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Amino Acids Regulation of Autophagy as a Sufficient Method to Disturb HCV Replication

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ABSTRACT

Hepatocellular carcinoma (HCC) is a growing clinical problem, being the second leading cause of cancer deaths worldwide. Inflammation of the liver is known as hepatitis and that can be caused by alcohol, drugs, and infection with bacteria or viruses. There are so many hepatitis viruses, so named because of the similar symptoms they have, however they are very different in structure and replication. Hepatitis C virus (HCV) is a blood borne virus which contains positive sense, single-stranded RNA and belongs to the *Flaviviridae* family. HCV infection induces a variety of cellular pathways that are either suppressing viral replication or maintaining viral infection. Interferon signaling is the most common pathway that prevents HCV infection while cellular autophagy has been found to ensure viral replication. Autophagy is a cellular process that responsible for degradation event via delivering the unnecessary proteins to lysosomes. In the current work, the effect of some individual amino acids, including glycine, glutamine, lysine and proline have been investigated for its possible influence on HCV replication using HepG2 cell lines. Additionally, the possible regulation of cellular autophagy was assessed in response to each amino acids treatment. Interestingly, our results indicated that glycine and glutamine pre-treatment showed competitive inhibition of HCV replication indicated by the expression of viral NS5A at both RNA and protein levels. Likely, both amino acids regulate autophagosome formation indicated by LC3 expression; however glycine treatment activated apoptotic signaling pathway and subsequently cell death program. Collectively, the current data suggest that treatment with either glycine or glutamine have the ability to prevent HCV replication may via disturbance of cellular autophagy.

INTRODUCTION

Hepatitis C virus (HCV) is a positive sense, single-stranded RNA virus in the Flaviviridae family (Khalil et al., 2017; Manns et al., 2017). The genome of HCV was identified in 1989, and the name hepatitis C was subsequently applied to the human infection caused by this single-strand ribonucleic acid (RNA) virus of positive polarity (3). Six different genotypes have been identified based on differentiation in particular regions of the viral genome. HCV contains structural proteins, the envelope glycoprotein E1 and E2 heterodimer, which are surround the viral particles with endsomal membrane and responsible for viral-host attachment and fusion. The virion nucleocapsid poses multiple copies of a small basic core protein (C) and contains the RNA genome which release into cytoplasm positive sense RNA strand. The nonstructural (NS) proteins of HCV include P7, NS2 protease, NS3-4A complex harboring protease, NS4B, NS5A, and NS5B polymerase (4). HCV is a major causative agent of acute and chronic liver diseases. HCV chronically infects more than 12 million of Egyptian population and causes almost half million deaths each year by inducing liver failure or hepatocellular carcinoma (5,6). Chronic HCV infection can progress to severe liver disease including cirrhosis and hepatocellular carcinoma. The hepatitis C virus is a blood borne virus and the most common modes of infection are through exposure to small quantities of blood (7). This may happen through injection drug use, unsafe injection practices, unsafe health care, and the transfusion of unscreened blood and blood products (8). HCV is a major risk factor of hepatocellular carcinoma (HCC). HCC is a multi-step process that may progress over 20-40 years and involves a number of steps chronic damage to hepatocytes, such as HCV infection, leads to a release of inflammatory and fibrotic mediators such as reactive oxygen species, cell death signals that contribute to stellate cell activation. The activated hepatic stellate cell promotes liver scarring through proliferation, contractility, fibrogenesis, matrix degradation and inflammatory signaling (9). HCV infection is treated with a combination of pegylated IFN-α and ribavirin for 24–48 weeks clears the virus-referred to herein as sustained viral response (SVR) in 50%–60% of genotype 1 and 4 patients. IFN-α provides the primary antiviral effect and can clear HCV when used alone. When ribavirin is taken with IFN-α, it roughly doubles the clearance rate (4-6). There are no effective therapies for patients who fail to clear virus following IFN-α plus ribavirin therapy, and the reasons for the high rate of therapeutic failures are unknown (10,11). Additional, Amino acids have critical role in our body, such as activation of the mammalian target of rapamycin (mTOR) kinase which regulates protein translation, cell growth, and autophagy. Amino acids also

