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Nitric oxide level and CD3- ζ expression in response to Interferon/ Ribavirin Therapy in chronic Hepatitis C Egyptian patients

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Abstract

Hepatitis C virus (HCV) infection is one of the major causes of liver diseases all over the world; it is considered one of the leading causes of cirrhosis, hepatic failure and hepatocellular carcinoma (HCC) in developed countries including Egypt. Since, the discovery of the virus, the main drug used in all antiviral protocols was interferon- α (IFN- α) but, which is not effective in 60% of these patients. The goal of this study was to measure nitric oxide synthase (NOS), and CD3- ζ in chronic HCV patients which could explain the failure from therapy. 5ml of peripheral blood were collected from 30 patients with chronic HCV infection and 10 healthy control volunteers. Patients were categorized in to responders and non-responders according to viral titre upon IFN- α treatment. CD3- ζ expression was measured in the peripheral blood by using flow cytometry and NOS levels were assessed in the sera. Significant decreases ($P < 0.001$) in the expression of CD3- ζ in IFN- α non-responder was recorded when compared to responder patients and with healthy volunteers. In contrast, there were significant increases ($P < 0.001$) in the expression of NOS in IFN- α responder as compared to non-responder patients and healthy volunteers. Conclusion: these findings can be suggested Nitric oxide level and CD3- ζ expression that have immune suppressive function can be reversed and enhance responsive of HCV patients to interferon- α and ribavirin.

Key words: Hepatitis C virus (HCV); IFN- α ; ribavirin; Nitric oxide synthesis (NOS); CD3 Zeta.

1 Introduction

Hepatitis C virus (HCV) is considered a main cause of chronic hepatitis and it may leads to cirrhosis, hepatic failure and hepatocellular carcinoma (Mohd Hanafiah et al., 2013; Afdhal et al., 2014). Chronic HCV patients are subjected to treatment with ribavirin and interferon- α (IFN- α) but, which is not effective in 60% of these patients (Trembling et al., 2013; Vasudevan and Lubel 2015). So that, Sovaldi is considered a new drug which has been discovered in the recent years, that prevent proliferation of the virus by act directly on the life cycle of the virus (Dhingra et al., 2014).

Nitric oxide (NO), which is synthesized by inducible NO synthase iNOS, plays a critical role in regulation of immune cells by functioning as an antimicrobial agent which decreases microorganism replication (Atik et al., 2008). Furthermore, NO is considered as one of the main mediators in chronic inflammatory infections, where it is generated by liver non-parenchymal and parenchymal cells from L-arginine via iNOS (Iwakiri 2012; Alam et al., 2015). Previous studies has reported that there was a positive correlation between expression of iNOS and both liver injury and hepatic viral load, where unlimited and copious amount NO is produced by iNOS as a result of liver damage leading to inflammation and tumor development (Atik et al., 2008).

The altered levels of Indoleamine 2, 3- dioxygenase (IDO) and NO are often associated with the emergence of immunoregulatory cells, including regulatory T cells and myeloid derived suppressor cells (MDSCs). The suppressive

function of MDSCs exerted in many ways where, arginase-1 (Arg 1) and iNOS are considered critical two enzymes for the suppressive function of these cells through conversion of L-arginine to either urea or L-ornithine (Tadmor et al., 2011). Reduction of essential amino acid such as L-arginine arrests T cells in G0- G1 phase of the cell cycle and down regulates the T cell CD3 zeta- chain that leads to inhibition of the T cell activities. CD3 zeta- chain, a part of T cell receptor (TCR), plays an interesting role in conjugation of antigen recognition to many intracellular signal-transduction pathways to the effector function of T cell (Ostrand-Rosenberg 2010). As such, alteration in IDO and iNOS impacts will alter CD3-zeta, and three pathways results in T cell dysfunction immune failure. The presents tudy aimed to measure the expression of iNOS and CD3- ζ in a population of Egyptian patients with chronic HCV in both responders and non-responders to IFN- α and ribavirin therapy.

