UMA INVESTIGAÇÃO SOBRE O EFEITO DO VÍRUS INFLUENZA H9N2 NA APOPTOSE DE CÉLULAS TESTICULARES EM EMBRIÃO DE GALINHA USANDO SYBR VERDE PCR EM TEMPO-REAL**AN INVESTIGATION INTO THE EFFECT OF H9N2 INFLUENZA VIRUS ON APOPTOSIS OF TESTICULAR CELLS IN CHICKEN EMBRYO USING SYBR-GREEN REAL-TIME PCR**

بررسی تاثیر ویروس آنفلوآنزای اچ 9 ان 2 به روی آپوپتوز سلولهای بافت بیضه ی جنین جوجه ی مرغ با استفاده از تکنیک مولکولی ریل تایم پی سی آر- سایبرگرین

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RESUMO

Os vírus da gripe podem induzir a morte celular em seu hospedeiro por apoptose ou necrose. O H9N2 é um subtipo do vírus da gripe aviária (AIV) que pode causar graves danos aos órgãos reprodutivos das galinhas poedeiras. O presente estudo teve como objetivo investigar o efeito do vírus influenza H9N2 na apoptose de células testiculares em embriões de galinha. Para este fim, A/Chicken/Tehran/ZMT-101/99(H9N2) foi inoculado em 210 ovos embrionados com quinze dias de idade postos por galinhas SPF. Em seguida, de acordo com o desenho do experimento, os embriões vivos foram dissecados no 19º e no 21º dia de vida fetal e no 25º dia após o nascimento para estudos patológicos e moleculares e avaliação da expressão gênica no tecido testicular. Os tecidos dissecados foram corados com hematoxilina e eosina (H&E) e estudados sob um microscópio óptico com aumento de 400x. Para estudos moleculares, o RNA viral foi extraído dos tecidos testiculares, replicado por RT-PCR e, finalmente, avaliado o H9N2. Em seguida, a expressão dos genes envolvidos na apoptose das células do tecido testicular foi avaliada por PCR em tempo-real. Estudos patológicos indicaram que o H9N2 causou lesões na orquite do tecido testicular, seminífero e nefrite do hospedeiro. Estudos moleculares também mostraram que a replicação de H9N2 no corpo hospedeiro aumenta a expressão de BAX e Caspase 3 e reduz a expressão de Bcl-2 e Bcl-X_L. Essas mudanças na expressão gênica aumentaram a apoptose e induziram a morte celular no hospedeiro. Em resumo, os resultados do estudo sugeriram que o H9N2 pode aumentar a expressão de genes pró-apoptóticos e reduzir a expressão de genes anti-apoptóticos, resultando em destruição severa do tecido testicular causado pela apoptose celular.

Palavras-chave: Apoptose, H9N2, Vírus da gripe A, PCR verde em tempo real SYBR, Tecido testicular.

ABSTRACT

Influenza viruses can induce cell death in their host through apoptosis or necrosis. The H9N2 is a subtype of the Avian Influenza Virus (AIV) that can cause severe damage to reproductive organs of laying hens. The present study aimed to investigate the effect of the H9N2 influenza virus on apoptosis of testicular cells in chicken embryos. To this end, A/Chicken/Tehran/ZMT-101/99(H9N2) was inoculated to 210 embryonated fifteen-day-old

eggs laid by SPF hens. Then, according to the experiment design, live embryos were dissected on the 19th and 21st days of fetal life and on the 25th day after birth for pathological and molecular studies and evaluation of gene expression in testicular tissue. Dissected tissues were stained with hematoxylin and eosin (H&E) and studied under an optical microscope at 400x magnification. For molecular studies, viral RNA was extracted from testicular tissues, replicated by RT-PCR, and finally evaluated H9N2. Then, the genes expression involved in the testicular tissue cells apoptosis was evaluated through real-time PCR. Pathological studies indicated that H9N2 caused lesions in testicular tissue orchitis, seminiferous, and nephritis of the host. Molecular studies also showed that H9N2 replication in the host body increases BAX and Caspase 3 expression and reduces the expression of Bcl-2 and Bcl-X_L. These changes in gene expression increased apoptosis and induced cell death in the host. In summary, the study findings suggested that H9N2 can increase the expression of pro-apoptotic genes and reduce the expression of anti-apoptotic genes, resulting in severe destruction of testicular tissue caused by cell apoptosis.