regulate other inter-mediators metabolic catalysts that involved in eukaryotic cells reactions and regulation of the immune system (12). For instance, branched chain amino acids (BCAAs) inhibits the proliferation of liver cancer cells in vitro and plays critical role in lymphocyte proliferation and dendritic cell maturation (13). Importantly, amino acids treatment prevent new blood vessel formation and reduce production of growth factors such as (HGH) which activates cell proliferation, and reduces the amount of certain amino acids such as Gly which is necessary for DNA replication in carcinogenic cells. Notably, type-2 diabetes is embedded in the inability of beta cell to secrete sufficient insulin essential for rising metabolic requirements that associated with insulin resistance and obesity (14). Strong evidence suggests that besides fatty acids, elevated dietary protein consumption may also contribute to this medical condition (15). This is consistent with the association between high protein intake and impaired glucose tolerance (IGT), IR, and the rising incidence of T2D. Interestingly, obesity and insulin resistance are also associated with increased fasting levels of a number of amino acids, for instance, leucine (Leu), isoleucine, proline (Pro), glutamine (Glu), alanine (Ala), and valine (Val). In a recent study with relatively few participants, increased fasting levels of Val and Leu were also observed in subjects with IGT and T2D (16,17). In the current work, we aim to highlight and investigate the potential impact of exogenous treatment with individual amino acids in HCV replication. The molecular function of such amino acids including Gly, Lys, Pro and Glu has been tested *in-vitor* following HCV infection. Interestingly, our findings suggested that Gly and Glu amino acids have the ability to disturb HCV infection via regulation of autophagosome formation and program of cell death in HepG2 cells.

MATERIALS AND METHODS

Cells lines

Human liver cancer cells, HepG2 cell line (VACSERA, Giza, Egypt) was propagated in RPMI medium that contains 4 mM L-glutamine, 4 mM sodium pyruvate, 100 U/ml penicillin/streptomycin and 10% bovine calf serum (BCS), and cells were incubated at 37°C and 5% CO2 incubator.

HCV infection

Serum from a patient with HCV genotype 4 was identified and obtained from Ain Shames Specialized Hospital, Egypt. For infection, HepG2 cells were incubated for three days with the serum of derived sample in multiplicity of infection (MOI) of 1 (18).

Amino acids treatment

HepG2 cell lines were seeded in 6-well plate in concentration of 200.000 cells per well in 2 ml of RPMI medium and were incubated overnight in CO2 incubator. Then cell were treated with (200ug/well) of individual amino acids, Gly, Glu, Lys and Pro. Finally treated cell were infected with HCV (MOI=1) for three days.

Cell viability and cytotoxic effects

Number of living cells and representative of cell images by inverted microscope have been performed for each amino acid treatment to state cell viability rate following HCV infection.

Assessing of LDH released into the media of treated cells was monitored in 96well plate using LDH production kit. According to the manufacture procedures, 40µl of samples was incubated with 40µl LDH buffer and 20µl LDH substrate for 1 hour then the relative activity of LDH was measured and calculated according to the indicated standard curve. Cell treated with 50 and 100µl of Triton x-100 was used as a positive control (19).

Quantitative real time polymerase chain reaction (qRT-PCR)

qRT-PCR is a sensitive method for the quantification of mRNA expression levels using specific primers. Q-RT-PCR will be used to detect the expression levels as one of mentoring methods to investigate the formation of LC3 in HebG2 cells. Usually, RT-PCR involves two steps: the reverse transcriptase (RT) reaction, followed by PCR amplification. RNA is first reverse transcribed into complementary DNA (cDNA) using reverse transcriptase. The resulting cDNA is used as template for subsequent PCR amplification using DNA polymerase enzyme and primers specific for one or more genes.

Total RNA isolation and cDNA synthesis

Cells were collected from cell culture plates in clean and RNase free tubes. Total RNAs were isolated from treated and untreated cells using TriZol (Invitrogen), chloroform methods.

