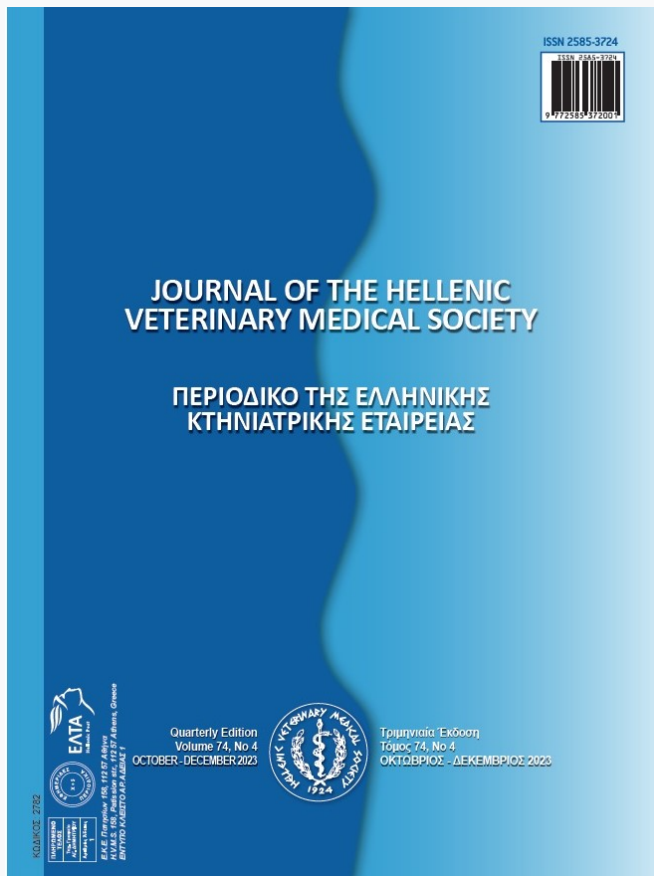


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## The effect of bromhexine on mucosal immune response against avian coronavirus infectious bronchitis vaccine in chickens

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**ABSTRACT:** Avian coronavirus infectious bronchitis (IB) is one of the most important and contagious diseases in chickens, all over the world. The administration of chemical compounds that improve the mucosal immune response to the IB vaccine can increase resistance against the virus. To examine the effect of bromhexine on the mucosal immune response against the IB vaccine, 360 one-day broiler chicks were allocated to eight groups, randomly. Group one was the control group with no vaccine administration, and only used bromhexine. Groups 2, 3, and 4 received the IB vaccine once (at 5 days old), twice (at 5 and 15 days old), and three times (at 5, 15, and 25 days old). The chickens in groups 2, 3 and 4 received bromhexine from 48 hrs before the vaccination to 24 hrs after receiving the vaccine in drinking water. Group five was the negative control group and did not receive bromhexine and IB vaccine. Groups six, seven, and eight received IB vaccine once (at 5 days old), twice (at 5 and 15 days old), and three times (at the 5, 15, and 25 days old) without bromhexine. The chickens were slaughtered 10 days after each vaccination time. Sampling in group five was at the ages of 15, 25 and 35 days old. The nose and trachea samples were collected and the mucosal surface of the nose and trachea was rinsed. Afterward, specific immunoglobulin A (IgA) level against IB vaccine in the mucosal respiratory surface was measured through ELISA using specific chicken IgA antiglobulin. The results showed that the use of bromhexine on the first vaccination had no effect on mucosal immune response. However, with the second and third vaccinations, antibody titer to IB vaccine was higher than chickens received the vaccine without bromhexine. It appears that using bromhexine in booster IB vaccination can improve specific mucosal immunity.

**Keywords:** Bromhexine, Mucosal immunity, Infectious Bronchitis, Broiler chicken.

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## INTRODUCTION

Avian coronavirus infectious bronchitis (IB) is one of the most important and contagious diseases in chickens, all over the world (Jackwood & de Wit, 2020). Some reports support a significant relationship between mucosal IgA titer and resistance to avian infectious bronchitis virus (IBV) infection (Okino et al., 2013; Toro & Fernandez, 1994). Although serum immunoglobulin G (IgG) has a role in neutralizing IBV in the general blood cycle, mucosal IgA is the first line of defense against IBV. It has a critical role in protecting chickens against IBV (van Ginkel et al., 2008). Therefore, antibodies against IBV in the upper respiratory system are imperative for immunity against IBV (Smialek et al., 2017). Therefore, compounds that can efficiently stimulate mucosal immune response to IBV can be used to improve resistance to the disease.

