A Newly Discovered Role of the Anti HCV Neutralizing Antibody as a Potential Risk in Influencing HCV Replication and Infection Reactivation

Salah SH1,2*, Ghaleb HA3, Ehab H4 and EL-HARIRI HM5
1Department of Immunology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt
2R&D Center Vaccine Immunology and Immunogen Discovery, Egypt
3Department of Pharmacology, Cairo University, Egypt
4Department of Internal Medicine, National Research Center, Egypt
5Department of Community Medicine, National Research Centre, Egypt

Abstract

Despite all intensive efforts and widespread use of Direct-Acting Antivirals (DAA), that achieve a high Sustained Virological Response (SVR) rate and clearance of Hepatitis C Virus (HCV) RNA from serum, the mechanism of late relapse, is still unknown. Previous observational studies have shown the rapid onset of anti-HCV antibodies to be associated with a higher likelihood of clearance but this study introduced a controversial role of neutralizing antibodies, as it influence HCV reactivation and replication, by coating its structural and nonstructural protein particles in antigen/neutralizing antibody immune-complex form (Ag/nAbs) comprising HCV antigen (E1, E2, C and its specific bounded neutralizing antibody as C1, C2 and C3) for a long time preventing its attacks by CD8+ cytotoxic T-cells and anti-viral drugs. A screening biomarker was designed to detect this Ag/nAbs complex in serum samples of infected patients. In a pilot study comprised 76 patients with chronic HCV Genotype 4, 22 patients were treated before with interferon/Ribavirin for 48 weeks and didn’t respond to this type of therapy as (Group I), 24 patients received Sofosbuvir 400 mg and Daclatasvir 60 mg daily for 24 weeks, as (Group II), 14 patients were diagnosed positive HCV RNA and detectable anti HCV antibody in their sera (Group III) 6 patients were negative HCV RNA and detectable anti HCV antibody as (Group IV) none of the patients in group 3, 4 had been previously treated with antiviral drugs. A total of 10 sera collected from healthy human subjects having no history of any liver complications, undetectable anti HCV antibodies and negative HCV RNA by RT-PCR in their sera were included as negative controls (Group V). Serum samples were collected 3 times at 3, 6 and 12 month intervals for quantitative measuring of (HCV RNA), circulating unbounded neutralizing (anti-E1), (anti-E2), (anti-C antigen), (E1 antigen/bounded anti E1 complex as C1), (E2 antigen/bounded anti E2 complex as C2), (C antigen/bounded anti C antibody complex as C3). We concluded that there is a marked association between the increase levels of circulating immune-complex C1, C2, C3 and the clearance of HCV RNA from blood, also we identified significant correlation between the relapse of HCV RNA after achieving SVR for a long time and the diminish in levels of these immune-complex. These results have important implications for the development of real therapeutic and prophylactic vaccine and also raise the great possibility for developing a serological screening method for monitoring HCV treatment.

Keywords: HCV envelope glycoproteins (E1); HCV envelope glycoproteins (E2); HCV core antigen (c); Neutralizing antibody (nAbs); Antigen/antibody complex (Ag/Abs); Type I interferon-a (IFN-a); Direct-acting antivirals (DAA)

Introduction

Hepatitis C Virus (HCV) is a major cause of liver disease and hepatocellular carcinoma worldwide [1]. HCV establishes a chronic infection in the majority of cases. HCV has been classified into seven major genotypes, which differ by 30% at the nucleotide level, and this positive sense, single-stranded RNA virus has a capacity for rapid evolution of variant viruses during persistent infection. HCV contains 9.6-kb RNA genome that is translated as a single poly-protein and then cleaved by viral and host proteases into structural proteins (core, E1, and E2), p7, and nonstructural
proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [2]. Viral attachment and entry are mediated by the envelope glycoproteins, E1 and E2 [3,4]. Four attachment or entry receptors that are required for infection of hepatocytes have been identified, including CD81. The structural proteins of HCV include the capsid (core) protein and two envelope glycoproteins (E1 and E2) [6,7]. Around 80% of infected individuals develop chronic infections, more than 2% of the globe is chronically infected and infection is the main etiological agent of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma among the mechanisms the virus exerts to persist infection are, down regulating expression of its glycoproteins on cell surface [8-10], thus reducing the possibility of antibody recognition and destruction of infected cells.

