Plant Archives Vol. 19, Supplement 1, 2019 pp. 538-544 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EFFECT OF ATORVASTATIN AGAINST NEWCASTLE DISEASE VIRUS IN CHICKEN EMBRYO FIBROBLAST CELLS

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Abstract

This study carry out to investigate the cytotoxicity and antiviral activity of atorvastatin against Newcastle disease virus (NDV) in chicken embryo fibroblast. The cytotoxicity was tested on chick primary fibroblast cells by MTT assay while antiviral activity was determined through infected these cells with NDV simultaneously treated with different concentrations of atorvastatin then measurement the cytopathic effect and real time reverse transcription-polymerase chain reaction (rRT-PCR). The result showed that atorvastatin concentrations 2 mg/ml was safety and no toxic, also has good antiviral activity against NDV in chick primary fibroblast cells. The results suggest that atorvastatin is expected to be a new alternative control measure for NDV infection.

***Key word*:** Atovastatin, NDV, antiviral activity, chick primary fibroblast cells

Introduction

Poultry industry is expose to many infectious threats. One of them is Newcastle disease (ND) which is an acutely, highly infectious viral disease, infected most avian species, regardless of variation in sex and age (Alexander *et al*., 2012 and Iram *et al*., 2014). ND causes severe economic losses in poultry industrialism world-wide due to high mortality and decline in growth performance of broiler chickens as well as, deteriorates the quantity and quality of eggs in layers (Yan *et al.,* 2011; Miller and Koch, 2013).

The causative agent of ND is Paramyxovirus type 1 (APMV-1) which also, called Newcastle disease virus (NDV) which is a negative sense non segmented single strand RNA virus belong to the family Paramyxoviridae, genus Avulavirus (Mayo, 2002). According to virulence of the virus NDV strains are classified into velogenic, mesogenic and lentogenic (Orsi *et al.,* 2009). While as NDV velogenic strains divided to neurotropic velogenic NDV (NVNDV) and viscerotropicvelogenic NDV (VVNDV) which cause severe clinical signs and high mortality (Huang *et al*., 2004; Piacenti *et al.,* 2006).

Strict biosecurity together with vaccination is only commercial control measure for precluding and controlling ND in chickens farms (Miller and Koch, 2013). Despite that, outbreaks of ND still continue in immunized birds (Zhang *et al*., 2010, 2011; Wang *et al*., 2015). Furthermore, absenteeism antiviral agents against NDV in poultry medicine hence new replacement controlling procedures are demandable to prevent the replication of NDV or decrease its drastic effects on an infected flock (Dortmans *et al*., 2012; Miller *et al*., 2013). Once of these new alternative control measures is investigate about antiviral agent.

Atorvastatin drug belong to statinsfamily, which also, well-known as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor are widely utilized worldwide for treating hypercholesterolemia (Hennessy *et al*., 2016). Statins inhibit the mevalonate which is rimming step in the cholesterol synthesis pathway by competitive bindingly to HMG-CoA reductase in a dose-depended manner, that lead to diminishing cholesterol production and other intermediate product likedolichol, geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (Young *et al.,* 2014). Beside cholesterol reduction, statins also, have others multiple effects called pleiotropic effects like antithrombotic, antioxidant, antiplatelet, endothelial protection, immunomodulatory, anti-inflammatory and neutrophil extracellular trap (NET) production, all these effects are cholesterol-independent through reduce importance isoprenoid intermediating like as (GGPP) and (FPP) that leading to decreasing cell signaling proteins such as Ras, Rac, and Rho (Chow *et al.,* 2010; Gazzerro *et al*., 2012; Kozarov *et al*., 2014).

Many studies referred that statins have an antimicrobial potential against different infectious agent like different bacterial species and several pathogenic fungi in human (Chamilos *et al*., 2006; Macreadie *et al*., 2006; Bergman *et al*., 2011; Lopez-cortes *et al*., 2013; Kozarov *et al*., 2014). While as, other studies indicated to that statins have confluent activities against several virus infection causes by different viral species such as Respiratory Syncytial Virus (RSV) *in vivo* and *in vitro*

(Tara *et al*., 2001), Human Immunodeficiency Virus (HIV) (Kelesidis, 2012), Highly Pathogenic Avian Influenza H5N1, seasonal and H1N1virus infection in BALB/c mice (Yohichi *et al*., 2012).