Bromhexine is a derivative of vasicine and an alkaloid derived from *Adhatodavasisca*. The compound is used to alleviate the symptoms of respiratory diseases such as IB in poultry (Rayees et al., 2014). Bromhexine increases the secretion of mucus and lowers viscosity of respiratory secretions, so it is a mucolytic agent, while it does not affect lung function (Yuta & Baraniuk, 2005). Diluting and mucoactive agents have a notable role in accelerating ciliary activity and in return increasing the discharge of secretions. These features make them an option for controlling respiratory diseases symptom and IB signs in particular (Rogers, 2007). The role of mucociliary system in creating immunity and protection against bacteria is highly important so it is considered an element of the general immunity system (Whitsett & Alenghat, 2015). The ciliary activity and increasing mucus gland secretion accelerate the discharge of pathogens from respiratory organs and prevents the spread of pathogens to the respiratory epithelium (Carrillo et al., 2016).

There are several mucolytic herbal and chemical drugs. These compounds are available in the pharmacopeia that can dilute the secretions of the mucociliary system (Gholami-Ahangaran et al., 2019, Gholami-Ahangaran et al., 2021). Bromhexine is one of the most important chemical drug that it can increase lysosomes activity and discharge secretions (Shaban et al., 2019). Bromhexine hydrolyzes glycoprotein strains in the secretions, decreases the adhesiveness of the secretions, and accelerated the flow of secretions (Gil et al., 2020). Bromhexine is absorbed through the digestive system and maximum plasma concentration occurs in one hour after ingestion. The bromhexine

metabolites are mostly discharged through urination and a very small portion of the drug can be excreted via feces. For instance, Ambroxol is one of the metabolites of bromhexine (Yuta & Baraniuk, 2005).

There are a few studies on the effects of bromhexine on the chicken respiratory system. Carrillo et al. (2016) examined the rheometry of mucus secretions in chickens and showed that bromhexine can lower the rheometry of mucus secretions (Carrillo et al., 2016). Another study highlighted the role of bromhexine in increasing the intracellular concentration of antibiotics in chickens affected by infectious coryza. They showed that simultaneous use of tilmicosin and bromhexine increased tilmicosin concentration in the cells of target tissue. It also decreased re-isolation of the bacterium and the severity of respiratory signs and tissue side effects (Shaban et al., 2019). Although bromhexine is known as a mucoactive agent in chicken, its efficiency in terms of the effect on the mucosal immune response has not been studied yet, which is the aim of the present experimental study. The stimulating effect of bromhexine on mucosal immune response as a mucolytic agent was examined to highlight the use of bromhexine as a supportive medication for respiratory diseases.

## MATERIALS AND METHODS

### Grouping of chickens

To examine the effect of bromhexine on mucosal immune response, 360 one-day broiler chicks of Ross 308 were randomly allocated to eight groups with three replications (with 15 chickens in each repeat and 45 chickens in each group). All the chickens had a similar growing condition with *ad libitum* access to feed and water. Group one was the control group with no vaccine administration, and only used bromhexine. Groups 2, 3, and 4 received the IB vaccine once (at 5 days old), twice (at 5 and 15 days old), and three times (at 5, 15, and 25 days old), respectively. The chickens in groups 2, 3 and 4 received bromhexine (1 mg/kg) from 48 hrs. before the vaccination to 24 hrs. after receiving the vaccine in drinking water. Group five was the negative control group and did not receive bromhexine and IB vaccine. Groups six, seven, and eight received the IB vaccine once (at 5 days old), twice (at 5 and 15 days old), and three times (at the 5, 15, and 25 days old), respectively, without bromhexine. Nine chickens in each group were euthanized 10 days after each vaccination time and trachea-nose lavages were prepared for assaying of mucosal IgA

titer. Sampling in group five (without vaccine) was at the ages of 15, 25 and 35 days old.