2 Materials and Methods

2.1 Subjects:

Thirty patients with chronic HCV infection (mean age= 44 \pm 4.5 years; male/female: 21/9) were recruited from The Tropical Medicine & Infectious Diseases Department, Tanta University (Tanta, Egypt) and Ten healthy volunteers (mean age = 39.5 \pm 4.51 years; male/female: 8/2) were also recruited to compare the results. Patients enrolled in the present study were treated with pegylated IFN- α (long acting interferon; Pegassys or Peg Intron) once every week for 48 weeks plus daily treatment with 800-1200mg ribavirin. According to the clinical response, the treatment was stopped after 24 weeks if no response occurs. The research study was approved by the ethics committee, Faculty of Medicine, Tanta University and the informed consent was obtained from all patients before participation.

2.2 Inclusion and exclusion criteria:

The inclusion criteria included the evidence for HCV infection using PCR (viral titer) and liver function tests. The exclusion criteria included any concomitant infectious diseases such as HBV, HIV, schistosomiasis, auto-immune diseases or prior chemotherapy.

2.3 Measurement of viral load:

HCV levels in serum were detected by reverse transcription PCR nucleic acid extraction using COBAS AmpliPrep/COBAS TaqMan HCV Test (Roche Diagnostic, Basel, Switzerland) according to the manufacturer's procedure.

2.4 Reagents:

Lymphocyte Separation Medium (LSM) was purchased from Corning Cellgro® (Mediatech, Inc., Manassas, VA, USA), and BD FACS® lysis buffer was purchased from BD Bioscience (San Diego, USA). The following human monoclonal antibodies (hmAbs) were purchased from eBioscience (San Diego, CA): anti-CD4 (clone: RPA-T4), anti-CD8 (clone: RPA-T8) and anti-CD247 (TCR zeta CD3 zeta) (clone: 6B10.2). Cadmium powders were purchased from (BDH, Germany) and 0.1 g/l N-1-

naphthylethelenediamine from (sd fine-chem- limited (SDFCL) India). Sodium nitrite, 30% zinc sulfate, hydrochloric acid (0.1mol/l), 0.1mol/l PH 9.6 ammonium hydroxide buffer, 1 g/l sulfanilamide and 25 g/l phosphoric acid were purchased from (Sigma- Aldrich).

2.5 Flow cytometric analysis:

For measure the expression of CD3- ζ , fresh venous peripheral blood samples were collected in sodium heparin tubes. Briefly, 100 μ L of blood was stained with human mAbs. Using concentrations recommended by the manufacturers of each antibody in staining tubes, the tubes were incubated in cold dark conditions for 20 minutes then BD FACS lysing solution (1X) was added for 15 minutes for RBCs lysis. Samples were then centrifuged at 1250 rpm for 5 minutes; the supernatant was discarded to remove the lysed RBCs. The cells then were washed twice using PBS to remove any remained debris or RBCs, the pellets then re-suspended in PBS. Negative stained samples were used as internal controls all over the experiments. FACSCalibur or FACSCanto II (BD Biosciences, SanJose, CA, USA) were used for acquisition. FACSDiva, CellQuest (BD Biosciences) and Flowjo software were used for data analysis(El-Awady et al., 2005).

2.6 Detection of total NOS in serum:

Serumsamples (50 μ l) were diluted with 200 μ l dist. H₂O then (50 μ l) of Zn- Sulphate was added to complete the volume 300 μ l, (dilution 1:6). The diluted samples were mixed and incubated at room temperature for 15 minute then centrifuged at 4000 r.p.m for 5 minutes. After that (230 μ l) of supernatant was added to the washed dried cadmium then incubated for 2 hours and centrifuged for 5 minutes. The supernatant (200 μ l) was added into micro-titer plate then (200 μ l) of each of dist. H₂O (Blank) was added. After that, (50 μ l) of color reagent was added to all wells then, the contents of the wells were gently mixed for 1 minute. The absorbance values were read at 490 nm then; the absorbance of the blank well was subtracted from the absorbance of all wells to calculate the concentration of an unknown sample. Finally, nitric oxide calculated by used standard curve (Yüksel et al., 2014).