There is no study used statins as antimicrobial in chickens, for that, the present study was aimed to investigate the effect of statins against NDV in chicken embryo fibroblast cells.

Materials and Methods

Atorvastatin

Atorvastatin Lipitor® (Pfizer Inc., New York, NY, USA) tablets, each tablet is containing 20 mg of Atorvastatin, were pulverized and suspended in phosphate buffered saline (PBS) (Sigma–Aldrich, St. Louis, MO, USA).Median lethal dose (3.8mg/egg) and effective dose (0.1 mg/0.2 ml/egg) of Atorvastatin used in this study were determined previously (data do not published).

NDV Strain Used for Infected Cell

NDV (MH407212 strain) used in this study was provided by Department of Pathology and Poultry diseases, Veterinary Medicine College/University of Baghdad (Iraq). Viruses were propagated in 9-day old chicken embryo eggs and the 50% egg infectious dose (EID50) was measured as $10^{7.48}$/mL according to (Reed and Muench, 1938).

Atorvastatin Cytotoxicity Assay

The cytotoxicity of the atorvastatin was examined according to a procedure used for general screening of cytotoxic agents. Based on metabolic cell viability, this was performed using a modified MTT [3-(4, 5-Dimethyl-2-thiazolyl)- 2, 5-diphenyl-2H-tetrazolium bromide)] assay which affects the mitochondrial reductase activity of viable cells (Mosmann, 1983). Primary chicken fibroblast cell which prepared as described by (Zhao *et al.,* 2011) was cultivated for 24 hours in 96-well microplates with 2 x 10^6 cells/mL initial concentration. Cultured cells were then treated with different concentrations of Atorvastatin (0, 0.1, 0.2, 0.5, 1, 2, 4, 8 mg/ml) and incubated for 48 hours at 37ºC under a 5% CO_2 atmosphere after that, 5 mg/ml in 0.1M PBS of the MTT solution was added into the 96 well plates and incubated at 37ºC for 4 h. (Xu *et al*., 2007; Bai *et al*., 2008). Thereafter, supernatants were aspirated and100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan and incubated for 1 h. Optical density (OD) was then measured at 570 nm, with a reference wave length of 690 nm by an ELISA plate reader (Bio Tek µQuant, USA). The percentage cell viability was calculated by utilizing the equation below.

$$\text{Cell viability}\% = \frac{\text{mean absorbance of treated cells}}{\text{mean absorbance of control cells}} \times 100$$

Methyl thiazol tetrazolium is a yellow water-soluble tetrazolium dye, that when reduced by viable cells turns into a purple water insoluble formazan product.

In Tissue Culture Atorvastatin Cytotoxicity Assay

The chicken embryo fibroblast cells that prepared from 9-11 chicken embryo according to (Zhao *et al.,* 2011)were seeded for 24 hours in plastic culture plate contain 24 wells with 2 x 10^6 cells/ml in each well as initial concentration, then, these wells were divided to 6treatment groups, where each 4wells represent once of treatment groups, the 1st group inoculated with 0.5mg/ml atorvastatin, 2nd group inoculated with 1mg/ml of atorvastatin, 3rd group inoculated 1.5 mg/ml of atorvastatin, 4th group inoculated with 2 mg/ml of atorvastatin, 5th group inoculated 4mg/ml of atorvastatin and the 6th group consider as control negative(chicken embryo fibroblast primary cells only). The occurrence of anycytopathic effect (CPE) was observed under the inverted microscope at 8 h intervals (Freshney, 2010).

Antiviral Activity of Atorvastatin in Tissue Culture

After preparation primary chicken embryo fibroblast cells, these cells were seeded in 24-wells plate with growth median until obtain to confluent monolayer cells, the plate were divided to 6 groups each group include 4 wells, as following:

- 1st group included cells with maintenance media as control group.
- 2nd group the cells were infected with NDV only.
- 3rd group the cells were infected with NDV simultaneously treated with 0.5mg/ml Atorvastatin.
- 4th group the cells were infected with NDV simultaneously treated with 1mg/ml Atorvastatin.
- 5th group the cells were infected with NDV simultaneously treated with2mg/ml Atorvastatin
- 6th group the cells were infected with NDV simultaneously treated with4mg/ml Atorvastatin

