Iranian Journal of Veterinary Science and Technology

Received: 2018-Jul-10
Accepted after revision: 2018-Dec-11
Published online: 2019- Mar- 10

RESEARCH ARTICLE

DOI: 10.22067/veterinary.v2i10.74060

Evaluation of the antiviral effects of aqueous extracts of red and yellow onions (*Allium Cepa*) against avian influenza virus subtype H9N2

Saba Ahmadi[a], Zolfaghar Rajabi[a], Mehdi Vasfi Marandi[b]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran. [b] Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

ABSTRACT

Avian influenza virus subtype H9N2 causes important economic losses in industrial poultry worldwide. Biosecurity and vaccination have not completely prevented the outbreak of avian influenza virus subtype H9N2 in poultry, and there are no appropriate medicines available. Onion is one of the plants used from the ancient times both as food and medicine. The purpose of this study was to evaluate the antiviral effects of aqueous extract of red and yellow onion against avian influenza virus subtype H9N2. First, a study was performed to evaluate the toxic effects of the extracts on the embryonated chicken eggs. For antiviral evaluation, three mixtures were prepared: mixture of the virus and the red onion extract, mixture of the virus and the yellow onion extract, and mixture of the virus and PBS, as a control group. The mixtures were separately inoculated to the chorioallantoic sac of the embryonated eggs after 2, 8 and 24 hours incubation at room temperature. Mortality rate and hemagglutination assay titers were recorded. The results indicated that the red onion extract decreases mortality of the embryos and the yellow onion extract increases the life of the embryos, and both of the extracts decrease HA titers. In conclusion, it seems that both extracts especially aqueous extract of the red onion not only destroys the avian influenza virus subtype H9N2, but also they probably decrease the propagation of the virus in the embryonated chicken eggs.

Keywords

Avian Influenza, H9N2, Onion, Aqueous extract, Antivirus

Abbreviations

HA: Hemagglutination
AI: Avian Influenza
CAF: Chorioallantoic Fluid

Corresponding author:
Zolfaghar Rajabi
Tell/Fax: +984136378734
Email: rajabi@tabrizu.ac.ir

Introduction

Avian influenza (AI) virus subtype H9N2 causes important economic losses in industrial poultry worlwide [1]. Subtype H9N2 which is a low pathogenic avian influenza virus was first isolated and identified in the 1960s [2]. This subtype rapidly spread in the industrial poultry of Iran after the first report in 1998 [3]. Biosecurity and vaccination have not completely prevented the outbreak of AI virus subtype H9N2 in the poultry and at the present, there is no appropriate anti-influenza drug for the treatment of infected food animals including commercial poultry [4].

Common onions (*Allium Cepa*) are perennial plants which are cultivated throughout the world [5]. These plants are used medicinally, as well as for food [5]. Onions have been used in folk medicine for thousands of years [1]; furthermore, different studies have revealed that onions have several therapeutic properties such as antimicrobial activity [6], antiparasitic [7], antiviral [8], antifungal [2], antioxidant and anti-inflammatory effects [5]. Limited available data exhibit inhibitory effects of onion against human immunodeficiency virus (HIV), herpes simplex virus type 1, poliovirus type 1, para-influenza virus type 3, and potato virus [8, 9, 10].

As no data are available regarding the antiviral effects of onion against avian influenza virus subtype H9N2, the purpose of this study was to evaluate the antiviral effects of the aqueous extract of onion against this virus. Red and yellow onions have had different antibacterial effects [11], therefore, in this study the anti-influenza effect of the two types of onions were compared.

Results

Cytotoxicity of the aqueous extracts of the red and yellow onions

Diluted and even undiluted aqueous red and yellow onion extracts had no adverse effect on embryo's viability. No mortality rates were recorded among embryos.

