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THE EFFECT OF IN-OVO INJECTION OF BEE VENOM ON HATCHABILITY AND SOME IMMUNOLOGICAL PARAMETERS OF ALEXANDRIA CHICKS' STRAIN AT HATCH

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ABSTRACT: Researchers are trying to boost the immune system of poultry birds through the use of natural immune-stimulants including bee venom. There is a scarcity in literature about the use of bee venom for *in ovo* injection studies. The aim of this study is to investigate the effect of *in ovo* injection of two levels of freeze-dried bee venom (BV) (10 and 20 µg/egg) into eggs of Alexandria breeder hens at 18-day of incubation on the hatchability percentage, hatching weight per egg weight, and immune response of the young chickens. The doses of BV used in the current work were based on a pilot trial obtained directly before the main experiment of this work. A total number of 900 fertile eggs at day 18 of incubation were used in this study. Eggs were weighed and randomly allocated into three experimental groups as: control (C), low dose of BV and high dose of BV with 300 eggs each. Results showed that in-ovo injection with 10 µg/egg BV significantly increased hatchability percentage (89.45%) over the control (87.5%) and 20 µg/egg BV (77.73%). The highest relative chick weight was observed with eggs of control group followed by lower and higher dose of BV groups. The increase of dose has reduction effect on the relative chick weight. The total protein and albumin values decrease significantly ($P \leq 0.001$) in 10 and 20 µg BV/egg groups compared to control group. Results for triiodothyronine (T3), and thyroxine (T4) concentrations of chick's blood at hatch showed that in-ovo injection of BV decrease significantly both of T3 and T4 concentrations in Alexandria chick serum at hatch. In addition, results of showed that the spleen relative weight increased significantly in 20 BV dose in-ovo group compared to control group. Also, the same trend was observed with bursa relative weight compared to the control group. In conclusion, the in-ovo injection with 10 µg BV/egg resulted in better hatchability percentage with Alexandria breeder eggs.

Key words: In ovo injection - BV - Hatchability - Immunological Parameters

INTRODUCTION

Rearing farms should receive quality chicks from hatcheries to avoid relapse in the growth that may arise from weak chicks due to their exposure to pathogens or other stressors (Schaal & Cherian, 2007). In recent years, incubation and embryonic development towards hatch are becoming more relevant to the successful rearing of poultry (Foye *et al*., 2007). In-ovo injection for hatching eggs with different nutrients may help to overcome the problems that occurs during embryonic development and the hatching process, resulting in healthier and better productive chicks (Ellen, 2004). The in ovo injection offers promising and sustaining progress in production efficiency of commercial poultry (Stephanie Roto *et al*., 2016).

The usage of all bee products, including BV and honey, dates back thousands of years for their medicinal properties were cited in religious books like the Bible and the Quran (Fratellone, 2015; El-Wahab & Eita, 2015). Apitherapy is a branch of alternative medicine that relies on the usage of honeybee products that consists of honey, pollen, propolis, royal jelly, and mainly BV, which is also known as apitoxin (Hellner *et al.,* 2007; Trumbeckaite *et al.,* 2015).

BV is produced by female worker bees and is known to contain many active components including: peptides like melittin, apamin and adolapin; enzymes, such as phospholipase A2 and hyaluronidase; and amino acids and volatile compounds (Lee *et al*., 2009; Moreno & Giralt, 2015; Bellik, 2015; Rim Wehbe *et al.,* 2019). Natural BV substance has strong anti-inflammatory, antibacterial, analgesic actions and contributes to the enhancement of immune responses (e.g. Rudenko & Nipot, 1996; Curcio-Vonlanthen *et al*., 1997; Rim Wehbe *et al.,* 2019), and has anti-cancer and anti-viral potential (Hood *et al.,* 2013; Rady *et al.,* 2017).

BV given either by injection or via drinking water increased performance and enhanced antioxidant capacity in broilers and pigs (Han *et al*., 2009; 2010). BV plays an important role in regulating the immune system, Perrin-Cocon *et al*. (2004) and Ramoner *et al*. (2005) reported that BV secretory phospholipase A2 induces maturation of dendritic cells and activates the dendritic cell immune response. This plays an important role in host defense against infection by microbial pathogens (Banchereau & Steinman, 1998; Samuel, 2001).

To take advantage of the BV characteristics in chicken embryos, this study was made to evaluate the effects of in-ovo injection of two levels of BV (10 and 20 µg/egg) into eggs of Alexandria breeder hens at 18-day of incubation, to evaluate their effects on hatchability percentage, hatchling traits, immune response and some blood biochemical parameter of hatched chicks.