Cellular immune responses, involving both CD8+ Cytoxic T Lymphocytes (CTL) and CD4+ T-helper cells, play an essential role in the control of HCV infection, as they do in other persistent viral diseases. Whereas CTLs are traditionally thought to be the main effector cells that eliminate HCV-infected cells [11], it is clear that HCV-specific CD4+ T cells also play a critical role. These cells can potentially act in multiple ways and are central to the initiation and maintenance of adaptive immunity [12]. Two likely major roles are in providing help for CD8+ T cells by cytokine production and activation of antigen-presenting cells, but there are multiple other mechanisms of action including direct antiviral effects, a role in B cell maturation, and regulatory functions [13].

The neutralizing antibody plays an essential role in controlling chronic viral infections and can mediate Antibody Dependent Cellular Cytotoxicity (ADCC), Complement-Dependent Cytotoxicity (CDC) or antibody dependent phagocytosis [14,15]. Our level of understanding on the role of neutralizing antibody activity in controlling HCV replication is limited. Most HCV seropositive patients are has viremia, and there is no correlation between clinical outcome and antibody response to any specific HCV antigen. Furthermore, the presence of HCV antibody did not prevent the chimpanzees from re-infection by homologous or heterologous viral strains in chimpanzee cross-challenge experiments [16,17]. When the body’s immune system raises antibodies against antigenic determinants of host or foreign substances that recognize and bind to the antigen molecules an immune-complex comprise these neutralizing antibody/Antigen complex can form. Normally, insoluble immune complexes that are formed are cleared by the phagocytic cells of the immune system, but when an excess of antigen-antibody are present, the immune complexes are often deposited in tissues [18,19], where they can induce the production of IgG- anti complex antibody. Immunized rabbits with complex A and B peptide: A 30 male New Zealand albino rabbits weighing 8-10 lb were classified into three groups (A), (B) and (C), every group contains 10 rabbits, group (A) were injected with Complex mixture C1 and group (B) injected with complex mixture C2 and groups (c) with complex mixture C3. All the rabbits in every group were injected with 0.20 ml of complex mixture intramuscular for 45 days, after six weeks blood samples were collected from the rabbits. 30 ml of immune serum (150 mg) was obtained from every rabbit. The obtained serum contains IgG-anti complex, were all rabbits of group A has IgG anti-C1, group B IgG anti-C2 and the group C has IgG anti-C3 antibodies. To obtain the anti-complex antibody from sera of every rabbit, 22 patients treated before with interferon/Ribavirin for 48 weeks (Group I) all the patients of this groups not responded to the therapy, 24 patients received Sofosbuvir 400 mg and Daclatasvir 60 mg daily for 24 weeks daily for 24 weeks as (Group II), 14 patients none of them had been previously treated with antiviral drugs as (Group III), 6 patients were negative HCV RNA and detectable anti HCV antibody as (Group IV) and a total of 10 sera samples collected from healthy human subjects having no history of any liver complications, undetectable anti HCV antibodies and negative HCV RNA by RT-PCR in their sera were included as negative controls (Group V).

Materials and Methods

Human sera

This study was carried out on a total 76 patients with chronic HCV Genotype 4, every patients wrote consent of acceptance to participate in this study, 22 patients treated before with interferon/Ribavirin for 48 weeks (Group I) all the patients of this groups not responded to the therapy, 24 patients received Sofosbuvir 400 mg and Daclatasvir 60 mg daily for 24 weeks daily for 24 weeks as (Group II), 14 patients none of them had been previously treated with antiviral drugs as (Group III), 6 patients were negative HCV RNA and detectable anti HCV antibody as (Group IV) and a total of 10 sera samples collected from healthy human subjects having no history of any liver complications, undetectable anti HCV antibodies and negative HCV RNA by RT-PCR in their sera were included as negative controls (Group V). Serum samples were collected 3 times at 3, 6 and 12 month intervals for quantitative measuring of HCV RNA, circulating free unbounded anti-E1, anti-E2, anti-core antibodies, E1 antigen/bounded anti E1 complex, E2 antigen/bounded anti E2 complex, c antigen/bounded anti core antibody complex. A new Enzyme-Linked Immunosorbert Assay (ELISA) was developed for the detection of immune-complex C1, C2 and C3.