Approximately 45 IU of NDV was inoculated in each well for infected cells then incubated at 37 ºC for 30 minutes for virus adsorption after that, re-fed with maintenance medium and re-incubated at 37 ºC till a good cytopathic effect (CPE) of the virus was appeared. The cytopathic effect (CPE) was examined daily under the inverted microscope for the virus growth and compared with control group. wells were showing good CPEs, their maintenance medium was collected with a rubber, pooled and stored at –70 ºC. After that, the total RNA was extracted with a Qiagen Kit according to the manufacturer's instructions. NDV RNA was quantified

usingreal time reverse transcription-polymerase chain reaction (rRT-PCR).

The matrix gene primer and probe previously described, and validated by (Wise *et al.*, 2004) was designed for amplification of matrix gene which used in detection NDV by real time RT-PCR as shown in Table (1).

Table 1: Primer and probe sequence of matrix (M) gene

Gene	Primer-Probe	Sequence	Size bp
Matrix	M+4100	5'- AGTGATGTGCTCGGACCTTC-3'	121
	M-4220	5'- CCTGAGGAGAGGCATTTGCTA-3'	

Results

The result of MTT assay **to** determined toxicity of different concentrations (0, 0.1, 0.2, 0.5, 1, 2, 4, 8) mg/ml for 48h of Atorvastatin to primary chicken embryo fibroblast cells (CEF) was explained in (Fig.1).

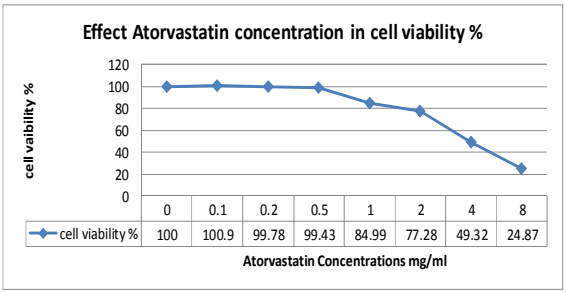

Fig. 1 : The effect Atorvastatin concentration mg/ml in cell viability%

Determine Cytotoxic Dose of Atorvastatin in Tissue Culture

The toxicity of Atorvastatin to primary chicken embryo fibroblast cells (CEF) was determined through observed CPE after inoculation with different concentration (0.5, 1, 1.5, 2 and 4) mg/ml of Atorvastatin. The concentrations of Atorvastatin (0.5, 1, 1.5 and 2) mg/ml do not have any cytotoxic effect on cell culture (Fig. 2). While CEF were inoculated with 4mg/ml Atorvastatin only showed many normal cells and some abnormal cells (Fig. 3).

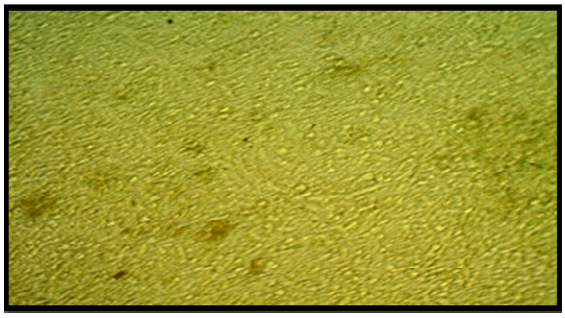

Figure 2: CEF cell monolayer inoculated with 2mg/ml Atorvastatin show many normal cells. (10X objective).

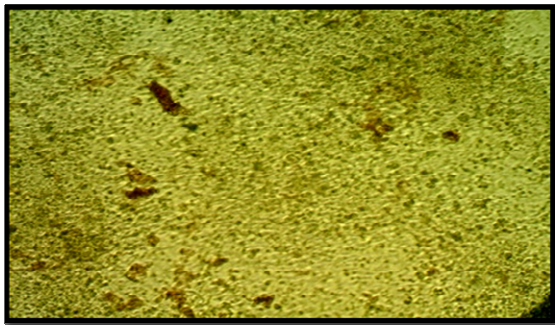

Figure 3: CEF cell monolayer inoculated with 4mg/ml Atorvastatin show many normal cells and some abnormal cells.

