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EVALUATION OF INTERLEUKIN-8 (IL-8) AND TOTAL NITRIC OXIDE LEVELS AND RT- PCR TECHNIQUE FOR DETECTION OF GENOTYPE (4) HEPATITIS C VIRUS

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ABSTRACT

The aim of this study was to investigate the serum levels of interleukin-8 and total nitric oxide in patients with genotype 4 hepatitis C virus before and after treatment with interferon and ribavirin compared against RT- PCR technique. The study was performed on 72 patients divided into 3 groups according to the diagnosis: Group I (Positive ELISA-antibodies - Negative PCR), Group II (Positive ELISA-antibodies - Positive PCR), and Group III after Treatment with interferon and ribavirin (Positive ELISA-antibodies - Negative PCR), in addition to a control group of 16 healthy volunteers. The results revealed that serum levels of interleukin-8 and total nitric oxide after treatment with interferon and ribavirin appeared to increase rather than before treatment compared against RT- PCR technique.

INTRODUCTION

Interleukin-8 is a chemokine produced by macrophages and other cell types such as epithelial cells (Utgaard, 1998) and is suggested to be a key parameter in localized inflammation (Vlahopoulos et al., 1999). The serum levels of IL-8 in HCV-infected patients have been demonstrated to be significantly elevated compared to levels in normal healthy volunteers; it serves as a chemical signal that attracts neutrophils at the site of inflammation, and therefore is also known as Neutrophil chemotactic factor. When first encountering an antigen, the primary cells elaborate it are the macrophages which phagocytose the particle. Upon processing, they release chemokines to signal other immune cells to come in to the site of inflammation. IL-8 is one of such chemokines (Stephen et al., 2001).

The biological activities of Nitric Oxide (NO) were first widely appreciated when it was identified as the endothelial-derived relaxing factor (EDRF) responsible for the potent vasodilating properties of stimulated endothelia (Ignarro et al., 1987; Palmer et al., 1987 and Furchgott and Zawadzki, 1980). Since then, the NO has been recognized as a pleiotropic biological mediator, regulating diverse activities ranging from neuronal function to immune system regulation. It is a gaseous free radical with a short half-life *in vivo* of a few seconds or less. Therefore, the levels of the more stable NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), have been used in the indirect measurement of NO in biological fluids

(Marletta et al., 1988; Wennmalm et al., 1992; Wennmalm et al., 1993 and Tsikas, 2005). Altered levels of NO have been shown to be associated with sepsis, reproduction, infection, hypertension, exercise, type 2 diabetes, hypoxia, and cancer (Ochoa et al., 1991; Evans et al., 1994; Rosselli et al., 1994; Manukhina et al., 2000; Lyamina et al., 2003; Newaz et al., 2003; Taysi et al., 2003; Maeda et al., 2004 and Yugar-Toledo et al., 2004).

NO is produced via the reaction of L-arginine, NADPH, and O_2 to NO and citrulline (Palmer et al., 1988a; Palmer et al., 1988b; Moncada et al., 1989 and Bredt and Snyder, 1990). NO catalyzed by enzymes of the nitric oxide synthase (NOS) family, Members of the NOS family include neuronal (nNOS/NOS1), endothelial (eNOS/NOS3), and inducible (iNOS/NOS2) (Alderton et al., 2001). As the name implies, nNOS is highly expressed in neurons of the central and peripheral nervous systems, and has also been described in other cell types including skeletal muscle myocytes, lung epithelial cells, and skin mast cells (Bredt et al., 1991; Dun et al., 1992; Nakane et al., 1993; Asano et al., 1994; Sugaya and McKinney, 1994; Reuss et al., 1995; Gath et al., 1996 and Shimizu et al., 1997). eNOS is highly expressed by endothelial cells and may also be found in neurons, dermal fibroblasts, epidermal keratinocytes, thyroid follicular cells, hepatocytes, and smooth muscle cells (Pollock et al., 1991; Dinerman et al., 1994; Comini et al., 1996; Wang et al., 1996; Colin et al., 1997 and Shimizu et al., 1997). iNOS is expressed in a wide range of cell types including chondrocytes, epithelial cells, hepatocytes, glial cells, and several cell types of the immune system (Geller et al., 1993; Asano et al., 1994; Maier et al., 1994 and Bogdan, 2001). In general, eNOS and nNOS are constitutively expressed and regulated by Ca^{2+} / calmodulin, while iNOS is induced by endotoxin and inflammatory cytokines, and exhibits a relative insensitivity to Ca^{2+} (Alderton et al., 2001 and Kone et al., 2003).

