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The Study of Histopathological Effect of H9N2 Sub-type Influenza Virus on SPF Chicks' Airway

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Abstract: Influenza is a viral disease which has been known in 1901AD in 1955, a kind of influenza virus was known as a factor caused disease that later on called avian plague because of high rate mortality. In the present study, the sub-type H9N2 of avian influenza virus (obtained from Razi Serum Producing and Research Center) which was cloned two times in embryonic eggs was inoculated to the SPF (Valo, Lohman, Germany) at their 3rd week by nasal drops: At first, SPF chickens were divided to 2 groups of 10 subjects; a group as a treatment group and another one as a control group. Then treatment group was infected by H9N2 sub-type of influenza virus with dosage of $10^{7.5}$ EID50. The control group obtained normal saline serum nasally equal to the inoculated viral solution volume. In histopathological studies of lung and pleura of treated chicks with H9N2 sub-type influenza virus, propagated pneumonia which accompanies hyperemia and severs hemorrhage was observed in to allele and pleurisy sacs. In microscopic view, there is sever hyperemia and hemorrhage in allelic sacs, edama infiltration of fibrin and inflammatory cells, especially heterophylus in pulmonary' tissue inter-space. In the present study, the apoptosis of primary bronchial cells was observable in some cases which demonstrate significant changes compared with control group. Probably, the expression of apoptosis induction channel which has been discussed in Brydon's studies is a suitable justification for these changes. So, it must be noted that there are various channels in occurrence of cell death following infection by influenza virus and knowing these channels needs extensive studies.

Key words: Histopathology, H9N2 influenza, SPF chicks', respiratory tract, channel, Iran

INTRODUCTION

Influenza is a viral disease which has been known in 1901AD in 1955, a kind of influenza virus was known as a factor caused disease that later on called avian plague because of high rate mortality. The importance of influenza viruses as a pathogen with worldwide spreading has been well known in human, domestic animals and birds and sometimes has been known as a pandemic disease among human being. Avian influenza viruses are members of orthomixoviridae family and genus *A*. Since 1994, H9N2 genome from A influenza virus has caused prevalence of the disease in birds and has led very much mortality in Korea and China; from 2001-2002, H9N2 viruses were isolated extensively from the meat and marrow of imported chickens from China in quarantine center of Yokohama, Japan. In March 1999, two case of influenza virus isolation from 1-4 years old girls was obtained in Hong Kong who recovered from influenza. In this regard, five H9N2 viral cases were obtained from human subjects in August, 1998.

Pathological aggravation of H9N2 of A virus which was isolated from chickens in China has been proven via coincidental infection by bacteria such as golden staphylococcus and hemophylus para-galinarome (Liu *et al*., 2003). Because of the hazard of prevalence of some pathological genera in human being as a zoonosis, information about functions of influenza viruses in cells area and host cells and about the induction of the mechanisms of in host cells death causes to increase the understanding about pathological process of virus and helps us about suitable attitude to the disease. Influenza virus causes some tissue changes in different body organs and respiratory system is one of those organs. Therefore, one of the aims of the present study has been the evaluation of tissue pathogenesis following H9N2 influenza virus incubation which can be helpful for recognizing some pathogenesis aspects of the disease. With regard to the importance of influenza and increasing prevalence of the disease among domestic animals as well as the risk of some pathogenesis sub-types incidence among human being as a zoonosis, it is needed that the

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pathogenesis of some viral genotypes such as H9N2 is examined in terms of tissue injuries. Today's fundamental studies can pave the way for identifying diseases pathogenesis, so the attempt is to examine the kind and severity of airway injuries of infected chicks by sub-type H9N2 influenza virus, experimentally; so economic and life-threatened losses to mankind will be prevented by recognizing occurred damages and presenting some preventive ways as well as suitable treatments.