Keywords: Apoptosis, H9N2, Influenzavirus A, SYBR Green Real-Time PCR, Testicular tissue

چکیده

ویروس های آنفلوانزا می توانند باعث القا مرگ سلولی در میزبان خود از طریق فرایند آپوپتوزیس یا نکروزیس شوند. تحت تیپ اچ 9 ان 2 ویروس آنفلوانزا پرندگان (AIV) می تواند باعث آسیب شدید در اندام های تولید مثلی مرغ های تخم گذار شود. در این مطالعه کوشش شده است که تاثیر ویروس آنفلوانزا تحت تیپ H9N2 به روی آپوپتوز سلول های بیضه جنین جوجه مورد بررسی قرار گیرد. برای این منظور سویه اچ 9 ان 2 به 210 تخم مرغ جنین دار اس پی اف 15 روزه تلقیح شد سپس طبق الگوی طراحی شده آزمایش ، جنین های زنده در روزهای 19 و 21 جنینی و در روز 25 پس از تولد جهت بررسی اثرات پاتولوژیکی و بررسی مولکولی و بیان ژن بافت بیضه تشریح شدند. بافت های تشریح شده با رنگ هماتوکسیلین و انوزین (H&E) رنگ آمیزی شدند و به وسیله میکروسکوپ نوری با بزرگنمایی X400 مشاهده شدند. جهت بررسی مولکولی ، RNA ویروسی از بافت های بیضه استخراج شد و تکثیر ویروس آنفلوانزا به وسیله تکنیک (ار تی - پی سی آر) با استفاده از پرایمر اختصاصی bp808 مورد بررسی قرار گرفت و در انتها بیان ژن هایی که ممکن است در القای فرایند آپوپتوز نقش داشته باشند و باعث تخریب سلول های بافت بیضه شده اند به وسیله تکنیک مولکولی ریل تایم پی سی آر- سایبرگرین مورد ارزیابی قرار گرفت. بررسی های پاتولوژیکی این آزمایش نشان داد که تحت تیپ اچ 9 ان 2 باعث ایجاد ضایعات بافتی در بافت بیضه میزبان می شود و در بررسی های مولکولی مشخص شد که تکثیر ویروس اچ 9 ان 2 در بدن میزبان باعث افزایش بیان ژن های بکس و کاسپاز 3 و کاهش بیان BCL-2 و BCL-X_L می شود که این تغییرات بیان ژنها ، باعث افزایش فرایند آپوپتوز و القا مرگ سلولی میزبان می شود. به طور خلاصه بررسی های حاصل از این مطالعه نشان داد که تحت تیپ اچ 9 ان 2 می تواند باعث افزایش بیان ژن های پروآپوپتوتیک و کاهش بیان ژن های ضد آپوپتوتیک شود و این امر باعث تخریب شدید بافت های بیضه در اثر القا فرایند آپوپتوز سلولی شود.

کلمات کلیدی : آپوپتوزیس ، اچ 9 ان 2 ، بافت بیضه

1. INTRODUCTION

H9N2 is a subtype of Influenza A virus that causes a contagious infectious disease. The virus belongs to the family of Orthomyxoviridae and the class of Low Pathogenic Avian Influenza Virus (LPAIV) (Abolnik, 2017). This virus has become prevalent among domestic birds in most Asian countries since the mid-1940s, as 5-30% of mortalities among birds have been reported to be caused by H9N2 (Abolnik, 2017; Alexander & Capua, 2008). Many studies have indicated that infection with H9N2 causes mild clinical symptoms in the gastrointestinal tract, respiratory system, and genital organs of infected chickens (Pantin-Jackwood *et al.*, 2015). H9N2 can apply specific effects on the immune system of infected birds that result in the immunity termination followed by a secondary infection (Bano, Naeem, & Malik, 2003). Different studies state that the avian influenza virus can greatly affect the host cell processes, including aging, proliferation, and autophagy, and cell apoptosis (Moon *et al.*, 2017; Qi, Zhang, Wang, & Wang, 2016; Yan *et al.*, 2017). Apoptosis may be induced in different types of

