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Serum IL-12p70 and TLR 7 gene expression in Egyptian patients infected with HCV and treated with Sofosbuvir, Ribavirin and/or Pegylated interferon

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Abstract

Chronic hepatitis C virus infection is one of the most important health problems in Egypt. The purpose of this study was to assess the efficacy, and tolerability of two therapeutic modalities, dual (Sofosbuvir+ribavirin)and triple (sofosbuvir + ribavirin + peg-interferon), in treating Egyptian patients infected with HCV genotype-4. In addition, we determined serum IL-12p70 and TLR7 gene expression in patients infected with HCV with and without treatment, compared to the normal volunteers. No significant changes in serum creatinine and fasting blood glucose concentrations were reported at the end of the treatment with dual and triple therapy and after 3 months of follow up. Pateints infected with HCV showed a non significant change in the relative quantitation of TLR7 gene expression, and a significant elevation in serum IL-12p70 concentration (161.73%, $p<0.0001$), compared to the volunteers group (II). In dual and triple therapy groups (III &IV), there was a significant elevation in the relative quantitation of TLR7 gene expression (250.53 and 506.32%, respectively, $p<0.0001$), compared with HCV group, but these elevations returned to the normal level after 3-months of follow up period. On the other hand, the concentration of serum IL-12p70 was significantly reduced in all treated groups, compared to both healthy volunteers and HCV groups , except group III (SOF+RBV) showed a non significant change compared to healthy volunteers. The treatment modalities, either with Sofosbuvir plus ribavirin or Sofosbuvir plus ribavirin and pegylated Interferon is highly effective in our cohort of Egyptian patients infected with HCV genotype-4. Further investigations should be done to increase the time after the end of treatment modalities to ensure complete remission of HCV infection.

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Introduction

Chronic hepatitis C virus (HCV) infection affects an estimated 170 million people worldwide (Lavanchy, 2009). Data from the Egypt Demographic and Health Survey (Guerra *et al.*, 2012; Waked *et al.,* 2014)estimated the prevalence of HCV viraemia to be 7.3% in 2013 with predominance of genotype 4(Gower *et al.,* 2014).Optimal therapy for patients with hepatitis C virus genotype 4 (HCV-4) infection is changing rapidly; the standard of care for a long time has been a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV),with modest response rates and considerable adverse events (Abdel-Razek and Waked, 2015).

Recently, because of direct-acting antiviral agents (DAAs) development, more treatment modalities have become available. Current guidelines for infection with HCV genotype 4 include both interferon-containing and IFN-free regimens; the latter consisting of DAAs with or without ribavirin (Moreno *et al.*, 2015). Moreover, in Egypt new regimens of DAAs in combination with PegIFNα/RBV are approved to reduce the incidence of viral breakthroughs and relapse due to resistance mutations (Barth, 2015).

Sofosbuvir (Sovaldi®, Gilead Sciences) is a direct-acting pyrimidine nucleotide analog; the first NS5B HCV polymerase inhibitor (Herbst and Reddy, 2013). It was approved by the US Food and Drug Administration and the European Medicines Agency and has become commercially available for the treatment of CHC in the US in late 2013 and in several European countries in early 2014 (Pawlotsky, 2014). It is administered at a dosage of 400 mg once daily, taken with or without food, and has a potent activity against all HCV genotypes (Cholongitas and Papatheodoridis, 2014).

Individuals infected with HCV have two possible consequences of infection, clearance or persistent infection, detected by a complex set of virus-host interactions (Rehermann, 2009).

The previous work of Hiroishi *et al.* (2008) showed that in HCV infection, distinct patterns of protective or immune pathological responses can be triggered by the inflammatory/regulatory cytokines and therefore are involved in the clearance or the establishment of chronic HCV. Replication of HCV occurs in hepatocytes as well as peripheral blood mononuclear cells (PBMCs) and bone marrow cells (Revie and Salahuddin, 2011), resulting in viral persistence and chronic immune activation. The phagocytic and cytolytic activities of PBMCs are critical for pathogen clearance. After activation, increased production of cytokines such as interleukin-12 is vital for enhancing the adaptive immune response (Alhetheel *et al.*, 2017). One of the most important proinflammatory cytokines is interleukin-12 (IL-12) which is produced upon stimulation by antigen presenting cells (Del Vecchio *et al.*, 2007) and presented with the initiation of immune response, so IL-12 can be considered as one of the most well known factors determining T-helper 1 and 2 (Th1and Th2) differentiation (Watford *et al.*, 2003).

Toll-like receptors (TLRs) are one of the pattern recognition receptors (PRRs) and are important for the innate immune response. Upon the activation by the pathogen-associated molecular patterns (PAMPs), PRRs enhance various cytokines expression. Some TLRs are present on the cellular surface and sense extracellular PAMPs, where some TLRs are located in the endosomes to detect internalized pathogens (Blasiusand Beutler, 2010). TLR7 can detect ssRNA molecules, in addition, the presence of a GU-rich sequence in the HCV genome can be detected by TLR7 (Zhang *et al.*, 2009). TLR7 is expressed in the intracellular endosomal compartment where viral genomes can be degraded to RNA oligonucleotides and activate immune responses. Following the demonstration that agonistic engagement of TLR7induces the production of type I interferon with anti-viral activity in antigen-presenting cells, many studies have focused on its possible therapeutic use in hepatitis C virus (HCV) infection (Funk *et al.*, 2014).