### **IB vaccine preparation**

The IB vaccine used in the study belonged to the H120 strain supplied by Razi Vaccine and Serum Research Institute (Iran). The vaccine is an attenuated IBV (H120 strain of Massachusetts serotype), which was produced in SPF eggs and lyophilized. Each dose of the vaccine contained  $10^{3.5}$  to  $10^4$  EID<sub>50</sub> after being solved in drinking water.

### **Bromhexine preparation**

The elixir of Bromhexine contained 0.8mg/ml hydrochloride bromhexine supplied by Tolid Darou Pharmaceutical Co. (Iran). The dosage was 1mg/Kg of body weight (Shaban et al., 2019).

### **Respiratory Mucosal Lavage**

The nose and trachea samples were collected and the mucosal surface of the trachea and nose was rinsed three times with one ml of phosphate buffer saline (PBS) solution (pH=7.4) containing 0.1% bovine serum albumin (BSA) (Amiri et al., 2017).

### **Assaying of IgA titer**

Immediately after the collection of lavage samples, the specimens were centrifuged to obtain the coating supernatant solution (Amiri et al., 2017; Shahabi-Ghahfarokhi et al., 2016). To measure specific IgA against IBV in the respiratory mucosal surface, the specimens were examined using the ELISA method (Synbiotic Co.). Goat anti-chicken IgA, conjugated with horse radish peroxidase (HRP) was separately prepared from Bethyl Laboratories (Cat. No. A 30-103P).

The method of Gelb et al. (1998) and Thompson et al. (1997) were followed for calculating specific IgA titer in the lavage samples. For this, the optical density (OD) of the negative control group was obtained and added three times the standard deviation (SD) (Gelb et al., 1998; Thompson et al., 1997). To calculate the titer of each specimen, negative control and positive-negative threshold were used (Amiri et al., 2017; Gelb et al., 1998; Thompson et al., 1997). The IgA of each sample was obtained based on negative control and positive-negative threshold using KPL software and expressed based on logarithm 2.

### **Assaying of respiratory mucosal viscosity**

The viscosity of mucosal respiratory lavages

was determined by a rheometer (Brookfield, UK). All the measurements were conducted at 5 °C and 25R.P.M. The lavage samples were gently stirred for 20 s before testing. Triplicate measurements were performed for each sample.

### **Statistical analysis**

All data were analyzed by Sigma State (2.0) software program using one-way analysis of variance (ANOVA) statistical method. The means that showed significant differences in ANOVA, were compared using the Tukey test at  $p < 0.05$ .

## **RESULTS**

Results revealed chickens that received the IB vaccine had a significantly higher IgA titer compared to the chickens that did not receive the vaccine ( $P < 0.05$ ). Among the chickens that received IB vaccine, the highest mucosal IgA titer was on the 10<sup>th</sup> day after three vaccinations at the age of 5, 15, and 25 days old. The difference was significant with other chickens that received one vaccine in 5 or twice vaccine in 5 and 15 days old, respectively ( $p < 0.05$ ). A comparison of mucosal IgA titer 10 days after vaccination in chickens that received bromhexine showed that the highest IgA level was in the chickens that received three vaccines at 5, 15 and 25 days old and bromhexine, simultaneously ( $P < 0.05$ ).

A comparison of vaccinated chickens and the chicken that were vaccinated and received bromhexine showed that the use of bromhexine along with the 2<sup>nd</sup> and 3<sup>rd</sup> vaccinations increased mucosal IgA titer, but the only significant difference was seen between the chickens received three IB vaccines by bromhexine and chicken that received three IB vaccine, a lonely ( $P < 0.05$ ) (Table 1).

There was no significant difference in the viscosity of mucosal respiratory lavage samples in all chickens who received vaccine a lonely or simultaneous with bromhexine, in all vaccination programs (Table 2).