Because it is lipid soluble, NO is not stored but is synthesized de novo and freely diffuses across lipid membranes. NO has the potential to mediate its effects on target cells via several different mechanisms. For instance, NO-mediated activation of the enzyme guanylyl cyclase (GC) catalyzes the formation of the second messenger Guanosine 3',5'-cyclic Monophosphate (cGMP). cGMP is implicated with a range of biological functions such as regulating smooth muscle contractility, cell survival, proliferation, axon guidance, synaptic plasticity, inflammation, angiogenesis, and the activity of cyclic nucleotide-gated channels (Zhuo et al., 1994; Arancio et al., 1996; Lev-Ram et al., 1997; Hood and Granger, 1998; Suhasini et al., 1998; Kronemann et al., 1999; Arancio et al., 2001; Guzik et al., 2003 and Hamad et al., 2003). NO also functions as an anti-tumor and anti-microbial agent via mechanisms that include its conversion to peroxynitrite ($ONOO^-$), the formation of S-nitrosothiols, and the depletion of arginine (Bogdan, 2001). Another putative role for NO includes the suppression of mitochondrial respiration through the inhibition of cytochrome oxidase (Antunes et al., 2004). No may also modify protein activity through post-translational nitrosylation via the attachment of an NO moiety to the thiol side chain of

cysteine residues (Gaston et al., 2003 and Hess et al., 2005).

2. Material and methods

2.1. Patients

The study was performed on 72 patients attended to outpatient clinic of Mansoura University. Patients agreed to be admitted into the study after they had been informed about the requirements and aims of the study. The patients were divided into three groups, according to their test diagnosis (Table 1). A control group of 16 healthy volunteers was also used.

Table 1 Demographic characteristic of the patients.

Group	Test Diagnosis	No. of patients	Age	Gender (F/M)
I	+ve (ELISA-antibodies) & -ve (RT-PCR)	24	35 - 52	9/15
II	+ve (ELISA-antibodies) & +ve (RT-PCR)	24	40 - 54	11/13
III (After treatment)	+ve (ELISA-antibodies) & -ve (RT-PCR)	24	41 - 56	11/13

2.2. Chemical used

- Human Interleukin-8 ELISA Kit (KOMA BIOTECH INC.): Catalog No: K0331216, Quantity: 96 tests, Storage: 4 C^o.
- Total NO/Nitrite/Nitrate Assay Kit Catalog No: KGE 001 (R&D Systems, INC.): For the quantitative determination of Nitric Oxide concentrations in cell culture supernates, serum, plasma, and urine.

2.3. Methodology :

- ELISA-antibodies standard kit for HCV (anti-HCV) (Abbott II anti-HCV ELISA, Abbott Lab, IL, USA).
- Quantitative PCR, Extraction of HCV-RNA from serum was carried out using QIAamp Viral RNA Mini Kit - for viral RNA purification from plasma, serum, cell-free body fluids, and cell-culture supernatants.
- TaqMan® RT-PCR Kit for HCV detection, TaqMan®, Assay reagents, Part number 4324018, Lot number M 10770, Stored at -20 C^o, For Laboratory R&D use only, Applied Biosystems, Manufactured by Roche, Branchburg, New Jersey USA. TaqMan® and AmpErase® are registered trademarks of Roche Molecular Systems, Inc.

- Genotyping for HCV by PCR using type-specific primers. Optimization of typing assay Type-specific PCR was described originally by (Ohno et al., 1997) which used type-specific primers to amplify the core region of HCV for determination of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a.