The purpose of this study was to assess the efficacy, and tolerability of two therapeutic modalities (Sofosbuvir + ribavirin as well as sofosbuvir + ribavirin + peg-interferon.) in treating Egyptian patients infected with HCV.

Materials and methods

Patients

The study included 140 subjects who were divided into four groups *viz*. Group I (Normal Control):49 healthy volunteer subjects (31 males and 18 females) with no past history for liver diseases, age (36.33±11.02) years old as a normal control group. Group II (HCV): 91 Egyptian patients infected with HCV (53 males and 38 females). This group served as positive control. Group (III): (Dual therapy), 41 Egyptian patients (21 males and 20 females) infected with HCV were treated with sofosbuvir (SOF; Pharco, Egypt) in combination with RBV (Rebetol, Schering-Plough, Germany) for 24 weeks. Subgroup (III): (Dualtherapy follow up), Follow up was performed for 26 Egyptian patients (19 males and 7 females) from Group III after 3 months from the end of the treatment period. Group (IV): (Triple therapy), 50 Egyptian patients (32 males and 18 females) infected with HCV were treated with Sofosbuvirplus RBV and peg-interferon (Peg-Intron, Schering-Plough, Germany) for 12 weeks. Subgroup (IV): (Triple therapy follow up), Follow up was performed for 19 Egyptian patients (11 males and 8 females) from Group IV after 3 months from the end of the treatment period.

Sofosbuvir 400 mg was administered orally once-daily in the morning, peg interferon 160 µg was subcutaneously injected once a week, and ribavirin was administered orally twice-daily in the morning and evening with food (doses were determined according to body weight. 1000 mg daily in patients with a body weight of <75 kg and 1200 mg daily in patients with a body weight of ≥75 kg).

Inclusion criteria

Men and non-pregnant women >18 years of age were enrolled with a body mass index ≥18 kg/m^2. HCV-infected patients (genotype 4a) with viral load ≥10^4 IU/ml and positive HCV antibodies for more than 6 months detected by the Enzyme Immunoassay (EIA) technique were eligible. Fibrotic HCV patients with fibrosis score < 3 were taken in the study.

Exclusion criteria

Patients were excluded from this study if they had previously taken direct-acting antivirals targeting the HCV NS5B polymerase, alcohol intake, uncontrolled diabetic patients with high glycated-hemoglobin level, co-infection with the human immunodeficiency virus, other etiologies of chronic hepatitis (e.g. autoimmune, hepatitis B virus infection and drug-induced liver injury) and presence of any chronic systemic illness. Also, patients with platelet levels at baseline ≤ 50,000/mm^3, autoimmune diseases (positive antinuclear antibodies), thyroid disorders, hepatocellular carcinoma, and serum creatinine> 2.5mg% were excluded.

Sample collection

Ten milliliters of venous blood were collected from volunteers and HCV-infected patients under complete aseptic conditions and divided as follows:

- A volume of 3 ml was placed in a sterile tube containing ethylene diamine tetra acetic acid (EDTA); for performing CBC and determining TLR7 gene expression mRNA in peripheral total leukocytes using q-PCR technique.
- Precisely 2 ml was placed in sterile tube containing 3.2 % sodium citrate to perform prothrombin time.
- The remaining 5 ml were put into a sterile gel tube, left at 37^{o}C for 30 min for complete clot formation and then centrifuged for 10 min to obtain serum.

Blood analysis

All healthy volunteers and patients were subjected for full history and the following analyses:

- HCV viral load was quantified by RT-PCR (Step One ™RT-PCR, Applied Biosystems, Qiagen USA), and HCV antibodies were assayed by ELISA.
- HBV and HIV were tested by ELISA, levels of fasting blood glucose (FBG), creatinine, alpha fetoprotein level (AFP), thyroid stimulating hormone (TSH) and antinuclear antibody (ANA) were determined in sera.

● Determination of Complete blood count, prothrombin time were performed automatically.

● Activities of ALT and AST as well as concentrations of albumin and total bilirubin were estimated in sera.

● Analysis of Serum Interleukin-12 p70 Immunological Parameter.

Serum IL-12 p70 was assayed in serum by sandwich ELISA (Quantikine HS High sensitivity, R& D Systems, USA). The concentration of IL-12 p70 in the samples was then determined by comparing the optical density of the samples to the standard curve.

● RNA Extraction from Whole Blood, cDNA Synthesis and qPCR

RNA was extracted from blood total leukocytes using QIAamp RNA Blood Mini Kit supplied by Qiagen, Germany. During the QIAamp procedure for purification of RNA from blood, erythrocytes were selectively lysed and leukocytes were recovered by centrifugation. Leukocytes then were lysed using highly denaturing conditions that immediately inactivated RNases, allowing the isolation of intact RNA. After homogenization of the lysate by a brief centrifugation through a QIA shredder spin column, ethanol was added to adjust binding conditions and the sample was applied to the QIAamp spin column. RNA was bound to the silica membrane during a brief centrifugation step. Contaminants were washed away and total RNA was eluted in RNase-free water for direct use. The quantity and purity of the RNA were assessed by measuring absorbance at 260 and 280 nm, using a UV-spectrophotometer (Photo Biometer, Eppendorf, Germany) and then calculating A_{260}/A_{280} ratio.