Isolated RNA was dissolved in RNase free water and the concentration of all samples was adjusted to final concentration of 100 ng/ul. Then 10 ul from each isolated and purified total RNA was used to generate cDNA using cDNA synthesis kit (Qiagen). According to the manufacturer protocol, total RNA was incubated with reverse transcriptase and poly (dT) primers at 45°C for one hour followed by 5 min incubation at 95°C. The cDNA was then incubated at -20°C until used.

qRT-PCR investigation parameters

qRT-PCR was used to detect the expression levels of HCV-NS5A, LC3 and Casp-3 genes to investigate both viral replication and cellular immune response in HepG2 cells. The resulting cDNA was used as template for subsequent PCR amplification using DNA polymerase enzyme and primers specific for one or more genes. The relative gene expression of viral NS5A, LC3 and Casp-3 were detected using the QuantiTect SYBR Green PCR Kit (Qiagen, USA) and oligonucleotides specific for each individual gene, NS5A-For-5´-ATTCGTTCGTAGTGGGATCCA -3´, NS5A-Rev-5´- AAGAGTCCAGTATTATCACCTT -3´, LC3-For-5´-CGTCCTGGACAAGACCAAGT-3´, LC3-Rev-5´-CCATTCACCAGGAGGAAGAA-3 and Casp3-For-5´- CAGCTCATACCTGTGGCTGT -3´, Casp3-Rev-5´- CTGAGGTTTGCTGCATCGAC -3. Levels of GAPDH were amplified using specific oligonucleotides, GAPDH-For-5'-TGGCATTGTGGAAGGGCTCA-3' and GAPDH-Rev-5'-TGGATGCAGGGATGATGTTCT-3' which was used for normalization as internal control. The following parameters have been used in RT-PCR program, 95°C for 4 min, 40 cycles (94°C for 45 seconds, 58°C for 30 seconds and 72°C for 45 seconds) and hold at 4°C. The indicated Ct values have been analyzed using ΔΔ Ct equations (20,21).

Flowcytometry

Follow cytometry (FCM) is a quick expanding technology gives the ability to analyze many properties of cells in very short time in FCM stained cells. HepG2 cell lines were seeded in 6-well plate in concentration of 200.000 cells per well in 2 ml of RPMI medium and were incubated overnight in CO2 incubator. Then cell were treated with (200mg/well) of individual amino acids. Finally treated cell were infected with HCV (MOI 1) genotype 4 following by 3 days incubation. The old media was removed and treated cells washed using PBS, trypsinized, collected in PBS and centrifuged at room temperature (RT) at 5,000 rpm for 5min. The supernatant was removed and the pellet has been resuspended in PBS that

contains triton X-100 (permeabilization step) followed by centrifugation as previously described. The supernatant was removed and the pellet has been resuspended again in PBS that contain 1% BSA, 1:100 diluted primary antibody (mouse monoclonal antibody for LC3 and rabbit monoclonal antibody for NS5A) followed by one hour incubation at RT. After centrifugation, the pellet has been washed three times using PBS and the cells were incubated with secondary antibodies (either goat anti rabbit or goat anti-mouse) in dilution of 1:100 followed by incubation in dark for 30 min at RT. Finally, the cells were centrifuged, the supernatant was removed and the cells have been washed as previously described. The pellet was finally re-suspended in 500 µl PBS and FCM (Becton Dickinson Facs-caliber Device) has been used to investigate the relative protein expression as recommend protocol.

STATISTICAL ANALYSIS:

Microsoft Excel was used to perform final graphs and histograms for our data. Student's two tailed t-test has been used to investigate the significance of all data that provided by real time PCR analysis. SDS2.2.2 software was used to analyze the qRT-PCT data to drive the Ct values for potential gene expression using equations indicated by (20,22).

RESULTS AND DISCUSSION

Individual amino acids treatment have no cytotoxic effects on HepG2 cell lines

To investigate the potential cytotoxic effects of each amino acids on HepG2 cells, number of living cells and cell representative images have been monitored in pre-treated cells. Additionally, Lactate dehydrogenase (LDH) production from pre-treated cells has been measured in order to investigate cell viability rate. Accordingly, the HepG2 cell line was seeded in 6-well plate in concentration of 2 x 10^5 cells per well following by overnight incubation. Then the cells have been pre-treated with the indicated amino acids in concentration of 200µg per well. Finally, the pre-treated cells were infected with HCV genotype 4 (MOI of 1) followed by 3 days incubation with the same concentration of each individual amino acids. Interestingly, both proline and glutamine showed negligible cytotoxic effects on treated cells indicated by cell images and number of living cells. However, glycine and lysine showed significant influence on cellular shape and number of living cells (Figure 1A and B). Importantly, production level of LDH has been increased up to 4 fold in pre-treated cells with either Gly or Lys that further confirms the negative effect of these amino acids on cell proliferation upon HCV infection. Nevertheless, treatment with Pro

and Glu showed low production level of LDH in treated cells (Figure 1C). These findings indicate that Pro and Glu amino acids are safe in used during HCV infection while treatment with Gly and Lys have the ability to stimulate program of cell death during HCV infection on HCC cells.