Mortality rate

Mortality rate of embryos was similar in eggs that were inoculated with the mixture of the AI virus and the aqueous extracts of red or yellow onions after 2, and 8 hours incubation at room temperature. The two mixtures that were incubated for 2 hours, caused death of 60% and 40% of the embryos on the second and third day post inoculation, respectively. Although the mortality rate of the control group on the second day post inoculation was 100%, statistically there was no significant difference between the control group and treatment groups (p = 0.30). The two other mixtures that incubated for 8 hours caused the death of 80% and 20% on the second and third day post inoculation, respectively. While the Mortality rate of the control group was 100% on the second day post inoculation. The statistical comparison of the three groups, two treatment groups, and one control group, showed that there was no significant differences between them (p = 0.62), (Tables 1 and 2).

The mortality rate of embryos in eggs that were inoculated with the mixture of the AI virus and the aqueous extracts of red onion after 24 hours incubation at room temperature was only 40% on the third day post inoculation. The mortality rate of embryos in eggs that were inoculated with the mixture of the AI

Table 1
Mortality rate of embryos after day 2, and 3 of inoculation of the mixture of the AI virus and aqueous extract of red or yellow onion or PBS, after 2 hours incubation at room temperature.

Groups	Mortality rate (%)	
	Day 3	Day 2
A	60 ± 24.5	40
B	60 ± 24.5	40
C	100	0
p value	0.300	NA*

*The data were not analyzed. Group A, received the mixture of the virus and the red onion extract. Group B, received the mixture of the virus and the yellow onion extract. Group C, received the mixture of the virus and PBS.

Table 2
Mortality rate of embryos after day 2, and 3 of inoculation of the mixture of the AI virus and aqueous extract of red or yellow onion or PBS, after 8 hours incubation at room temperature.

Groups	Mortality rate (%)	
	Day 3	Day 2
A	80 ± 20.0	20
B	80 ± 20.0	20
C	100	0
p value	0.619	NA*

*The data were not analyzed. Group A, received the mixture of the virus and the red onion extract. Group B, received the mixture of the virus and the yellow onion extract. Group C, received the mixture of the virus and PBS.

virus and the aqueous extract of yellow onion after 24 hours incubation at room temperature was 40%, 40%, and 20% on the second day, the third day, and seventh day post inoculation, respectively. The mortality rate of embryos in eggs that were inoculated with the mixture of the AI virus and PBS after 24 hours incubation at room temperature was 100% on the third day post inoculation (Table 3). The statistical analysis of mortality rate of treatment groups and the control group on the second and third day of incubation showed that there was no significant differences between them, and the *p* value was 0.4 and 0.09, respectively.

Table 3
Mortality rate of embryos after day 2, 3, and 7 of inoculation of the mixture of the AI virus and the aqueous extract of red or yellow onion or PBS, after 24 hours incubation at room temperature.

Groups	Mortality rate (%)		
	Day 3	Day 2	Day 7
A	0	40 ± 24.5	0
B	20 ± 20	40 ± 24.5	20
C	0	100	0
p value	0.397	0.088	NA*

*The data were not analyzed. Group A, received the mixture of the virus and the red onion extract. Group B, received the mixture of the virus and the yellow onion extract. Group C, received the mixture of the virus and PBS.

Hemagglutination titers

Mean HA titers of Avian Influenza Virus subtype H9N2 in chorioallantoic fluid (CAF) of embryonated eggs that were inoculated with the mixture of the virus and the aqueous extract of red onion, the mixture of the virus and the aqueous extract of yellow onion, and the mixture of the virus and PBS after 2 hours incubation at room temperature were 1.1, 3.5, and 4.9, respectively (Table 4). Statistically, there was a significant difference between the positive control group and group A that received the mixture of the virus and the extract of the red onion ($p = 0.013$). Mean HA titers of the virus in CAF of embryonated eggs that were inoculated with the mixture of the virus and the extract of red onion, mixture of the virus and the extract of yellow onion, and mixture of the virus and PBS after 8 hours incubation were 4.1, 4.8, and 6.6, respectively (Table 4). A statistical comparison showed that there is a significant difference between positive control group and the group that received mixture of the virus and the extract of the red onion, and also between the two treatment groups ($p = 0.000$). Mean HA titers of the virus in CAF of embryonated eggs that were inoculated with the mixture of the virus and the extract of red onion, mixture of the virus and the extract of yellow onion, and mixture of the virus and PBS after 24 hours incubation were 6.3, 4.8, and 6.1, respectively (Table 4). Statistical analysis showed that there was no significant differences between the groups ($p = 0.082$).