A total of 1µg of RNA was reverse transcribed into single-stranded complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific, Belgium. cDNA synthesis was performed using Gene Amp PCR System 9700, Applied Biosystems by life technologies, USA. cDNA products were analyzed on 1% agarose gel. Detection of amplification product was done via quantitative real time-PCR (ViiA™ Real-Time PCR System, Thermo Fisher Scientific, Belgium) using SYBR Green PCR master mix (Applied Biosystems by life technologies, USA) for detecting TLR7 gene expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization of the amount of mRNA expression of the gene of interest. Primers for TLR7 and GAPDH were designed according to (Hornung *et al.*, 2002) obtained from Invitrogen, Thermo Fisher Scientific, Belgium and used as follows:

For TLR7 the Forward primer was: TTTACCTGGATGGAAACCAGCTA and Reverse primer: TCAAGGCTGAGAAGCTGTAAGCTA and The GAPDH-forward primer was ATGGCT ATG ATG GAG GTC CAG and GAPDH reverse was TTG TCC TGC ATC TGC TTC AGC Briefly, (400 ng/reaction) of cDNA was used for each PCR with 80 µmol/L of forward and reverse primers in a total volume of 50µL. The thermal cycling conditions comprised of 15 minutes at 95°C, followed by 50 cycles at 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. To control for the specificity of the amplification products, after 50 cycles a melting curve was generated in the range of 55°C to 95°C. The relative quantity of the target mRNA was normalized to the level of the internal control GAPDH mRNA level. Gene expression was expressed in relative units ($RQ = 2^{-\Delta\Delta CT}$) where ΔCT is the difference between the gene of the target and the housekeeping gene, and ΔΔCT is the change between the ΔCT for each sample and the control group. TLR7 gene was then described as the fold change from the control group.

Statistical analysis

The results were analyzed by the SPSS v.16.0 software (SPSS Inc, Chicago, IL, USA). Results are presented as mean ± SD and median. We used independent sample t-test for comparison of the individual data between the groups. P values of less than 0.05 were considered statistically significant.

Results

Data shown in Table 1 report that patients with chronic HCV infection had 1.09 x 10^6 IU/ml viral load with significant increases in serum ALT and AST activities (75 and 65.6%, respectively, $p<0.0001$),

compared to the healthy volunteers. Serum ALT and AST activities at the end of treatment of HCV-infected patients with SOF+RBV (24 weeks) and 3 months after the end of the treatment period (follow up) did not changed significantly, compared to HCV group, while there was no evidence for HCV viral load. On the other hand, treatment of HCV patients with SOF+RBV+IFN-α for 12 weeks and after 3 months from the end of treatment elevated serum ALT and AST activities (%34.61&22.9% and 47.7&41.8%, respectively, $p<0.0001$), compared to HCV group, without any viremia.

Table 1. Viral features; liver enzymes, kidney function and blood glucose of the volunteers and patients in the studied groups.

Parameters / Groups	Viral Load (x 10^6IU/ ml)	Serum ALT (IU/L)	Serum AST (IU/L)	Creatinine (mg%)	FBG (mg%)
Normal Control	0.0±0.0	28.4±8.45	28.6±8.0	0.61±0.28	102±19.18
Mean± SD	0.0	27.0	27.0	0.50	106.0
Median					
HCV	1.09 ±1.72	49.73±2.48	47.38±2.63	0.60±0.23	97.66±22.04
Mean± SD	0.38	42.0	42.0	0.60	95.00
Median					
Change%, P	Viremia, 0.0001	75.0%,0.0001	65.6%,0.0001	1.6%, NS	4.2%, NS
HCV+SOF+RBV	0.0±0.0	50.29±1.15	51.56±1.36	0.99±0.25	111.22±23.50
Mean± SD	0.0	49.00	54.00	0.96	110.50
Median					
Change%, P<	0%, NS	77%, 0.0001	80.2 %,0.0001	6.2%, NS	8.8%, NS
Change%, p^a<	-100%, 0.0001	1.18%, NS	8.8%, NS	65%, NS	14.4%, NS
HCV+ SOF+RBV F	0.00±0.0	52.88±6.83	55.57±8.21	0.83±0.20	103±1.39
Mean ± SD	0.00	51.50	58.00	0.84	1.00
Median					
Change%, P<	0%, NS	86.19%,0.0001	94.3%,0.0001	36%, NS	0.09%, NS
Change%, p^a<	-100%, 0.0001	6.3%, NS	17.2%, NS	38%, NS	5.46%, NS
Change%, P^b<	0%, NS	5.1%, NS	7.7%, NS	16.1%, NS	7.3%, NS
HCV+ SOF+RBV+IFN-α	0.00±0.0	66.94±2.14	70.02±2.17	0.76±0.27	104.90±19.85
Mean± SD	0.00	65.00	74.00	0.81	107.00
Median					
Change%, P<	0%, NS	135%,0.0001	144.83%, 0.0001	2.4%, NS	2.8%, NS
Change%, p^a<	-100%, 0.0001	34.61%, 0.0001	47.7%,0.0001	26.6%, NS	6.49%, NS
Change%, P^b<	0%, NS	33.0%, 0.0001	35.8%,0.0001	23.2%, NS	6.3%, NS
HCV+ SOF+RBV+IFN-αF	0.0±0.0	61.15±1.86	67.21±1.78	0.91±0.36	99.21±2.20
Mean± SD	0.0	64.00	65.00	0.89	98.00
Median					
Change%, P<	0%, NS	115%, 0.0001	134%,0.0001	49.1%, NS	2.9%, NS
Change%, p^a<	-100%, 0.0001	22.9%, 0.05	41.8%,0.0001	8.0%, NS	2.7%, NS
Change%, P^c<	0%, NS	-8.6%, NS	4.0%, NS	19.7%, NS	5.4%, NS

-Results are Mean± SD. -SOF= Sofosbuvir, RBV= Ribavirin, F= 3 Months Follow Up, IFN= Interferon, NS= non significant. -Change% and P: versus normal control group, Change% and P^a: versus HCV group, Change% and P^b: versus HCV+SOF+RBV group, Change% and P^c: versus SOF+RBV+IFN-α group. -The mean difference is significant at $p<0.05$.

