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CYTOTOXICITY AND ANTIVIRAL ACTIVITY OF ATORVASTATIN AGAINST NEWCASTLE DISEASE VIRUS IN CHICKEN EMBRYOS

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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. The cytotoxicity was tested on chick primary fibroblast cells by MTT assay while antiviral activity was determined on 9- day old chicken embryos through measurement the heamagglutinating titer of NDVin allantoic fluid, survival of embryo and percentage of mean embryo weight, the result showed thatAtorvastatin concentrations less than 2mg/ml was safety and no toxic, also Atorvastatin has good antiviral activity when administration before challenge. The results suggest that Atorvastatin is expected to be a new alternative control measure for NDVinfection.

Key words : Atorvastatin, Antiviral activity, Newcastle disease virus

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 a*l,2012, Iram *et a*l,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 viscerotropic velogenic 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, out breaks 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.

Statin drugs, 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 a*l,2006, Macreadie *et a*l, 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.

MATERIALS AND METHODS

Antiviral activity of Atorvastatin in ova

In-ovo antiviral activity of Atorvistatin against NDV was evaluated in 9 days old embryonated chicken eggs (ECEs) following the protocol described by (Chollom *et al* 2012). 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 (0.1mg/0.2 ml/egg) of Atorvastatin used in this study were determined previously(data do not published). NDV (MH407212strain) used in this study was provided by Departmet of Pathology and Poultry, Veterinary Medicine College /University of Baghdad (Iraq). Viruses were propagated in 9-dayold chicken embryo eggs and the 50% egg infectious dose (EID50) was measured as $10^{7.48}$/mL according to (Reed and Muench,1938). Atorvastatin and NDV were inoculated into allantoic cavity of embryonic eggs. In this study, 25 nine-day-old chicken embryos(without anti-NDV antibody) were brought from a local hatchery to carry out this experiment, the eggs were divided to 5 equal groups as follow:

1st group: the eggs were inoculated with 0.2ml PBS as control group.

2nd group: the eggs were inoculated with0.2ml NDV.

3rd group: the eggs were inoculated with Atorvastatin 0.1mg/0.2ml.

4th group: the eggs were inoculated with Atorvastatin 0.1mg/0.2 ml 6hour before challenge with 0.1ml NDV.

5th group: the eggs were inoculated with 0.1ml NDV then after 6 hour inoculated with Atorvastatin 0.1mg/ 0.2ml of Atorvastatin.

According to each group treatment, the eggs were inoculated by the allantoic route with sterile disposable 1ml syringes. Eggs were then incubated at 37^0C and candled daily for five days to check for embryo death. Embryo death post inoculation was recorded daily then chilled at 4 for at least two hours and the Amniotic-allantoic fluid aseptically harvested from each of the eggs and tested for the presence of NDV by heamagglutinating (HA) test which conducted according to (Murakawa *et al,* 2003).

The parameters were used to measured antiviral activity of atorvastatin against NDV in present study, are reduction in HA titer, survival of embryo and percentage of mean embryo weight as described by (Mabiki *et al*, 2013).

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 37! under a 5% CO2 atmosphere after that, 5mg/ml in 0.1M PBS of the MTT solution was added into the 96 well plates and incubated at 37 ! for 4 h. (Xu *et al*, 2007; Bai *et al*, 2008). Thereafter, supernatants were aspirated and100 ìL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan and incubated for 1 h. Optical density (OD) was then measured at 570nm, with a reference wavelength of 690nm by an ELISA plate reader (Bio Tek µQuant, USA). The percentage cell viability was calculated by utilizing the equation below.

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

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

RESULTS AND DISCUSSION

The results in table (1) showed that all embryos in group 3 which inoculated with NDV only were died within 48 hours compared with other groups that have different