MATERIAL AND METHODS

The pilot experiment:

This primary study aimed to observe the effect of BV in-ovo injection on hatchability percentage to determine the range of the experimental dosages. The high median lethal dose of BV estimated according to adult human is 2.8 mg/kg (Schumacher *et al.,* 1989). Therefore, the safe dose is up to 45 µg of BV/egg (based on chick's weight at hatch is 30-35g of Alexandria strain). A total of 140 eggs were collected from Alexandria breeder hens and incubated under optimal conditions at the Poultry

Research Center, Faculty of Agriculture, Alexandria University. Eggs containing viable embryos at day 18 of incubation were used. Eggs were distributed into seven treatments: control; control positive (saline) and five different BV treatments (5, 15, 25, 35 and 45 µg/egg). The hatchability percentages were 84.73, 83.92, 85.00, 87.33, 75.53, 71.73 and 63.09%, respectively. The higher doses (25, 35, 45 µg/egg) has significantly lower hatchability percentages compared to the lower doses and controls groups.

The experiment of study:

A total of 900 fertile eggs of Alexandria breeder hens at day 18 of incubation were used. Before setting, eggs were weighed and divided into 3 experimental groups with nearly equal weight (each treatment has five replicates, with 60 eggs for each). The 3 treatment groups as following: 1) control (C), non-injected, 2) low dose group injected with 10 µg BV/egg and 3) high dose group injected with 20 µg BV/egg. Fertile eggs were set in an electric forced draft incubator for the first 18 days of incubation under 37.8 ^{o}C and 53±3% relative humidity. The eggs were turned automatically 24 times per day with angles ±45^{o} after that were removed to the hatcher, in covered trays after injection process, for the last three days of incubation under 37.5 ^{o}C and 70±3 % relative humidity. Throughout the incubation period, all eggs were incubated according to the common routine procedures.

In-ovo injection solutions and procedures:

The in-ovo injection solutions of the present study include two levels (10 and 20 µg/egg) of BV (BV freeze-dried 95%) produced by (Ghada freeze-drying facility, Alexandria, Egypt) were dissolved in saline solution (NaCl 0.9%). The solution was prepared immediately before injection and gently warmed to reach the incubation temperature.

All eggs (including control) were taken out of the incubator for nearly 20 min to equalize the conditions for the injection process. A mini grinder was used to make a proper hole on the broadside of the eggshell. Using 21 needle-gauge at 432 h (18 d of incubation), eggs from all injection groups were injected from the top of the egg with in-ovo injection solutions (0.2 ml/ egg) into the amniotic fluid as described by Uni & Ferket (2003) and Uni *et al*. (2005). The site of injection was sanitized with ethanol 70% and sealed by using the wax gun after injection. In parallel, eggs of the control group were taken out of the incubator and kept in the same environmental conditions of injected eggs.

Day 20 and 21 hatchability/ total hatchability percentage were calculated (day 20 and 21 hatchability/ total hatchability percentage × 100) was calculated in each treatment. On the day of the hatch, the hatchability percentage and chick's body weight were measured. The hatchability percentage of the fertile egg (Scientific Hatchability % = number of chicks/ numbers of fertile eggs × 100) was calculated in each replicate. The relative hatchlings weight to egg weight was estimated (chick weight mean/ eggs weight mean × 100) in each replicate.

At 21-day of incubation period, three chicks taken randomize from each treatment were slaughtered to record spleen, bursa, and yolk sac weights then expressed as percentages of a chick's live body weight.

Blood samples were collected from slaughtered chicks via the jugular vein of a total of 3 chicks from each

treatment. It collected in dry clean centrifuge tubes without anticoagulant for serum separation. The clear serum samples were carefully drawn and transferred to Eppendorf tubes and stored at -20°C in the deep freezer until the time of chemical determinations. The biochemical characteristics of blood were determined calorimetrically on Hitachi 901 spectrophotometer.

Serum total protein (g/dl) measured using special kits delivered from sentinel CH Milano, Italy according to guidelines of Armstrong & Carr (1964). Serum albumin (g/dl) was determined using special kits delivered from sentinel CH Milano, Italy according to the method of Doumas *et al*. (1977). Serum globulin level (g/dl) was calculated by the difference between total protein and albumin, since the fibrinogen usually comprises a negligible fraction (Sturkie, 1986). Serum glucose concentration (mg/dl) was measured by the method of Trinder (1969). Serum tri-iodothyronine (T3) and thyroxine (T4) were determined measured using specific kit for poultry according to (Darras *et al*., 1991).