Antiviral Activity of Atorvastatin Against NDV in Tissue Culture

The result of Atorvastatin antiviral activity against NDV in tissue culture appeared typical cytopathic effects of NDV were observed in positive control group(chicken embryo fibroblast primary cells inoculated with NDV only) after 72 hour, included increased granularity, rounding and vacuolation of infected cells (Fig. 4), while (Fig. 5) explains normal cell observed in uninfected group (uninfected chicken embryo fibroblast primary cells), whereas, the effect of Atorvastatin in different concentrations (0.5, 1, 1.5, 2 and 4) mg/ml against ND is present in Figures (6, 7, 8, 9 and 10) respectively. The results of this experiment found out that 0.5 mg/ml concentration of statin has a mild effect on the viral replication where, there are many rounded cells and huge of cells destruction (Fig. 6), while 1mg/ml concentration of Atorvastatin showed a moderate antiviral activity against NDV replication, where, there are a normal spender cells associated with a lot of the rounded cells, and some plaque formation but less than in 0.5mg/ml concentration (Fig. 7), while 1.5mg/ml of statin showed more less cytopathic effect of NDV compared with 1mg/ml concentration as, showed many of normal cell in section (Fig.8), while, 2 mg/ml concentration of statin appeared the best antiviral activity where few rounded cells, observed in this concentration with high percent of normal cells (Fig.9), although, 4 mg/ml concentration of statin showed better antiviral activity against NDV where, no cytopathic effect appeared in this concentration, but, this concentration has some cytotoxic effect induce by statin which represented by infiltration of brown substance in cells that lead to abnormalities of cells morphology (Fig.10).

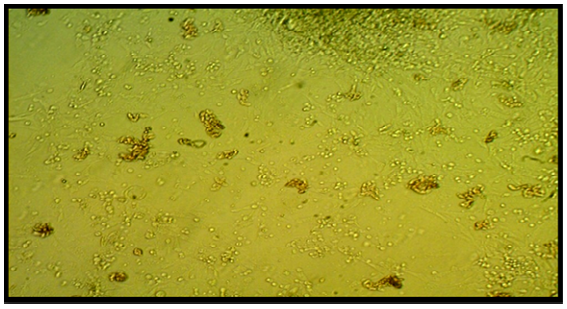

Figure 4: Rounding of infected cells in CEF cell monolayer following infection with NDV (10X objective).

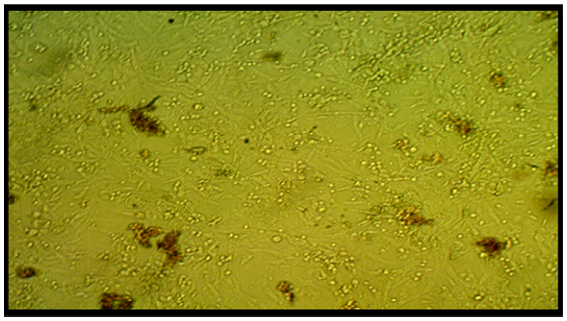

Fig. 5 : Uninfected CEF cell monolayer (10X objective).

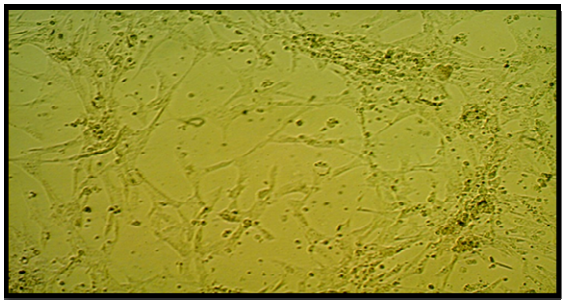

Fig. 6 : CEF cell monolayer following infection with NDV then inoculated with 0.5mg/ml Atorvastatin show many of rounded cells. (10X objective)

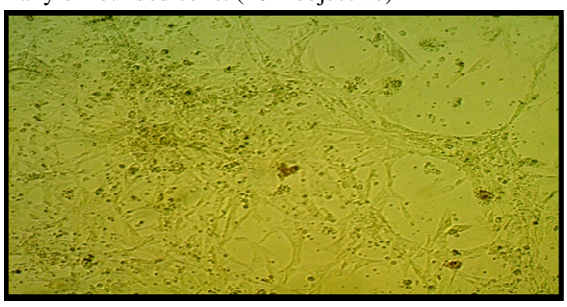

Fig. 7 : CEF cell monolayer following infection with NDV then inoculated with 1 mg/ml Atorvastatin show some rounded cells. (10X objective).