2.7 Statistical analysis:

The patients were divided into responders and non-responders according to the viral titer and collected the clinical data along the study and analyzed for each patient, each value was calculated as the mean \pm SD. Experimental differences over the controls were analyzed by the Student's t-test. P values < 0.05 were considered statistically significant. Linear correlation coefficient was used for detection of correlation between two quantitative variables in one group and Analysis of variance [ANOVA] test was used for comparison among different times in the same group in quantitative data.

3 Results

3.1 NOS expression in chronic HCV patients:

Significant increases ($P < 0.001$) in the levels of NOS were found in IFN- α responder CHC patients when compared to non-responder patients and healthy control volunteers; 66.7

± 1.7 , 55.6 ± 2.8 and 44.9 ± 3.8 , respectively as shown in Table 1 and Figure 1.

3.2 Expression of CD3 zeta in chronic HCV patients

The percentage of CD3 zeta chain in CHC patients were determined by using the gating strategies as shown in Figures 3. Significant decrease ($P < 0.001$) in the percentages of CD3 zeta were found in IFN- α non-responders patients than in responders and healthy controls; 49.8 ± 3.6 , 82.4 ± 2.4 and 97.1 ± 0.8 , respectively as shown in Table 2 and Figure 2.

4 Discussion

To shed a light on some of the mechanisms associated with the failure of chronic HCV patients to IFN-based therapy, the expression of NOS and CD3 zeta were measured in both responder and non-responder chronic HCV patients who were treated with interferon and ribavirin. Overall, we found increases in NOS associated with decreases in the expression of CD3 zeta in patients regardless the viral response to IFN/ribavirin therapy. Interestingly, the non-responders showed higher NOS and lower CD3 zeta when compared to responders. Taken together, these data indicate to the presence of immunosuppressive mechanisms in HCV patients in general and in non-responders in particular. These data are of significant importance to the therapeutic approaches of HCV since it opens a new avenue to utilize or design drugs that can target these molecules as adjuvant therapy with the conventional therapy of HCV.

We found a significant increase in the expression of NO in IFN- α responder and non-responder patients when compared with healthy volunteers. This increase in serum NO in IFN- α responders patients can be due to the positive correlation between the NO and viral response which in agreement with previous studies which reported that patients subjected to treatment with pegylated IFN- α and ribavirin, where, interferon was found to stimulate the production of NO (Ibrahim et al., 2010).

CD3- ζ is a critical receptor involved in the functionality of T cells. Several studies in different disease settings reported the down regulation of CD3- ζ is related to chronic inflammation and results in significant dysfunction in T cells and exacerbations of the disease (Zeng et al., 2014; Appleby et al., 2015). We found that there were significant decreases in the expression of CD3- ζ in IFN- α non-responder and responder patients as compared with healthy volunteers. The down regulation of CD3- ζ was due to the increase in the NO expression which in turn results in the increase of l- arginine that limit the proliferation and expression of CD3- ζ .

Our findings are in line with (Maki et al., 2003 and 2004) who showed a decreased expression of CD3- ζ chain and CD28 in HCV patients without or without Hepatocellular carcinoma (HCC) and there was no correlation between the levels of CD3- ζ expression and the

HCV-RNA quantity or the vitality of hepatitis. Moreover, Tumino et al., (2015) showed alteration in the CD3- ζ expression during HIV infection due to the production of iNOS, ARG1, IDO, and ROS.

Several previous studies proposed that degradation of CD3- ζ can be induced selectively by tumor cells and/or tumor-associated myeloid cells such as myeloid derived suppressor cells (MDSCs) (Mizoguchi et al., 1992; Otsuji et al., 1996; Baniyash 2004). In addition, (Nagaraj et al., 2010) showed an alteration in the CD3 ζ expression that induced by MDSCs which in turn down regulate antigen-specific T cell response. In addition, the dissociation of TCR: CD3 ζ occur upon the encounter with MDSCs.

Furthermore, our and other recent studies indicated the suppressive role of MDSCs in different human malignances, chronic HCV and HIV infection (Diaz-Montero et al., 2009; Vollbrecht et al., 2012; Cai et al., 2013]. For example, (Zeng et al., 2014) demonstrated the correlation between the persistence of HCV and MDSCs via decreasing the levels of CD3 ζ chain expression on cytotoxic T cells in chronic HCV patients.