Table 4
Mean hemagglutination titers (HA) of Avian Influenza Virus subtype H9N2 in chorioallantoic fluid of embryonated eggs inoculated with the mixture of the virus and PBS or aqueous extracts of red and yellow onion after 2, 8, and 24 hours incubation at room temperature.

Groups	Mortality rate (%)		
	24 hours	8 hours	2 hours
A	$1.1 \pm 0.60^{a*}$	4.1 ± 0.31^{a}	6.3 ± 0.25
B	3.5 ± 1.8^{ab}	4.8 ± 0.49^{a}	4.8 ± 0.60
C	4.9 ± 0.19^{b}	6.6 ± 0.10^{b}	6.1 ± 0.24
p value	0.013	0.000	0.082

*The data with the same superscript letters are not significantly different. Group A, received the mixture of the virus and the red onion extract. Group B, received the mixture of the virus and the yellow onion extract. Group C, received the mixture of the virus and PBS.

Discussion

AI viruses have pathological effects on chicken embryos by induction of apoptosis and necrosis. All highly pathogenic and low pathogenic AI viruses, are embryo lethal, and hatching of internally contaminated eggs has not been reported [4]. Therefore, the mortality rate of the contaminated embryos is 100%. In this study although comparison of the results of the mortality rates between treatment and control group indicated that there was no statistical difference between them ($p > 0.05$), but the results clearly showed that when the mixture of the AI viruses and the aqueous extract of red onion incubated for 24 hours could decrease the mortality rate to 40%. 20% of the embryos that received mixture of the H9N2 virus and the aqueous extract of yellow onion survived approximately a week, while in the control group, mortality rate was 100% three days post inoculation. Therefore, both extracts had antiviral effects against AI virus subtype H9N2, although the antiviral effects of red onion were more than that in yellow onion. Organosulfur compounds and flavonoids are important antimicrobial and antiviral compounds that are extracted from onions [5, 13]. A study shows that allicin, as an organosulfur compound, has antiviral activity in addition to its antibacterial and antifungal activities [6, 14]. Flavonoid compounds including hesperetin, reduce

intracellular replication of some viruses, for example, human immunodeficiency virus [8], herpes simples virus type 1, poliovirus type 1, respiratory syncytia virus, and parainfluenzavirus type 3 [9]. There is also a study that indicates onion stems can reduce the *in vitro* and *in vivo* infectivity of potato virus Y [10]. Therefore, anti-influenza subtype H9N2 properties of the aqueous extracts of the red and yellow onions may be due to their allicin and flavonoid compounds [8]. There is a published document that shows that the antibacterial properties of red onion extracts are igher than those in yellow onion [11]. In our study, the anti-influenza virus activity of the aqueous extract of red onion was more than that in the aqueous extract of yellow onion. The reason could be due to the differences in the amount of their anti-influenza components [13].

For propagation of AI viruses, it is preferred that the viruses to be inoculated to 9-11-day-old embryonated chicken eggs via the chorioallantoic sac [4]. In order to get higher viral titers and higher hemagglutination titers, it is necessary that the stock infectious viruses to be diluted to 10^{-4} for virus propagation or to 10^{-9} for titration [15]; otherwise, embryos die sooner and virus titers do not increase. Therefore, if the two extracts have anti-influenza H9N2 properties, they should reduce the amount of the stock virus. Thus, if the diluted virus is inoculated into the embryonated eggs, its proliferation will increase. [16]. In our study, comparison of the results of mean HA titers indicated that by increasing the duration of exposure of the virus and the extracts at room temperature, the amount of virus titers in the chorioallantoic fluid of the chicken embryonated eggs increases. We expected that mean HA titers of treated groups to be more than the control group [17], but mean HA titers of the control group were more than treatment groups. These results could be due to the direct effect of the extracts on the propagation of the H9N2 viruses. [18]. Mean HA titers of AI Virus H9N2 in the chorioallantoic fluid of embryonated eggs that were inoculated with the mixture of the virus and the aqueous extracts of red onion after 2 and 8 hours post incubation at room temperature was less than those of the mixture of the virus and the aqueous extract of yellow onion. This finding probably indicates that the red onion extract inhibits the propagation of the virus more than the yellow onion extract. In conclusion, according to the results of the mortality rate of the embryos and mean HA titers, it seems that both extracts, especially aqueous extract of red onion, not only destroys the avian influenza virus subtype H9N2, but also decrease the propagation of the virus in chicken embryonated eggs.