Figure 1

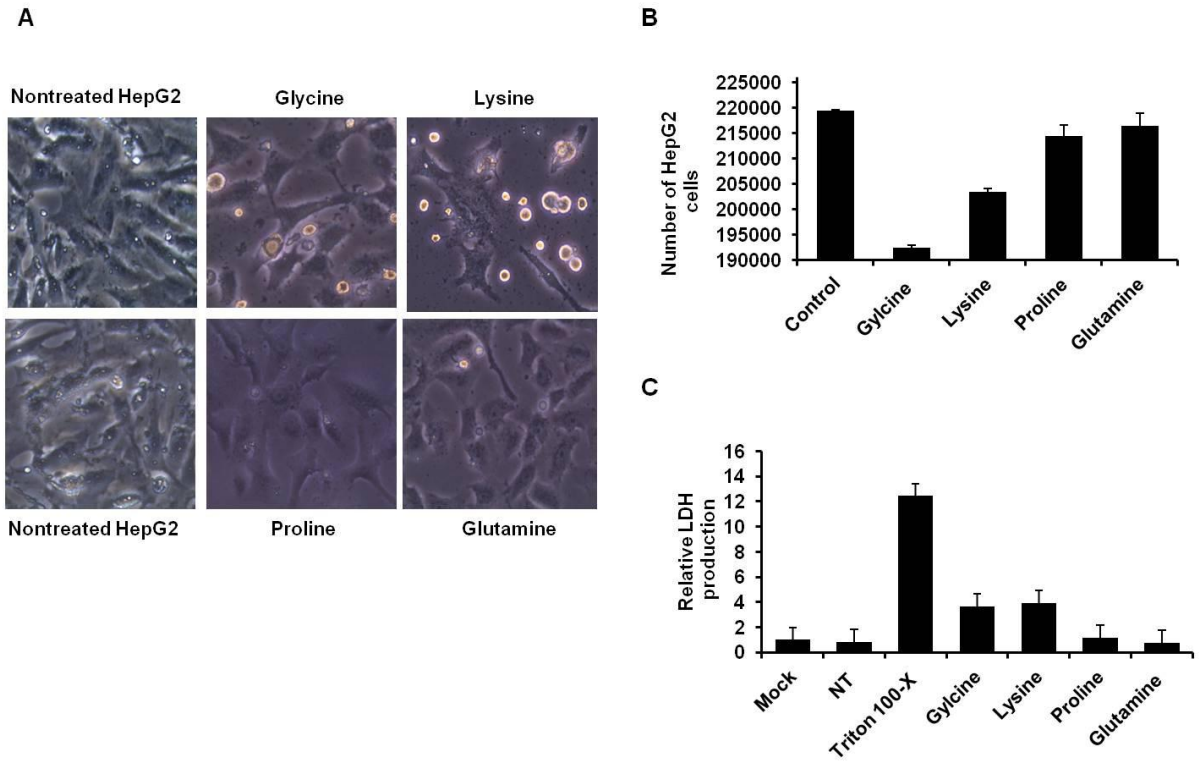

Figure 1: The indicated cytotoxic effects of individual amino acids treatment (A) The representative images of HepG2 cells that were treated with individual amino acids by using inverted microscope (40x). **(B)** Number of living cells upon indicated amino acids treatment that were manually calculated **(C)** Relative LDH production from treated cells compared to Triton X-100 treated cells and non-treated cells (NT). Error bars indicate the standard deviation (SD) of two independent experiments.