Immune-complex preparation: HCV antigens [E1, E2 and c] of different concentrations (0.05, 1.5, 2.0 ng/mL) of (SIGMA-ALDRICH) were obtained to make in vitro serial concentration of agglutinate immune-complex C1, C2, C3 (antigen/antibody). An equal concentration of antigens and its specific antibodies were mixed and allowed them to agglutinate and precipitated to obtain three specific different complexes structure, C1 (E1 antigen/anti E1), C2 (E2 Ag/anti E2) and C3 (c antigen/anti core antibody) with these serial concentrations; 0.05, 0.5, 5, 10, 15, 20 ng/mL. Every concentration of antigen is incubated to its similar concentration of Abs, a polyethylene glycol was added to maintain the activity of the structural protein, then the mix form centrifuged at 2500 g for 10 minutes, the supernatant are poured and the obtained sediments were added to 3 ml of Freund’s complete adjuvant to immunize the rabbits groups with these immune-complexes to induce the production of IgG- anti complex antibody.

Immunized rabbits with complex A and B peptide: A 30 male New Zealand albino rabbits weighing 8-10 lb were classified into three groups (A), (B) and (C), every group contains 10 rabbits, group (A) were injected with Complex mixture C1 and group (B) injected with complex mixture C2 and groups (c) with complex mixture C3. All the rabbits in every group were injected with 0.20 ml of complex mixture intramuscular for 45 days, after six weeks blood samples were collected from the rabbits. 30 ml of immune serum (150 mg) was obtained from every rabbit. The obtained serum contains IgG-anti complex, were all rabbits of group A has IgG anti-C1, group B IgG anti-C2 and the group C has IgG anti-C3 antibodies. To obtain the anti-complex antibody from sera of every rabbit in every group, ammonium sulfate 40% saturated solution was used to precipitate the antibody, we centrifuging the precipitate at 15,000 rev/min for 5 minutes in a Beckman 152 microfuge, the supernatant was removed and the precipitate was washed twice with half-saturated (NH4)2SO4, then we carefully remove and discard the supernatant, we re-suspend the precipitate with PBS, another centrifugation was done to remove any remaining depriv, we determine the IgG concentration (mg/ml) by estimating the absorbance reading at 280 nm.
ELISA Assay was prepared to detect (C1), (C2) and (C3) immune-complex in serum sample of infected patients in all examined groups (96-well plates): Description: ELISA Assay Kit for the quantitative measuring of (Anti C1), (Anti C2) and (Anti C3) in serum samples of examined patients in all groups as follow, the IgG anti (C1, C2, C3) antibody obtained from the rabbits blood and precipitated by ammonium sulphate 40% saturated solution has been used as capture antibody for capturing the different complex (C1, C2, C3). We designed the supplied components in every kit to sufficient the assay in 96-well

1. ELISA plate’s kit components for detection of C1, included a component A coated plate with 50 μl anti C1 and component B: Complex C1 standard calibrators (0.05, 0.5, 5, 10, 15, 20 ng/ml).

2. ELISA plates kit components for detection of C2, included a-Component A coated plate with 50 μl anti C2 and component B: C2 standard calibrators (0.05, 0.5, 5, 10, 15, 20 ng/ml).

3. ELISA plates kit components for detection of C3, included a-Component A coated plate with 50 μl anti C4 and component B: C3 standard calibrators (0.05, 0.5, 5, 10, 15, 20 ng/ml).

And the other kits components included component C: HRP-conjugated anti-rabbit IgG 25>l, component D: TMB solution A (3, 3’, 5, 5’-tetramethylbenzidine) 15 ml, component E: TMB solution B (H2O2) 15 ml, component F: TMB Stop Solution 30 ml.