Materials and methods

Avian influenza virus

Avian influenza virus subtype H9N2, A/Chicken/Iran/ZMT-101(101)/98 (H9N2), was used for this study. HA titers of the virus were 27. This virus was kindly provided by the Department of Poultry Diseases, Faculty of Veterinary Medicine, University of Tehran.

Preparation of aqueous onion extracts

Extracts of red and yellow onion were prepared separately. Briefly, the bulbs of red or yellow onion were washed and grated with a grater. The pulp was pressed through a clean cloth and then was centrifugated at 2000 rpm for 5 minutes at 24 ̊C. The extract was passed through a 0.45 µm filter for sterilization.

Cytotoxicity of the extracts

A study was performed to evaluate the toxic effects of the extracts. For this purpose, the onion extracts were separately diluted with sterile PBS solution at a ratio of 1/2, and 1/4. Fifteen 10-day-old embryonated chicken eggs were divided into three groups, 5 eggs/group. 0.3 ml of the undiluted extract was inoculated to the chorioallantoic sac of each egg in the first group; the other two groups received diluted extracts (1/2,and 1/4).

Antiviral effects of the aqueous extracts of the red and yellow onions

The same method used for the evaluation of the two extracts. In order to evaluate each extract, the avian influenza virus subtype H9N2 was diluted with sterile PBS solution to 10^{-1} and then was mixeds with the extracts separately or PBS in 1/2 ratio. For each extract, fifteen 10-day-old embryonated chicken eggs were divided into three equal treatment groups. Fifteen other 10-day-old embryonated chicken eggs were divided into three equal positive control groups.

Group A, which includes three subgroups A1, A2, and A3, received mixture of the virus and the red onion extract after 2, 8, and 24 hours incubation at room temperature (25° C), respectively. Group B, which includes three subgroups B1, B2, and B3 received mixture of the virus and the yellow onion extract, respectively. Group C, which includes three subgroups C1, C2, C3 received mixture of the virus and PBS, respectively (Table 5). A 0.1 ml amount of the mixtures were inoculated to the chorioallantoic sacs of the eggs, and then the eggs were incubated at 37°C. Mortality rate of embryos and HA titers of AI virus in the chorioallantoic fluid were recorded for evaluation.

Hemagglutination (HA) assay

The chorioallantoic fluid of eggs whose embryos were dead on the second and third day of inoculation, harvested and titers of AI Virus subtype H9N2 were measured as recommended previously[12]. Briefly, 50 µl sterile PBS placed in every 12 wells in a microtiter plate. In the first wells, 50 µl of the chorioallantoic fluid of eggs were added and serially diluted. Then, 50 µl of 0.5 % washed chicken erythrocytes was added to each well, and incubated at room temperature for 30-40 min. HA activity determined and expressed as the reciprocal of log2.

Statistical analysis.

One-way ANOVA and Duncan multiple range tests were used for analysis of the data. The SPSS statistics, version 22, was used for statistical analysis.

