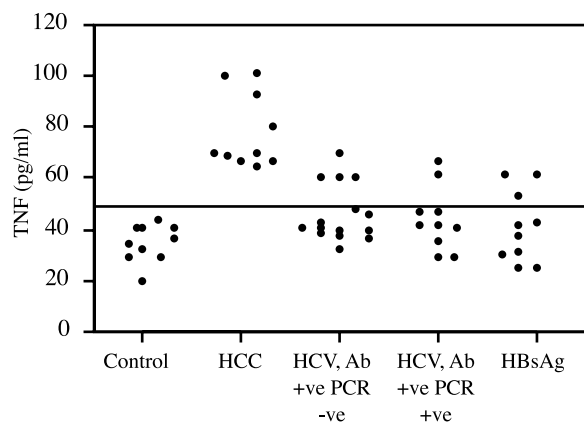


Fig. (3): Scatter diagram of TNF- α (pg/ml) levels in all the studied groups. The horizontal line represents the cut-off value 43.48 pg/ml (mean \pm 2SD of controls). The sensitivity is 100%, 35.3%, 40% and 30% for the four groups respectively at a specificity level of 100%.

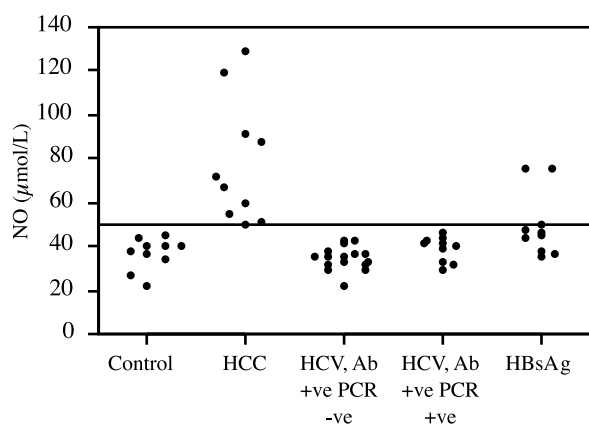


Fig. (2): Scatter diagram of NO (μ mol/L) levels in all the studied groups. The horizontal line represents the cut-off value 44.12 μ mol/L (mean \pm 2SD of controls). The sensitivity is 100%, 7.14%, 4.34% and 9.09% for the four groups respectively at a specificity level of 100%.

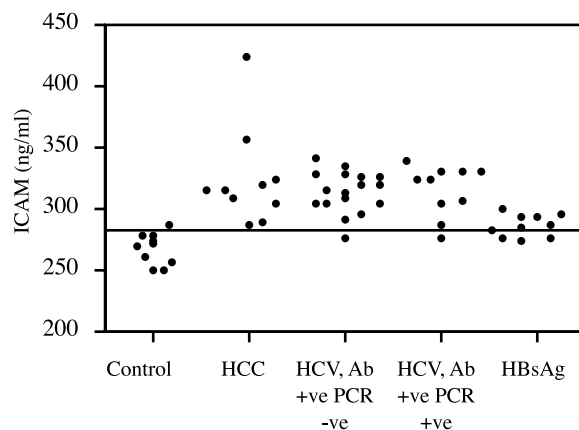


Fig. (4): Scatter diagram of ICAM (ng/ml) levels in all the studied groups. The horizontal line represents the cut-off value 292.1 ng/ml (mean \pm 2SD of controls). The sensitivity is 90%, 94.11%, 80% and 40% for the four groups respectively at a specificity level of 100%.