Investigate the presence of different immune-complex C1, C2 and C3 in serum samples of HCV infected patients: We run the three ELISA Assay to detect each specific complex in serum sample for each patient in all groups (I, II, III, IV, V). We diluted the complex C1, C2 and C3 standard (component B) and every examined sample with PBS to ten concentrations, then 100 μl of diluted standard and samples were added to the specific well (component A) in the three designed kits. Incubate at room temperature for at least 1 hr, dilute 10 μl of HRP-conjugate anti rabbit IgG (component C) with 10.5 ml of dilution solution then add 100 μl into each well anti-rabbit IgG peroxidase: Aspirate and wash plate 4 times. Incubate at room temperature for 30 min, TMB peroxidase substrate solution preparation: Mix equal volumes of TMB Solution A (Component D) and TMB solution B (component E) in a clean, preferably HDPE, and polypropylene or glass container immediately prior to use at room temperature. The wells were blocked, and each well was estimated at absorbance 450 nm using an ELISA reader (Biotec, USA). The mean OD readings of complex C1, C2, and C3 were significantly higher than (P<0.05) considered positive among all infected HCV patients.

Detection of free neutralizing unbounded antibody to HCV (E1, E2 and core antigens) in serum samples by ELISA

The immunoreactivity of the mixture of core and E2, E1 recombinant proteins to its specific circulating antibodies in the HCV positive sera was observed with micro titer immune plates, a special device comprising three plates connecting to each other, were every plate coated with 50 μg/ml of specific antigen (core & E2 and E1). We let the antigens to adsorb on to the micro ELISA plates for 48 hrs at 4°C. Wells were blocked following washing with PBST, A 100 ml of each serum sample for all groups (I, II, III, IV, V) of HCV was added for every triple wells, then we incubated at 37°C for 1:30 hr. The plates were washed and incubated with 100 ml rabbit anti-human IgG conjugated (HRP) at 37°C for 30 min. The ultimate immune complexes were seen by adding 100 ml TMB substrate solution, the wells were blocked, and then the absorbance at 450 nm of each well well was read using an ELISA reader (Biotek, USA). To distinguish between positive and negative HCV sera the cutoff value was calculated as the mean value of the OD of HCV 150 negative samples plus 2 Standard Deviations (SD) equal to 0.05 mg/ml.

HCV RNA levels were determined by bDNA.

Blood samples for all tested groups were collected three times within 48 weeks for HCV- RNA were to evaluate the quantitation of the HCV-RNA in serum samples of infected subjects by the by Amplicor HCV version 2.0 (sensitivity limit, 15 viral copies/mL).

Results

Identify the levels of anti- HCV (E1, E2, and core) antigens in all groups

The outcomes OD readings were obtained from ELISA assays for the titer of the nAbs of anti-core, E2 and E1 antigens in the sera of all examined patients during the three successive samples, in group I patients which had viral count less than or equal to (10 × 109 copies/ml) the levels of the circulating unbound nAbs showed marked decrease in nAbs titer ranged from (1.2 mg/ml to 3.5 mg/ml) (reference range 0.05 mg/ml), patients which had viral count less than or equal to (10 × 109 copies/ml) the levels of the circulating free unbound nAbs showed marked increase in nAbs titer reached to (5.5 mg/ml to 12.3 mg/ml), patients which had viral count (10 × 109 copies/ml) the levels of the circulating nAbs recorded increase in levels of nAbs titer 12.2 mg/ml to 20.5 mg/ml, in group II all three samples reading denoted decreasing in levels of these free unbounded nAbs to (1.1 mg/ml to 3.0 mg/ml) except the 4 patients relapsed
after they achieved SVR for 6 months were their last sample showed increase in levels of free nAbs (5.7 mg/ml to 8.9 mg/ml), in group III were all of them had not been previously treated with antiviral drugs the levels of free nAbs showed levels ranging from (4.8 mg/ml to 7.7 mg/ml) in 40% of them and from (7.0 mg/ml to 11.9 mg/ml) in 60% while patients in group IV showed slightly positive for anti E2 and core antigens with value ranging from (0.07 mg/ml to 1.1 mg/ml), group V showed complete undetectable anti-E1, E2 and core antigens during the study course.