MATERIALS AND METHODS

In the present study, the sub-type H9N2 of avian influenza virus (obtained from Razi Serum Producing and Research Center) which was cloned two times in embryonic eggs was inoculated to the SPF (Valo, Lohman, Germany) at their 3rd week by nasal drops: At first, SPF chickens were divided to 2 groups of 10 subjects; a group as a treatment group and another one as a control group. Then treatment group was infected by H9N2 sub-type of influenza virus with dosage of $10^{7.5}$ EID50. The control group obtained normal saline serum nasally equal to the inoculated viral solution volume. After 3 days of inoculation, understudied chickens (Treatment and control group) were autopsied and their trachea and lung were sampled. In order to obtain tissue pathological sections, the samples were sent to the Pathological Laboratory of Tabriz, Veterinary Science University in 10% formalin. Under studied samples were dried, transparent, smeared with paraffin and molded followed by cutting in 5-6 micron thicknesses and staining by hematoxilin and eozine.

Data statistical analysis: In order to analysis the damage occurred in chicks' airway following challenge with H9N2 influenza virus, the severity of observed pathologic cases like hyperemia, edema, inflammatory cells' infiltration, hemorrhage, necrosis, apoptosis, degeneration of respiratory cilia, formation of cartilaginous nodules and separation of respiratory epithelial tissue were graded in Table 1. In order to evaluate the relationship between occurred changes in lung tissue and H9N2 influenza virus, SPSS software and one-way Multi-variable Variance Analyses test (MANOVA) was used.

Table 1: Grading of the severity of various pathologic cases observed following experimental infection by H9N2 influenza virus

Damage severity grading	Pathologic cases: Hyperemia	Edema	Inflammatory cells	Hemorrhage	Necrosis	Apoptosis	Loss of cilia	Cartilaginous nodules	Separation of epithelial tissue
1	Lack of hyperemia	Lack of edema	Lack of inflammatory cells	Lack of hemorrhage	Lack of necrosis	Lack of apoptosis	Lack of cilia Loss	Lack of cartilaginous nodules	Lack of epithelial tissue separation
2	Hyperemia at most to 25% of observed capillaries in 10 microscopic fields	Edema at most to 25% of observed inter-tissue space in 10 microscopic fields	Inflammatory cells at most to 25% of observed surface in 10 microscopic fields	Hemorrhage at most to 25% of observed surface in 10 microscopic fields	Necrosis at most to 25% of observed cells in 10 microscopic fields	Apoptosis at most to 25% of observed cells in 10 microscopic fields	Loss of cilia at most to 25% of observed epithelial tissue in 10 microscopic fields	Cartilaginous nodules at most in 3 of 12 microscopic fields	Separation of epithelial tissue at most to 25% of epithelial cells in 10 microscopic fields
3	Hyperemia in 25 to at most 50% of observed capillaries in 10 microscopic fields	Edema in 25 to at most 50% of observed inter-tissue space in 10 microscopic fields	Inflammatory cells in 25 to at most 50% of observed surface in 10 microscopic fields	Hemorrhage in 25 to at most 50% of observed surface in 10 microscopic fields	Necrosis in 25 to at most 50% of observed cells in 10 microscopic fields	Apoptosis in 25 to at most 50% of observed cells in 10 microscopic fields	Loss of cilia in 25 to at most 50% of observed epithelial tissue in 10 microscopic fields	Cartilaginous nodules at most in 3 to at most 6 of 12 microscopic fields	Separation of epithelial tissue in 25 to at most 50% of epithelial cells in 10 microscopic fields
4	Hyperemia in 50-75% of observed capillaries in 10 microscopic fields	Edema in 50-75% of observed inter-tissue space in 10 microscopic fields	Inflammatory cells in 50-75% observed surface in 10 microscopic fields	Hemorrhage in 50-75% of observed surface in 10 microscopic fields	Necrosis in 50-75% of observed cells in 10 microscopic fields	Apoptosis in 50-75% of observed cells in 10 microscopic fields	Loss of cilia in 50-75% of observed epithelial tissue in 10 microscopic fields	Cartilaginous nodules in 6 to at most 9 of 12 microscopic fields	Separation of epithelial tissue in 50-75% of epithelial cells in 10 microscopic fields
5	Hyperemia in 75-100% of observed capillaries in 10 microscopic fields	Edema in 75-100% of observed inter-tissue space in 10 microscopic fields	Inflammatory cells in 75-100% observed surface in 10 microscopic fields	Hemorrhage in 75-100% of observed surface in 10 microscopic fields	Necrosis in 75-100% of observed cells in 10 microscopic fields	Apoptosis in 75-100% of observed cells in 10 microscopic fields	Loss of cilia in 75-100% of observed epithelial tissue in 10 microscopic fields	Cartilaginous nodules in 9 to at most 12 of 12 microscopic fields	Separation of epithelial tissue in 75 to 100% of epithelial cells in 10 microscopic fields