2.4. Statistical analysis

All statistical analysis were done by a statistical software package (SPSS 12.0 for win-

dows, SPSS Inc.). All results are expressed as mean ± SD. Significance was established at a P level < 0.05.

RESULTS

3.1. Detection of HCV antibodies

All serum patients were screening to HCV-antibodies ELISA, The screening were performed on 72 patients divided to 3 groups: Group I, Group II and Group III after Treatment with interferon and ribavirin. All of these groups were positive for HCV-antibodies. A control group of 16 healthy volunteers who were negative for HCV-antibodies.

3.2. Determination of HCV by RT-PCR quantitative

All serum patients were screening to HCV by RT-PCR quantitative, The screening was performed on 72 patients divided to 3 groups : Group I (Negative PCR), Group II (Positive PCR) and Group III (Negative PCR) after Treatment with interferon and ribavirin.. A control group composed of 16 healthy volunteers was negative for RT-PCR. Fig. 1 shows that the sample was read as positive for HCV-RNA, and Fig. 2 shows that the sample was read as negative for HCV-RNA.

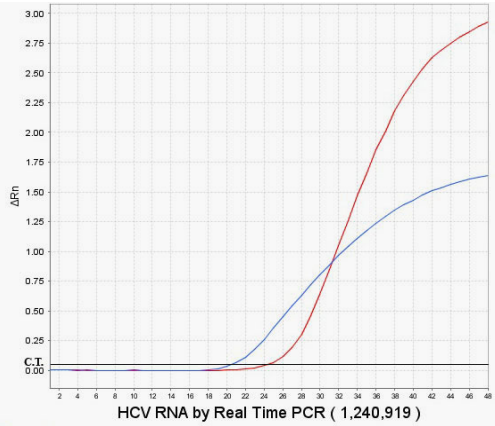

Fig (1) : Showed the sample was read as positive for HCV-RNA.
Patient Sample. Internal Positive control (IPC) **C.T.:** Threshold Cycle at 0.09

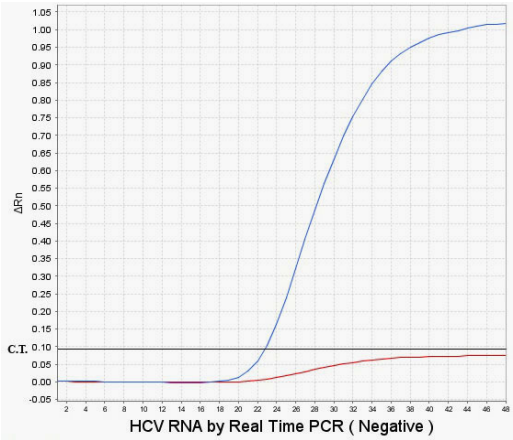

Fig (2) : Showed the sample was read as negative for HCV-RNA
Patient Sample Internal Positive control (IPC) C.T.: Threshold Cycle at 0.09

3.3. Determination of genotype 4 HCV to all serum patients :

As can be seen in Fig. 3 (Mix-2 for Genotyping) typical electrophoresis patterns of PCR products from HCV genotype 4, a visible band corresponding to the expected size (99 bp) was demonstrated, and the sample was read as positive for subtype 4 (Fig. 3 lanes 1 to 13). On the other hand, sera which did not demonstrate this visible band by PCR, was read as negative for HCV subtype 4.