Table 5
Experimental design of the study of the antiviral effects of aqueous extracts of red and yellow onions against avian influenza virus subtype H9N2

[a] Duration of Incubation (hr)	10-day-old embryonated chicken eggs								
	Group A			Group B			Group C		
	A1	A2	A3	B1	B2	B3	C1	C2	C3
2	*			*			*		
8		*			*			*	
28			*			*			*

[a] The duration of incubation of the mixtures of the virus with PBS or aqueous extract of red or yellow onions at room temperature. *Embryonated chicken eggs that received the mixtures after incubation at room temperature. Group A, received the mixture of the virus and the red onion extract. Group B, received the mixture of the virus and the yellow onion extract. Group C, received the mixture of the virus and PBS.

Acknowledgments

The authors would like to thank the Research Council of the University of Tabriz for funding this research.

Author Contributions

Designed the experiments: Z.R. Performed the experiments: S.A. Scientific counseling: M.V.M

Conflict of Interest

The authors declare that they have no competing interests.

References

1- Vahabpour-Roudsari R, Shamsi-Shahrabadi M, Monavari SH, Sajjadi SE. Evaluation of potential antiviral activity of hydroalcoholic extract of Lemon Balm L. against Herpes Simplex Virus type-I. Iran. J. Virol. 2007;1:28-32.

2- Harper SA, Fukuda K, Uyeki TM, Cox NJ, Bridges CB. Prevention and control of influenza.

3- Vasfi Marandi M, Bozorgmehri Fard MH. Isolation of H9N2 subtype of avian influenza viruses during an outbreak in chickens in Iran. Iran. Biomed. J. 2002 Jan 15; 6(1):13-7.

4- Swayne DE, Suarez DL, Sims LD. Influenza. Diseases of poultry. 2013 Oct 4:181-218.

5- Bouvier NM, Palese P. The biology of influenza viruses. Vaccine. 2008 Sep 12;26:D49-53..

6- Suleria HA, Butt MS, Anjum FM, Saeed F, Khalid N. Onion: nature protection against physiological threats. Crit. Rev. Food. Sci. 2015 Jan 2;55(1):50-66.

7- Mohamed EF. Antiviral properties of garlic cloves juice compared with onion bulbs juice against potato virus Y (PVY). J. Am. Sci. 2010;6(8):302-10.

8- Lanzotti V. The analysis of onion and garlic. J. Chromatogr. A. A. 2006 Apr 21;1112(1-2):3-22.

9- Centers for Disease Control and Prevention (CDC). "Avian influenza (bird flu)." (2007).

10- Lamb RA, Choppin PW. The gene structure and replication of influenza virus. Annu. Rev. Biochem. 1983 Jul;52(1):467-506.

11- Wilson JC, Itzstein M. Recent strategies in the search for new anti-influenza therapies. Curr. Drug. Targets. 2003 Jul 1;4(5):389-408.

12- Killian ML. Hemagglutination assay for the avian influenza virus. Avian Influenza Virus. 2008:47-52.

13- Liguori L, Califano R, Albanese D, Raimo F, Crescitelli A, Di Matteo M. Chemical composition and antioxidant properties of five white onion (Allium cepa L.) landraces. J. Food. Quality. 2017 Jan ;2017.

14- Bayan L, Koulivand PH, Gorji A. Garlic: a review of potential therapeutic effects. Avicenna. J. Phytomed. 2014 Jan;4(1):1.

15- Spackman, E., and D. Suarez. Avian influenza virus. Humana Press, Totowa, NJ. 2008. Crossref, Google Scholar

16- Barjesteh N, Brisbin JT, Behboudi S, Nagy E, Sharif S. Induction of antiviral responses against avian influenza virus in embryonated chicken eggs with Toll-like receptor ligands. Viral. Immunol. 2015 May 1;28(4):192-200.

17- Brauer R, Chen P. Influenza virus propagation in embryonated chicken eggs. J. Vis. Exp: JoVE. 2015(97(

18- Ding Y, Zeng L, Li R, Chen Q, Zhou B, Chen Q, leng Cheng P, Yutao W, Zheng J, Yang Z, Zhang F. The Chinese prescription lianhuaqingwen capsule exerts anti-influenza activity through the inhibition of viral propagation and impacts immune function. BMC Complem. Altern. M. 2017 Dec;17(1):130.