RESULTS AND DISCUSSION

In histopathological studies of lung and pleura of treated chicks with H9N2 sub-type influenza virus, propagated pneumonia which accompanies hyperemia and sever hemorrhage was observed in to allele and pleurisy sacs (Fig. 1). In microscopic view, there is sever hyperemia and hemorrhage in allelic sacs, edama, infiltration of fibrin and inflammatory cells, especially heterophylus in pulmonary' tissue inter-space (Fig. 2) in microscopic studies, the formation of cartilaginous nodules in various parts of lung parenchyma was observable as separated and scattered points (Fig. 3). In microscopic observations, hemorrhage in para-bronchial space and cell death, degeneration of epithelial cells as well as deciliation were observable clearly. Also, muscular fibers' necrosis under epithelial tissue were attracted the attention (Fig. 4-6). In histopathological studies of pulmonary paranchyma's vessels, besides sever hyperemia plenty of inflammatory cells were observed in intravas cular space. Endothelial damages were thoroughly apparent in the form of acute swelling of cells which has led to ballooning degeneration (Fig. 7). Also, aggregation of inflammatory cells around damaged blood vessels of pulmonary

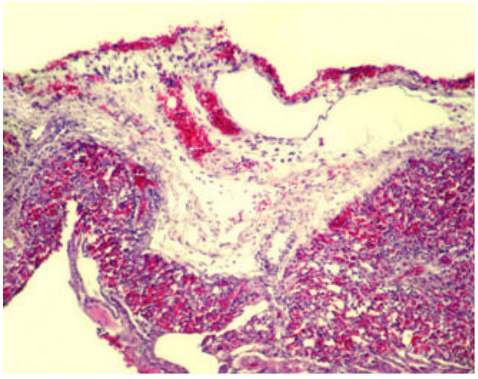

Fig. 1: Microscopic view of lung and pleura of treated chicks with H9N2 sub-type influenza virus, propagated pneumonia which accompanies hyperemia and sever hemorrhage are observed in to allelic sacs: Infiltration of inflammatory cells which is accompanied by hemorrhage, edema and fibrin deposition has caused to increase the thickness of pleura (H&E& x120)

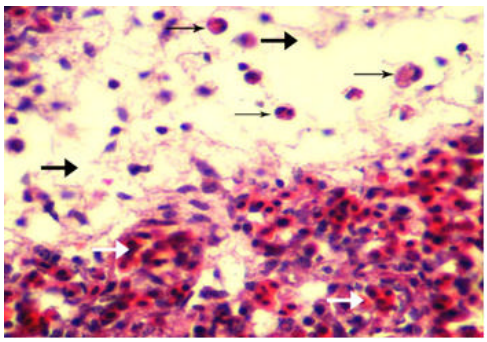

Fig. 2: Magnification of a part of H9N2 sub-type influenza virus treated chicks' lung: There is sever hyperemia and hemorrhage in allelic sacs (light arrows), edama, infiltration of fibrin (dark arrows) and inflammatory cells, especially heterophylus (thin arrows) in pulmonary tissue inter-space (H&E& x400)