Exploring the change in the level of HCV-RNA quantitation viral load

HCV RNA levels below the detection limit were found in all patients of group IV and control group in the three obtained sera, in groups I (16) patients showed high viral count over (10 × 10^5 copies/ml) in most of them, all patients in group III showed viral load from (5 × 10^4 copies/ml) to (1.8 mg/ml) and (C3 1.2 mg/ml to 2.1 mg/ml), patients with moderate viral count showed increase in levels of the complex were C1 (2.2 mg/ml to 5.1 mg/ml) and C2 (2.7 mg/ml to 4.6 mg/ml) and C3 (3.1 mg/ml to 7.0 mg/ml), while patients of group I with mild viral load of serum HCV RNA showed decrease in levels of C1 and C3 were (C1 0.6 mg/ml to 2.1 mg/ml) and C2 (0.7 mg/ml to 1.6 mg/ml) and (C3 1.1 mg/ml to 2.6 mg/ml), patients with moderate viral count showed increase in the levels of the complex were C1 (2.2 mg/ml to 5.1 mg/ml) and C2 (2.7 mg/ml to 4.6 mg/ml) and C3 (3.1 mg/ml to 7.0 mg/ml), while patients of group I with mild viral load of serum HCV RNA showed marked elevation in quantity of complex levels were C1 ranged from (5.7 mg/ml to 9.0 mg/ml), C2 (6.1 mg/ml to 10.2 mg/ml) and C3 (4.8 mg/ml to 9.5 mg/ml). In patients of group II the quantity measure for the three successive samples revealed a marked increase in levels of the three complex were C1 (7.6 mg/ml to 11.6 mg/ml), C2 (8.2 mg/ml to 13.0 mg/ml) and C3 (9.9 mg/ml to 14.8 mg/ml) except the 4 patients which showed relapse at 48 weeks the levels of the three complex recorded severe decrease in quantity of the three complex were C1, became (0.9 mg/ml to 1.2 mg/ml), C2 (1.0 mg/ml to 1.8 mg/ml) and C3 (1.2 mg/ml to 2.1 mg/ml). in group IV all the results showed undetectable absorbance for all complex C1, C2 and C3 in comparing its results with the group V (The patients considered positive if his OD reading yielding absorbance over 0.05 mg/ml).

Correlations between anti-E1, E2, core antibody and the viral load: The obtained results for all examined sera of all infected HCV patients during the three successive samples revealed a positive correlation between increasing in viral load of serum HCV RNA and the increase in level of the neutralizing free unbounded anti-HCV (E1, E2 and core antigen) as showed in Figure 1, also with decreasing in the level of the viral count a marked decrease in levels of the these free unbounded nAbs.

Correlations between the quantity of unbounded anti-E1, E2, core antibody and the complex C1, C2, C3: A strong positive correlation between increasing in quantity measure of circulating immune-complex C1, C2, C3 and the decreasing in the level of free unbounded neutralizing anti-HCV (E1, E2 and core antigen) antibody, respectively a decreasing in the level of these immune-complex C1, C2, C3 showed in opposite a marked increase in levels of the these free unbounded nAbs.

Correlations between the quantity of complex C1, C2, C3 and the viral count: Investigating the last correlation between the levels of circulating immune-complex C1, C2, C3 and the viral count revealed a positive correlation between the decreasing in quantitative measure of the immune-complex and the increasing in the titer of viral count, consequently a decrease in viral count followed by an increase in levels of circulating immune-complex as showed in Figure 3.

Discussions

Hepatitis C Virus (HCV) is a major national health problem

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<tr>
<th>Group</th>
<th>Anti-HCV antigens</th>
<th>Complex</th>
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<tbody>
<tr>
<td></td>
<td>E1</td>
<td>E2</td>
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<tr>
<td>Group-I</td>
<td>8.3 ± 2.0^a</td>
<td>9.4 ± 2.2^a</td>
</tr>
<tr>
<td>Group-II</td>
<td>1.7 ± 0.4^a</td>
<td>1.8 ± 0.6^a</td>
</tr>
<tr>
<td>Group-III</td>
<td>5.9 ± 2.0^b</td>
<td>6.2 ± 2.2^b</td>
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<tr>
<td>Group-IV</td>
<td>0.7 ± 0.3^c</td>
<td>0.7 ± 0.2^c</td>
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<tr>
<td>Group-V</td>
<td>Undetected</td>
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*p <0.001*
in Egypt due to its high prevalence and associated morbidity and mortality. The Egyptian Ministry of Health is making a great effort to treat the HCV patients and is also conducting a medical survey to identify unknown infected patients. HCV consists of a lipid membrane envelope with embedded 2 glycoproteins; E1 and E2, serving in viral attachment and entry into the cell. The hyper variable region 1 (HVR1), found on the E2 glycoprotein, shields the virus from the immune system. It prevents CD81 from latching onto its respective receptor on the virus. In addition, E2 can shield E1 from the immune system. Within the envelope is an icosahedral core; core (C) protein, inside which the viral RNA is located. Treatment of HCV had evolved from Interferon alpha (IFN-a), Ribavirin with INF-a, to a newer era of Direct-Acting Antiviral (DAA) agents. However, despite successful treatment options for HCV which could achieve over 90% Sustained Virological Response (SVR); undetectable HCV PCR six months after the end of therapy, a possible late relapse or re-infection still exist. While the current end point of therapy depends on undetectable HCV PCR, all patients remain positive for HCV broadly neutralizing Antibody (nAb). Referring to HIV; another RNA virus, patients also remain positive for HIV Ab and deteriorate despite undetectable HIV RNA during Antiretroviral Therapy (ART). Thus elimination of viral RNA does not equal cure from it. Also, it is still unclear till now if the HCV nAb which formed against the core and E1, E2 envelope glycoproteins, play a protective or a pathogenic role in HCV infection.