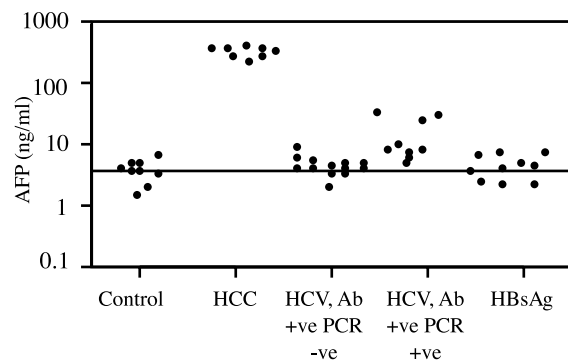
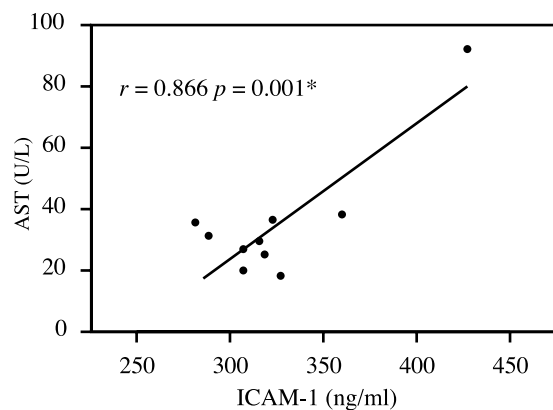
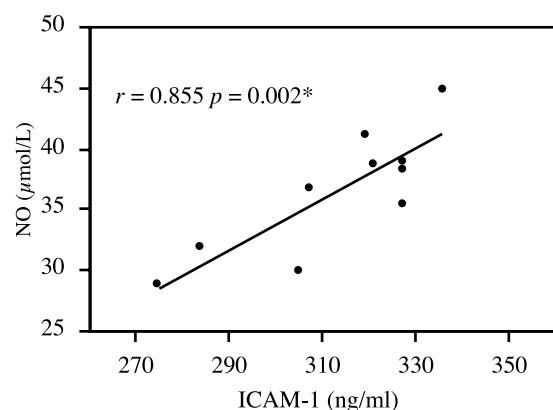


Fig. (5): Scatter diagram of AFP (ng/ml) levels in all the studied groups. The horizontal line represents the cut-off value 5.28 ng/ml (mean \pm 2SD of controls). The sensitivity is 100%, 11.76%, 100% and 20% for the four groups respectively at a specificity level of 100%.



r = Correlation coefficient, * Highly significant.

Fig. (6): Correlation between serum AST and ICAM-1 levels among HCC group of patients.



r = Correlation coefficient, * Highly significant.

Fig. (7): Correlation between serum NO and ICAM-1 levels among group of patients infected with HCV and positive for both antibodies and PCR tests.

DISCUSSION

In this study, we aimed at the evaluation of serum levels of NO, TNF- ∞ , ICAM-1 and AFP markers among Egyptian farmers infected with HBV and HCV in addition to HCC patients and healthy control subjects. The levels of serum transaminases (AST and ALT) and albumin were measured and ultrasonography examination of the liver of these patients was also done. To assess the possible correlation of hepatitis B and C viruses among Egyptian farmers and their relation to HCC.

The present study showed that serum level of NO was significantly increased in HCC, HBsAg seropositive and HCV-antibody seropositive and HCV-RNA positive groups. Whereas in patients with HCV-antibody seropositive and RT-PCR negative, insignificant changes were observed [13]. NO sensitivity levels were 100% for HCC, 50% for HBs-Ag and 10% for HCV-Ab and HCV-RNA positive group and zero% for HCV-Ab positive, at a specificity level of 100% Fig. (2). These results were supported by the finding of Ali et al. [2] who showed NO over production in liver cirrhosis and with the finding of Moussa et al. [15] who reported similar results in HCC patients.

