Effects of *in ovo* supplementation with nano-nutrition (L-Arginine conjugated with Ag NPs) on muscle growth, immune response and heat shock proteins at different chicken embryonic development stages

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Simple Summary

In the current study, we have analyzed that the *in ovo* injection of conjugated L-arginine (L-Arg) with Silver nanoparticles (Ag NPs) at three different embryonic developments (eighth day (d), 14th day, and 18th day) could be positive effects on survival rate, hatching rate. In addition, the immunoglobulin (IgM) levels in serum and heat shock proteins such as HSP-60 and 70 as well muscle development markers mainly myoD and myogenin expressions were significantly up-regulated in 14d injection. Moreover, the levels of SGOT and SGPT were significantly retained to the normal level at 14d injection when compared to 8d and 18d injection. Hence, 14th day would be suitable day for injection of L-Arg with Ag NPs to promote the survival rate, hatching rate, immune system and muscle growth. Also, it can be a better choice to make nano-encapsulated nutrients to carriers of nutrients without any toxicity on 14d injection.

Abstract

The aim of the study was to analyze the *in ovo* injection of chemically and biologically synthesized silver nano-particles (Ag NPs) using *Brassica oleracea L. var capitate f. Rubra,* (BOL) conjugation with L-Arginine (L-Arg) on the immune, muscle growth, survivability and hatchability of the broiler chickens. L-Arg (100 µg) conjugated with 1000 µg of Ag NPs synthesized by (BOL)-extract and L-Arg (100 µg) conjugated with 100 µg of Ag NPs chemically synthesized were injected into fertile eggs at 8d, 14d and 18d of incubation. Living embryo and hatched chicks were calculated. Survivability and hatchability were not affected by the injected dose of L-Arg (100 µg) with 1000 µg (BOL-Ag NPs) and L-Arg (100 µg) with 100 µg (C-Ag NPs) but it significantly improved when the eggs were injected on day 14 of incubation compared with those injected on days 8 or 18. Moreover, the protein expression of muscle development markers

such as myogenin and myoD were significantly up-related in 14 d of incubation whereas the heat shock proteins (HSPs) such as HSP-60 and HSP-70 were significantly up-regulated in 18 d incubation. In addition, the *in ovo* injection on 18 d significantly increased the serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) as well the immunoglobulin (IgM) levels were increased in 14d incubation period in serum at the same concentration.

Keywords: nanoparticles; silver; L-arginine; conjugation; and muscle growth

1. Introduction

Chickens have been genetically improved for increased body weight gain, growth rate, and breast muscle mass to meet demands of consumers [1]. Muscle growth and development are closely linked to body weight which is consist into two mechanisms such as hyperplasia and hypertrophy both are increased during embryogenesis. During embryonic muscle development are three distinct stages: the formation of myoblasts, the fusion of myoblasts to form myotubes, and the conversion of myotubes to form myofibers [2].

Immediate growth performance can be improved by in-ovo feeding, a technique that can add exogenous nutrition [3]. Similarly, the survival, hatchability, and growth performance of chickens can be increased by *in ovo* injection [4]. In recent years, the administration of amino acids into fertilized broiler eggs via *in ovo* feeding has provided poultry companies with an alternative method to increase hatchability and muscle growth weight of newly hatched chicks [5,6]. L-Arginine (L-Arg) is classified as an essential amino acid. It has been reported that *in ovo* administration of L-Arg in the embryonic phase could increase the growth rate and muscle mass because L-Arg can stimulate the release of growth hormone [7,8]. Amino acids are important substrates for glycogen synthesis which may limit the availability of amino acids for protein synthesis. This condition is believed to occur because of the optimal development of the embryo during embryogenesis. However, chicken embryo development occurs inside the egg. Thus, we need a right method to add exogenous nutrients into the egg.

Nanoparticles (NPs) are of interest due to their use in the prevention of infectious avian influenza viruses which can spread rapidly to poultry flocks. They need further research, particularly with regard to their effects in biological systems. Recent studies have shown that plant

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mediated synthesized Ag NPs are superior choices to other amino acids or proteins with the aim to produce a new method of *in ovo* nano-nutrition that avoids the toxicity of chemically synthesized Ag NPs [9]. *In ovo* supplementation of Ag NPs conjugated with glutamine (100 mg/L.) can improve the growth and immune status of embryos and chicks. Furthermore, Ag NPs conjugated with hydroxyproline can enhance the development of blood vessels [10]. *In ovo* injection of Ag NPs and amino acid complexes can enhance both innate and adaptive immunity in chicken [11].