In order to study the toxic effects of the treatment modalities, creatinine and fasting blood glucose levels were estimated in sera of the patients. Treatment with SOF+RBV and SOF+RBV+IFN-α at the end of the treatment period and after 3 months of follow up did not cause any significant changes in serum creatinine and fasting blood glucose concentrations (Table 1). Regarding to hemostatic and chemistry characteristics in the different studied groups (Table 2), infection of patients with HCV caused significant reductions in blood platelet count (38.83%, $p<0.001$), plasma clotting factors activity (13.29%, $p<0.001$) and serum albumin concentration (16.59%, $p<0.0001$), while elevated serum total bilirubin value (142.4%, $p<0.0001$), compared to the healthy volunteers. On the other hand, all treatment modalities resulted in more significant reduction in blood platelet count, compared to HCV group.

Table 2. Hemostatic and Chemistry Characteristics of the Volunteers and Patients in the Different Studied Groups.

Parameters / Groups	Platelets Count ($x10^4/mm^3$)	Clotting Factors Activity (%)	Serum Albumin Conc. (g%)	Serum T. Bilirubin Conc. (mg%)
Normal Control	26.58±8.79	93.10±5.20		0.59±0.21
Mean± SD	25.4	94.0	4.22±0.35	0.60
Median			4.20	
HCV	16.26±3.98	80.73±7.51		1.43±0.43
Mean± SD	16.2	83.00	3.52±0.51	1.40
Median			3.60	
Change%, P	-38.83%, 0.001	-13.29%, 0.0001	-16.59%, 0.0001	142.4%, 0.0001
HCV+SOF+RBV	5.52±2.1	79.5±1.00		1.15±0.32
Mean± SD	5.10	79.00	3.5±0.57	1.10
Median			3.60	
Change%, P<	-79.2%,0.0001	-14.6%,0.0001	-17%,0.0001	94.92%, 0.0001
Change%, p^a<	-66.05%,0.0001	0.11%, NS	-0.56%, NS	-19.58%,0.0001
HCV+ SOF+RBV F	5.86±2.83	83.23±8.23		1.15±0.21
Mean± SD	5.00	83.00	3.68±0.48	1.10
Median			3.60	
Change%, P<	-77.95%,0.0001	-10.60%, 0.0001	-12.7%, 0.0001	94.92%,0.0001
Change%, p^a<	-63.96%, 0.0001	3.09%, NS	4.5%, NS	-19.58%,0.0001
Change%, P^b<	6.16%, NS	0.50%, NS	-16.74%, NS	0%, NS
HCV+ SOF+RBV+IFN-α	6.25±3.30	82.5±9.7		0.98±0.52
Mean± SD	5.60	82.00	3.69±0.74	0.88
Median			3.90	
Change%, P<	76.49%,0.001	-10.50%,0.0001	-12.56%,0.0001	66.10%,0.0001
Change%, p^a<	61.56%, 0.0001	3.21%, NS	4.83%, NS	-31.47%, 0.0001
Change%, P^b<	-13.22%,0.05	0.6%, 0.05	-16.52%, NS	-14.78%, NS
HCV+ SOF+RBV+IFN-αF	4.93±1.96	80.94±8.03		1.01±0.47
Mean± SD	4.70	81.00	4.13±0.55	0.94
Median			4.20	
Change%, P<	-81.45%,0.0001	-13.06%, 0.0001	-2.13%, NS	71.19%,0.0001
Change%, p^a<	69.68%, 0.0001	0.26%, NS	17.33%, 0.0001	-29.37%, 0.0001
Change%, P^c<	21.12% , NS	-2.86%, NS	11.92%, 0.01	3.06%, NS

-Results are Mean ± SD. -PT= Prothrombin Time, SOF= Sofosbuvir, RBV= Ribavirin, F= 3 Months Follow Up, IFN= Interferon, NS= nonsignificant.

-Change% and P: versus normal control group, Change% and P^a: versus HCV group, Change% and P^b: versus HCV+SOF+RBV group, Change% and P^c: versus SOF+RBV+IFN-α group. -The mean difference is significant at $p<0.05$.

In addition, they reduced significantly serum total bilirubin concentration, compared to HCV group, but still significantly higher than that of normal volunteers. Plasma activity of clotting factors was significantly decreased in the treatment groups, compared to the healthy volunteers. Serum albumin concentration was non significantly changed in all treatment groups, compared to HCV , except for Subgroup (IV SOF+ RBV+INF F) it was significantly increased (17.33%, $p<0.0001$).

Table 3. Hematologicfeatures in the different studied groups.