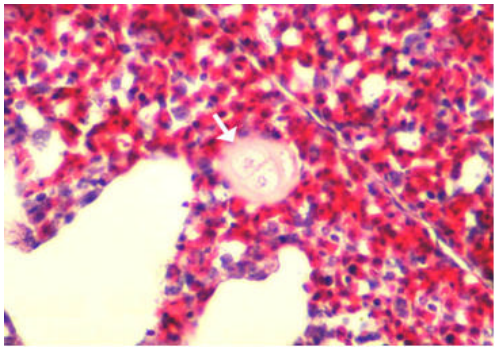

Fig. 3: Microscopic view with further magnifying of a part of H9N2 sub-type influenza virus treated chicks' lung: The formation of cartilaginous nodules (arrow) in various parts of lung parenchyma is observable (H&E& x400)

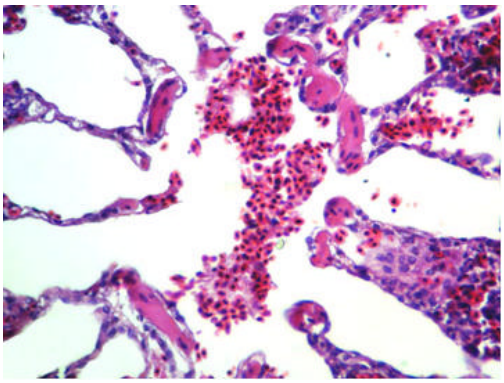

Fig. 4: Microscopic view of para-bronchitis of H9N2 sub-type influenza virus treated chicks' lung: Hemorrhage in para-bronchial space and cell death, degeneration of epithelial cells; muscular fibers' necrosis under epithelial tissue is observable clearly (H&E& x250)

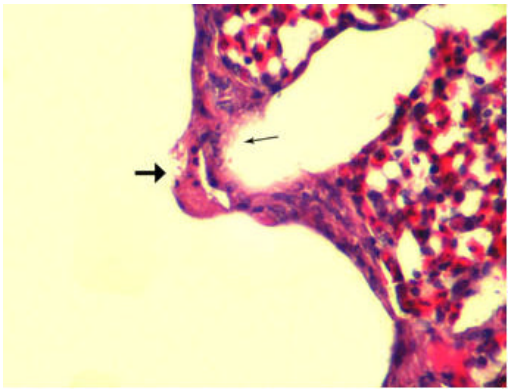

Fig. 5: Microscopic view with further magnification of para-bronchitis wall of H9N2 sub-type influenza virus treated chicks' lung: Damaged epithelial tissue in allelic sac (thin arrow) as well as changes caused by necrosis of muscular fibers (thick arrow) under the para-bronchitis epithelial tissue (H&E& x400)

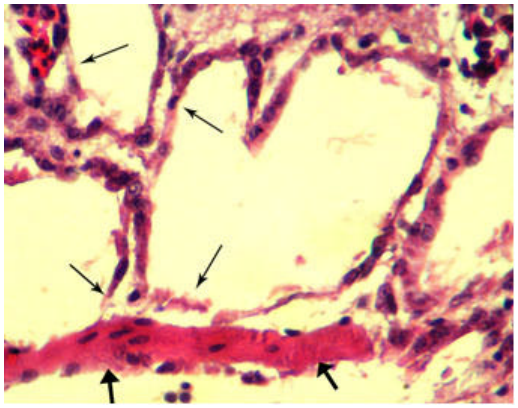

Fig. 6: Microscopic view with further magnification of para-bronchitis wall of H9N2 sub-type influenza virus treated chicks' lung: The changes caused by epithelial tissue's cell death of allelic sacs (thin arrows) and cell death of muscular fibers (thick arrows) under the para-bronchitis epithelial tissue an eosinophylic increase of cytoplasm are clear completely (H&E& x400)

parenchyma which was accompanies edema and sever infiltration of fibrin was significant (Fig. 8). In microscopic point of view, sever bronchitis was significant in the form of inflammatory cells' infiltration in bronchitis epithelium, expanded deciliation on epithelial tissue along with sever hemorrhage and degeneration of epithelium following epithelial cells death. Accumulation of damaged tissue