Statistical Analysis: Data from all response variables was analyzed using the GLM procedure of Statistical Package for Social Sciences (SPSS®) software program (SPSS, 2016). Data were analyzed in one-way ANOVA in CRD. The significant differences among treatment means were tested according to Duncan (1955).

The statistical model used was as follows:

$Y_{ij} = \mu + T_i + e_{ij}$

Where:

Y_{ij}= Observed value of the dependent variable.

μ= Overall mean.

T_i = Effect of the treatments.

e_{ij}= experimental random error.

RESULT AND DISCUSSIONS

Hatchability:

In respect to day 20 and 21 hatchability the results showed highly significant ($P \leq 0.001$) differences among treatments on total hatchability percentages of Alexandria strain eggs **(**Table 1). In-ovo injection of lower BV dose has higher significant hatchability percentage compared to the corresponding values of the control and higher dose groups (89.45, 87.50 and 77.73%, respectively). The present results confirm the previous findings showed that the in-ovo injection of different nutrients, to make good nutritional status for the embryos, improve hatchability percentage of broiler breeder eggs (Al-Shamery & Al-Shuhaib, 2015; Edwards *et al*., 2016). Al-Asadi (2013) reported that in-ovo injection of arginine and lysine in broilers eggs at 18d of incubation increased hatchability percentages. Also, Hassan *et al*. (2018) reported that in ovo administration of indicated that the in-ovo injection of L-arginine or royal jelly enhanced hatchability percentages of Hubbard eggs.

Relative Hatchling weight and Yolk sac:

The significant ($P \leq 0.001$) highest relative chick weight was observed with eggs of control group (75.85%), followed by lower (72.35%) and higher (69.41%) in-ovo BV dose groups. The increase of dose has reduction effect on the relative chick weight. The Bee products has different results with broiler chick weight, since chick weight at hatch improved due to in-ovo injection with royal jelly (Aljumaili, 2012; Moghaddam *et al*. 2014; Hassan *et al*., 2018), while the in-ovo injection of

pollen extract tended to reduce the hatching broiler chicks' weight (Coskun *et al*., 2014). However, Bozbay *et al*. (2016) found that in-ovo injection of propolis has not affected hatchling weight.

The results of table (1) indicated that in-ovo injection of BV doses has acceleration effect on chick hatching time, since the hatching rate at 20-day was more than at 21-day, also the increase of dose increase this rate. This may be explaining the reduction of relative chick weight in both BV treatments. Also, this finding confirms by the observed relative weight of yolk sac, chicks of in-ovo 10 and 20 µg BV/egg doses has lower yolk sac weight percentages compared to control group (17.71, 14.70 and 19.70%, respectively), meaning the yolk consumption was higher in BV treatments.

The early chick hatchers had lower body weight compared with midterm and late hatchers as showed by Lamot *et al.* (2014) with Ross 308 chicks and Wesam Fares *et al.* (2017) with Gimmizah chicks from different flock age. However, El-Nagar (2018) showed insignificant differences in that respect with Alexandria chicks.

In addition, the chick hatching weight is determined by many factors such as genetic, incubator environment, storage length, weight loss in incubator (Schmidt *et al.*, 2009; Wondmeneh *et al.,* 2011) and egg weight has direct effect in that respect (e.g. Alkan *et al.*, 2008; Schmidt *et al.*, 2009; Alabi *et al.*, 2012).

In explanation, the chicken embryo generally needs 21 days (504 h) to complete incubation, including the drying of down (Etches, 1996). The first-hatched chicks remain in the incubator until most of chicks have emerged from the shell. As a consequence, they can spend without feed and water in the hatcher under suboptimal conditions, exposing the chicks to dehydration (Willemsen *et al.*, 2010; Bergoug *et al.,* 2013).

Blood biochemical parameters:

The results of total protein (TP), albumin (Alb), globulin (Glob) and glucose (Glu), concentrations of chick's blood at hatch are shown in Table (2). The TP and Alb values decrease significantly ($P \leq 0.001$) in 10 and 20 BV dose in-ovo groups compared to control group (TP values were 2.60, 2.84 and 3.20 g/dl, and Alb values were 1.54, 165 and 2.07 g/dl, respectively). Whereas the differences being insignificant for Glob values.

There were no significant effects of BV supplementation via drinking water of broiler chicks on TP, Alb, and Glob values as noticed by Han *et al*. (2010). Moreover, Elshater *et al*. (2014) stated that rats after oral administration of BV treatments for 40 days, there was a significant increase in Alb level compared to control group.