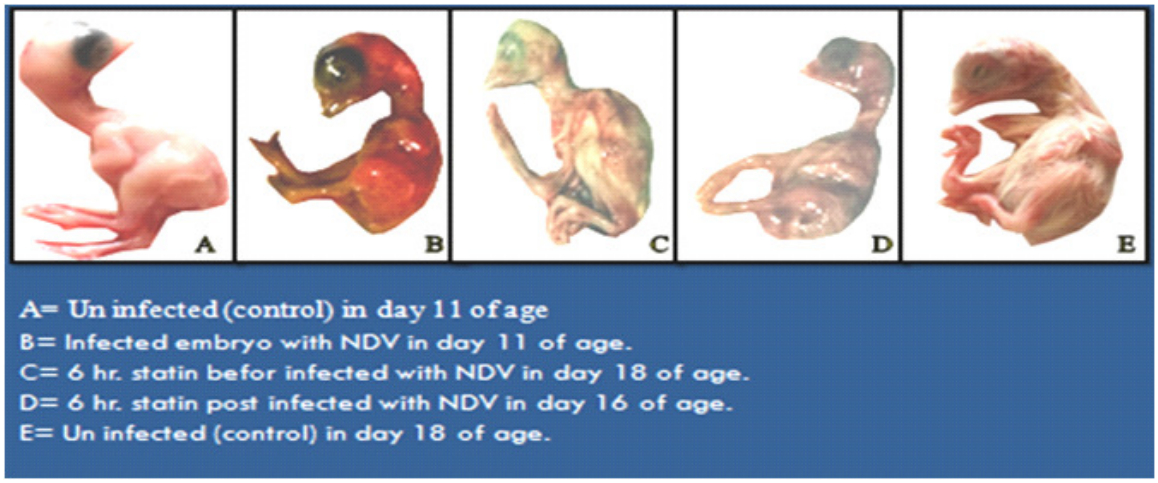

Figure 1 : Gross lesions in chick embryoes in defferent experimental groups.

mortality rates (0, 0, 20, 60)% in groups (G1,G2,G4 and G5) respectively.

The gross lesions were observed in embryos in G2 which inoculated with NDV only revealed haemorrhage on the whole body of embryo including cranium, legs, wings and back compared with control that has no lesion and Atorvastatin group which showed in (fig.1).

The weight of chickens embryos consider importance measured to antiviral activity against NDV, table (2) explains weight of chicken embryos and relative mean embryos weight(MEW) post inoculation with Atorvastatin 0.1mg/0.2ml/egg and 0.2 ml NDV at day 9 of age according to each treatment group of experimental design, the results indicate to higher MEW (100,93,90,60)% recorded in G1,G4,G3,G5 respectively while less MEW recorded in G2.

Other parameter dependent to measure the antiviral activity of Atorvastatin against NDV in this study is haemagglutenation(HA) and relative mean reduce haemagglution percentage (HA%), the result referred to higher mean HA and mean reduce HA% was recorded in G2 which inoculated with NDV only was $2^9$and 100% respectively, after that come G5 which recorded $2^6$and

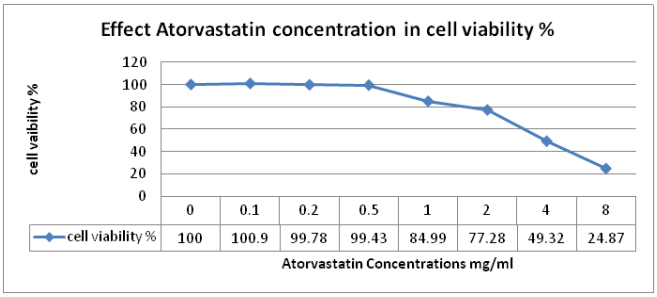

Figure 2 : The effect Atorvastatin concentration mg/ml in cell viability%.

13% while the other groups G1,G3 and G4 were 0 in each group as show in (table 3).

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. 2).

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

Table 1 : Mortality rate among experiment groups

Groups	No. egg	Mortality of chicken embryos			Mortality(%)
		24hr.	48hr.	72hr.	
G1 0.1ml normal saline control	5	0	0	0	0
G2 0.1 ml of NDV only	5	0	3	2	100
G3 0.1mg/0.2ml Atorvastatin only	5	0	0	0	0
G4 0.1 mg/0.2ml Atorvastatin before challenge with 0.1 ml of NDV 6hr.	5	0	1	0	20
G5 0.1 mg/0.2ml Atorvastatin post challenge with 0.1 ml of NDV 6hr.	5	0	1	2	60

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 Infiuenza 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.

The cytotoxicity of Atorvastatin in primary chicken embryo fibroblast cells (CEF) was tested by the MTT assay which confirm that Atorvastatin concentrations less than 2mg/ml are not affect in a viability cells percent as shown in (fig.2) that mean Atorvastatin cytotoxic depend on its concentration**.**

Also, in this study, the chick embryo selected to check antiviral activity of Atorvastatin against NDV because the chick embryo has chorioallantoic membrane (CAM) which represent the outer most extra-embryonic membrane that is highly vascularized for gaseous exchange and calcium transportation between the embryo and its environment. Valdes *et al*, (2002), Lokman *et al*, (2012) referred to that CAM provides a technically simple way

Table 2 : Weight of chicken embryos and relative mean embryoes weight (MEW%)

Groups	No. egg	Mean embryo weight (g)					MEW (%)
		1	2	3	4	5	
G1 0.1ml normal saline control	5	12	12.3	11.7	12	12.5	100
G2 0.1 ml of NDV only	5	5	4.8	6	7	5.4	47
G3 0.1mg/0.2ml Atorvastatin only	5	10.5	11	11.6	10.8	11.4	91
G4 0.1 mg/0.2ml Atorvastatin before challenge with 0.1 ml of NDV 6hr.	5	10.4	11.2	11.6	11	12	93
G5 0.1 mg/0.2ml Atorvastatin post challenge with 0.1 ml of NDV 6hr.	5	7	6.6	6.8	7.2	8.4	60