hosts by a wide variety of viruses. Type A (e.g., avian, horse, swine, and human) and also Type B of influenza viruses are capable of inducing apoptosis in different cell lines (Hinshaw, Olsen, Dybdahl-Sissoko, & Evans, 1994; Ito, Kobayashi, Morita, Horimoto, & Kawaoka, 2002; Lowy, 2003). Due to the activation of the apoptotic gene (Fas antigen) in the early stages of infection, type A and B influenza underlie the process of apoptosis induction (Ito *et al.*, 2002). Viruses induce apoptosis in two ways as follows: the intrinsic pathway (mitochondrial/cytochrome C) and the extrinsic pathway (receptor death) (Lowy, 2003). These two mechanisms involve receptors stimulation, kinase/phosphate activation, and release of transcriptional regulatory factors. The main regulators of apoptosis include the Bcl-2 genes, tumor suppressor p53, mitochondria, and caspases (Krajewska *et al.*, 2002). The apoptosis process depends on the release of pro-apoptotic proteins from the intermembrane space of mitochondria and thereby the destruction of the mitochondrial outer membrane, controlled by the Bcl-2 gene (Lalier *et al.*, 2007). Studies show that H9N2 induces apoptosis in chicken's macrophage

cells by non-structural protein NS1 through the receptor's death-inducing pathway (Fas or FasL-mediated) (Xing *et al.*, 2008; Xing *et al.*, 2011). Other studies have also shown that H9N2 causes tissue damage and excessive apoptosis in chicken's oviduct. Influenza virus causes different pathological changes in host tissues, including testicular tissue. However, it is not yet clearly specified how H9N2 triggers apoptosis in testicular tissue of the chicken embryo. Therefore, it is necessary to scrutinize the molecular mechanisms of H9N2-induced apoptosis. Hence, the aim of the present study was to investigate the effects of avian influenza virus (H9N2) on the expression of pro-apoptotic and anti-apoptotic genes in testicular tissue of the chicken embryo.

All experiments of this project have been performed with the approval of the Ethics Committee for the Protection of Laboratory Animals of Shiraz Islamic Azad University. (IR.IAUSHIRAZ.8346.95.942)

2. MATERIALS AND METHODS

2.1. Preparation of virus strain

Virus A/chicken/Tehran/ZMT-101/99 (H9N2) is a strain of influenza isolated from infected birds in Iran. The virus was propagated by inoculating 11 day-olds embryonated Specific Pathogenic Free (SPF) chicken eggs for 48h at 35°C. The virus was screened to exclude the presence of the other infected agent such as bacteria and common virus poultry, as previously described. The allantoic fluid was harvested and subsequently titrated according to the Reed and Muench method (OIE, 2012). In this study, H9N2 was provided by Razi Vaccine and Serum Research Institute Iran- South Branch (Shiraz).

2.2. Preparation of specific-pathogen-free (SPF) embryonated eggs

For this study, 210 SPF embryonated eggs were purchased from a company SPF chicken center of Venky, India. These eggs were free from any pathogenicity and even the maternal antibody titer. The eggs were kept in a hatching machine under optimal temperature (35.6 °C) and humidity (65%) for 15 days, and then they were used for AIV inoculation (OIE, 2012).

2.3. EID50

Before performing any test, the EID50 of AIV (H9N2) was determined. For this purpose, ten consecutive dilutions of the virus from 10^{-1} through 10^{-10} were prepared, and 200 mL of each dilution

was injected into five embryonated eggs aged 9-11 days. After five days, the EID50 level was calculated based on REED & MUENCH table (OIE, 2012).