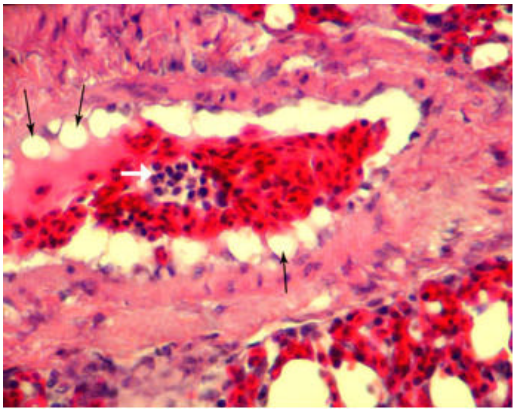

Fig. 7: Sectional microscopic view of a pulmonary paranchyma's blood vessel of H9N2 sub-type influenza virus treated chicks: Sever hyperemia as well as inflammatory cells presence (light arrow) was observed in intravascular space; endothelial damages (thin arrow) in the form of acute cell swelling which has led to ballooning degeneration were observed completely (H&E& x400)

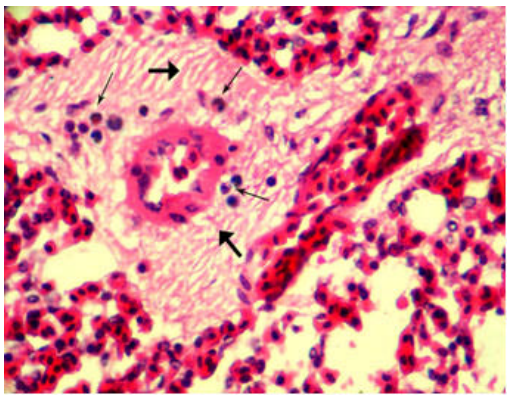

Fig. 8: Microscopic view of a part of H9N2 sub-type influenza virus treated chicks' lung: There is edema and permeation of fibrin (thick arrows) and accumulation of inflammatory cells (thin arrows) in one of damages vessels of pulmonary's parenchyma are clear (H&E& x400)

debris accompanied with inflammatory cells and red blood cells were clear in intra-bronchial space (Fig. 9-11). Edema, hyperemia, hemorrhage, sever infiltration of inflammatory cells and vast mucosal damage were observed in tissue histopathological study of chicks' trachea treated with H9N2 sub-type influenza virus (Fig. 12). Sever hemorrhage and the mass presence of separated cells as well as debris of damaged tracheal tissue was clear in tracheal space (Fig. 13).

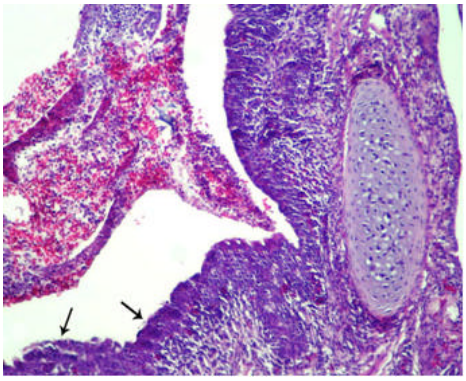

Fig. 9: Microscopic view of inside and part of mucous of primary bronchitis wall of H9N2 sub-type influenza virus treated chicks: Sever bronchitis in the form of inflammatory cell infiltration in bronchitis mucous, vast deciliation (arrows) on epithelial tissue accompanied with hemorrhage and mucosal degeneration as well as tissue, inflammatory cell and red blood cell debris inside of bronchitis are clear (H&E& x400)