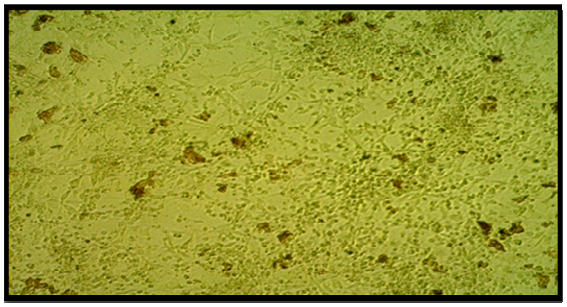

Fig. 8 : CEF cell monolayer following infection with NDV then inoculated with 1.5 mg/ml Atorvastatin show few rounded cells with increase number of normal cells. (10X objective)

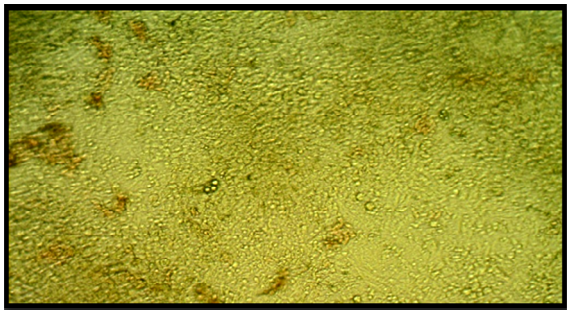

Fig. 9 : CEF cell monolayer following infection with NDV then inoculated with 2 mg/ml Atorvastatin show few rounded cells with many normal cells. (10X objective)

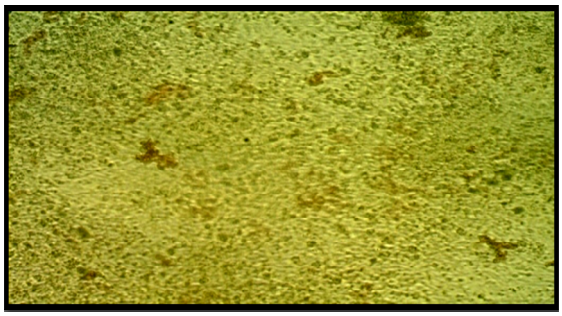

Fig. 10: CEF cell monolayer following infection with NDV then inoculated with 4 mg/ml Atorvastatin show no rounded cells with many normal cells beside appear some abnormal cells (10X objective)

Real Time RT-PCR

After collecting sample from monolayer chicken embryo fibroblast infected with NDV only(control positive) also, from other groups that infected with NDV then treated with different concentrations (0.5, 1, 2 and 4 mg/ml) of Atorvastatin and from group that uninfected untreated (control negative) to determine activity of statin against NDV, all samples tested by

rRT-PCR positive control (C+) had C_T value of 16.55 where other treated group (23.17, 19.15, 18.74 and 17.32) respectively as shown in (Fig. 11).

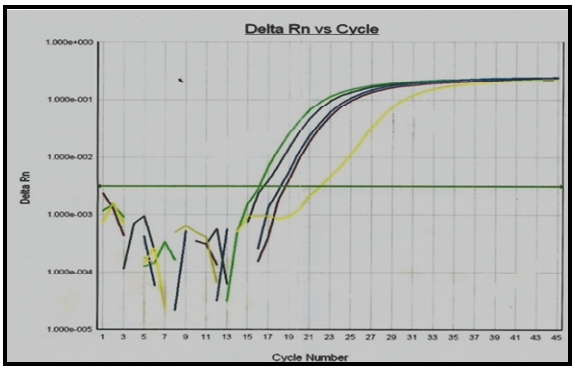

Fig. 11 : The rRT-PCR in four tested samples shows the Logarithmic fluorescence plots versus cycle number resulting from the determination of NDV RNA. The Fig. shows the yellow, brown, blue, and black curves represented the four treatment groups (G3, G4, G5, G6) respectively of C_T value 17.32, 18.74, 19.15 and 23.17 respectively where the green curve represented the positive control (G2) 16.55 C_T value.