2.4. Experiment design

H9N2 was administered to 180 embryonated fifteen-day-old eggs laid by SPF hens, 30 of which were regarded as the control. In the test group, 100 μ L of the virus (with a dilution of 10^{-4}) was injected into eggs. In addition, 30 embryonated eggs in the control group received 100 μ L of PBS.

After inoculation, the eggs were examined daily to determine whether the embryo was alive and the dead embryos were discarded. On the 19th day of embryonic life, 140 embryos in the test group remained alive, 70 of which were removed from the egg and dissected. In addition, 12 out of 24 alive embryos in the control group were dissected. The eggs with alive embryo were kept in a hatching machine until the 21st day when the chicks were born. Then testicular tissue of 37 male embryos from the test group and ten male embryos from the control group was sampled for molecular and pathological studies (Table 1). The eggs were hatched on the 21st day, and the chickens were kept under standard temperature and humidity for 25 days. After this period, the chickens of both groups were euthanized, and their testicular tissue was removed. (Ito *et al.*, 2002; xing *et al.*, 2008)

2.5. Pathological examination of embryo and chicken testicle

To study the pathological characteristics of testicular tissue of embryos and chickens after a viral infection, it is necessary to prepare the tissue based on standard methods (Bonfante *et al.*, 2017). To this end, uninfected and infected chicken tissues were cut into 5 μ m blocks and fixed in 10% buffered formalin solution for 48h, routinely processed, and embedded in paraffin wax. In the end, testicular sections were stained with Hematoxylin and Eosin (H&E). Finally, samples were studied under an optical microscope (Bonfante *et al.*, 2017).

2.6. RNA extraction

To perform Real-Time PCR, viral RNA was extracted from testicular tissue (0.19) by using an RNA extraction kit (High pure viral RNA kit, Roche Applied Science Mannheim, Germany) according to the manufacturer's instructions then reverse

transcribed into cDNA. In order to ensure the optimum quality of the extracted RNA and determine its concentration, the absorbance of each sample at 260 and 280 nm was determined using a Picodrop Microliter UV/Vis Spectrophotometer (Sigma-Aldrich Corporation).

2.7. cDNA synthesis

cDNA of the extracted RNA was synthesized using the Ki622 Fermentas kit. To this end, the reaction was incubated at 25°C for 5 minutes and then at 42°C for 60 minutes. Finally, reaction tubes were placed at 70°C for 5 minutes in order to inactivate the RT enzyme

2.8. RT-PCR and H9N2 detect

The definitive presence of H9N2 in tissue samples was confirmed using RT-PCR (Reverse Transcriptase Polymerase Chain Reaction). For this purpose, using a primer that produces 808 bp after the RT-PCR process if there is H9N2.

2.9. Evaluation of apoptotic genes expression using SYBR-Green real-time PCR

The expression of desired genes was measured through SYBR-Green real-time PCR. To this end, all primers were designed by Allele ID-7.8. In this reaction, beta-actin was used as the internal control gene. Real-time PCR was performed using ABI Step One Applied Biosystem (USA).

The components of the real-time PCR reaction were prepared and adjusted according to the instructions of the device kit. The final volume of the reaction mix was considered to be 25 µL that contained 12.5 µL of PCR Master Mix, 1 µL of forwarding primer, 1 µL of reverse primer, 30 ng (6 µL) of cDNA, and 4.5 µL of RNase-free water. The PCR program included initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 60 seconds, and extension at 72°C for 30 seconds. The ABI Step One also delivered us the melting curve at the end.

3. RESULTS AND DISCUSSIONS

3.1. Virus EID50

Considering the protocol provided by OIE and the REED & MUENCH calculation table, the EID50 value for H9N2 was obtained $10^{-6} \log_{10}$. None of the embryo and chicken testicular tissue samples in the control group showed significant

microscopic changes.

