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Experimental Study of Renal Tubular Cells Apoptosis Subsequent to Infectious by Influenza Virus (H9N2) in SPF Chickens

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Abstract: Influenza virus produces cell death in animals and human. Since cell death can be caused by either necrosis or apoptosis. We investigated the types of cell death that occur in chickens infected with avian influenza virus, A/chicken/Iran/772/2000(H9N2). In experimental study 60 SPF chickens at 3 weeks old were divided to two groups. The first group was infected with $10^{7.5}$ EID50 titer of the virus intranasaly and the second group was treated with saline normal. Following 72 hrs, renal tissues were collected and fixed in 10% formalin solution. The prepared microscopic sections with the thickness of 5-6 micron were stained using TUNEL method. In comparison to the control group, there were significant mean difference of apoptotic cells in renal tubular cells of the infected group ($p<0.005$). We demonstrated that A/chicken/Iran/772/2000 (H9N2) is able to induce apoptosis in renal tubular cells.

Key words: Avian influenza, apoptosis, renal apoptosis

INTRODUCTION

Influenza is a viral disease which has been known in 1901A.D. in 1955 a kind of Influenza virus was known as a factor caused disease that later on called fowl 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 some times has been known as a pandemic disease among human being. Fowl 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 to 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 one to four year 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 (Barber, 2001).

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 hemaphilus paragalinarum (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 our understanding about pathological process of virus and helps us about suitable attitude to the disease. Being aware that eukaryotic cell death can be resulted from necrosis or apoptosis and necrosis has important role in pathological responses such as impairing viral disease, recent studies have demonstrated that many viruses such as influenza causes to apoptosis or programmed death of host cells (Brydon *et al*., 2005; Ito *et al*., 2002; Morris *et al*., 2005). Influenza virus causes some tissue changes in different body organs and kidney is one of those organs; examination of cell death changes following incubation of H9N2 serotype virus is one of the aims of the research. Influenza virus has numerous genotypes and the examination of cell apoptosis in SPF chickens' renal tissue infected by H9N2 serotype is the aim of the present study which can be very useful in Influenza disease pathogenesis studies. With regard to the importance of Influenza and increasing prevalence of the disease among human and domestic animals it is needed that the pathogenesis of some viral genotypes such as H9N2 are examined in terms of cell injuries. To days fundamental studies can pave the way for identifying diseases pathogenesis so our attempt is to suggest effective treatment methods to the clinical experts by experimental studies and examining the ways of cell injuries.

MATERIALS AND METHODS

In this experimental study the bird Influenza virus namely H9N2 which was cloned two times in embryonic eggs was intra venous inoculated to the SPF (Valo, Lohman, Germany) at their third week: at first, SPF chickens were divided to two groups of 30 subjects; a group as a

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treatment group and another one as a control group. Then treatment group was infected by H9N2 Influenza virus with I.V inoculation dosage of $10^{7.5}$ EID50. The control group obtained normal saline serum nasally equal to the inoculated viral solution volume. After three days of inoculation, understudied chickens (treatment and control group) were autopsied and their kidneys were sampled. In order to obtaining 5-6 micron sections the samples were sent in 10% formalin to the pathological laboratory of Tabriz veterinary science university. Under studied samples were dried, transparent, smeared with paraffin and molded followed by cutting in 5-6 micron thicknesses and staining by TUNEL method. The method of the present study is experimental and statistical analysis using SPSS software version 13 and T test and considered variable in the study was quantitative changes of programmed cell death or apoptosis.

Executive method of TUNEL technique

Executing of TUNEL technique:

1. The sections are cleaned out of paraffin, they are rinsed followed by closing to the K proteinase, then incubated for 30 min in 37°C, consequently rinsed with phosphate buffer solution.
2. The tissue sections are Closed to 50 micro liter reactive solution of TUNEL for 60 min in 37°C and rinsed with phosphate buffer solution.
3. The tissue sections are incubated with 50 micro liter POD converter solutions then rinsed with phosphate buffer solution for 30 min in 37°C; afterwards is closed to tetra chloride di-amino Benzedrine solution and re-incubated for 20 min in 25°C.
4. The sections were rinsed with phosphate buffer and stained with toloidine blue (Barber, 2001; Ito *et al*., 2002).