HCV replication is significantly interrupted by Gly and Glu treatment

The nonstructural (NS) protein is a proline-rich, predominantly hydrophilic protein without obvious trans-membrane helices. HCV-NS5A is a multiple phosphor-isoform found in cytoplasmic compartments of infected cells such as endoplasmic reticulum and Golgi complex. This location of NS5A facilitates the interaction with various cellular and viral

proteins during HCV replication (23). Therefore, the relative expression of NS5A at RNA level and its clearance at protein level have been used in our statement of amino acids treatment on HCV replication. Accordingly, HepG2 cells were seeded in 6-well plates in concentration of $2X10^5$ cells per well and were incubated overnight. Then the cells were pre-treated with the indicated concentration of each amino acids for two hours and cells were infected by HCV in MOI of 1followed by incubation overnight at CO2 incubator. Primary monoclonal antibody against NS5A and Alex-Flour secondary antibody were used to stain and detect viral NS5A in living cells using flow cytometry. Total RNA and complementary cDNA have been prepared for qRT-PCR to detect the relative expression of viral NS5A using specific oligonucleotides. Interestingly, the relative gene expression of HCV-NS5A was significantly decreased in HepG2 cell that were pre-treated with either Gly or Glu amino acids (*P=0.02* in both). Meanwhile, the treatment with other amino acids showed negligible and nonsignificant differentiations of NS5A relative expression compared to untreated cells (Figure 2A). Moreover, in flow cytometry, the expression of viral NS5A corresponding protein has been detected on 10% of cells population regarding Gly pre-treatment. In case of Glu pre-treatment, 20% of investigated cells showed positive signaling for NS5A protein while Pro and Lys pre-treated cell showed 40% and 60%, respectively (Figure 2B). Taken together, these data demonstrate that treatment with Gly and Glu amino acids could prevent HCV replication indicated by the expression profile of viral NS5A. Further, our data suggest that, as supplementary materials, Gly and Glu amino acids are attractive and helpful candidates in therapeutical strategy against HCV.

