Hepatitis C virus (HCV) is the main cause of chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC) worldwide. The risk for the development of HCC increases with the severity of liver inflammation and fibrosis. Inflammatory cytokines are critical components of the immune system and influence cellular signaling. In this study, we demonstrated that TNF-α, IL-2 & IL-8 levels were significantly elevated in PBMC– HCV in-vitro model. We tested the hypothesis whether Epicatechin (EC) and/or 6-gingerol (GING) could inhibit such elevation in those cytokines or not. We found that both compound could significantly inhibit the inflammatory cytokines and the use of combined treatment is more effective than single treatment (EC or GING), assuming a possible synergistic effect. In conclusion, the use of anti-inflammatory compounds such as EC and GING as combined treatment may offer a pharmacological approach of targeting TNF-α, Il-2 and IL-8 production, which may provide a potential novel strategy for the development of anti-HCV therapy.

Keywords: Pro-Inflammatory Cytokines, HCV, Chemokine, Drug Development, Epicatechin, 6-Gingerol

Introduction

Hepatitis C virus (HCV) is a major health burden infecting 170-210 million people worldwide. Additional 3-4 million are newly-infected annually and the highest prevalence occurs in Egypt. HCV slowly progresses to serious complications as cirrhosis (1%-2%) and hepatocellular carcinoma (HCC). The goals of treatment in individual patients are virus eradication, prevention of cirrhosis and HCC, and thus decreasing the global burden of HCV. It has been hypothesized that tumor necrosis factor-alpha (TNF-α) is the central mediator of the inflammatory process in HCV. Serum levels of TNF-α have been correlated with elevated AL Tand increased severity of fibrosis in HCV patients. Decreased TNF-α concentration has been noted in patients with sustained response to interferon therapy. Therefore, blocking TNF-α could play a protective role in the progression of HCV-related liver fibrosis.

Anti-TNF-α therapy has been demonstrated to be effective and well tolerated in the setting of hepatic C virus (HCV) infection. On other hand, the important side effects that have been most extensively related to TNF inhibitors include lymphoma, infections, congestive heart failure, demyelinating disease, lupus-like syndrome, induction of auto-antibodies, injection site reactions, and systemic side effects. However, many synthetic chemical compounds and herbs are commonly used by practitioners of complementary and alternative medicine as antiviral agents and potent reducers of TNF-α. Based on our preliminary results we observed that ginger and grape seed extracts showed anti-HCV effect. Therefore, we chose to examine the most active ingredient (epicatechin [EC] and 6- gingerol [GING]) from both extracts as separate and synergetic inhibitors of TNF-α. In chronic hepatitis C, intra-hepatic expression of both IL-2 and IL-8 are increased with fibrosis and inflammatory activity. Positive correlations were...
found between IL-8 and other cytokines and between cytokines themselves. These findings suggest that these interacting cytokines play an active role in the pathogenesis of CHC. In the current work, we screened the IL-2 and IL-8 levels in the presence and absence of treatments (EC&GING) on PBMC culture infected with HCV.

The goal of the current investigation was to determine whether EC and GING would induce synergistic interaction in inhibiting HCV. Hence, we examined the effect of each compound alone and in combination on TNF-α, IL-2 and IL-8 cytokines.

**Experimental Section**

**Isolation of PBMCs by standard density gradient technique**

Peripheral blood is the primary source of lymphoid cells for investigation of the human immune system. Its use is facilitated by Ficoll-Hypaque density gradient centrifugation, a simple and rapid method of purifying PBMC was carried out according to the method of Berthold.

**Cytotoxicity assay**

A cell suspension of 6 x 10⁴ cells ml⁻¹ was collected and seeded in 96-well plates (100 μl cell suspension per well). The plates were incubated at 37°C in humidified 5% CO₂ for 24 hr. After obtaining a semi confluent cell layer, the exhausted old media were discarded and 100 μl of different treatment concentrations (previously prepared in RPMI media) or RPMI 1640 medium (as a negative control) were added. The cell plates were incubated at the same growth conditions for 3 days. After 3 days, the culture medium was discarded, 100 μl of neutral red stain (100 μg ml⁻¹) was added to each well and incubated at 37°C in humidified 5% CO₂ for 3 h.

**Antiviral assay**

The antiviral assay was performed according to the method of El-Fakharany et al. with some modifications. Pre-treatment assay: PBMC cells were grown in 6 well plates for 24 h. Before virus inoculation, non-cytotoxic concentration of the treatment was added to the cells and incubated for 90 min. Then the treatment was removed and the cells were washed twice with PBS. Hepatitis C virus (HCV) was inoculated onto the cells for 90 min. The virus was removed and the cells were washed twice with PBS then Dulbecco’s modified Eagle’s medium (DMEM) was added supplemented with 10 % fetal calf serum (FCS) to the cells. The cells were then incubated for 96h at 37°C and 5% CO₂. Post treatment assay: PBMC were grown in 6 well plates for 24 h. Cells were inoculated with HCV onto near confluent PBMC monolayers for 1 h then the cells were washed twice with PBS. Treatment was added to the cells that are then incubated for 96h at 37°C and 5% CO₂. In case of co-treatment, the non-toxic concentration of GING and EC were added to the cells at the same time with HCV inoculation. The reduction in viral load due to the treatment was determined from results of RTqPCR.