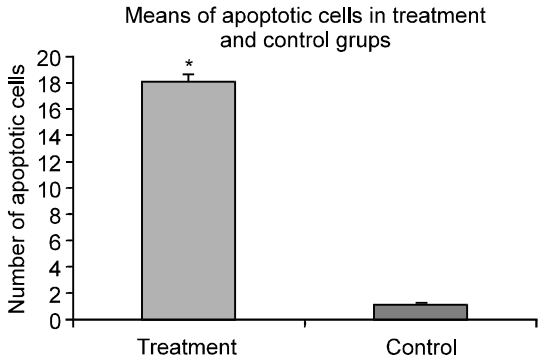
RESULTS

After obtaining microscopic section from SPF chickens' renal tissue of treatment group (T) and control group (C), they were examined in the regard of apoptotic cell existence. For this purpose, the numbers of apoptotic cells were counted in 5 microscopic fields with x 40 magnification rates and provided at Table 1. Also, statistical analysis and obtained diagram are provided in table and diagram with regard to Mean±SD = 18±1.79 and Mean±SD = 1±0.56 which have been obtained by T test for treatment and control groups, respectively; these data demonstrate that always there is significant difference ($p<0.005$) in apoptotic cells numbers between treatment and control groups.

Qualitative results of apoptosis changes in treatment and control groups' renal tissue: In this part of study, the renal tissue images obtained by optical microscope from treatment and control groups have been provided. As revealed in these pictures, numerous forms of positive TUNEL apoptotic cells in treatment group renal tissue sections are observable. Apoptotic cells is recognizable in microscopic observation by nucleolus fragmentation which are observed from light to dark brown in nucleolus TUNEL staining and fragmented sections (Fig. 2 and 3). In microscopic view the numbers of renal tubules apoptotic cells in treatment group are more compared with control group.

Table 1: Microscopic quantitative results of renal tissue in control and treatments groups

Group	Mean	Standard deviation	Standard errors
Treatment	18.1	18.1±1.790	18.1±0.566
Control	1.1	1.1±0.567	1.1±0.179



Diag. 1: Mean of apoptotic cells in treatment and control groups of five microscopic fields by TUNEL method obtained from SPF chickens' renal tissue (n = 30) received disease virus (treatment group) and normal saline (control group)

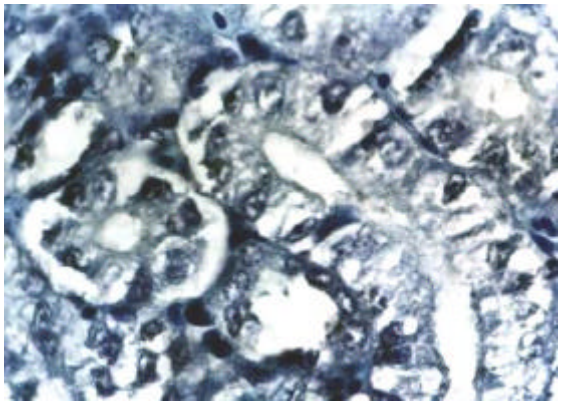


Fig. 1: Sectional microscopic view of control group's kidney in which apoptosis changes are not observable (TUNEL staining with x100 magnification)

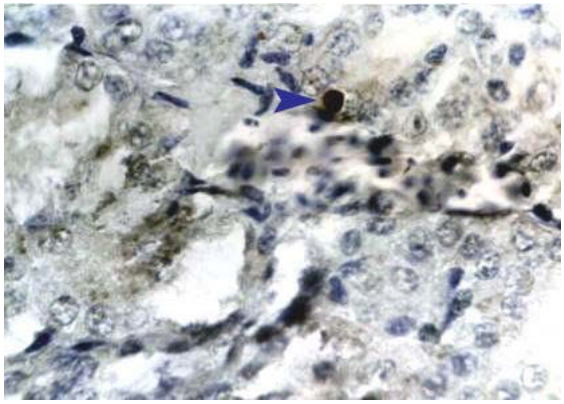


Fig. 2: Sectional microscopic view of treatment group's kidney in which numerous forms of apoptosis cells with positive TUNEL (arrow) are observable (TUNEL staining with x100 magnification)

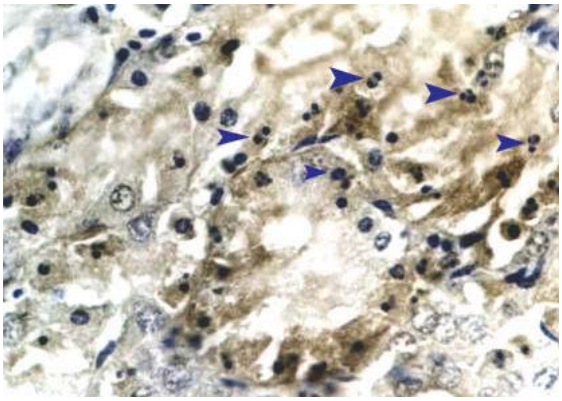


Fig. 3: Sectional microscopic view of control group's kidney in which numerous apoptosis changes in tubular cells (arrow) are observable (TUNEL staining with x100 magnification)