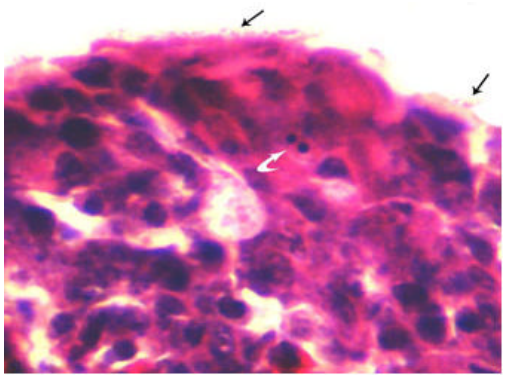

Fig. 10: Microscopic view with further magnification of superficial parts of primary bronchitis of H9N2 sub-type influenza virus treated chicks: Deciliation (thin arrow) on epithelial tissue and apoptotic cells (bent arrow) with symbolic characteristics of accumulated apoptosis and fragmentation of chromatin are clear (H&E& x1200)

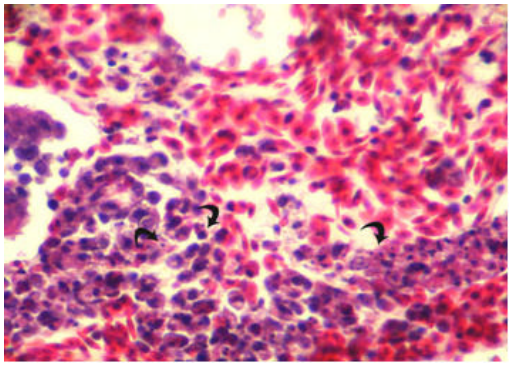

Fig. 11: Further magnification of superficial parts of epithelial cell debris and hemorrhage in inside the primary bronchitis space of H9N2 sub-type influenza virus treated chicks: The presence of red blood cells as a result of hemorrhage, separated necrotic cells of bronchitis epithelial tissue as well as the presence of apoptotic cells (bent arrow) inter-debris in bronchitis are clear completely (H&E& x600)

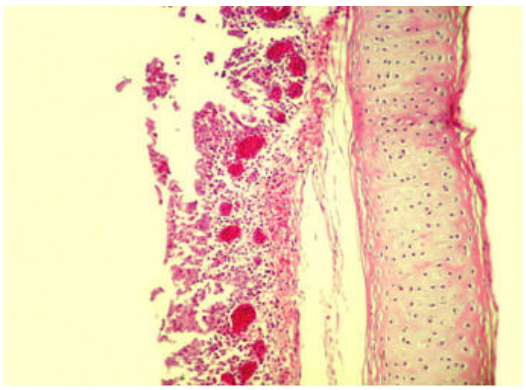

Fig. 12: Microscopic view of a part of H9N2 sub-type influenza virus treated chicks' tracheal wall: Edema, hyperemia, hemorrhage, sever infiltration of inflammatory cells and vast degeneration of tracheal mucous are observable (H&E& x120)

The results of data statistical analysis: Based on multi-variable one-way variance analysis, in general, there is a significant relationship between the histopathological changes occurred in pulmonary tissue and H9N2 influenza virus ($p<0.05$). Based on conducted complementary Analysis by one-way Analysis of Variance (ANOVA), there was a significant difference about each of pathologic cases between control and treatment groups ($p<0.05$) but there was no significant difference between treatment and control groups about cartilaginous nodules (Fig. 14).