Kassim [11] reported also an increase of cytosolic nitrite and nitrate as a result of increased production of NO in ovarian cancer cells. It was reported that NO released by the inducible NO synthase (iNOS) was cytostatic/cytotoxic for a variety of microorganisms as well as tumor cells [12]. It was found also that NO expression preceded necroinflammatory liver and maximal immunofluorescence reaction was coincident with tissue injury, supporting the hypothesis that NO contributes to hepatic cytotoxic mechanism [18]. Cytokine-mediated death of tumor cells could be achieved through endogenous NO released from tumor cells or as exogenous NO from activated macrophages and endothelial cells [29]. The present work showed normal level of NO in group of patients positive for HCV-antibodies and negative for HCV by RT-PCR, this might explain that the overproduction of nitric oxide might be actually dependent on the degree of affection and the severity of HCV infection.

TNF- ∞ levels, were found to be significantly increased in the groups of patients with

HCC, HCV-antibody seropositive and RT-PCR negative and in HCV-antibody seropositive and RT-PCR positive. Statistically the presented results indicated that the sensitivity of TNF- α measurement was 100% in HCC, 40% in HCV-antibody seropositive and RT-PCR positive group, 35.3% in HCV-antibody positive and RT-PCR negative, while the HBsAg group of patients was less sensitive (30%) and was insignificantly changed at a specificity level of 100%, Fig. (3). These results were supported by the findings of Yuan et al. [30] who reported that TNF- α participated in the activity process of liver disease. Zang et al. [32] reported that TNF- α was significantly correlated with the elevated serum ALT and bilirubin and insignificantly correlated with alfa-fetoprotein. TNF- α is an important mediator in the pathogenesis of liver necrosis and failure of circulation in HCV and/or HBV infection. However, the presented results showed insignificant increase of TNF- α in HBs-Ag patients group.

As regards the changes in the levels of serum ICAM-1, there was significant increase in all infected groups under investigation. Also, a significant correlation between serum AST and ICAM-1 ($r = 0.866$, $p = 0.001$) was observed among the HCC patients group, Fig. (6). In addition, there were high sensitivity levels of ICAM-1 in all groups, 90% in HCC, 94.11% in HCV-antibody seropositive and RT-PCR negative, 80% in HCV-antibody seropositive and RT-PCR positive groups while the HBs-Ag group of patients showed 40% at a specificity level of 100%, Fig. (4). Our results were in accordance with those reported by Hyodo et al. [10] and Shimizu et al. [23] who reported that intercellular adhesion molecule-1 was a useful marker for the determination of the severity of liver disease and hepatic fibrosis as well as a marker for HCC progression and prognosis. However, the presented results recorded significant correlation between NO and ICAM-1 levels ($r = 0.855$, $p = 0.002$) in the group of patients infected with HCV and positive for both antibodies and RT-PCR, Fig. (7). Thus; this might explain the high severity of liver disease in this group than the other infected groups.

Concerning the changes induced in the level of AFP, it was found that the HCC and the HCV groups with HCV-Ab positive and RT-

PCR negative or positive showed significant increase, while in the HBs-Ag group insignificant change was observed. A significant correlation was observed between AFP and hepatomegaly in the group of patients infected with HCV and positive for antibody and RT-PCR ($r = 0.764$, $p = 0.001$). Also, this group exhibited high percentage of liver cirrhosis (40%) obtained by ultrasonography examination compared to the other groups.

AFP a relatively specific marker for HCC [33] was reported to be elevated in up to 80% of patients with HCC as well as in patients with chronic active hepatitis and in cirrhotic patients [4]. However, in the present study, the sensitivity was 20% in the HBs-Ag infected group while in HCV-antibody seropositive group the sensitivity was 11,7% at specificity level of 100% Fig. (5).

In the group of patients infected with HCV and were positive for both antibodies and RT-PCR, the elevation of AFP and its correlation to the percentage of hepatomegaly, the high percentage of cirrhosis observed by ultrasonography examination and the positive correlation levels of NO and ICAM-1 led us to conclude that the group of patients infected with HCV and positive for both antibodies and RT-PCR are strongly associated with HCC among Egyptian farmers than others infected with HBV and/or HCV and positive only for antibodies and negative for RT-PCR.

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