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Insights into Replication of HCV-Genotype 4 in C6/36 Mosquito Cell-Line

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Hepatitis C virus (HCV) is the main cause of chronic hepatitis worldwide. This study inspected one of the possible endemic natural transmission route of HCV viral infection; transmission via mosquito. *Aedes albopictus* clone (C6/36) and HepG2 cells (HCV natural host cells) were infected with high titer HCV infected-serum. Cells and culture supernatants were collected at 4h, 3- and 5- days post-infection. Quantitative Reverse transcription polymerase chain reaction (qRT-PCR), microscopic examination and Transmission Electron Microscopy (TEM) were applied. qRT-PCR results showed a gradient increase in HCV RNA in C6/36 and HepG2 cells 3- and 5- days post-infection with complete disappearance after 4h. The maximum viral load was observed 5 days after infection. While HCV RNA was detected in both types of cells, a remarkable elevation was found in HepG2 cells. Although we approve the existence of the virus in mosquito cells, there is no increase in HCV RNA in C6/36 cells comparing with HepG2, which displayed a remarkable rise in viral load. Severe cytopathological effect and the presence of virus particles in both HCV infected cells were confirmed. Thus, HCV could invade C6/36 cells and may replicate, causing cytopathic effects. In conclusion, our presented data provide evidences about the possible ability of HCV to infect not only human hepatic cells but also mosquito ones causing severe cytopathic damage. To the best of our knowledge, this study regarded as the first in vitro study deals with HCV genotype 4 providing actual experimental evidences that this genotype could indeed infect and exist in Aedes mosquitoes.

Keywords: HCV; *Aedes albopictus*; C6/36; Genotype 4; RT-PCR

INTRODUCTION

Hepatitis C (HCV) is a positive-stranded RNA virus and the most prominent member of the genus Hepacivirus in the Flaviviridae family. HCV is known as a major cause of chronic hepatitis, represents a global health burden, often lead to severe liver damage, cirrhosis, and liver cancer with up to 3% global dominance in the world's population and there are at least 21.3 million HCV patients in Eastern Mediterranean nations (Al Enziet al.2011;Alazard-Danyet al. 2019). Egypt has the highest prevalence in the world (Amer et al.2015). Egypt Demographic and Health Surveys (EDHS) measured antibody prevalence among population aged 15–59 years at 14.7%in 2009 and at 10.0% in 2015 (El-Zanaty and Way, 2013; Kandeel et al. 2017).

Two potentially ubiquitous routes of endemic HCV transmission are previously suggested (Pybus et al., 2007). The first has been fueled by the inadvertent behaviors of human (blood transfusion, use of blood products, hemodialysis, non-sterile administration of medicines by injection, intravenous drug abuse, acupuncture.

Etc.) combined with sexual, vertical and intra-familial transmission. The second suggested route that could account for the hidden HCV epidemic is by a vector. The natural transmission routes of HCV remain unknown, mainly (30-40%) of infected cases are without perceptible route. Endemic HCV appears to be concentrated in the tropics and sub-tropics, where human populations are subject to higher biting rates by a wide range of abundant arthropods. Furthermore, the rest of the human pathogenic flaviviruses are vector-borne. Currently, available results regarding the spread of HCV remain contradictory, and their interpretation is a matter of intense debate (Shepard et al., 2005; Tarishand Ghanim,2014).

In vivo, HCV has been isolated from bodies or heads of mosquitoes collected from the houses of HCV-infected individuals and from mosquitoes experimentally fed with infected blood (Chang et al., 2001; Hassan et al., 2003). Many investigators have suggested that the mechanical transmission of HCV by mosquitoes is plausible (Germi et al., 2001; Hassan et al., 2003; Pybus et al., 2007). Corroborative data were obtained and indicated the existence and survival of HCV in the mosquitoes for many days (Silverman et al.,1996; Chang et al., 2001; Hassan et al., 2003). As a consequence, these hidden HCV epidemics merit further consideration. C6/36 cell line has been described as one of the most sensitive mosquito cell lines to arbovirus infection and its susceptibility to infection by viruses might occur in low concentrations in viremic sera (White, 1987). Therefore, in this stud, we investigated the possible HCV replication and transmission in C6/36 cell line compared with human hepatocellular carcinoma cells (HepG2).