Parameters / Groups	RBC Count (million/mm³)	Hb Conc. (g%)	HCT (%)	MCV (*fl*)	MCH (pg)	MCHC (%)
Normal Control	4.37±0.49	13.12±1.4	39.39±4.48	90.05±0.38	30.01±0.12	33.3±0.0
Mean± SD	4.37	13.1	39.3	90	30	33.3
Median						
HCV	4.49±0.55	13.45±1.62	43.96±4.90	97.51±1.95	29.69±2.06	30.88±1.35
Mean± SD	4.55	13.65	44.20	97.500	30.00	30.74
Median						
Change%, *P*	2.75%, NS	2.5%, NS	11.6%,0.0001	8.28%,0.0001	-1.07%, NS	-7.27%,0.0001
HCV+SOF+RBV	2.66±0.55	7.80±1.59	26.85±4.73	99.78±3.87	29.30±1.45	28.99±1.20
Mean± SD	2.68	7.60	26.00	100	29.6	29.10
Median						
Change%, P<	-39.13%,0.0001	-40.5%,0.0001	-31.84%, 0.0001	10.81%,0.0001	-2.37%,0.003	-12.94%, 0.0001
Change%, p^a<	-40.76%, 0.0001	-42%, 0.0001	38.9%, 0.0001	2.33 %, 0.0001	-1.33%, NS	-6.12%, 0.0001
HCV+ SOF+RBV F	2.86±0.42	9.27±1.56	29.9±5.0	104±3.6	32.24±1.92	30.9±1.03
Mean± SD	2.82	9.45	29.1	105	32.9	31.25
Median						
Change%, P<	-34.55%, 0.0001	-29.3%, 0.0001	-24%,0.001	15.5%, 0.0001	7.43%, 0.001	-7.2%, 0.01
Change%, p^a<	-36.3%, 0.0001	-31.07%, 0.0001	-39.7%, 0.0001	6.65%, 0.001	8.59%, 0.0001	0.064%, NS
Change%, P^b<	7.5%, NS	18.8%, 0.01	11.3%, 0.05	4.22%, 0.01	10.03%, 0.001	6.5%, 0.0001
HCV+ SOF+RBV+IFN-α	3.45±0.36	10.63±1.10	35.21±3.39	101.0±3.48	30.66±0.79	30.17±0.66
Mean± SD	3.50	10.70	35.50	100	30.30	30.18
Median						
Change%, P<	-21.05%, 0.0001	-18.9%,0.0001	-10.61%, 0.0001	12.16%, 0.0001	2.17%, 0.0001	-9.40%, 0.0001
Change%, p^a<	-23.16%, 0.0001	-21.00%,0.0001	19.9%, 0.0001	3.5%, 0.0001	3.27%, 0.0001	-2.30%, 0.0001
Change%, P^b<	29.70%, 0.0001	36.28%,0.0001	31.1%, 0.0001	1.2%, 0.05	4.64%, 0.0001	4.0%, 0.0001
HCV+ SOF+RBV+IFN-αF	2.90±0.39	9.38±1.22	31.57±3.67	108.0±2.9	32.36±0.81	29.72±0.56
Mean± SD	3.01	9.90	33.10	108	32.08	29.90
Median						
Change%, P<	-33.64%, 0.0001	-28.5%, 0.0001	-19.85%, 0.0001	19.9%, 0.0001	7.83%, 0.0001	-10.75%, 0.0001
Change%, p^a<	-35.4%, 0.0001	-30%, 0.0001	28.1%, 0.0001	10.7%, 0.01	9.00%, 0.0001	-3.76%, 0.0001
Change%, P^c<	-15.94%, 0.0001	--11.76%, 0.0001	10.3%, 0.001	6.9%, 0.001	5.54%, 0.0001	-1.49%, 0.01

-Results are Mean ±SD. -SOF= Sofosbuvir, RBV= Ribavirin, F= 3 Months Follow Up, IFN= Interferon, NS= nonsignificant. -Change% and P: versus normal control group, Change% and P^a: versus HCV group, Change% and P^b: versus HCV+SOF+RBV group, Change% and P^c: versus SOF+RBV+IFN-α group. -The mean difference is significant at $p<0.05$.

Hematologic features of all the studied groups were significantly varied and differed from that of the healthy volunteers (Table 3). In HCV group, there were significant elevations in HCT (11.6%, $p<0.0001$) and MCV (8.28%, $p<0.0001$) in HCV- infected patients, in addition to a significant reduction in MCHC (7.27%, $p<0.0001$), compared to volunteers group, On the other hand, SOF+RBV group caused significant reductions in all the studied CBC parameters except for MCV where it was significantly increased, compared to both volunteers and HCV groups. SOF+RBV F, SOF+RBV+IFN-α and SOF+RBV+IFN-α F groups produced significant reductions in all the studied CBC parameters except for MCV and MCH where they were significantly increased, compared to volunteers and/or HCV groups.

Table 4 demonstrated that, Infection of Egyptian patients with HCV decreased body immunity, which was reported by significant reductions in total WBC count (13.69%, $p<0.05$) and the absolute numbers of segmented neutrophils (35.31%, $p<0.0001$), eosinophils (40.41%, $p<0.001$) and monocytes (39.28%, $p<0.001$), compared to the normal volunteers, without any significant changes in the absolute numbers of staff neutrophils and lymphocytes. The treatment modalities (SOF+RBV, SOF+RBV F, SOF+RBV+IFN-α and SOF+RBV+IFN-α F) produced more significant reductions in the total WBC count and the absolute numbers of segmented neutrophils, eosinophils, monocytes and lymphocytes compared to HCV group. On the other hand, there were non-significant changes in WBC count and the absolute numbers of segmented neutrophils, eosinophils, monocytes and lymphocytes when comparing SOF+RBV F with SOF+RBV. But in comparing SOF+RBV+IFN-α F with SOF+RBV+IFN-α, there were significant reductions ($p<0.05$) in the absolute count of eosinophils and lymphocytes only.