Table 3 : HA and relative mean reduce HA%

Groups	No. egg	HA	Mean reduce HA(%)
G1 0.1ml normal sline control	5	2^0	0
G2 0.1 ml of NDV only	5	2^9	100
G3 0.1mg/0.2ml statin only	5	2^0	0
G4 0.1 mg/0.2ml statin before challenge with 0.1 ml of NDV 6hr.	5	2^0	0
G5 0.1 mg/0.2ml statin post challenge with 0.1 ml of NDV 6hr.	5	2^6	13

of studying complex biological systems with well-developed vascular tissues. It also has high reproducibility, a small footprint, inexpensive and easy to handle. The CAM model is recognized as an intermediate model that can bridge the gap between cell-based and animal-based assays; other than showing similar patterns of cellular toxicity as *in vitro* models, the CAM also gives tissue responses similar to those in mammalian models (Lokman *et al*, 2012).

The results of the antiviral activity of statin against NDV in ova measured according to three parameters which are reduce mortality rate, mean embryos weight and reduce of haemagglutination (HA) activity of the virus, these parameters were dependent by(Mabiki *et al,* 2013). The mortality rate in chickens embryos in this experiment as shown in table (1) referred to high mortality rate was in G2 were inoculated with 0.2ml NDV, where all embryos in this group were died during 48hours, while, the mortality rate in G4 which treated with Atorvastatin 6 hour before inoculated with NDV was 20% this result may be due to effect of Atorvastatin in prevent replication of NDV in early stage of life cycle, while the mortality rate in G5 which treated with Atorvastatin 6 hour after inoculated with NDV was 60% this may be due to NDV replicated in the embryos before injection of Atorvastatin, whereas, the mortality rate in G1(control) was 0% also, in G3 (treated with 0.1mg/0.2ml Atorvastatin) was 0% that confirm that this dose of statin has no toxic effect on embryos.

The second parameter dependent in this experiment to determent the efficiency of statin as antivirus against NDV in chicken embryos is mean embryo weight percentage (MEW%) post five days of virus inoculation as shown in table (2), the high MEW% observed in G1(control) was 100% , G3 (treated with Atorvastatin only) was 91% this may be contributed to continuous growth of chicken embryos which confirmed that effective dose of Atorvastatin do not toxic. The MEW% in G4 (treated with Atorvastatin 0.1mg/0.2ml/egg 6hour before inoculated with NDV) was 93% that may be referred to effect of Atorvastatin in prevent replication of the virus lead to continuous growth of chicken embryos and increase of their body weight, while in G5 (treated with Atorvastatin 0.1mg/0.2ml/egg 6hour after inoculated with NDV)was 60% that may be due to NDV inoculated 6hours before Atorvastatin that lead to give a chance to virus attach, penetrate and replicate after that killed chicken embryos before the Atorvastatin work, or the efficiency of Atorvastatin decreased when injected post NDV inoculation. While as the less MEW% recorded in G2 (NDV only) was 47% that may be due to that NDV killed the embryos which lead to stop embryos growth.

The virus antigen titer in different treatments was determined by hemagglutination test is the third parameter dependent in this experiment to evaluate anti-NDV activity of Atorvastatin as explained in table (3), the higher HA percentage present in G2(inoculated with NDV only) was (100%) this reflect the ability of virulent NDV to replicate in chicken embryos, the presence of NDV, in the allantoic fluid can be detected by slide HA test and microtitre plate HA and HI test following the standard procedure (Alexander, 2009). Whereas, the lower HA% recorded in G1(control) and G3 (treated with Atorvastatin only) this due to these embryos do not infected with NDV. While HA% in G4 (treated with Atorvastatin 0.1mg/0.2ml/egg 6hour before inoculated with NDV) was 0% in spite of, this group inoculated NDV the reason may be attributed to effect of treatment with Atorvastatin lead to reduced the virus infectivity by decreasing the virus HA titer this result agrees with result obtained by (Mehrbod *et al*,2012) who confirmed the ability of Atorvastain in combination treatment with virus (influenza virus H1N1), reduced the virus infectivity by decreasing the virus HA titer in comparison with the virus treatment. While HA% in G5(treated with Atorvastatin 0.1mg/0.2ml/egg 6hour after inoculated with NDV) was 13% that may be some virus can replicated before the Atorvastatin inoculated, or the Atorvastatin activity cannot prevent virus replication completely when Atorvastatin injected post NDV inoculation. This indicate that atorvastatin is safety and no toxic to living cells in concentration less than 2mg/ml also, has good antiviral activity against NDV when administration before challenge. This study recommended to conduct more studies on atorvastatin that may lead to discover new antiviral drug.

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