Discussion

NDV remains a continuous threat to the poultry industry worldwide due to the capability of the virulent strains to cause high mortality. Since 1968 Iraq has been endemic for NDV when the initial isolates made by Kaschula from infected chickens at Abu Graib, designated AG68 (Borland and Allan, 1980). From this date poultry industry was exposed to severe economic loss due to infected with a virulent NDV. In Iraq as other endemic countries in the world, the great challenge facing the poultry industry is controlling of ND. The main control system of ND via vaccination programmers beside high bio-security measurements (Miller *et al.,* 2013). Several intense vaccination programs applied in the veterinary field add extra stress to birds flocks from hatch via introducing many different combinations of NDV vaccine strains which consider as a load on flocks health. In addition, the vaccine could take a long time to initiate the protective immune system. Therefore, different strategies to either prevent the replication of NDV or to decrease its drastic impact on infected flocks are needed (Park *et al.,* 2014). One of those potential strategies is to use antiviral agents against NDV, although, fewer substances are available for the treatment of viral infections in poultry when compared with the large amount of the available antibiotics for the treatment of bacterial and fungal infections (Huber, 1998). One of the chemotherapy agent is statin which used as antiviral against different viruses, Respiratory Syncytial Virus (RSV) (Tara *et al*., 2001), Human Immunodeficiency Virus HIV (Kelesidis, 2012), Highly Pathogenic Avian Influenza H5N1, seasonal and H1N1virus infection (Yohichi *et al*., 2012) in vitro and vivo. The antiviral activity of statins dependent on pleiotropic effects which encompass modification of endothelial function, plaque stability and thrombus formation, and anti-inflammatory and immunomodulatory properties (Liao, 2002), beside to, the essential effect as lipid-lowering agent through inhibitor of HMG-CoA reductase, which catalyzes the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) an essential enzyme in cholesterol biosynthesis to mevalonate (Lennernas, 2003). Hence, these pleiotropic effects of statin have not yet been evaluated against any viral infection of poultry, for that, in this study, the antiviral activity of Atorvastatin against NDV is examined in chicken embryo fibroblast.

Antiviral activity of statin against NDV *In-vitro* assays usually rely on the virus ability to infect and replicate in chick embryo fibroblast primary cell, where this cell culture systems provide a rapid and reliable method to grow viruses at higher titers, to apply reverse genetics, and to test antiviral compounds.

In the present study, the primary chicken embryo fibroblast were inoculated with different concentrations of Atorvastatin (0.5, 1, 1.5, 2, 4 mg/ml) simultaneously with NDV, the results referred to that Atorvastatin has antiviral activity against NDV, that appeared through viability of cells and absence or decrease the cytopathic effect of NDV compared with control positive which infected with NDV only which characterized by decrease viability cells and appear characteristic cytopathic effect (CPE) characterized by rounding, vacuolation, syncytia formation and cell death (Ravindra *et al.*, 2009; Mehrabanpour *et al.*, 2010). The cause beyond decrease cytopathic effect and increase the viability cells in groups that infected with NDV and treatment with atorvastatin, may be due to the ability of atorvastatin in depletion the cellular cholesterol which may play important role in NDV entry and penetrate the cell through caveolae-mediated endocytosis, that cholesterol depletion exerts an inhibitory effect on NDV interaction with the target cell (Cantin *et al*., 2007; Martín *et al*., 2012). The present finding corresponded with finding obtained by (Martín *et al*., 2012) who found that Depletion of cellular cholesterol by treatment with methyl-β-cyclodextrin (MβCD) inhibited NDV binding, fusion and infectivity. This inhibition was almost completely compensated by replenishing cellular cholesterol levels, suggesting that the effect of MβCD treatment on virus activities would be due to the removal of cholesterol. The antiviral activity increase

gradually with increase Atorvastatin concentration, but, the best concentration given antiviral activity was 2mg/ml which appear more of viable cells as well as, this concentration did not appear any cytotoxic effect (Fig. 9), in contrast to 4mg/ml which has good antiviral activity but, in same time has cytopatic effect of cytotoxicity as show in figure (10). These results were confirmed by rRT-PCR was appeared that Atorvastatin has antiviral activity and this activity increase with increase concentration of Atorvastatin as shown in Figure (11).

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