Table 5 shows that, infection of HCV does not cause any significant change in the relative quantitation of TLR7 gene expression with a significant elevation in serum IL-12p70 concentration (161.73%, $p<0.0001$), compared with the volunteers group.

In SOF+RBV and SOF+RBV+IFN-α groups, the treatments produced a significant elevation in the relative quantitation of TLR gene expression (250.53 and 506.32%, respectively, $p<0.0001$), compared with HCV group, but these elevations returned to the normal level after 3-months of follow up period. On the other hand, the concentration of serum IL-12p70 was significantly reduced in all treatment groups, compared to both volunteers and HCV groups , except group III (SOF+RBV) showed anon significant change compared to volunteers.

Discussion

Chronic hepatitis C virus (HCV) infection is a major global public health problem, with recent estimates suggesting that 64-103million people are infected worldwide (Gower *et al.*, 2014; Coppola *et al.,* 2014). For many years, Pegylated interferon-alpha (peg-IFN) plus ribavirin (RBV) combination therapy was the standard treatment for HCV infection (Coppola *et al.,* 2008). However, the low rate of virological response and the frequency and severity of the side-effects (flu-like symptoms, depression, cytopenia and hemolytic anemia) made this therapy arduous for patients with cirrhosis (Borgia *et al.,* 2003; Miller *et al.*, 2014).

Recently, regimens without interferon, which combine several classes of directly acting antiviral agents (DAAs), have improved the response rate and tolerability, particularly in difficult-to-treat patients such as the patients with an advanced liver disease. In fact, these IFN-free regiments yield sustained virological response (SVR) rates of approximately 90%, even in patients with cirrhosis. However, in difficult-to-treat patients, such as cirrhotics and nonresponder patients, the international guidelines, still suggest the combination of DAAs and Ribavirin to achieve a high SVR rate (European Association. 2015).

Data presented in this study reported a significant decrease in serum albumin with a significant increase in serum total bilirubin concentrations in HCV group. These results agreed with Afify *et al.,* 2017and Morsy *et al.,* 2016.

Table 4. Blood WBC count and Its Differential absolute number in the Studied Groups

Parameters / Groups	WBC Count (thousands/mm³)	Segmented Neutrophils (cells/mm³)	Staff Neutrophils (cells/mm³)	Eosinophils (cells/mm³)	Monocytes (cells/mm³)	Lymphocytes (thousands/mm³)
Normal Control	6.43±1.96	4.22±1.37	65.79±63.44	216.1±105.64	222.1±145.89	2.47±0.98
Mean± SD	5.85	4.52	72.75	227	178.5	2.38
Median						
HCV	5.55±1.78	2.73±1.06	74.53±49.43	128.77±78.25	134.86±80.14	2.48±0.97
Mean± SD	5.300	2.54	67.5	114	123	2.3
Median						
Change%, P	-13.69%, 0.05	-35.31%,0.0001	13.28%, NS	-40.41%, 0.001	-39.28%, 0.001	0.40%, NS
HCV+SOF+RBV	2.91±6.81	1.48±0.48	36.63±23.87	63.85±33.45	57.71±32.81	1.28±0.44
Mean± SD	2.90	1.42	31	62	52	1.26
Median						
Change%, P<	-54.7%, 0.0001	-64.93%, 0.0001	-44.32%, NS	-70.45%, 0.001	-74.02%, 0.01	-48.18%, 0.0001
Change%, p[a]<	-47.5%, 0.0001	-45.79%, 0.0001	-50.85%, 0.0001	-50.42%, 0.0001	-57.21%, 0.0001	-48.39%, 0.0001
HCV+SOF+RBV F	2.80±8.15	1.39±0.53	28.92±24.05	78.75±49.40	60±32.9	1.24±0.410
Mean± SD	2.80	1.41	28.5	70	59.0	1.87
Median						
Change%, P<	-56.4%, 0.0001	-67.06%, 0.0001	-56.04%, NS	-63.56%, 0.001	-72.9%, 0.001	-49.4%, 0.001
Change%, p[a]<	-49. %, 0.0001	-49.08%, 0.0001	-61.20%, 0.0001	-38.84%, 0.01	-55.8%, 0.0001	-49.8%, 0.0001
Change%, P[b]<	-3.78%, NS	-6.08%, NS	-21.05%, NS	23.34%, NS	3.9%, NS	-3.12%, NS
HCV+ SOF+RBV+IFN-α	3.28±9.15	1.57±0.56	37.48±23.15	76.22±49.3	77.86±45.61	1.54±0.57
Mean± SD	3.15	1.44	35	63	69	1.47
Median						
Change%, P<	-48.9%, 0.0001	-62.80%, 0.0001	-43.03%, NS	-64.73%,0.0001	-64.94%, 0.0001	-37.65%, 0.0001
Change%, p[a]<	-40.9%, 0.0001	-42.49%, 0.0001	-49.71%, 0.0001	-40.81%, 0.0001	-42.27%, 0.0001	-37.90%, 0.0001
Change%, P[b]<	12.71%, 0.05	6.08%, NS	2.32%, NS	19.37%, NS	34.92%, 0.05	20.31%, 0.05
HCV+ SOF+RBV+IFN-α	2.89±9.69	1.51±0.71	40.16±32.42	50.21±27.73	67.42±57.12	1.24±0.42
FMean± SD	2.69	1.25	29	38	56	1.33
Median						
Change%, P<	-55.0%, 0.0001	-64.22%, 0.0001	-38.96%, NS	-64.73%, 0.0001	-69.64%, 0.0001	-49.80%, 0.001
Change%, p[a]<	-47.9%, 0.0001	-44.69%, 0.0001	-46.12%, 0.0001	-40.81%, 0.0001	-50.00%, 0.0001	-50.00%, 0.0001
Change%, P[c]<	-11.90%, NS	3.82%, NS	7.15%, NS	-34.12%, 0.05	-13.41%, NS	-19.48%, 0.05

-Results are Mean± SD.