MATERIALS AND METHODS

Cell culture

Two cell lines were tested; *Aedes albopictus* mosquito cell (C6/36) and liver cancer cell (HepG2) lines. They were grown in RPMI-1640 medium (GIBCO, Scotland) with 10% heat-inactivated mycoplasma- and virus-free fetal bovine serum (FBS), antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) and 10 mMN-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES). Cell cultures were incubated at 37°C(HepG2) and 28°C(C6/36) in humidified 5% water and 95% air grown incubator. The media were changed every 48hr.

Viral inoculation and sample collection

Serum samples were collected from 25 Egyptian patients with chronic HCV genotype 4 infection who were recruited from the Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City (USC). All patients were positive for HCV and negative HbsAg and HBV-DNA. Viral titration was quantified by real-time polymerase chain reaction (qRT-PCR), and it was $\cong 1.7\times 10^5$ IU/ml.

C6/36 and HepG2cell lines have been seeded in 6-well plates with 100,000 cells/ml(2ml/well) then incubated. Both cell lines were grown to semi-confluence (≅80%) in complete medium. Cells were rinsed twice with FBS-free medium then100 µl of HCV infected serum sample pool /well were added as previously described **(**El-Awadyet al.2006; Sakai et al. 2007) with slight modifications. After 2h incubation; complete RPMI-1640 was added to infected cells (2ml/well**).** HCV-infected cells were incubated with periodic observation for evidence of morphologic effect and harvested at different intervals (4h, 3- and 5-days post-infection). Blank wells consisted of cells cultured alone with complete medium. Both supernatant and cells were collected for investigations; the number of copies of HCV virus (viral load) was determined by qRT-PCR in collected cells and supernatant; also electron microscope examination (TEM) of collected cells was performed. Cells cultured with complete RPMI-1640 (without serum samples) were used as negative controls. Each experiment is consisted of 6 replicates and performed for 3 times.

Detection of viral load in collected samples by qRT-PCR

Total RNA was extracted from each cell line supernatant and cell pellets according to the manufacturer's protocol (QIAGEN; QIAamp® Viral RNA Mini Kit, Cat No./ID: 52904. Hilden, Germany). Positive HCV RNA strand was analyzed by qRT-PCR agreeing with the manufacturer instructions protocol (QIAGEN; artus® HCV RT-PCR Kit; Hilden, Germany).

Transmission Electron Microscopy (TEM)

TEM was performed to detect ultra-structural changes and viral particles replication in C6/36 and HepG2cells. Both infected cells were washed with phosphate buffered saline (PBS) and fixed in 2.5% glutaraldehyde in 0.035 picric acid and 0.05 M cacodylate buffer at pH: 7.4. Cells were then post-fixed in 1% osmium tetroxide in 0.05M

cacodylate buffer for 15 min, washed in 0.1M maleate buffer at pH: 5 and finally dehydrated in ethanol, infiltrated with propylene oxide, and ended by embedded in epoxy resin**.** Ultrathin sections were stained with a saturated solution of Uranyl acetate diluted to 50% in acetone and lead citrate(Barbosa et al.2014). Microscopic examination was performed under a JEOL-JEM/1010 TEM at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

Statistical analysis

All statistical analyses were performed using SPSS version 17 (SPSS, Inc., Chicago, IL). Data were presented as means ± SD. Comparisons between different groups were performed by Paired T-test. The level of significance was set at $P<0.05$.

RESULTS

HCV RNA detection by qRT-PCR

Intracellularly, no HCV RNA was detected in HepG2 4h post-infection. Meanwhile, HCV RNA concentration showed a gradient significant ($P<0.01$) increase in collected cells (3078±140 and 6951±508) 3- and 5- days post-infection; respectively, with the maximum titer at 5 days. Similarly, HCV RNA wasn't detected in C6/36 cells 4h from infection. A significant increase ($P<0.001$) in viral load was observed 3-(1993±85) and 5- (4953±172) days post-infection. In contrast, HCV RNA was detected in extracellular supernatant of both cell lines at all-time intervals with different concentrations; 16360±574, 100±10 and 17238±527 for HepG2 and16375±339, 440±90 and 10432±1964 for mosquito cell line at 4h,3- and 5-days post-infection; respectively. Maximum significant elevation was observed at 5 days ($P<0.001$) when compared with 3 days post-infection. The comparison between both cell lines (Figure 1 and 2) revealed a reduction in viral load from extracellular with time. HCV RNA was diminished from 16375IU/ml after 4h to 10432IU/ml at 5days post-infection in C3/36 mosquito cell line. In contrary, HCV RNA was increased in HepG2 cells after 5 days of infection to be 17238 IU/ml compared with 16360 IU/ml at 4h.We found no HCV RNA in uninfected cell lines (either extra- or intracellular).