In general, Alb concentrations may be used as an indicative of hepatocyte injury (Baghbanzadeh & Decuypere, 2008). Glob levels have been used as source of antibody production and an indicator of immune responses (Javed *et al.*, 1995).

The results of Glu values in table (2) showed that the 20 µg BV in-ovo group has significantly ($P \leq 0.01$) highest value compared to the corresponding values of 10 µg BV and control groups

Energy required for hatching activities comes from glucose provided from glycogen in liver, yolk sac membrane, and muscle, resulting in an increase in plasma glucose between pipping and

hatch (e.g. Christensen *et al.,* 2001; Yadgary & Uni, 2012; van de Ven *et al*. 2013). Also, van de Ven *et al.* (2013) and Lamot *et al.* (2014) suggest that early hatched chickens physiologically differ from midterm and late hatched chickens. However, van de Ven *et al*. (2013) showed that blood glucose level was not affected (ranged 201.67 to 214.18 mg/dl) for the three hatching chick categories (early, midterm and late groups). Based on previous observations, the in-ovo injection of BV helps to enhance the increase of blood Glu level of chicks at hatch.

Thyroid hormones:

Data for triiodothyronine (T3) and thyroxine (T4) concentrations of chick's blood at hatch are shown in table (3). The results showed that in-ovo injection of BV decrease significantly both of T3 and T4 concentrations in Alexandria chick serum at hatch.

The thyroid gland is an endocrine organ secretes hormones (T3 and T4) and several factors known to affect thyroid function such as ambient temperature, food intake, time of day or other environmental factors (Dawson *et al*., 1992). Normal thyroid hormone levels are necessary for adequate development, maintenance and function of both the antibody and cell-mediated immune responses (Cremaschi *et al*., 2000; Klecha *et al*., 2000), and also the basal metabolic rate of mammals and birds (Hulbert & Else, 2004).

Immunological status:

Results of table (4) showed that the spleen relative weight increased significantly in 20µg of BV/egg dose in-ovo group compared to control group. Also, the same trend was observed with bursa relative weight. Whereas, the 10µg of BV/egg dose in-ovo group has insignificant differences on spleen percentage and significant differences in bursa percentage compared to control group.

These two organs are essential to the ontogenetic development of adaptive immunity in chickens (Cooper *et al.*, 1966), and among other of lymphatic system (Ciriaco *et al.,* 2003) are responsible for chicken body defense (e.g. Júnior *et al.,* 2018; Singh, 2019; Miller, 2020), which improve the health condition. The lymphoid system plays a great role to provide the protection from infections with different etiological agents (Kannan *et al.,* 2015).

CONCLUSION

It concluded that the in-ovo injection with 10 µg of BV/egg has better hatchability percentage with Alexandria breeder eggs. The findings of this research call for further studies of BV in-ovo injection levels (less than 10 µg) to determine the optimum concentration of BV for better hatchability and chick quality.

In ovo injection - BV - Hatchability - Immunological Parameters

Table (1): Effect of in-ovo injection of Bee Venom (BV) in fertile eggs of Alexandria breeder on 20- and 21- day hatchability total hatchability, relative chick and yolk sac weights (Means ± SE).

Treatments	Day 20 hatchability/ total hatchability (%)	Day 21 hatchability/ total hatchability (%)	Total hatchability (%)	Relative chick weight/ egg weight (%)	Yolk sac (%)
Control (Non-injected)	64.00	36.00	87.50^{b}±0.26	75.85^{a}±0.18	19.70^{a}±0.35
10 µg BV/egg	82.40	17.60	89.45^{a}±0.20	72.53^{b}±0.21	17.71^{a}±0.78
20 µg BV/egg	85.70	14.30	77.73^{c}±0.45	69.41^{c}±0.23	14.70^{b}±0.85
P-value			0.000	0.000	0.000

[a,b,c] Means having different letters in the same column are significantly different (P≤0.05).

Table (2): Effect of in-ovo injection of Bee Venom (BV) on some blood biochemical parameters on Alexandria chick strain at hatch.

Treatments	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Glucose (mg/dl)
Control (Non-injected)	3.20^{a}±0.10	2.07^{a} ±0.03	1.13±0.07	133.67^{b} ±3.90
10 µg BV/egg	2.60^{b}±0.03	1.54^{b} ±0.03	1.07±0.02	138.67^{b} ±16.08
20 µg BV/egg	2.84^{b}±0.13	1.65^{b} ±0.09	1.19±0.04	196.67^{a} ±17.34
P-value	0.002	0.000	0.282	0.01

[a,b] Means having different letters in the same column are significantly different (P≤0.05).

Table (3): Effect of in ovo injection of Bee Venom (BV) on thyroid hormones of Alexandria chick strain at hatch.