-Results of basophils percentage are not shown because they were 0 in all the studied groups. -SOF= Sofosbuvir, RBV= Ribavirin, F= 3 Months Follow Up, IFN= Interferon, NS= nonsignificant.

-Change% and P: versus normal control group, Change% and P[a]: versus HCV group, Change% and P[b]: versus HCV+SOF+RBV group, Change% and P[c]: versus SOF+RBV+IFN-α.

On the other hand, all the treatment modalities showed no HCV viral load with a significant increase in serum total bilirubin, compared to the volunteers. This emphasizes that hyperbilirubinemiais because of RBC hemolysis due to ribavirin treatment (Morsy *et al.*, 2016).

Table 5. Relative TLR7 Gene Quantitation (RQ) and Serum Interleukin-12 p70 (IL-12 p70) Concentration in the studied groups.

Parameters / Groups	Relative TLR7 Gene Quantitation (RQ)	Serum IL-12 p70 (pg/ml)
Normal Control	0.954±0.74	10.27±1.35
Mean± SD	0.70	9.94
Median		
HCV	0.952±0.76	26.88±3.13
Mean± SD	0.82	26.74
Median		
Change%, p	-0.20%, NS	161.73%, 0.0001
HCV+SOF+RBV	3.33±0.72	10.99±1.71
Mean± SD	3.0	10.10
Median		
Change%, P<	250%, 0.0001	7.01%, NS
Change%, p[a]<	250.53%, 0.0001	-59.1%, 0.0001
HCV+SOF+RBV F	0.75±0.35	8.90±1.05
Mean± SD	0.75	8.92
Median		
Change%, P<	-21.0%, NS	-13.34%, 0.05
Change%, p[a]<	-21.05%, NS	-66.89%, 0.0001
Change%, P[b]<	-77.48%, 0.0001	-19.02%, 0.001
HCV+ SOF+RBV+IFN-α	5.76±2.75	7.37±0.56
Mean± SD	5.25	7.35
Median		
Change%, P<	506%, 0.0001	-28.24%, 0.0001
Change%, p[a]<	506.32%, 0.0001	-72.58%, 0.0001
Change%, P[b]<	163.06%, 0.01	-32.94%, 0.0001
HCV+ SOF+RBV+IFN-αF	0.94±0.61	8.21±1.20
Mean± SD	0.67	8.23
Median		
Change%, P<	-1.05%, NS	-20.06%, 0.001
Change%, p[a]<	-1.05%, NS	-69.46%, 0.0001
Change%, P[c]<	-83.68%, 0.0001	11.40%, NS

Results are Mean ± SD of 10 values.

-TLR7= Toll Like Receptor 7, SOF= Sofosbuvir, RBV= Ribavirin, F= 3 Months Follow Up, IFN= Interferon, NS= nonsignificant.

-Change % and P: versus normal control group, Change% and P^a: versus HCV group, Change% and P^b: versus HCV+SOF+RBV group, Change% and P^c: versus HCV+ SOF+RBV+IFN-α -The mean difference is significant at $p<0.05$.

After the end of the double therapy "SOF+RBV; Gr. III", nonsignificant change in serum albumin concentration compared HCV group was noticed which was then reduced significantly by 12.7% at$p<0.0001$ after a follow up period of 3 months (SOF+RBVF; Subgroup III) compared to normal volunteers. On the other hand, serum albumin concentration remained low at the end of triple therapy (Gr. IV), compared to volunteers.

Serum albumin concentration returned to the normal level after 3 months of follow up (Subgroup IV). From the results presented in this piece of work it is clear that the regimen of pegylated interferon, sofosbuvir plus ribavirin is safe and effective in treatment of Egyptian patients with hepatitis C virus as well as sofosbuvir and ribavirin.

The current study revealed that, Egyptian patients infected with HCV have hemostatic alterations involving a significant decrease in platelets count and clotting factors activity, compared to the volunteers, which agreed with Leticia *et al.,* 2014.

The study of Hyer *et al.*, 2001 had returned the cause of the PT prolongation after exposure to HCV to dysfibrinogenemia and thrombocytopenia. Kuter and Begley, 2002 explained the cause of marked thrombocytopenia in HCV infections by impaired hepatic synthesis of thrombopoietin protein.

HCV has also been reported to bind to platelet membranes by multiple cell surface receptors. Garcia-Suarez *et al.*, 2000 and Rajanand Liebman, 2001 documented that platelet membranes of patients with high HCV viral loads may be heavily coated with HCV, therefore anti-HCV antibodies will bind to the platelet surface-associated HCV, leading to phagocytosis of the antibody-coated platelets.

Our different treatment modalities produced more pronounced decrease in the platelets count, compared to HCV group. A reduction in clotting factors activity was reported in Gr III –IV and subgroups III- IV, compared to normal volunteers. These results agreed with those obtained by El Raziky *et al.*, 2013.

TLRs are a family of receptors that play key roles in innate immunity. Recent research indicates that the ligand for TLR7 can inhibit HCV replication in HCV-infected patients, suggesting that these TLRs may play a role in regulating HCV infection (Thomas *et al.*, 2007). Mele, 2016 documented that in addition to HIV-1, HCV capable of causing chronic infection interacts with TLR7 in CD4 T cells, inhibiting their function.