HCV effect on infected cell lines morphology

The inverted microscope observations showed similarities in both types of cells, mainly in their morphology and proliferative effects at different three intervals (4h,3- and 5- days post-infection). Untreated cells showed an ideal appearance and confluence ofC6/C36 (Figure 3A) and HepG2 (Figure 3E).The confluence appears to be reduced from 80% in untreated cells to 30% in treated cells (Figure 3B, 3F) and clusters of cell aggregations © were formed after 4h of infection. Three days post- infections, cells from both cell lines illustrated significant recovering with abnormal hypertrophic nucleated cells (h) (Figure 3C, 3G). At fifth-day post viral infection (Figure 3D, 3H); cell confluence decreased again with different cytopathic effects, cell granulation (g), vacuolated cells (v), ghost (gh), shrinkage (sh) and fragmented destroyed cells(f).

TEM of HCV particles on infected cell lines

Qualitative changes in infected cells were detected by TEM. Our results showed typical characteristics of normal C6/C36 (Figure 4A) and HepG2 (Figure 4E) in uninfected cells. In infected C6/C36 cells, electron micrographs displayed the presence of abundant virus-like particles predominantly in cytoplasmic vesicles or vacuoles, giving the appearance of virion transport through the endoplasmic reticulum secretory pathway of the cells. These virus-like particles containing structures (Figure 4C, 4D and Figure 5) were not observed in control mosquito C6/36 or control HepG2 cells. Vesicles containing virus-like particles with a follicular membranous envelope with irregular structures were clustered in the cytoplasm. The observed unevenly distributed electron-dense structures; suggestive of possible nucleocapsids 40–60-nm in diameter, were diversely polymorphic in appearance. Visualization of these features in C6/36 cells was identical to the structure and morphology of viral particles in HepG2 infected cells (Figure 4G and 4H). Virus particles were perceived in both cell lines on 3-(Figure 4C and 4G) and 5-(Figure 4D and 4H) day's post-infection. Higher magnification of the electron micrographs (Figure 4C and 4G) displayed release of several virus-like particles from C6/36 and HepG2 cells. Figure4D and 4Hshow desertion of almost all cell organelles in demolished cells at 5-day post-infection.

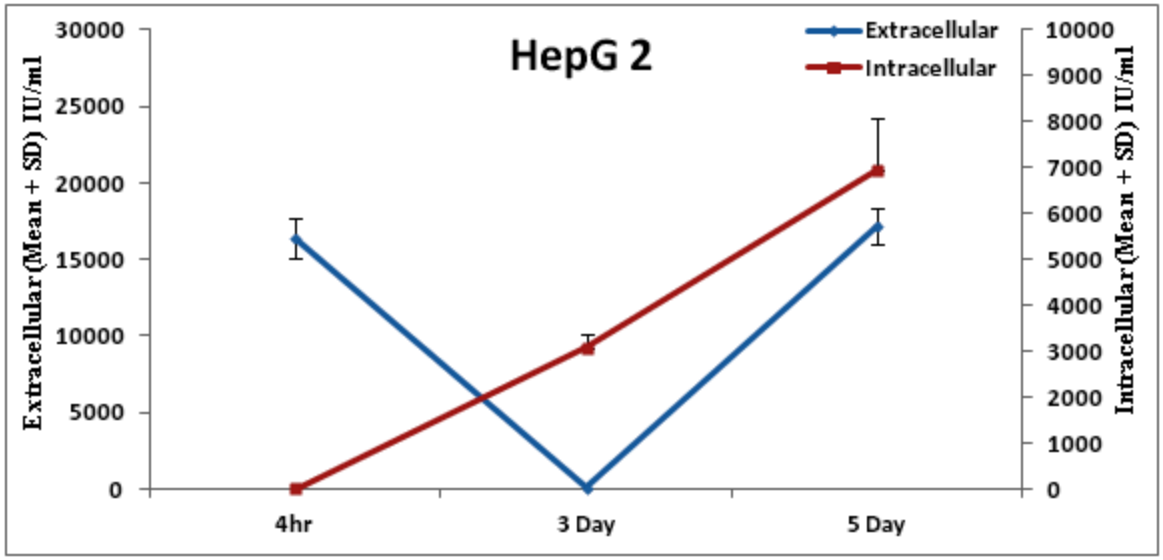

Figure 1: Mean values (M±SD) of HCV RNA extracted from HepG2 cells and media at the three investigated periods following treatment (4 h,3 Day and 5Day), detected by (RT-PCR).