3.2. Pathological study of embryo and chicken testicular tissue

H&E staining technique was employed for the pathological study of tissue samples. The results showed that replication of H9N2 leads to tissue lesions such as orchitis and seminiferous with the accumulation and infiltration of inflammatory mononuclear cells (Figure 1).

3.3. RT-PCR for the definitive detection of the virus exists in the host tissue

RT-PCR was used to definitively determine the existence of the virus in the testicular tissue of chicken. The formation of specific bands in the range of 808 bp indicated the presence of the virus in testicular tissues (Figure 2). Then, the best annealing temperature for this reaction was determined through the gradient PCR technique at 60°C.

3.4. Determination of the RT-PCR and standard curve efficiency

After confirmation of primers and definitive report of the presence of H9N2 in the host testicular tissue, the DNA concentration for evaluation of genes expression was determined to be 25 ng for each reaction. In all reactions, equal amounts of DNA and internal control gene (beta-actin) were used depending on the desired gene type. Hence, genomic RNA was used as the sample extracted from the H9N2 isolate to determine the test accuracy. The optimal temperature of annealing was adjusted to 60°C, and the optimal concentration of primers was determined to be 1 µL in 12.5 µL of the reaction mix. After 40 cycles of replication, the melting curve (T_m) was obtained 86.3°C for caspase 3, 87.1°C for Bcl-X_L, 83°C for BAX, 85.4°C for Bcl-2, and 78.5°C for beta-actin. Then, the standard curve was plotted in order to determine the line slope and coefficient of determination (R^2) for each gene. According to the line slope and coefficient of determination (R^2), the efficiency of reactions was greater than 90%.

3.5. Expression of apoptotic genes after virus inoculation

The findings showed that injection of the H9N2 virus could significantly increase the expression of BAX and caspase3 genes in embryos and chickens; so that the BAX gene was expressed greater than the control gene by 1094

times in embryos ($p=0.014$) and 1.92 times in chickens ($p=0.007$) and the caspase three gene was expressed greater than the control gene by 3.34 times in embryos ($p<0.001$) and 1.25 times in chickens ($p<0.001$) (Figure 3). The results also indicated that the injection of the virus caused a reduction in the expression of Bcl-2 and Bcl-X_L, representing the increased apoptosis process. Compared to the control gene, the reduction in gene expression was lower by 0.29 times in embryos ($p<0.001$) and 0.52 times in chickens ($p=0.012$) in Bcl-2 and by 0.53 times in embryos ($p<0.001$) and 0.64 times in chickens ($p=0.045$) in Bcl-X_L (Figure 3). As shown in (Figure 3), the inoculation of H9N2 increases the expression of apoptotic genes and reduces the expression of anti-apoptotic genes in embryos and chickens.

3.6. Discussions

Apoptosis is an important physiological mechanism for homeostasis and mitochondrial pathways. The apoptosis is generally induced by two pathways: the mitochondrial pathway and the death receptor pathway (Hinshaw *et al.*, 1994). An important point about the process of cell apoptosis is that the host cells take advantage of apoptosis to limit the activity of the influenza A virus by removing infected cells. However, viruses use apoptosis to maintain their own survival and proliferation in the host (Wurzer *et al.*, 2003). Shahsavandi *et al.* showed that H9N2 induces apoptosis in human cells through the mitochondrial pathway, and NS1 from the avian influenza virus (H9N2) can induce death receptor-mediated apoptosis in macrophage of chicken (Shahsavandi, Ebrahimi, Sadeghi, Mosavi, & Mohammadi, 2013).