**RNA extraction from PBMC**

*Total RNA was extracted using QIAamp RNA mini kit (Qiagen, USA)*

**Reverse transcription-nested PCR of genomic and anti-genomic RNA strands of HCV**

Reverse transcription-nested PCR was carried out according to the method of Lohr with few modifications. Primer sequences used were as follows: 1CH: 5’-cggtgcacggtctacgagacctc-3’, 2CH: 5’-aactactgtcctcagcagaa-3’, P2: 5’-tgctcatggtgeagtctca-3’, D2: 5’-actcggctagcagtctcgcg-3’ and F2: 5’-gtgcagcctccaggaccc-3’. To control false detection of negative-strand HCV RNA and known variations in PCR efficiency, specific control assays and rigorous standardization of the reaction were employed: (1) cDNA synthesis without RNA templates to exclude product contamination; (2) cDNA synthesis without RTase to exclude Taq polymerase RTase activity; (3) cDNA synthesis and PCR step done with only the reverse or forward primer to confirm no contamination from mixed primers. These controls were consistently negative.

**Strand-Specific RT-qPCR**

The real time quantitative (RTq) PCR was done to the final PCR product based on the SYBR Green I dye and Light Cycler fluorimeter using a standard HCV infected serum samples.

**Quantitative assay of cytokine levels**

The concentration of TNF-α, IL-2 and IL-8 were determined with assay kits from following the manufacturer’s instructions. Briefly, Cytokines were determined using ELISA Kit. This assay employs the quantitative sandwich enzyme immunoassay technique. Polyclonal antibody specific for interleukins have been pre-coated onto a micro plate. Standards and samples were pipetted into the wells and any interleukins present were bounded by the immobilized antibody. After washing away any unbounded substances, an enzyme-linked polyclonal
antibody specific for interleukins were added to wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of interleukins bound in the initial step. The color development was stopped and the intensity of the color was measured.

Statistical analysis
Statistical analysis of data is represented by the mean of triplicate group ± standard deviation. One way analysis of variance (ANOVA) and two way analysis of variance were used according to Bishop. Probability test (P) was carried out to show the significance degree, P≤0.0001 highly significant, P≤0.005 significant and P>0.005 non-significant. LSD (at α = 0.05) is the least significant differences.

Results
Cytotoxicity assay
GING was subjected to cytotoxicity test on PBMC line. Figure 1 showed that GING showed safety up to 10mg/l/100 µl DMEM. Accordingly, 10 mg/100 µl DMEM of GING would be the selected concentration for the antiviral activity assessment. Regarding the EC, the cytotoxicity assay was carried out previously by our group.

Antiviral assay
The viral load of HCV was assessed as a result of HCV amplification by RT-PCR, nested-PCR followed by quantitative real time PCR which provides the viral load in the form of copy/ml as shown in Table (1). Both treatments have a significant different influence on the initial viral load (P < 0.0001). Co-treatment has achieved more reduction of the initial viral load than that achieved in the case of post-treatment.

Effect of EC and GING on cytokines levels
Antiviral load
In case of PBMC infected with HCV: figure-2 showed that the highest inhibition (57.9% in comparison with positive control [infected PMB cells]) was recorded for EC, when the EC was incubated with PMB cells before HCV infection (pretreatment), whereas, both treatments after HCV infection (post treatment: 12.56%) or at the same time of HCV infection (Co-treatment: 11.59%) caused a very low viral inhibition. Moderate inhibition was recorded for combined EC and GING at co-treatment (29.95%) and GING at Co-treatment (27.95%)

followed by pretreatment of both (20.28%) and GING at pretreatment (18.84%). Very low inhibition was recorded for EC (12.56%), GING (9%) and combined EC and GING (8.69%) after HCV infection (post treatment).
Pro-inflammatory Cytokines and chemokine levels

- TNF-α: As shown in Figure 3a, high inhibition was recorded for GING and combined EC and GING at co-treatment (99.979%, 99.41% respectively), followed by pre-treatment with combined EC and GING and then GING (98.99%, 98.51% respectively). A slight decrease in inhibition was recorded for the co-treatment with EC (93.11%). A little lower inhibition for GING and combined EC and GING at post-treatment (89.99% and 89.02% respectively). The least effect was observed with EC at co and post-treatment (80.58%, 73.31% respectively).

- IL-2: As shown in Figure 3b, Moderate inhibition was recorded for epicatechin with 6-gingerol at pre-and post-treatment (56.54% & 56.08%, respectively), lower inhibition was recorded when both (EC and GING) were added at the same time of HCV infection (co-treatment: 35.86%). In case of single treatment as GING or EC the inhibition was rather higher for co-treatment (49.92% & 39.36%, respectively), followed by pre-treatment (44.17% & 23.38% respectively) and the least with post-treatment (20.95% & 14.9%, respectively).