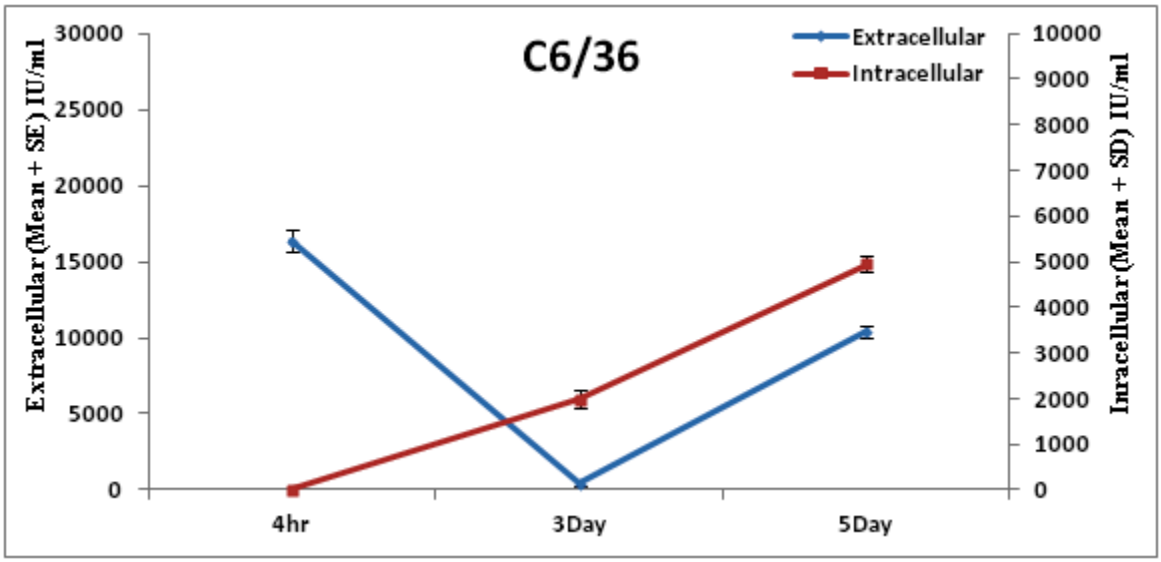

Figure 2: Mean values (M±SD) of HCV RNA extracted from insect cell line(C6/36) cells and media ,identified by (RT-PCR),at three different periods of time (4 h,3 Day and 5Day), after infection .