Modern broiler lines are intensively selected for a higher growth rate with increased size of muscles, including pectoral muscles [12]. This leads to an enhanced requirement of chicken embryos for energy and protein, consequently leading to imbalance between requirement and reserves of nutrients stored within eggs. They may limit maximal (according to genotype) growth and development of chicken embryos. Some authors have indicated that concentrations of certain amino acids in eggs are insufficient to fully support embryonic development [13,14]. Furthermore, because of limited carbohydrate storage in eggs, amino acids are important substrates for glycogen synthesis which may limit the availability of amino acids for protein synthesis [15]. It has been demonstrated that supplying embryos with extra nutrients and energy could enhance *in ovo* nutrition [16,17]. Recently, it has been shown that *in ovo* administration of L-glutamine (25 mg/ml) to chicken embryos can increase mRNA and protein levels of vascular endothelial growth factor (VEGF-A) [18] which is responsible for endothelial cell proliferation. In addition, VEGF-A can stimulate vasculogenesis and angiogenesis[21] and affect pectoral muscles morphology [10].

Several genes associated with cellular interactions and differentiation during organogenesis of the eye, ear, brain, skin, and tissues such as bones and cartilages are either transiently expressed or initiate expression during later stages of embryogenesis [19].

This pattern of development is regulated and controlled by a variety of genes. It depends on the availability of nutrients stored within eggs. Consequently, an adequate supply of nutrients is critical for embryonic development. To assure an adequate nutrient content in eggs, *in ovo* administration of nutrients is one method that can increases hatching weight and the size of breast muscle [16,17,20]. However, this method has drawbacks, including interference with embryo homeostasis and the risk of microbial hazards. In addition, it can affect the proper transport and distribution of supplemented nutrients [10].

Muscle development is mainly determined during embryogenesis. Consequently, the final number of muscle fibers is accomplished in prenatal and early post hatch periods [2]. Moreover, muscle maturation during embryogenesis is dependent on the development of a vessel network, which provides cells with oxygen and nutrients. Cu may indirectly interfere with the molecular status of muscle maturation during embryogenesis by affecting myoblast determination protein 1 (MyoD1) and paired box protein 7 (Pax7) [21].

Administration of L-arginine at a dose of 0.7% in turkeys[20] and 1.0% in quail by *in-ovo* feeding could increase body weight and post-hatch performance. Up to date, there have been no attempts to address obstacles that impede the manipulation of stages of chick embryos *in ovo* by treatment with conjugated silver nanoparticles (Ag NPs) with L-Arginine (L-Arg). Therefore, the aim of the present study was to investigate the effect of *in ovo* injection of conjugated L-Arg to eggs after different incubation time on hatchability, survivability, and muscle growth markers of 1-d-old chicks.

2.   Materials and Methods

2.1. Ethics Statement

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) with the approval from ethical committee of Chonbuk National University, Korea Republic (CBNU 2015048).

2.2. Chemicals

Silver nanoparticles (Ag NPs) and L-Arg, were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Antibodies were purchased from ENZO Life Science (Farmingdale, NY, USA). Chemiluminescent for band detection was bought from Thermo Scientific (Rockford, IL, USA). All laboratory glassware was acquired from Falcon Lab ware (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.3. Synthesis of Ag NPs using BOL extracts

Ten grams of fresh and healthy *Brassica oleracea L. var capitate f. Rubra* (BOL) leaves were cleaned with tap water, followed by cleaning with distilled water (D.H_2O) several times to remove any external particles adhered onto their surface. They were then boiled in 100 ml of D.H_2O for 5 min in a microwave oven by the method of [22]. The resulting extract was filtered through a Whatman filter paper. The reaction mixture containing 6 ml of extract and 44 ml of D.H_2O was added to 1 mM $AgNO_3$ to a final reaction volume of 50 ml and kept in dark place for four hours at room temperature. A control setup with only leaves' extract was also maintained throughout the experiment.

2.4.Synthesis of Ag NPs' composites using polyvinylpyrrolidone (PVP)

For the synthesis of Ag nanoparticles, a Sharp microwave oven (model R-259) was used. In a typical procedure, 10 ml of 1% (w/v) ethanolic solution of PVP and 0.2 ml of 0.1 M AgNO3 in a 25 ml closed conical flask were placed in a microwave oven operating at 100% power of 800 W with a frequency of 2,450 MHz for 5s. The colorless solution instantaneously turned into a characteristic pale yellow color, indicating the formation of Ag NPs. The advantage of a microwave-mediated synthesis over conventional heating is that it has improved reaction kinetics generally by a magnitude of one or two due to rapid initial heating and the generation of localized high-temperature zones at reaction sites [23].