## **DISCUSSION**

Serum antibody level, the re-isolation of virus from the respiratory tract, clinical signs, histological lesions and trachea ciliary activity are indices that can be used to evaluate the resistance of chickens to IBV (Legnardi et al., 2020). Although serum antibody has a role in the resistance to IBV, it has no direct relationship between serum antibody and protection against the virus. In some cases, chickens with a high level

**Table 1:** Mean IgA titer in the respiratory lavage ( $\log_2$ ) in chickens received IB vaccine

Groups	Vaccination program (age)	Sampling age		
		15 days old	25 days old	35 days old
Chickens received only IB vaccine	No vaccine	1.05±0.55 <sup>c</sup>	0.22±0.11 <sup>f</sup>	0.40±0.23 <sup>f</sup>
	Single vaccination (5 days old)	3.07±0.37 <sup>d</sup>	1.27±0.26 <sup>e</sup>	0.31±0.12 <sup>f</sup>
	Double vaccination (5 and 15 days old)	3.18±0.55 <sup>d</sup>	3.45±0.61 <sup>cd</sup>	1.38±0.33 <sup>e</sup>
	Triple vaccination (5, 15 and 25 days old)	2.97±0.37 <sup>d</sup>	3.67±0.56 <sup>c</sup>	4.97±0.72 <sup>b</sup>
Chickens received IB vaccine + Bromhexine	No vaccine	1.17±0.45 <sup>e</sup>	0.45±0.31 <sup>f</sup>	0.34±0.23 <sup>f</sup>
	Single vaccination (5 days old)	3.15±0.67 <sup>d</sup>	1.57±0.26 <sup>e</sup>	0.52±0.12 <sup>f</sup>
	Double vaccination (5 and 15 days old)	3.05±0.55 <sup>d</sup>	4.02±0.71 <sup>c</sup>	2.89±0.43 <sup>d</sup>
	Triple vaccination (5, 15 and 25 days old)	3.00±0.37 <sup>d</sup>	4.11±0.46 <sup>bc</sup>	5.90±0.52 <sup>a</sup>

\*The different words in superscript of data represents a significant difference ( $p < 0.05$ ). All data were statistically analyzed with each other's

\*Data expressed as mean±SD

**Table 2:** The viscosity of mucosal respiratory lavage samples in chickens received IB vaccine

Groups	Vaccination program (age)	Sampling age		
		15 days old	25 days old	35 days old
Chickens received only IB vaccine	No vaccine	0.11±0.09	0.12±0.11	0.14±0.08
	Single vaccination (5 days old)	0.12±0.07	0.12±0.06	0.12±0.08
	Double vaccination (5 and 15 days old)	0.12±0.08	0.12±0.08	0.13±0.07
	Triple vaccination (5, 15 and 25 days old)	0.12±0.07	0.13±0.06	0.14±0.05
Chickens received IB vaccine + bromhexine	No vaccine	0.11±0.09	0.12±0.07	0.14±0.05
	Single vaccination (5 days old)	0.12±0.07	0.13±0.08	0.15±0.10
	Double vaccination (5 and 15 days old)	0.12±0.08	0.14±0.07	0.15±0.09
	Triple vaccination (5, 15 and 25 days old)	0.12±0.07	0.14±0.08	0.16±0.10

\* All data were statistically analyzed with each other's

\*Data expressed as mean±SD

of serum antibody titer demonstrated no resistance to the pathogenic virus and *vice versa* (Gillette, 1981). On the other hand, the local immune system in the respiratory system is the first line of defense against IBV (van Ginkel et al., 2008). Thus, the resistance against IBV might be due to the local immune system in the trachea or cellular immunity (Maslak & Reynolds, 1995). In this regard, some reports support a significant relationship between IgA titer and resistance to IBV (Smialek et al., 2017). Therefore, instead of measuring serum IgG, measuring local IgA can be a suitable alternative for monitoring chicken flocks af-

ter vaccination or viral infection.

In the present study, all the chickens that received the IB vaccine were euthanized 10 days after vaccination and examined for specific mucosal IgA against the IB vaccine through the ELISA method. Previous studies have reported that IBV antibodies reach the highest level in the respiratory system approximately 10-14 days after intra-trachea/eye vaccination. In present study, a comparison of local IgA titers 10 days after vaccination in all chickens showed that vaccination via drinking water can stimulate mucosal immune response in the trachea and nose of the chick-