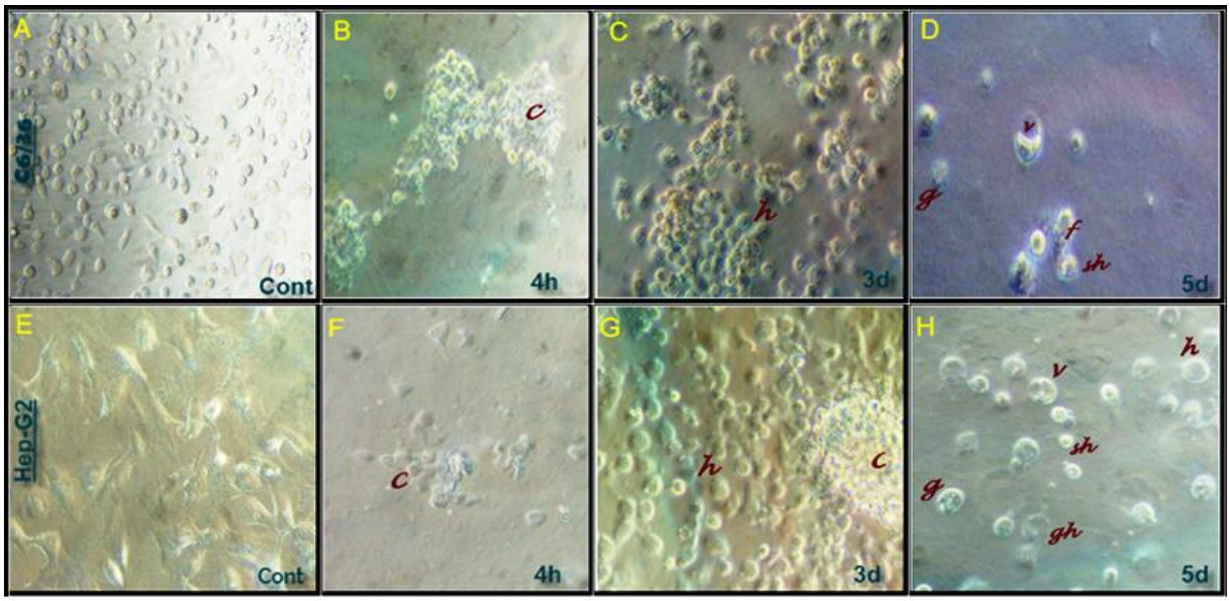

Figure 3: Morphological images by an inverted microscope at 400× magnification. All panel images are electronically resized to some degree. C6/36 and HepG2 cell lines were untreated (A and E) and treated with HCV-infected serum (≅1.7× 105 IU/ml) (B-D for C6/36; F-H for HepG2) and examined at different time intervals (4h, 3d and 5d post infection). Cell granulation (g), vacuolated cells (v), ghost (gh), shrinkage (sh) and fragmented destroyed cells (f).

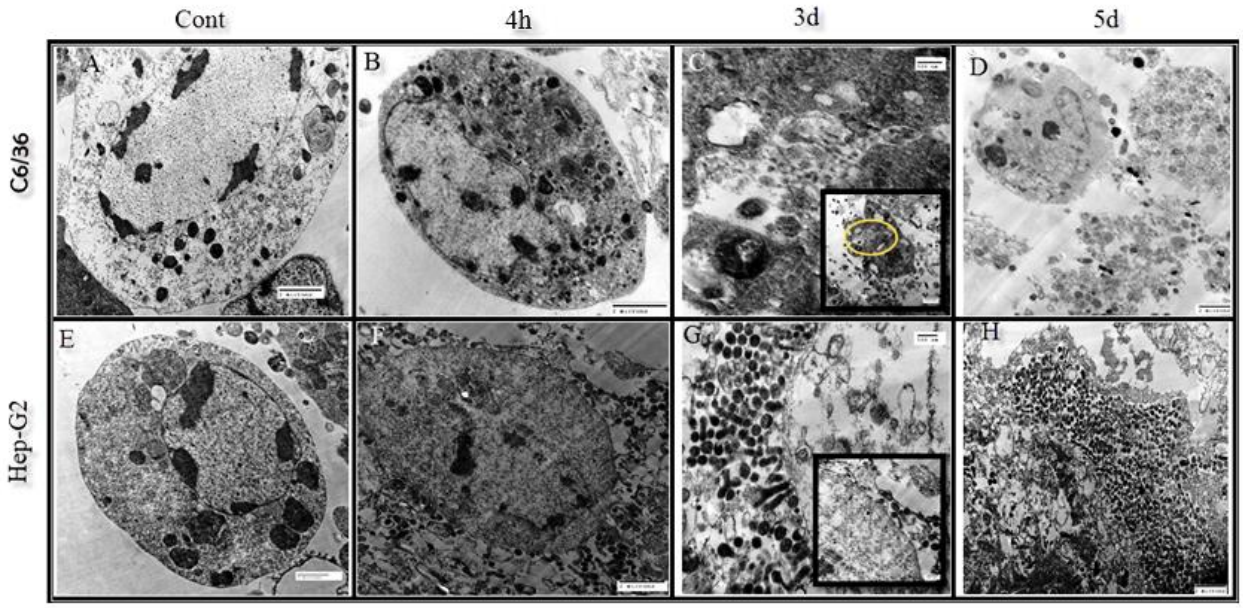

Figure 4: Standard thin TEM sections of C6/36 and HepG2 cells harvested at 4h, 3- and 5-days post-infection (B, C, D for C6/36 and F,G, H for HepG2; respectively) comparing to control (A, E). All images have scale of 2 microns and are electronically resized to some degree; squares inside C and G images are 500 nm scales).