- IL-8: As shown in Figure 3c, high inhibition (92.0%) was achieved when pre-treated with EC and when co- and pre-treated with combined EC and GING (90.96% & 90.27%, respectively). Moderate inhibition was recorded for GING starting with pre-treatment (79.22%), then at co-and post-treatment (70.73% & 70.11%, respectively), in addition to EC at post-treatment (74.6%), combined EC and GING at post-treatment (66.99%) and lower to 61.9% for EC at co-treatment.

Discussion

For all treatments (single or combined treatments), the lowest inhibition was recorded at post-treatment assuming that the mode of inhibition might depend only on the viral entry process and not on the viral replication. Although, the highest inhibition for viral load recorded for EC was 57.9% at pre-treatment (Figure 2), very low inhibition was recorded at both co- and post-treatment. These results might indicate that the EC acts only on the cell receptors of viral entry and it could not be an efficient blocker for the viral capsid proteins (envelope) or an inhibitor for viral replication. The current results agree with previous reports in which they investigated the effect of green tea catechins and its derivatives, such as epigallocatechin-3-gallate (EGCG), epigallocatechicine (EGC), epicatechingallate (ECG) and epicatechin (EC) and found that only, EGCG inhibited binding of HCV to target cells. However, the other derivatives had no effect as blocker for viral attachment. Moreover, Calland et al22 also tested green tea catechins and reported that EGC, ECG and EC did not display any anti-HCV at co-treatment revealing that EC doesn't block the HCV envelope. For single treatment with GING, the highest inhibition was recorded for co-infection (27.05%) and then pre-treatment (18.84%), such records might be interpreted as viral inhibitions at two levels: (i) GING might act as blocker for viral capsid proteins, (ii) GING might bind to the cell receptor(s) for viral entry. In case of combined treatments (EC and GING), there is somehow a synergistic effect, where the highest inhibition was recorded for co-infection (29.95%) and then pre-treatment (20.28%). Those kinds of
inhibitions are similar to the previous inhibitions recorded for single treatment with GING, in which we assumed that, the combined treatments act on both viral capsid proteins and cell receptors for viral entry. To the best of our knowledge, no previous study was carried out on the effect of GING on HCV, inhibition the current results is the first recorded in this issue. However, early induction of TNF-α apparently required endocytosis of HCV. Several toll-like receptors (TLRs) including TLR3, TLR7, TLR-8 and TLR-9 reside in the endosomes and their activation lead to the induction of various cytokines including TNF-α. Among these TLRs, TLR-8 is activated by single-stranded RNA of HCV. The induction of TNF-α by HCV was further confirmed by current study. The results in figure 3 reveal that, no significant difference was recorded between the single treatments of GING and combined treatment in each mode (co-, pre- and post-treatment) respectively. In addition, no significant difference between co-infection and pre-infection was observed. The single treatment with EC recorded a significant lower inhibition in all modes in comparison with GING single treatment and combined treatment. Our experimental IL-2 results of PBM infected cells (positive control) are in agreement with the in vivo infection study of Kasprezak et al who reported augmented expression of IL-2 in livers of patients with chronic HCV. The in vivo study of El-Desouky reported that, the IL-2 serum level was significantly high in HCV patients. The single treatment with EC recorded a lower inhibition in IL-2 expression for all modes (39.36%, 23.38% and 14.9%) in comparison with GING (49.92%, 44.17% and 20.95%). Moreover, both treatments have the same TNF-α inhibition profile (The highest inhibition was for co-treatment followed by pre- and then post-treatment). For combined treatment, the inhibition was the highest recorded at pre- and post-treatment (56.54% and 56.08%), while the lowest inhibition was recorded at co-treatment (35.86%). The reason for this is however unclear and may be due to a steric hindrance between EC and GING in the presence of viral particles at the same time. IL-8 is an important inflammatory mediator in response to viral and bacterial pathogens. The elevated IL-8 expression has been observed in HCV patients as well as in vitro infected cells. IL-8 was considered as the potential biological marker in fibrosis scores and ALT level. In the present study, we demonstrated that HCV infection up-regulate IL-8 expression in PBMC. In this context, the EC and GING were investigated to determine their roles not only in HCV inhibition but also in the regulation of IL-8 expression. The highest inhibition was at pre-treatment for EC (92.0%), followed by combined treatment (EC& GING) at co-and pre-treatment (90.96% & 90.27% respectively), whereas the lowest inhibition was for single treatment with EC at co-treatment (61.94%). These results suggest that both single and combined treatment could modulate the inflammatory response by down regulating the IL-8 on which they might bind or compete at the IL-8 receptor. Taken altogether, our results suggest that the use of combined treatment is more effective than single treatment (EC or GING), assuming a possible synergistic effect. In conclusion, our results indicate the promising potential of anti-inflammatory compounds such as EC and GING, which could protect host cells and / or limit inflammation, unlike current HCV therapy that target infection and replication. Inflammation may contribute to the acquisition or spread of HCV infection. Thus, our results suggest that the use of anti-inflammatory compounds such as EC and GING as combined treatment may offer a pharmacological approach of targeting TNF-α, IL-2 and IL-8 production, which may provide a potential novel strategy in adjuvant development for anti-HCV therapy.

References