The apoptosis process depends on the release of pro-apoptotic proteins from the intermembrane space of mitochondria and, thereby, the destruction of the mitochondrial outer membrane, controlled by Bcl-2 genes (Lalier *et al.*, 2007). The Bcl-2 protein family includes the genes encoding anti-apoptotic proteins, including Bcl-2, Bcl-X_L, Bcl-W, MCL-1, BFL-1, and Bcl-B and the genes encoding pro-apoptotic proteins such as Bid, Bik, Bok, Bad, Bak, and BAX (Krajewska *et al.*, 2002). Pro-apoptotic proteins like BAX located on the mitochondrial outer membrane cause this layer to be permeable to apoptosis activating factors, including cytochrome C (Gross, 2016; Lalier *et al.*, 2007). Another category of pro-apoptotic proteins is activated by extracellular or intracellular access, which is divided into 2 types of activator and sensor, based on the stress. Activators play a role in apoptosis induction (Gross, 2016). Another factor contributing to the

induction of apoptosis is caspases. By cleaving the activators, active caspases cause further mitochondrial permeability, the release of cytochrome C, and finally, the formation of the apoptosome complex (Elmore, 2007). Primary caspases, such as caspase 8 and caspase 9, activates downstream effector caspases, including caspase 3, caspase 6, and caspase 7, that cut a series of cell proteins, and then apoptosis takes place (Iwai, Shiozaki, & Miyazaki, 2013; Krajewska *et al.*, 2002; Xing *et al.*, 2011). Studies have reported that Poly (ADP-ribose) polymerase (PARP) is one of the main cleavage sites of caspase 3 in vivo (Schultz-Cherry, Dybdahl-Sissoko, Neumann, Kawaoka, & Hinshaw, 2001).

The present study aimed to investigate four categories of genes involved in H9N2-induced apoptosis in embryos and chickens of SPF laying hens. The studied genes were selected from two categories including anti-apoptotic proteins encoding genes (Bcl-2 and Bcl-X_L) and pro-apoptotic proteins encoding genes (BAX and caspase 3). In addition, beta-actin was employed as the internal control gene. In this study, H9N2 was inoculated to embryonated fifteen-day-old eggs laid by SPF hens, and testicular tissue of embryos and chickens were sampled at preset intervals in order to investigate pathologic effects and expression of the genes involved in cell apoptosis. It is noteworthy that the expression of genes was measured through SYBR Green real-time PCR. The findings corroborate the results of previous studies regarding the attack of influenza virus to tissues containing specific receptors, including epithelial cells, decreased expression of anti-apoptotic genes, and increased expression of pro-apoptotic genes, resulting in apoptosis and cell death in the host tissue. In a study conducted by Doustar *et al.* (2004), it was shown that the attack of H9N2 to SPF hens leads to defects in lymphoid tissues caused by apoptosis and necrosis in these areas, especially in the spleen tissue (Doustar, 2004). Wang J. also reported that H9N2-induced infection causes severe tissue damage to the oviduct of laying hens as a result of apoptosis and changes in the expression of inflammatory factors (Wang *et al.*, 2015).

Bonfante *et al.* investigated the pathologic effects of the H9N2 influenza virus on the reproductive organs of laying hens. They found that some regions of the oviduct tissue were necrotized and epithelial cells were damaged due to the influenza virus (Bonfante *et al.*, 2017). She was also reported that HPAI viruses could cause necrosis and inflammation in the trachea and lungs (Moon *et al.*, 2017). On the other hand,

Hadipour *et al.* found that the H9N2 influenza virus causes edema, hyperemia, secretion of heterophile antibodies, destruction of cilia, and hyperplasia of epithelial tissue in the trachea (Hadipour, 2010). According to the observations of Swayne *et al.* (2013), H9N2 can result in necrosis, edema, and hypertension of kidney tubules, especially proximal tubules; this was also confirmed by other authors (Swayne & Spackman, 2013). In this study, the effect of the influenza virus on testicular tissue was investigated for the first time, and the results indicated the infiltration of mononuclear inflammatory cells and hyperemia with necrosis in this area.

The findings suggest that the H9N2 influenza virus can cause pathological lesions in the testicles, although its effects were not as severe as those reported in the trachea and lung tissues. Perhaps the H9N2 influenza virus causes fewer lesions in the testicular tissue than respiratory tissue, due to its tissue tropism or tendency. In terms of the role of apoptosis in cell death, Ito *et al.* (2002) stated that apoptosis or necrosis are the main factors involved in cell death of hens infected with influenza A virus. They reported that apoptosis causes cell death in the liver, kidney, and brain of the host, which is corroborated by the occurrence of viral antigens in apoptosis-degraded organs (Ito *et al.*, 2002). Qi *et al.* reported that the NS1 protein of H9N2 could induce apoptosis through the mitochondrial pathway and finally causes the destruction of epithelial cells in the oviduct of hens (Qi *et al.*, 2016). Different tissues of chicken have been sampled for the examination of apoptosis in previous studies.