ens. In addition, mucosal immune titer in chicken with more than one vaccination showed that immunity titer had increased following booster vaccination. This increase was not significant in the chickens with one or two vaccination and there was no significant difference in primary and secondary responses of mucosal IgA immunity in any of the sampling stages. Apparently, the secondary immunity response was a repetition of the primary response and it only prevented the decline in primary specific IgA in the respiratory mucosal surface. Other studies on humans and mammals have shown that the local mucosal secretory system cannot stimulate a secondary antibody response (Porter, 1973). There are reports that chickens do not demonstrate a proper mucosal immune response followed with booster vaccination or even after challenge following by primary vaccination to IB (Gelb et al., 1998; Thompson et al., 1997). So that some cases had a decrease in mucosal IgA titer following challenge or vaccination after primary vaccination to IB (Saiada et al., 2019). Gelb et al. (1998) showed that vaccination of one-day chickens using the Connecticut strain of IB vaccine via eye drops following a challenge with the M41 strain led to stimulation of the same local IgA or even a decline in local IgA titer, while serum titer increased in the chickens (Gelb et al., 1998). In our present study the decrease of IgA titer in booster did not occur, but in some research the decrease in IgA titer after booster vaccines was reported. Gurjar et al. (2013) proved that after first vaccination, Gamma interferon expression in the Harderian gland was increased, while the expression of Gamma interferon in the mucosal compartment declined during booster vaccination. However, in the systemic immune compartment, gamma interferon expression increases in the primary and secondary vaccination responses (Gurjar et al., 2013). In addition, Orr-Burks et al. (2014) reported that primary vaccination increased IgA, while the booster vaccination increased IgG in mucosal surfaces (Orr-Burks et al., 2014).

In the present study, the results showed that the best mucosal IgA titer was obtained 10 days after vaccination following the administration of live IB vaccine for three times. Since the difference was significant with other chickens that received IB vaccine (one and two vaccination), the immune response to the third vaccination was more stimulated compared to the first and second vaccinations. The literature review revealed no information in this regard. We cannot claim that three vaccinations create better resistance against the pathogenic virus compared with one

or two vaccinations. However, the results showed that following three vaccinations, a significant increase in local immunity response can be seen, so better resistance to the virus is expected.

In the recent study, the IgA titer in the chickens with one and two vaccinations was not significantly different. Still, the use of bromhexine in the chicken with two vaccinations created a significant difference in IgA titer; between the chickens with one or two vaccinations. Apparently, the stimulation of mucosal immune response after receiving bromhexine was the reason for the significant increase in IgA following two vaccinations compared with one vaccination. This difference in the titer was evident in the chickens with three IB vaccines. Also, the chicken that received IB vaccine and bromhexine had a significantly higher titer compared to the chicken that received the vaccine without bromhexine. The difference in IgA titer in the chicken that received vaccine and the chicken that received vaccine and bromhexine was significant in the second and third vaccination, while no difference was observed with one vaccination. It seems that an incomplete immune system in very young chickens is an explanation for this finding. There are similar results regarding the inefficient response of mucosal response and even humoral immunity following IB vaccination in chickens less than one week old (Saiada et al., 2019).

To ensure whether the increase in IgA titer in chickens receiving bromhexine falsely occurred as a result of increased mucus secretion or not, the viscosity of the fluid obtained from washing the surface of the respiratory mucosa was analyzed with a rheometer and it was similar in all samples without significant differences. This shows that in all groups, the increase in IgA titer was not related to more secretion of mucus. The lack of effect of bromhexine on the viscosity of respiratory mucus may be due to the sampling time. In this study, tracheal samples were washed 10 days after vaccination, at which time the effect of bromhexine may have disappeared. In addition, the short period of bromhexine consumption (3 days) may not have a significant effect on the viscosity of mucous secretions.

In this study, the mechanism of increasing IgA against the IB vaccine was not clear. Some reports related to human coronaviruses demonstrated that bromhexine may enhance coronavirus uptake by host cells (Depfenhart et al., 2020; Habtemariam et al., 2020). Therefore, it seems increasing uptake of the IB

vaccine can stimulate mucosal immunity in chickens.

In general, the utilization of bromhexine before and after IB vaccination specially in boosters can improve the mucosal immune response of the respiratory system and it is expected to increase resistance against IBV.

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This study was performed according to principles of ethics in caring and growing of animal that approved by the Islamic Azad University Shahrekord Branch, Iran (Shk.Eth.95.123).

## CONFLICT OF INTEREST

None declared by the authors.

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