Figure 2

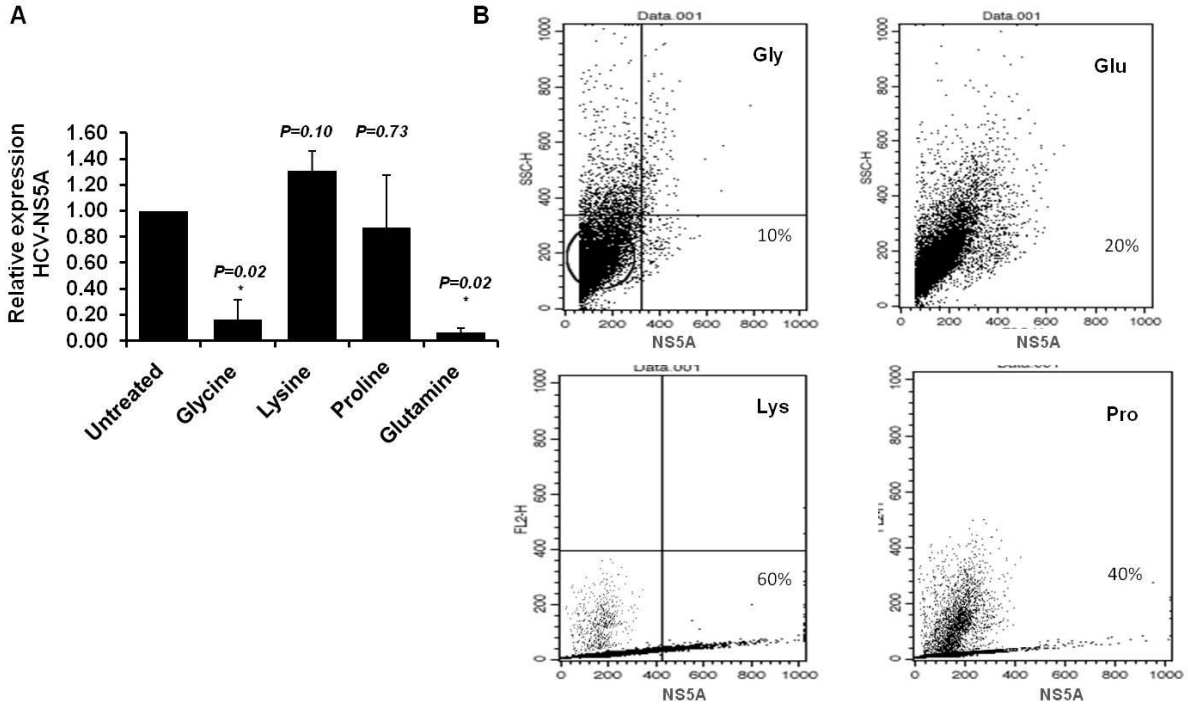

Figure 2: Gly and Glu treatment have the ability to disturb HCV replication (A) The relative expression of HCV-NS5A at RNA level indicated by qRT-PCR in HepG2 cells that were treated with the indicated amino acids in comparison with untreated and infected cells **(B)** FCM analysis of HCV-NS5A corresponding protein expression upon treatment with indicated amino acids. Error bars indicate the standard deviation (SD) of two independent experiments.

Regulation of autophagy and program of cell death by Glu and Gly treatment

Autophagy is a major intracellular digestive system that includes sequestration of dysfunctional and unnecessary proteins in a double membrane vesicle known as autophagosome for further degradation and recycling by lysosomes. In addition to maintaining cell survival, autophagy also regulates program of cell death and apoptotic signaling. Here we detected the relative expression of LC3 gene, as a biomarker for autophagy, and Casp-3, as an indicator for apoptosis, in all treated and infected cells. The results showed that both Gly and Lys amino acids pre-treatment lead to significant interruption of autophagic machinery indicated by LC3 gene expression. Meanwhile, Pro and Glu amino acids pre-treatment showed nonsignificant effects on autophagy compared to

untreated cells (Figure 3A). Conversely, the relative expression of Casp-3 was significantly increased (up to 4 fold) in cells that were pre-treated with either Gly or Lys amino acid in comparison with untreated and infected cells. Further, the relative expression of Casp-3 was reduced in cells that were pre-treated with either Pro or Glu amino acid (Figure 3B). Together, our findings indicate that pre-treatment with Gly activates program of cell death and regulates autophagosome formation during HCV replication. While Glu pre-treatment has the ability to prevent HCV replication without stimulation of apoptotic signaling or program of cell death. These data reveal the potential molecular function of Gly and Glu amino acids which facilitates its role to disturb HCV replication.

Figure 3

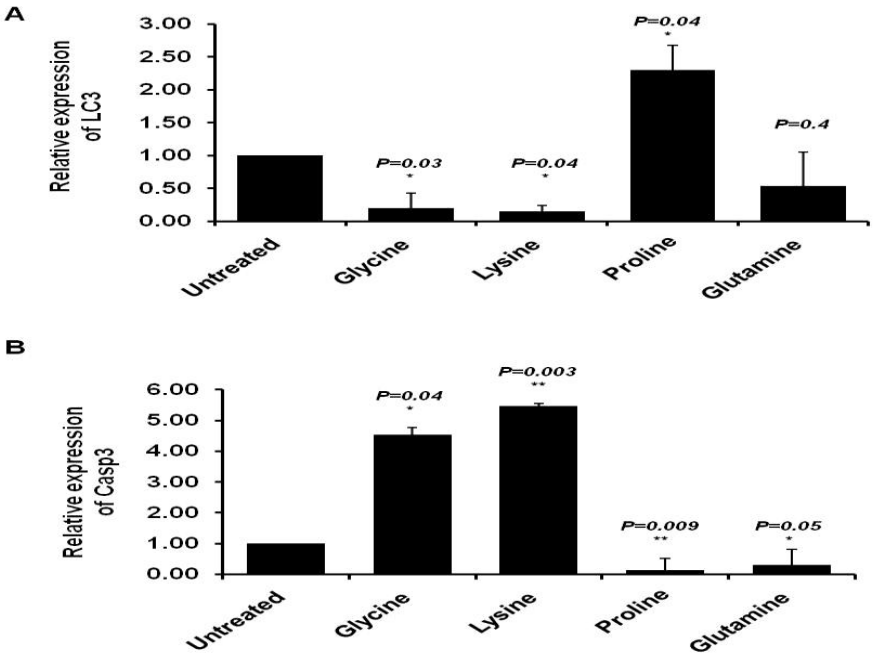

Figure 3: Regulation of autophagosome formation and apoptosis in treated cells (A) The relative expression of LC3 indicated by qRT-PCR in HepG2 cells that were treated with the indicated amino acids in comparison with untreated and infected cells **(B)** The relative expression of Casp3 and an indicator for apoptosis in HepG2 cells that were treated with individual amino acids compared to untreated and infected cells. Error bars indicate the standard deviation (SD) of two independent experiments.