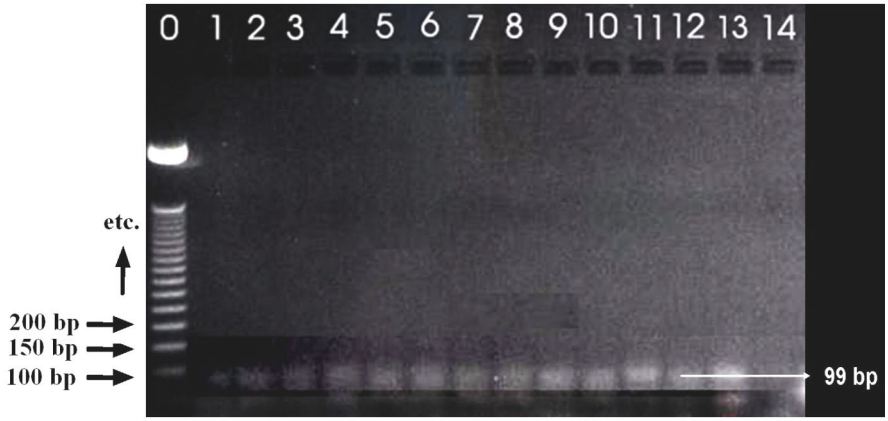

Fig. (3) : Typical electrophoretic patterns of PCR products from HCV genotype 4.
Lane (0) : Marker 50 bp DNA - ladder.
Lane (1 to 13) : Positive results for genotype 4.
Lane (14) : Negative Control.

3.4. Determination of Interleukin-8 in normal control group and in patients with HCV.

In Fig. 4, the mean serum level of IL-8 in the 16 samples of healthy control (was 2.5869 ± 0.0158 pg/ml) and in the 72 samples of patients with HCV (who were divided into 3 groups) the serum level of IL 8 were as follow : Group I (Positive ELISA- Negative PCR) (6.33 ± 0.9743 pg/ml). Group II (Positive ELISA- Positive PCR) (11.245 ± 3.5414 pg/ml) and Group III after Treatment with interferon and ribavirin (Positive ELISA- Negative PCR) (14.0 ± 21.818 pg/ml).

The results depicted in Table 2 show that there were highly significant differences in IL-8 levels between control (healthy volunteer) and each patient groups with HCV.

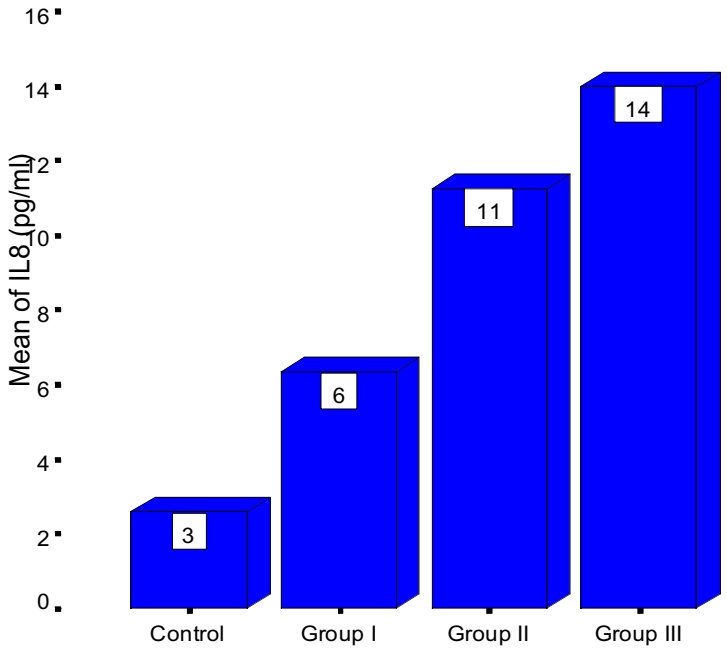

Fig. 4 : Mean serum level of IL-8 in 16 samples negative control and each separate group of 72 sample patients with HCV.
Control (healthy volunteer)
Group I (Positive ELISA- Negative PCR)
Group II (Positive ELISA- Positive PCR)
Group III after Treatment with interferon and ribavirin (Positive ELISA- Negative PCR)

Table 2 : Showed IL-8 is highly significant difference between control (healthy volunteer) and each patient groups with HCV.

	Control	**Group I**	**Group II**	**Group III**	**P1**	**P2**	**P3**
No. of Patients	16	24	24	24			
IL-8 (pg/ml)	2.5869 ± 0.0158 pg/ml	6.33 ± 0.9743 pg/ml	11.24 ± 3.5414 pg/ml	14.0 ± 21.818 pg/ml	0.000	0.000	0.017

P value is significant at the 0.05 level.
P1 value (Control versus Group I) = highly Significant difference.
P2 value (Control versus Group II) = highly Significant difference.
P3 value (Control versus Group III) = Significant difference.