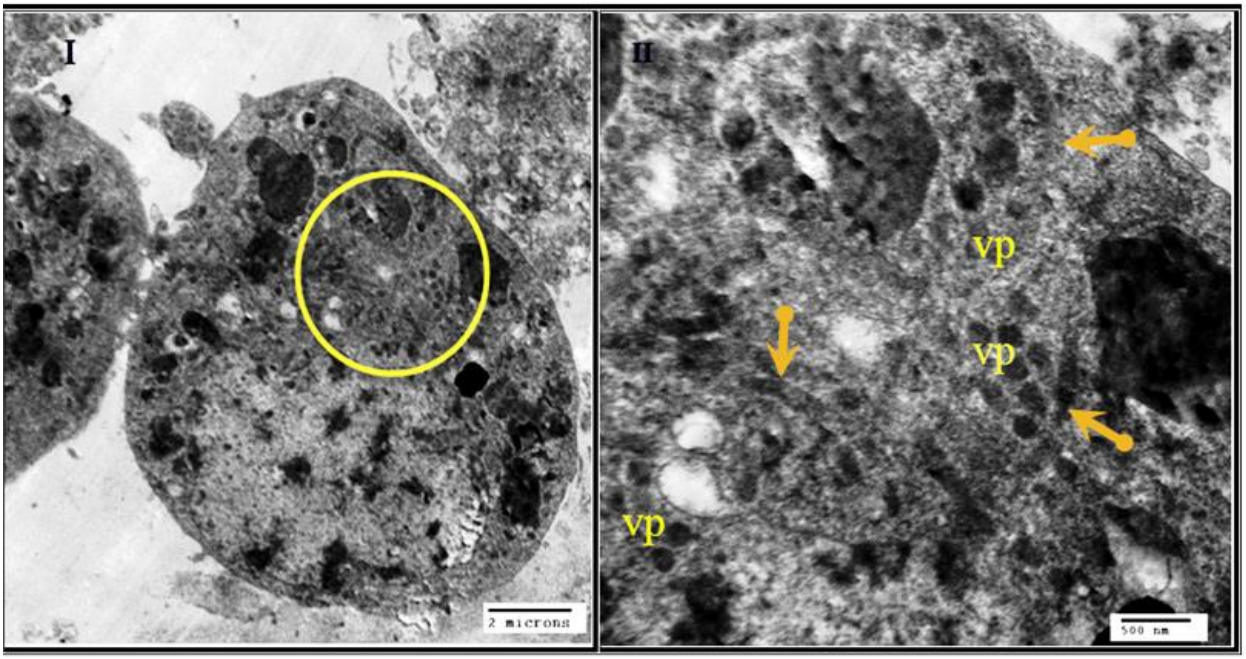

Figure 5: Standard thin TEM sections of C6/36 cell, in two different scales, showing 40–60 nm HCV virus-like particles (VP) in insect cells; which is absent in control preparation, embedded in large irregular cytoplasmic vesicles (so-called membranous web) (arrows)

DISCUSSION

An utterly open question endured how long-term endemic HCV transmission is sustained. There have been very few efforts to redress the lack of the available data which could clarify this critical issue. Limited data about the replication of HCV in mosquitoes or any other arthropod; even though various species are available (Silverman et al., 1996;Bellini et al., 1997; Chang et al., 2001; Hassan et al., 2003; Fallecker et al., 2017). As Aedes aegypti and Aedes albopictus are mosquito species cell lines which known as permissive cells and replicate different viruses from HCV family (Flaviviridae)(Walker et al., 2014), this study is designed to investigate whether Aedes albopictus (C6/36) cell line support HCV replication after in vitro experimental infection. As well-known, virus induces membrane alterations that are often generated in coordination with host factors and can be grouped into different morphotypes (Harak and Lohmann, 2015). We have studied the HCV infection in a suspect non-host cell line (C6/36) compared with the actual host cells (HepG2). Morphological and cytopathic changes accompanied by viral infection were investigated by inverted and electron microscopes.

Despite the similarities that C6/36 showed with Hep-G2 cell line in HCV RNA extracellular and intracellular curves indicating the appetence of HCV RNA in mosquito cells, there is no increase in HCV RNA concentration in C6/36 compared with that of HepG2 cell line, which displays unblemished replication of the virus. Thus, HCV has the ability to infect mosquito cells; although either this cell line was unable to replicate HCV, or that the virus cycle was abortive.