Our findings suggested that the immunological markers that have immune suppressive function can be reversed and enhance responsive of HCV patients to interferon- α and ribavirin.

5 References

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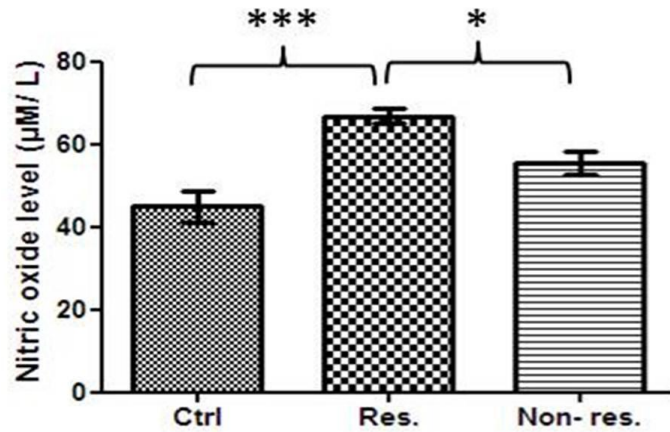


Figure 1

Figure 1: Nitric oxide concentration in CHC patients treated with IFN- α and ribavirin. Blood were collected from (n= 15) CHC patients respond to IFN- α and ribavirin treatment, (n= 15) CHC patients didn't respond and (n=10) samples for healthy control volunteers. * means $P < 0.05$: compared between responder and non-responder, *** means $P < 0.001$: compared with control group.

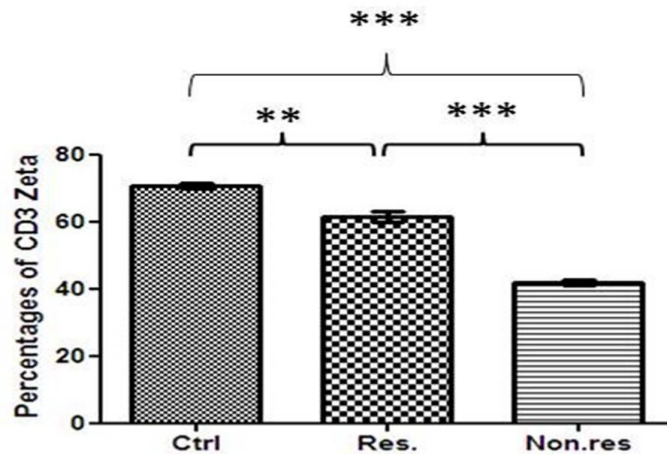


Figure 2

Figure 2: Statistical analysis of the expression of CD3 Zeta in CHC patient treated with IFN- α and ribavirin. Blood were collected from (n= 15) CHC patients respond to treatment, (n= 15) CHC patients didn't respond as well (n=10) samples for healthy control volunteers. *** means $P < 0.001$: compared between responder and non-responder and ** means $P < 0.01$: compared with control group.