The mechanisms by which HCV interacts with TLR7 *in vivo* are still poorly understood. TLR7 is located in the intracellular endosomal compartment. Replicating and non-replicating HCV genome fragments have been detected in several immune cell types during HCV infection, including T cells, which may directly affect T cell function (Kndo *et al.,* 2011).

HCV products are able to stimulate TLR signaling; however, HCV is able to simultaneously evade immune response through targeting and impairing TLR signaling (Chang *et al.*, 2010). It is suggested that HCV interferes with TLR signaling pathway via interaction with their signaling intermediates or altering their expression levels (Imran *et al.*, 2012). The data presented in this study indicated that HCV did not cause any alteration in the expression of TLR7 on total leukocytes. Our data are in accordance with Tarantino *et al.,* 2013 who reported that, there were no differences emerged in the TLR2 and TLR7 levels between patients with CHC and controls. There are contradictory results about TLR7 gene expression in patients with HCV; Dolganiuc *et al.*, 2006 reported that mRNA level for TLR7 extracted from peripheral blood mononuclear cells is up regulated in HCV-infected patients when compared with normal volunteers. Data reported by Mohammed *et al.*, 2013 and Motavaf *et al.*, 2014 showed that, chronically infection with HCV showed transcriptional down-regulation of TLR7 in their blood cells. Alternately, Ibrahim *et al.* (2016) showed a dramatic increase in TLR7 gene expression in HCV patients with F0-F1 fibrosis degree compared to healthy subjects, then its expression decrease in F2-F4 group when compared either to healthy subjects or F0-F1 groups.

Our treatment modalities Group III (HCV+SOF+RBV) and group IV (HCV+ SOF+RBV+IFN-α) showed significant elevation (250%, 506% respectively) in TLR7 gene expression with no evidence for viremia, compared to healthy volunteers or HCV-infected patients. Thomas *et al.*, 2007 reported that TLR7 agonists reduce effective lyviremia in hepatitis C patients via stimulating endogenous IFN production.

TLR7 showed lower expression in PBMCs (Taylor *et al.*, 2007) and monocytes (Boghdadi and Seleem, 2014) of chronic HCV patients who did not respond to exogenous IFN than in that of responder patients. Stimulation of TLR7 via different therapeutic modalities in treating virally infected hepatocytes may inhibit HCV replication through direct activation of antiviral genes independent of interferon regulation factors. Boghdadi and Seleem, 2014suggestedthat the TLRs- expression profiles of monocytes from patients with chronic HCV may be useful biomarkers for IFN therapy. Accordingly, TLR7 signaling is directly critical for the efficient control of HCV infection, not only by IFN induction, but also through IFN-independent mechanisms (Bader El Din *et al.*, 2016). On the other hand, our data showed that, the expression of TLR7 gene in total leukocytes returned to the normal and HCV-infected levels after 3 months from the end of treatment modalitie.

These results raise the possibility that targeting TLR7 with high affinity pharmacological stimulants may be able to control HCV infection by induction of IFN-α and direct activation of antiviral mechanisms in hepatocytes. Furthermore, they provide insight about the potential use of TLR7 as a new set of molecular markers of prognosis to response to therapy and outcomes of chronic HCV infection. Our data show that a differential mRNA expression of TLR7 is associated with different responses to different treatment groups. Triple therapy (SOF+RBV+IFN-α) showed the more efficient effect in gene expression than the double therapy. A larger scale clinical investigation should be undertaken to determine this novel therapeutic and prognostic approach.

IL-12 is one of the most important pro-inflammatory cytokines presented with the initiation of immune response, determining Th1 and Th2 differentiation (Youssef *et al.*, 2013). In the present study, chronic HCV-infected patients showed significantly higher serum levels of IL-12 p70 than normal volunteers, suggesting that a strong proinflammatory cytokine response could play an important role in the development of hepatic injury in patients with chronic hepatitis C, and therefore, apart from contributing to viral clearance, this polarized immunological profile may contribute to the pathogenesis of liver disease Gigi *et al.*, 2008;Capone *et al.*, 2010 and EL-Emshaty *et al.*, 2015.

In our treatment modalities, serum IL12p70was reduced significantly, compared to HCV-infected patients. Perperas *et al.* (2013) reported that IL-12 baseline levels are higher in patients who achieved sustained virological response, compared to patients who did not respond to the IFN+RBV combination treatment. Baseline serum levels of IL-12 higher than 3 pg/ml (cut-off) were found to positively predict patients who successfully responded to treatment. So, pre-treatment IL-12 levels seem to predict which patients will achieve SVR to treatment.

Patients with increased IL-12 serum levels were more likely to achieve SVR. Elsharkawy, *et al*, 2017 reported that, Sofosbuvir-based therapies, whether triple or dual showed higher rates of SVR compared to that of the previously used SOF.

Conclusion

The treatment modalities of Egyptian patients infected with HCV, either with Sofosbuvir plus ribavirin or Sofosbuvir plus ribavirin plus pegylated Interferon, showed no viremia immediately after the end of the treatment periods. Significant elevation in total leukocytes TLR7 gene expression as well as a significant reduction in serum IL12p70 can be used as an indication to patient response to therapy.

It is not possible to assess treatment effectiveness comprehensively as the rate of loss to follow-up is high. Further investigations should be done to increase the time after the end of treatment modalities to ensure complete remission of HCV infection.

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