There is a prodigious debate about the ability of HCV to replicate in the insect. Several in vitro studies supporting to our results demonstrated the expression of HCV structural proteins in a baculovirus-insect cell system but did not report HCV-like particle assembly (Hsu et al., 1993; Lanford et al., 1993;Matsuura et al., 1994). Results of Mitchell (1995) indicated that there was no HCV infection in Aedes albopictus mosquitoes after ingestion of HCV infected blood when mosquitoes were observed for 14 days after feeding. Silverman et al. (1996)confirmed that HCV does not readily infect Aedes albopictus. Baumert et al. (1998)showed the existence of HCV (HCV-J strain, genotype 1b) in both AP61 cells as well as Vero cells. In the same line, Germi et al. (2001)confirmed the binding and infection of HCV (genotype 1b) to Aedes pseudo-scutellaris (AP61cells) 28 days post-infection. More recently, Fallecker and his colleagues (2017) displayed the

ability of E1E2-expressing HCV (genotype3) pseudo particles to associate with the mosquito cell lines Aedes aegypti (Aag-2) and Aedes albopictus (C6/36) indicating that HCV could indeed occur in their cells. Nevertheless, these studies (Baumert et al.,1998;Germi et al. 2001;Falleckeret al.,2017) display replication of HCV within these cells that might be returned to the difference in genotypes or inoculum. Moreover, they applied for a longer experimental time extended to 28 days; although, they have noticed no replicative change 4 days post-infection (Germi et al., 2001; Fallecker et al., 2017) which indicated that HCV replication required a longer time to be detected.

The mechanism of positive RNA virus's replication must be taken in consideration; positive-strand RNA viruses replicate in cytoplasm of the infected cells and persuade intracellular membranous compartments harboring sites of viral RNA synthesis. These replication factories are assumed to concentrate the components of the replicase and to armor replication intermediates from the host cell innate immune defense (Harak and Lohmann 2015). Current TEM results indicate the appearance of viral particles in HepG2 and C6/36 cytoplasm which was confirmed by the qRT-PCR results. Thus, it appears that there may be common mechanisms to modulate host membranes and lipid homeostasis that identically revealed clearly by our TEM micrographs. Our results are in agreement with that of Harak and Lohmann (2015) who described the virus-induced membrane alteration, the so-called membranous web which was initially defined as an accumulation of vesicles embedded in a membranous matrix (Egger et al., 2002).

Analysis of HCV infected-cell pellets also reveal that the 40–60-nmenveloped virus-like particles in insect cells that were not seen in control preparation are similar to the features previously described for putative virions isolated from HCV-infected chimpanzee live, an HCV-infected human B-cell line and HCV cDNA-transfected HeLa cells (Shimizu et al. 1996; Iacovacci et al., 1997; Baumert et al., 1998).These 40–60-nm enveloped virus-like particles are recently described by Krol et al. (2019). Harak and Lohmann (2015) discussed and described the knowledge of the architecture of membrane rearrangements induced by different positive-strand RNA viruses. Our results agreed with their explanations that mainly focus on ultra-structural details of the viral replication complexes and the role of host factors that are hijacked to build and maintain those viral replication factories. In the same concept, the results of Shiokawa et al. (2014)showed that exogenous expression of a liver-specific microRNA; miR-122, in non-hepatic cell lines facilitates efficient replication but not particle production of HCV-derived from cell culture (HCVcc), suggesting that liver-specific host factors are required for infectious particle formation. These results indicate that hepatic differentiation participates in the expression of liver-specific host factors required for complete propagation of HCV that goes on the same track with our current results and confirm the importance of the cell origin, the virus identity and explain the different rate of viral multiplication and replication. Our results on HepG2 cells are in line with previously documented data which demonstrated that HCV cycle doesn't exceed week after infection to produce contagious HCV virions in mammalian cells (Chen et al. 2000; Germiet al.2001).

CONCLUSION

Taken together, this study showed that the mosquito cell line could acquire HCV infection, causing cytopathic damage. Presence of HCV RNA in C3/36 cells shed some light about the ability of HCV to infect cells other than human hepatic one. Our results stressed on the importance of tracking the possibility of transmitting HCV through insect vector, which might provide an assumption about the remarkable increase of HCV infection in Egypt. Future studies are mandatory to improve our results

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

OAK: Designed the experiment and reviewed the manuscript. AYG: Performed the experiments and wrote the manuscript. EAS: Performed the experiments and revised the manuscript. RMT: Performed statistical analysis and wrote manuscript. All authors read and approved the final version.

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