We assume that hepatocyte may be elected to originate this virus from itself and for it and thus it learned how to protect it, so infected hepatocytes send an immunological signals for the B-cells to produce a broadly specific nAbs for every structural and nonstructural proteins parts of HCV, their nature may reflect the immunity of liver cells. This Abs can negatively inhibit or positively stimulate both of CD8+ Cytotoxic T Lymphocytes (CTL) and CD4+ T-helper cells depending on the level of hepatocytes’ immunity. Immunocompromised hepatocytes activate the negative nAbs to attract the HCV antigenic structural and nonstructural proteins (E1, E2, and core) in a complex form protecting and controlling viral antigenicity for a long time. On the contrary, immunocompetent hepatocytes activate positive nAbs to stimulate both the CD8+ CTL and the CD4+ T-helper cells to clear the HCV RNA. This explains spontaneous viral clearance during acute infection in some individuals while others develop long-standing persistent infection. The results of this study support our hypothesis. Patients failed on IFN/Ribavirin and those with positive HCV Ab and positive HCV PCR, we found viral load to have a positive correlation with free unbounded nAbs and a negative correlation with immune-complexes C1, C2, and C3. Patients with mild viremia have a high level of complex and low level of free unbounded nAbs. While patients with high viremia, have low level of complex and high level of free unbounded nAbs. Patients treated with Sofo/dacla completely prove our hypothesis as during the 24 weeks of treatment they had a consistently negative viral load with a markedly high level of immune-complexes and a low level of nAbs. After 48 weeks post-treatment, four relapers had increased viral load and unbounded nAbs with decreased immune-complexes than their previous readings. Patients with spontaneous viral clearance; negative viral load and positive HCV Ab, have a completely negative immune-complex and a slightly positive free unbounded nAbs. So a positive immune response leads to complete clearance of the virus and its antigenic parts. This returned us to our opinion about the difference between the positive and the negative immune response. Current anti-HCV medications simultaneously clear the viral RNA and stimulate the negative immune response to form an immune-complexes with the viral structural and nonstructural proteins to protect and hide viral antigenicity for a long time. This mechanism introduce a mask results about the reality of the viral existence and clear answer about this huge positive results which reached to 90% with interferon-a (IFN-a) and the newer direct-acting antiviral agents. We are convinced that our results still needs sufficient evidence and we recommended extra studies to investigate the nature of these negative and positive antibodies.

Conclusion

These results carries an important implications for the development of real therapeutic interventions that depends on dissociating this immune-complex to facilitate its eliminations and engulfing with our positive immune cells and also it raises the possibility of developing serological assays for monitoring HCV treatment.

Acknowledgment

Doctor/Sherif out most of the experiments and drafted the manuscript, Doctor/Haider participated in the design and conceived of the study, Doctor/Ehab acts for thoughtful discussion and critical review of the manuscript and Doctor/El Hariri acts all the statistics and results revision. All authors read and approved the final manuscript. Ethics approval and consent to participate. The animal study was performed in accordance with regulation and guidelines of the Institutional Animal Care and Use Committee of the Cairo University.

Funding

This work was funded by R&D of Intelhealth center in Thailand.

References

10. Simonetti RG, Camma C, Fiorello F, Cottone M, Rapicetta M, Marino