Amino acids are considered as the basic housekeeping chemical during the whole process of biogenesis pathways and cellular signaling. The origin of protein known as proteogenesis is the key mediator of cellular signal transduction, mainly because proteins are unique factors able to maintain cell survival (24). Moreover, a few amino acids are required for successful

synthesis the folded essential proteins for first proteogenesis processes. Notably, the potential source of biotic organics contains number of α-amino acids including Glu, Pro, and Gly (25). Therefore, in the current work, we were interested to highlight and investigate the possible utilizing of such essential amino acids as supplementary materials during HCV replication. Accordingly, HepG2 cell line was pre-treated with each individual amino acids and infected with HCV genotype 4 followed by in-depth investigation of HCV replication and program of cell death. Interestingly, our findings indicate that either Gly or Glu amino acid has the ability to disturb HCV replication; however, each one could have different mechanism. While Glu amino acids has no cytotoxic effect on pre-treated cells, Gly amino acid showed significant toxic effects on pre-treated cells indicated by LDH secretion levels. Indeed, LDH is an enzyme found in all living cells and responsible for the conversion of lactate to pyruvic acid during Krebs's cycle in mitochondria. Importantly, secretion of LDH refers to a systemic toxic effect on cell proliferation that induces program of cell death (19). Importantly, both amino acid, Gly and Glu pre-treatment showed significant influence on HCV replication indicated by relative gene expression of NS5A and statement of its corresponding protein by flow cytometry. HCV-NS5A protein is one of the most common HCV research regarding its potential regulation of cellular immune response following infection. NS5A protein is translated from HCV genome as one of a large number of ploy-proteins that processed by NS3 protease (26). NS5A is known to modulate host cells interferon response via interaction with the cellular sensor protein for viral RNA that named retinoic acid-inducible gene I (RIG-I) protein resulted in blocking of interferon signaling pathway in infected cells (9). Additionally, NS5A plays a crucial role during HCV replication cycle and viral particles assembling through interaction with several viral and host proteins to insure HCV infection. Several evidences indicate that NS5A is localized in certain modified cytoplasmic membrane during HCV replication that facilitates its significant role in HCV replication complex and replicase (27). Here, the relative expression of NS5A has been detected by q-RT-PCR using novel designed specific oligonucleotides. On the other hand, the statement of NS5A protein in living cells has been monitored by flow cytometry using specific monoclonal antibody. Flowcytometry is used to provide a large number of population analyses in a short period of time. The data generated from this analysis is usually presented in histogram form or dot or contour plot. Here, flow cytometry has been used to investigate the clearance status of HCV-NS5A protein in HepG2 cells that were pre-treated with individual amino acids. Interestingly, in comparison with control infected cells, our findings reveal that the percentage of NS5A expression was 10% and 20% of infected cells that were pre-treated with Gly and Glu amino

acids, respectively. Meanwhile, this percentage was increased to 40% and 60% regarding pre-treatment with Pro and Lys, respectively. These data further confirm that HCV replication is potentially interrupted in HepG2 cells that were pre-treated with either Gly or Glu amino acid; however, each of them has its own molecular interaction in regard. Gly amino acids pre-treatment inhibits autophagy process and activates program of cell death via stimulation of apoptotic signaling and its related transcriptional factor Casp-3. In contradictory with this, Glu amino acid re-treatment regulates both autophagy and program of cell death indicated by relative gene expression of LC3 and Casp-3, respectively. Autophagy and apoptosis are cellular processes that genetically-regulate each other, control cell division and maintain cell survival. Despite the obvious differences between two processes, their regulation is intimately connected. Both autophagy and apoptosis are important in controlling cell fate (28–30) Collectively, our findings here reveal that regulation of autophagy and program of cells death by Gly and Glu amino acids provides a potential therapeutical strategy during HCV infection.

Declaration of Conflicting Interests

The Authors declare that there is no conflict of interest.

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