3.5. Determination of Total Nitric Oxide in normal control group and in patients with HCV.

The data presented in Fig. 5, show that the mean valus of Total Nitric Oxide level in the 16 control samples was (5.6563 ± 0.7891 μmol/L), while the mean values of No in 72 samples of patients with HCV were : in Group I (Positive ELISA- Negative PCR) (15.996 ± 2.7212 μmol/L); Group II (Positive ELISA-Positive PCR) (17.246 ± 9.7152 μmol/L) and Group III after Treatment with interferon and ribavirin (Positive ELISA- Negative PCR) (27.49 ± 26.174 μmol/L).

It is apparent of the results herein reported (Table 3) that Total Nitric Oxide level showed highly significant difference between control (healthy volunteer) and each patient groups with HCV.

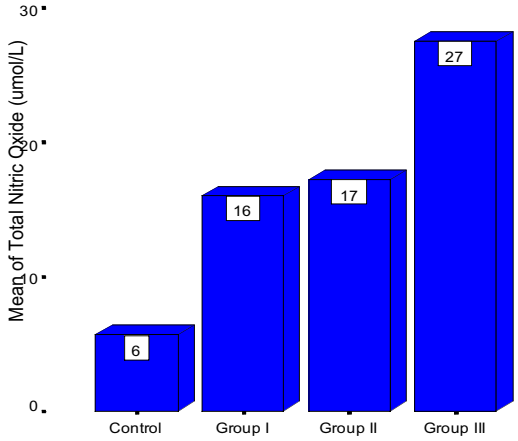

Fig. 5 : Mean of Total Nitric Oxide level in 16 samples negative control and each separate group of 72 sample patients with HCV.
Control (healthy volunteer)
Group I (Positive ELISA- Negative PCR)
Group II (Positive ELISA- Positive PCR)
Group III after Treatment with interferon and ribavirin (Positive ELISA- Negative PCR)

Table 3: Showed Total Nitric Oxide level is highly significant difference between control (healthy volunteer) and each patient groups with HCV.

	Control	Group I	Group II	Group III	P1	P2	P3
No. of Patients	16	24	24	24			
Total Nitric Oxide (μmol/L)	5.6563 ± 0.7891 μmol/L	15.99 ± 2.7212 μmol/L	17.24 ± 9.7152 μmol/L	27.49 ± 26.174 μmol/L	0.000	0.000	0.000

P value is significant at the 0.05 level.
P1 value (Control versus Group I) = highly Significant difference.
P2 value (Control versus Group II) = highly Significant difference.
P3 value (Control versus Group III) = highly Significant difference.

3.6. Correlations

There is no correlation between IL-8 and Total Nitric Oxide in 72 sample patients with HCV (Table 4).

Table 4 : Corralations between IL-8 and Total Nitric Oxide in 72 sample patients with HCV.