The occurrence of sever vascular and cell changes in lung has been one of the important observed cases following experimental infection by H9N2 influenza virus such that the resulted problems were observable as edema, hyperemia, necrosis, apoptosis as well as

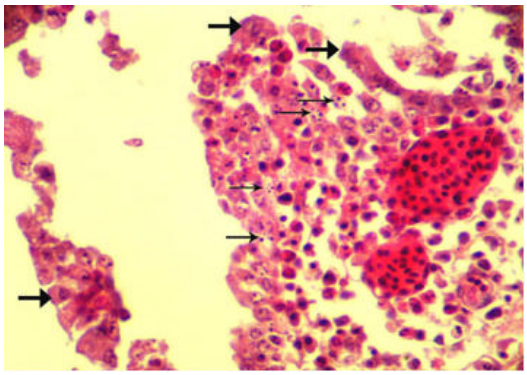

Fig. 13: Further magnification of sever degeneration of mucous and its inflow to the tracheal space of H9N2 sub-type influenza virus treated chicks: Sever hemorrhage and mass presence of separated and collapsed cells of damaged tracheal mucous which have been necrotic (thick narrow) and/or apoptotic (thic arrows) are clear (H&E& x400)

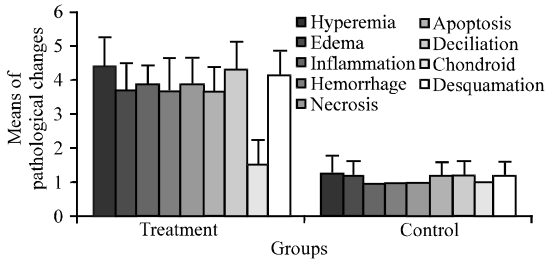

Fig. 14: Relationship between the histopathological changes occurred in pulmonary tissue and H9N2 influenza virus

infiltration of monocytes and heterophyls. Based on studies conducted by Brydon *et al.* (2005), it was clear that inflammatory cells inter the upper respiratory tract for overcoming to infection following infection by influenza virus; they cause to some vascular and cellular changes by releasing cytokines and inflammatory mediators in which mycrofages and monocytes are the main source of biochemical compounds. So, they cause to increase the activity of monocytes and heterophyles by producing and releasing alpha tumor necrotic factor cytokines, interleukin-1 and comokins. The presence of cytokines like alpha tumor necrotic factor and interleukin-1 lead to expression of binding molecules depended to excretion of monocytes and heterophyles, in one hand and induction of vascular changes by inflammatory mediators in other hand. The results obtained by Barber (2001), Suarez and Schultz-Cherry (2000) and Brydon *et al.* (2005) are conformed to the results of the present study from the vascular and cellular changes studies point of view; so, it can be said that vascular and cellular changes occur following infection by H9N2 sub-type influenza virus because of interactive between cytokines and inflammatory mediators in which the virus and dependant anti-genes have been the primary stimulant factors. Hemorrhage in various organs especially in pulmonary tissue is another questionable case in the present study. Endothelial cells' degenerative changes and sometimes their complete remove were observed that the main reason for these kinds of changes can be due to virus tendency to endothelial cells and induction of cytopathic effects in the cells which are observed as necrosis and apoptosis patterns; for this reason, vast hemorrhage was observed in the examination of affected chicks' pulmonary parenchyma. Ito *et al.* (2002), Schultz *et al.* (2002) and Frankfurt and Krishan (2001) conducted a study about the effect of influenza virus on liver and kidneys' vascular endothelial cells of affected chicks following experimental infection by H7N7, H5N1 and H5N3 sub-types influenza virus. They pointed out the role of viral proteins like NS^2 in apoptosis induction in endothelial and other tissue cells and obtained significant results in this relation. The focus on endothelial cell death and the role of the virus in inducing cell death following hemorrhage of pulmonary tissue asks the discussion about the reasons and accountings of cell death; since, epithelial cells, besides endothelial cells, of trachea and lung have had these changes.