DISCUSSION

Apoptosis or programmed cell death is in fact a set of predefined cellular events that accomplishes the damage of cells and their contents with full effectiveness. The main definition of apoptosis expresses that this kind of cell damage doesn't cause to inflammation but recently this hypothesis has been under reviewing and it is resulted that in some circumstances such as pathogen factor invasion, apoptosis causes to inflammation response induction so increases immunity response. Also, it was noted that apoptosis is excited by one of several ways that depends on primary motivation factor. In many of these ways, there are stimulating of protein kinas phosphates activation receiver as well as secretion of secondary messengers that acts as positive or negative factors in special genes interpretation (Barber, 2001; Hinshaw *et al*., 1994). One important factor in these routes is the activation of a series of cisteinic proteases (Caspase) which are divided in to two important groups: precursor caspase and effective caspase; precursor caspase involves the receivers of cell death as well as affecting other molecules which regulates cytoplasm and operates by photolytic. Theses activated caspase create a caspase cascade which leads to effective caspase. Effective caspase activate proteases and nucleases that cause to morphological change in apoptotic cells. The present study demonstrates the apoptosis process in influenza pathogenic virus and producing renal damages in the disease by quantitative examination and statistical analysis of apoptosis changes (cell death) in renal tubular cells following inoculation of H9N2 genotype of influenza virus (Chai *et al*., 2000; Liu *et al*., 2003; Zambon and Meduiro, 2001); so that there is always a meaningful difference between treatment and control groups considering quantitative changes of apoptotic cells ($p<0.005$). Therefore, our conception is that the reason of this meaningful difference can be arising from some mechanisms and involving factors in apoptosis induced by H9N2 genotype of influenza virus in renal tubular cells. Based on studies conducted by Ravi and Wrzar it is likely that apoptosis induced by the virus accomplished by activating of caspase 3 that the stages of activating of the enzyme is done by activating of NF-KB factor arising from inducing of TRAIL and FAS/FASL and inducing of pro-apoptotic genes transcription such as P53 and BAX that causes to induce the activation of caspase 9 by this way. The activation of caspase 9 has a relationship with viral proteins like NA and creating oxidative stress specially increasing the expression of the pathogenic proteins like HA, NA and NP; all of which cause to destroy the mitochondrial membrane and C cytochrome release. All of these interactions lead to activate caspase 9 enzyme which is as an important executive enzyme in inducing of apoptosis. They stated that nonstructural protein NS1 causes to start apoptotic passage and some meaningful results was obtained that the present results consistent with them (Chai *et al*., 2000; Keogh *et al*., 2000; Ravi *et al*., 2001; Schultz Cherry *et al*., 2003). Influenza virus can induce apoptosis by activating of apoptosis endogenous passages, i.e. by expressing BAX protein and controlling of BCL2 and forming of mitochondrial passages and discharging of C cytochrome, but we mustn't forget the role of cytokine that can induce apoptosis following the viral activation in infected cell and starting inflammatory processes in the presence of some cytokines such TNF.α and FAS/FASL passage. The studies of Ito and Suarez about immunological examination and cytokine relations have had meaningful results that the expression of involving mechanisms in apoptosis of renal tubular cells following influenza virus inoculation using their results

can explain our findings about cell death in renal tissue (Frankfurt and Krishan, 2001; Ito *et al*., 2002; Suarez and Schultz-Cherry, 2000; Wurzer *et al*., 2004). Schultus, Zambou and Morris conducted some studies and explained that H9N2 genotype influenza virus is likely cause to activate cell death passages by delayed activating of TGF-β via NA; they found a clear association between influenza virus and apoptosis by expressing endogenous mechanisms of apoptosis that we can note the effective role of these cases in expressing of inducing passages of tubular cells apoptosis in chickens and some meaningful differences (Schultz *et al*., 2002; Schultz Cherry *et al*., 2003; Schultz *et al*., 2003; Zambon and Meduiro, 2001). Numerous mechanisms have been proposed in other scholars' studies that each of which can be significant in apoptosis mechanisms following influenza virus invasion. In the current study it was found that H9N2 genotype influenza virus is able to apoptosis induction in tubular cells and causing renal damages. This case shows that H9N2 genotype influenza virus can likely be explained as a pathogen in human being that the examination and recognition of different aspects of pathogenesis of the virus in future studies help us in suitable attitude to influenza disease.

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