Treatments	T3 (ng/ml)	T4 (ng/ml)
Control (Non-injected)	2.59^{a} ±0.12	14.80^{a} ±0.38
10 µg BV/egg	1.93^{b} ±0.02	12.10^{b} ±0.24
20 µg BV/egg	1.87^{b} ±0.05	10.84^{c} ±0.22
P-value	0.000	0.000

[a,b,c] Means having different letters in the same column are significantly different (P≤0.05).

Table (4): Effect of in ovo injection of Bee Venom (BV) in fertile eggs of Alexandria breeder on slaughter traits at hatch (Means ± SE).

Treatments	Spleen (%)	Bursa (%)
Control (Non-injected)	0.01^{b}±0.000	0.13^{b}±0.002
10 µg BV/egg	0.03^{ab}±0.002	0.10^{c}±0.000
20 µg BV/egg	0.09^{a}±0.036	0.15^{a}±0.006
P-value	0.05	0.001

^{a,b,c} Means having different letters in the same column are significantly different (P≤0.05).

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الملخص العربي

تأثير حقن سم النحل في البيض على نسبة الفقس وبعض المعايير المناعية لكتاكيت سلالة الإسكندرية عند الفقس

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يحاول الباحثون تنشيط الجهاز المناعي للدواجن من خلال استخدام المنشطات المناعية الطبيعية بما في ذلك سم النحل. وهناك ندرة في الدراسات المرجعية عن استخدام حقن سم النحل في البيض. وتهدف هذه الدراسة إلى تقييم استخدام سم النحل المجفد (١٠ و٢٠ ميكروجرام/ بيضة) في حقن بيض التفريخ لسلالة الإسكندرية ودراسة تأثير حقنه عند اليوم الـ ١٨ للتحضين على نسبة الفقس، ووزن الكتكوت لوزن البيضة، والاستجابة المناعية للكتاكيت.
اعتمدت جرعات سم النحل المستخدمة في هذه العمل على دراسة تجريبية قبل التجربة الرئيسية مباشرة لتحديد الجرعة المناسبة. تم استخدام إجمالي عدد ٩٠٠ بيضة مخصبة في اليوم الـ ١٨ من الحضانة. تم وزن البيض وتوزيعه عشوائياً إلى ثلاثة مجموعات تجريبية على النحو التالي: مجموعة التحكم، وجرعة منخفضة من سم النحل وجرعة عالية من سم النحل تحتوي كل منها على ٣٠٠ بيضة (١٠٠ بيضة/ تجربة/ معاملة). وأظهرت النتائج: أن حقن البيض بـ ١٠ ميكروجرام/ بيضة أدى إلى زيادة معنوية في نسبة الفقس (٨٩.٤٥%) مقارنة بالكنترول (٨٧.٥%) وحقن البيض بـ ٢٠ ميكروجرام/ بيضة (٧٧.٧٣%). لوحظ أعلى وزن نسبي للكتاكيت مع مجموعة الكنترول يليها الجرعة الأقل ثم الأعلى من سم النحل، فزيادة الجرعة أدت لإنخفاض الوزن النسبي للكتاكيت. أدى الحقن بسم النحل بالمستويات المختلفة إلى تقليل كلاً من البروتين الكلي والألبيومين ($P \leq 0.001$)، كما انخفض أيضاً مستوى هرموني التراي أيودوثريونين (T3) والثيروكسين (T4) بشكل معنوي عن الكنترول في سيرم كتاكيت الأسكندرية عند الفقس. بالإضافة إلى ذلك، أظهرت النتائج زيادة ملحوظة في الوزن النسبي للطحال مع المجموعة المحقونة بمعدل ٢٠ ميكروجرام/ بيضة مقارنة بالكنترول. وأيضا، لوحظ نفس الاتجاه مع الوزن النسبي لغدة البرسا مقارنة بالكنترول.
الخلاصة: أن حقن بيض التفريخ بمعدل ١٠ ميكروجرام لكل بيضة ادى إلى تحسين نسبة الفقس بشكل ملحوظ بالنسبة لبيض سلالة الإسكندرية ويمكن أن نوصي باستخدامه كحقن في بيض التفريخ وذلك لزيادة نسبة الفقس. والنتائج المتحصل عليها من هذا البحث هي نتائج واعدة وتحتاج إلى دراسات مستقبلية على حقن بيض التفريخ بسم النحل بمعدلات أقل من ١٠ ميكروجرام لكل بيضة وكذلك باستخدام بيض تفريخ لبعض السلالات التجارية الأخرى.