		IL-8
Total Nitric Oxide	Pearson Correlation	0.020
	Sig. (2-tailed)	0.868
	N	72

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

DISCUSSIONS

Nitric oxide (NO), has been reported as free radical that is overproduced in liver cirrhosis. Hepatitis C virus (HCV) might increase NO levels via increased inducible NO synthase (iNOS) and Interleukin-8 is often associated with inflammation (Vlahopoulos et al., 1999). When first encountering an antigen, the primary cells to encounter it are the macrophages who phagocytose the particle. Upon processing, they release chemokines to signal other immune cells to come in to the site of inflammation. IL-8 is one such chemokine. It serves as a chemical signal that attracts neutrophils at the site of inflammation, and therefore is also known as Neutrophil Chemotactic Factor (Stephen et al., (2001). This work was carried out to study the effect of HCV-induced liver cirrhosis on IL-8 and NO levels among Egyptian patients before and after treatment with interferon and ribavirin. IL-8 levels showed a statistically significant increase among patients compared to the control group (P<0.000) and NO levels determined as the stable endproduct nitrate, showed a statistically significant increase among patients compared to the control group (P<0.000). Furthermore, IL-8 and NO levels increased proportionally with the severity of liver cirrhosis (P<0.000). IL-8 and NO levels is highly in patients after treatment with interferon and ribavirin (P<0.017) and (P<0.000) respectively. This is in conformity with the results of utgaard (1998); Maeda et al., (2004) and Tsikas (2005) who reported that both IL-8 and NO levels in patient with HCV are greally elevated as a result of virus activity as compared to that of healthy individuals. Based on the results of the present study and of other previous reports, we suggest the analysis of serum IL-8 and NO concentrations in order to detect present or absent of genotype (4) HCV after treatment with interferon and ribavirin compared with RT-PCR quantitative technique.

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الملخص العربى

تقييم مستوى الإنترلوكين 8 والنيترك أكسيد الكلى وتفاعل البلمرة المتسلسل الكمى للكشف عن النوع 4 لڤيروس إلتهاب الكبد الوبائى سى

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تناولت الدراسة تقييم العامل المناعى إنترلوكين - 8(IL-8) كأحد السيتوكينات الوسيطة (Proinflammatory Cytokines) فى عمليات الإلتهابات المناعية التى تحدث أثناء الإصابة بالڤيروس، وقد أوضحت النتائج إرتفاع مستوى إنترلوكين 8-14.0 (IL-8) فى مصل الدم للمرضى المصابين بالإلتهاب الكبدى الوبائى سى حيث إرتفع مستواه فى المجموعة الأولى إلى (6.33±0.9743pg/ml) وفى المجموعة الثانية إلى (11.245±3.5414pg/ml) وفى المجموعة الثالثة إلى (14.0±21.818pg/ml) فى مصل الدم لهؤلاء المرضى بينما كان مستواه (2.5869±0.0158pg/ml) فى مصل المجموعة الضابطة (Normal Control). كما تناولت الدراسة أيضاً تقدير كمية الـ Total Nitric Oxide كأحد الوسائط التى تلعب دوراً مهماً فى مقاومة الڤيروسات (The most versatile mediators in the control of viral infesxtions) كما يعمل أيضاً كـ (Apoptotic) فى بعض أنواع الخلايا أو كـ (Antiapoptotic modulator) فى بعض الخلايا الأخرى والتى منها الخلايا الكبدية فى حالات الإصابة بالڤيروس، وقد أوضحت النتائج إرتفاع مستوى Total Nitric Oxide فى مصل الدم للمرضى المصابين بالإلتهاب الكبدى الوبائى سى حيث إرتفع مستواه المجموعة الأولى إلى (15.996±2.7272µmol/L) وفى المجموعة الثانية إلى (17.246±9.7152µmol/L) وفى المجموعة الثالثة إلى (27.49±26.174µmol/L) فى مصل الدم لهؤلاء المرضى بينما كان مستواه يقدر بحوالى (5.6563±0.7891µmol/L) فى مصل المجموعة الضابطة (Normal Control). وجاءت هذه الدراسة متفقة مع دراسات سابقة من حيث أن النوع 4 هو السائد بين المرضى المصابين، كما تبين على ضوء هذه الدراسة يمكن تعيين ڤيروس إلتهاب الكبد الوبائى سى باستخدام طريقة الإدمصاص المناعى (ELISA) لـ Total Nitric Oxide والإنترلوكين -8(IL-8) وتتميز هذه الطريقة بأنها سهلة التطبيق وذات حساسية عالية وتكلفة أقل عند مقارنتها بطريقة الـ بى سى آر قبل وبعد العلاج بالإنترفيرون والريباڤيرين.

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EVALUATION OF INTERLEUKIN-8 (IL-8) AND TOTAL NITRIC OXIDE LEVELS AND RT- PCR TECHNIQUE FOR DETECTION OF GENOTYPE (4) HEPATITIS C VIRUS

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