4. CONCLUSIONS:

The present study was the first one that investigated the effect of apoptosis on testicular tissue in embryo and chickens. Molecular studies and evaluation of gene expression indicated that the expression of anti-apoptotic genes (Bcl-2 and Bcl-X_L) reduced, but the expression of pro-apoptotic genes (BAX and caspase 3) increased. This intensified the process of cell apoptosis in testicular tissue and caused testicular tissue destruction and tissue lesions such as orchitis and seminiferous with the accumulation and infiltration of inflammatory mononuclear cells. This study suggested that H9N2 induces apoptosis in the host body through BAX and caspase3 proteins. In other words, H9N2 induces apoptosis through the mitochondrial pathway by modulating the Bcl-2 proteins encoding gene family and cause tissue

lesions.

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Table 1. Experimental design

Group	Egg type	Antigen type	Antigen dilution	Injection dosage	Embryo age (day)	Embryo total number	Dead embryo number	Number of embryos at 19 days old			Number of embryos at 21 days old		Number of chickens at 25 days old	
Control	SPF	PBS	-	100 µL	15	30	6	Number of dissected embryos	Number of males	Number of remaining embryos	Number	Number of live births	Number	Number of males
								12	8	12	12	10	9	5
Test	SPF	H9N2	10 ⁻⁴	100 µL	15	180	40	Number of dissected embryos	Number of males	Number of remaining embryos	Number	Number of live births	Number	Number of males
								70	27	70	70	58	55	22

Table 2. Primer sequence used to detect H9N2 in RT-PCR

Genes	Primer Sequences	Sizes (bp)
H9 Serotype	Forward: 5'-AGCAAAAGCAGGGGAAGTCC-3'	808
	Reverse: 5'-CCATACCATGGGGCAATTAG-3'	

Table 3. Design of primers used in real-time PCR

Genes	Primer Sequences	Sizes (bp)
Bcl-2	Forward: 5'- TTCCGTGATGGGGTCAACTG-3' Reverse: 5'- CCACAAAGGCATCCCATCCT-3'	187
Bcl-xl	Forward: 5'- GATGCGCGAAAGGTCGGAT-3' Reverse: 5'- CCCGGTTACTGCTGGACATT-3'	141
Caspase 3	Forward: 5'- TGGTATTGAAGCAGACAGTGGA-3' Reverse: 5'- GGAGTAGTAGCCTGGAGCAGTAGA-3'	103
BAX	Forward: 5'- GTGATGGCATGGGACATAGCTC-3' Reverse: 5'- TGGCGTAGACCTTGCGGATAA-3'	90
Beta-Actin	Forward: 5'- TATTGCTGCGCTCGTTGTTG-3' Reverse: 5'- ACCAACCATCACACCCTGAT-3'	127

Figure 1. Lesions caused by H9N2 replication in testicular tissue of embryo and chicken (H&E staining, 400x magnification)

A: Orchitis (infiltration of inflammatory mononuclear cells)

B: Orchitis with seminiferous tubules necrosis.

Figure 2. Electrophoresis gel obtained from RT-PCR

Line 1: Non-infectious tissue of embryo and chicken

Lane 2: Non-infectious testicular tissue of embryo and chicken

Lanes 3 and 4: Testicular tissue of embryo and chicken infected with H9N2

Figure 3. Changes in the expression of genes associated with cell apoptosis in embryos and chickens. White charts belong to the control group, and colored charts represent the increase or decrease in gene expression compared to the control. A, B, C, and D represent the gene expression for the Bax, Bcl-2, Caspase3, and Bcl-xL genes, respectively.