Expanded studies about this issue have been conducted by Hinshaw *et al.* (1994), Ito *et al.* (2002) and Schultz *et al.* (2002); the results have been illustrative of the inducing effects of influenza virus on occurrence of apoptosis. They expressed that the stimulation of kinas protein receptors including tyrosine kinas, treonine kinas, mytogen kinas and secretion of secondary messengers as positive factor in interpreting some of especial genes cause to activate series of systeinic proteinase (Kaspasic enzyms); the enzymes have an important role in start and execution of apoptotic changes. According their idea, Wurzer *et al.* (2004) and Ravi *et al.* (2001) expressed that probably, the virus and kaspase-8 would cause phosphatase cascade of ELF2, activation of nuclear factor and NF-kB of immunoglobulin k locus in B cells as well as induction of apoptotic genes (like *FAS*, *P53* and *BAX*) transcription which cause ultimately to activate of kaspase-9 and the occurrence of apoptosis in respiratory epithelial cells. It is to be noted that dependant factors on virus also have effective role in cell death induction such that the studies of Morris *et al.* (2005) demonstrate that viral Noraminidase NA in MDCK culturing media leads to

form mitochondrial membrane channels and release of cytocrome C and as well as activation of kaspase-9 via creating oxidative reactions and activation of ROS channel due to excessive expression of HA, NA and NP. All of expressed mechanisms by Morris *et al.* (2005) in relation to sever apoptosis changes of respiratory epithelium make simpler and comprehensible the expression and justification of viral cytopathic changes in the present study. But tracheal epithelium necrosis, pulmonary alleles and vascular endothelium were significant in viral infection which was probably due to release of cytokines and various enzymes. Edvard *et al.* (2003) and Uiprasertkul *et al.* (2007) conducted studies in this line and expressed the role of cytokines like alpha tumor necrotic factor and released inflammatory mediators following infection by H1N1, H3N2 and H5N1 sub-types influenza virus. This is the issue that has been expressed in the results of the present study and demonstrates the inductive role of H9N2 in respiratory epithelium necrosis. Their significant results and the present study ($p<0.05$) demonstrate the inductive role of mentioned sub-types in cell death and tissue damages (Edvard *et al.*, 2003; Uiprasertkul *et al.*, 2007). Various nodules dependant on cartilage or cartilaginous nodules were observable in microscopic studies of the present study that there was no significant results in comparing between two, control and treatment groups but justification of their variant presence in some of tissue sections is significant. Sarango and Riddell (1985) have expressed various ideas about this issue and presumed its reason the chondrocytes embolies originated from abnormal cartilaginous tissues. They also justified that there is no relationship between the precense of these nodules and pathologic damages (Sarango and Riddell, 1985; Suarez and Schultz-Cherry, 2000).

Clear and focal removing of ciliated epithelium was another finding of the present study. Catherine in 2006 have conducted a study on the effect of H3N2 sub-type influenza virus; they have concluded that the base of respiratory epithelium ciliated cells in human being and birds is due to the presence of α-2-6-acid sialic which is as a receptor of virus for connecting and entering in to cell. They also could trace the virus in II type pneumocytes of pulmonary tissue and confirm its presence in the cells. In the present study, the ciliated respiratory epithelium degeneration and pneumocytes damage were observable that some comparisons were conducted with the results of Catherine studies and it was clear that H9N2 sub-type influenza virus affects on ciliated cells and pneumocytes. Brydon *et al.* (2005) have expressed that the occurrence of apoptosis in pulmonary broncheals epithelium cells in infection by influenza virus can be depend on CCL5 accompanied by phosphoralation of PKR dependant on eIF2. But it must be noted that the production of CCL5 in respiratory epithelium cells is in relation to the role of P38 and mitogene activated protein kinase and may be ROS^4 acts as a econdary messenger (Brydon *et al.*, 2005).

CONCLUSION

In the present study, the apoptosis of primary bronchial cells was observable in some cases which demonstrate significant changes compared with control group. Probably, the expression of apoptosis induction channel which has been discussed in Brydon *et al.* (2005) studies is a suitable justification for these changes. So, it must be noted that there are various channels in occurrence of cell death following infection by influenza virus and knowing these channels needs extensive studies.

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