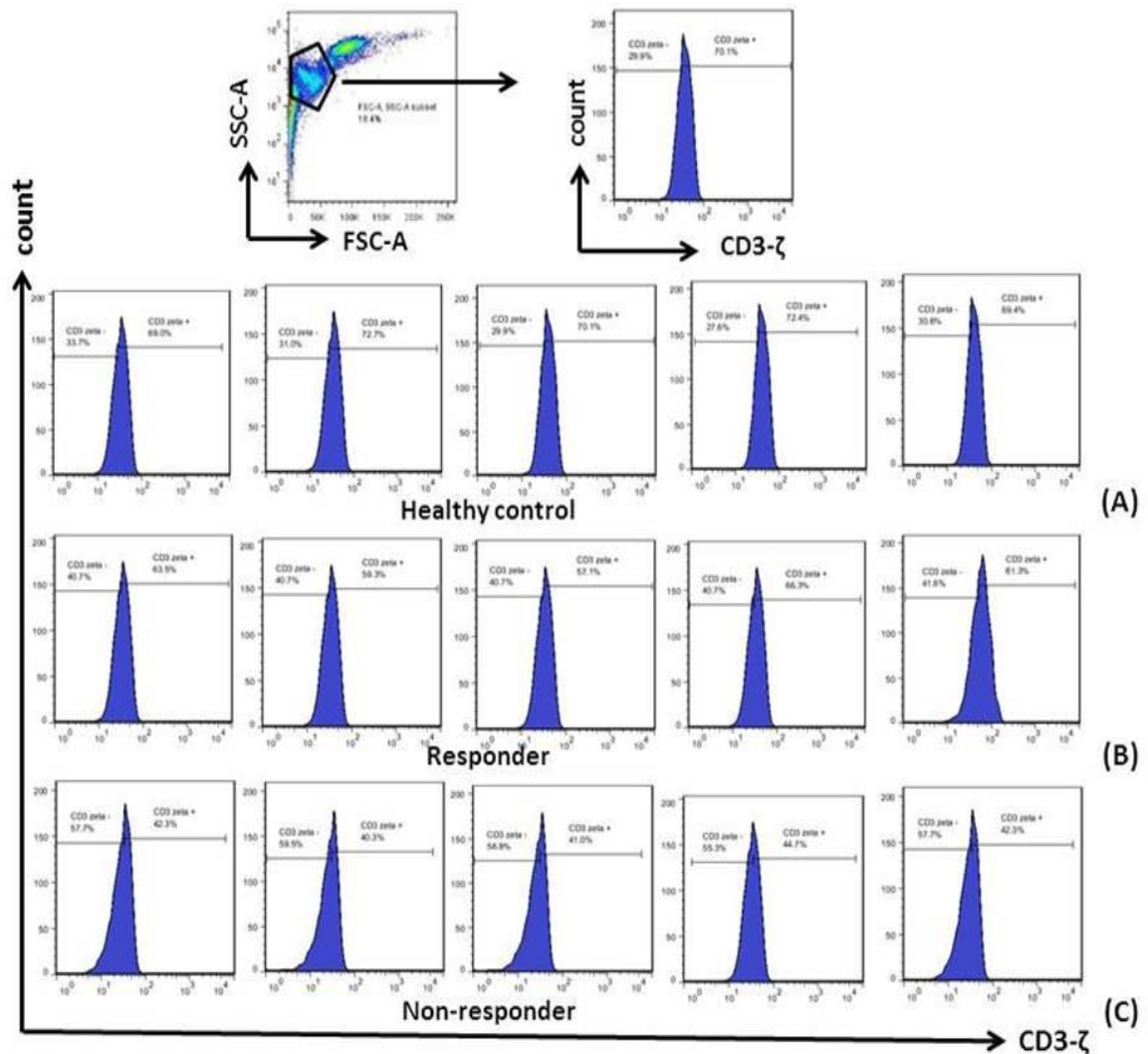


Figure 3

Figure 3:The gating strategy for CD3 Zeta in CHC patients. (A) Representative (n= 10) healthy control volunteers, (B) representative (n= 15) CHC patients respond to IFN- α and ribavirine treatment and (C) representative (n= 15) CHC patients didn't respond to the treatment.

Table 1: Comparison between CHC patient groups; responder and non-responder versus control group as regard to NOS level

Nitric oxide level ($\mu\text{M}/\text{L}$)	CHC patients		Healthy control
	Responders	Non-Responders	
Range	61.6 to 71.3	49.1 to 64.3	36.5 to 57.8
Mean \pm SD	66.74 \pm 1.744	55.60 \pm 2.783	44.98 \pm 3.886
P value	$P < 0.001$	$p < 0.05$	

Table 1

Table 2: Comparison between CHC patient groups; responder and non-responder versus control group as regard to the percentages of CD3- ζ

Percentages of CD3 Zeta	CHC patients		Healthy control
	Responders	Non-Responders	
Range	57.1 to 66.3	40.3 to 44.7	72.7 to 69
Mean \pm SD	82.44 \pm 2.395	49.76 \pm 3.579	97.14 \pm 0.751
P value	$P < 0.01$	$P < 0.001$	

Table 2

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