2.5.Characterization of Ag NPs

Preliminary characterization of Ag NPs done through visual observation for change in color. An aliquot of the reaction mixture was analyzed using UV-Vis spectroscopy (UV-1800, Shimazdu Corp., Kyoto, Japan) in wavelength range of 300 to 700 nm. The sample was centrifuged (15,000 rpm for 20 min) the supernatant was throw-out and the pellet was collected. For electron microscopic studies 25 µl of the Ag NPs was drop coated on a copper grid and the images of NPs were studied using a high-resolution transmission electron microscope (HR-TEM) (S-4800, Hitachi, Japan). For X-ray diffraction (XRD) studies, dried NPs were coated on XRD grid and the spectra were recorded using powder X-ray diffractometer (D/Max 2500, Rigaku, Japan).) and the resulting powder was used for further examinations.

2.6. Experimental Design and Incubation

In this study, fertile broiler eggs (1000) of *Ross* were obtained from Samhwa-Won Jong, South Korea. The eggs were numbered and weighed (60 ± 1.36 g) individually, abnormally weighted eggs were discarded from the experiment. The eggs were candling on 8th d for checking living embryos, the non-fertile eggs were discarded from the incubator at the 8th d of incubation. The 880 fertilized eggs were selected and randomly divided into ten groups ($4 \times 20 \times 3$ = replication × eggs × injection) (Table 1). On day 8, 14 and 18 of incubation, the blunt (air space/air sac) side of the egg was determined with a marker pen and disinfected (sterilization) with cotton swabs with 70% ethanol. After that *in ovo* administration of L-Arg (100 µg/100 µL/egg); conjugation of L-Arg (100 µg) with BOL extract synthesized Ag NPs (1000 µg/100 µL/egg); conjugation of L-Arg (100 µg) with chemical synthesis Ag NPs (100 µg/100 µL/egg) on eighth, 14th, and 18th day, respectively, through the air sac of the eggs using a 21-gauge needle. Immediately after the injection, the hole was sealed with liquid paraffin. Eggs were then placed in an incubator for 18 days under standard conditions (temperature of 37.8°C; humidity of 60%). Eggs in the setting compartment automatically turned 90∘ every 3 h (eight times a day). On the 18th day of incubation, eggs were moved to hatching boxes promptly placed in a hatching incubator with humidity maintained at 60% and temperature set at 37°C. Egg weight, chick weight, chick weight to egg weight ratio and hatchability were recorded on 21 d.

Table 1. Experimental design for dose (L-Arg and conjugate with BOL-Ag NPs and C-Ag NPs) with different embryonic stage (eighth day, 14th day, and 18th day).

Group	Dosage	No. of Eggs x No. of Replication	Total No. of Eggs
1C	Control	20×4	80

1C1 (8th day)	PBS/100 µL/egg	20 × 4	80
1Tb (8th day)	100 µg (L-Arg) +1000 µg (BOL-Ag NPs)/100 µL/egg	20 × 4	80
1Tc (8th day)	100 µg (L-Arg) +100 µg (C-Ag NPs)/100 µL/egg	20 × 4	80
2C1 (14th day)	PBS/100 µL/egg	20 × 4	80
2Tb (14th day)	100 µg (L-Arg) +1000 µg (BOL-Ag NPs)/100 µL/egg	20 × 4	80
2Tc (14th day)	100 µg (L-Arg) +100 µg (C-Ag NPs)/100 µL/egg	20 × 4	80
3C1 (18th day)	PBS/100 µL/egg	20 × 4	80
3Tb (18th day)	100 µg (L-Arg) +1000 µg (BOL-Ag NPs)/100 µL/egg	20 × 4	80
3Tc (18th day)	100 µg (L-Arg) +100 µg (C-Ag NPs)/100 µL/egg	20 × 4	80

Note: *In Ovo* Injection and Treatment Groups

2.7. Survival Rate Measurement

Embryos' survival rates during the incubation period were measured on the eighth day. After *in ovo* injection, the number of live eggs were recorded from the total number of each treatment group on 18d. The percentage of survival rate was calculated with the following equation (1):

$$\text{Survival rate \%} = \frac{\text{No. of live eggs}}{\text{No. of fertilie eggs}} \times 100 \qquad (1)$$

2.8. Hatching Rate and Body Weight Measurements

On the 21st day, hatched chicks were moved from the hatcher incubator to hatching boxes to determine hatching rates. The hatched chicks were kept without feed and water at 32°C and then the groups were weighed to record their live body and liver weight. The hatching rate was calculated with the following equation (2):

$$\text{Hatching rate \%} = \frac{\text{No. of chicks hatched on 21st day}}{\text{No. of fertilie eggs that were } in\ ovo \text{ fed}} \times 100 \qquad (2)$$

2.9. Biochemical Indices

At the end of the experimental period, three hatched chicks of each replicate group per treatment were randomly selected and then sacrificed under anesthesia (diethyl ether) for sampling. Blood was collected from the jugular vein into tubes for serum separation. The breast muscle was collected and washed in ice-cold saline. It was then homogenized with 0.1 M of cold phosphate buffer, pH 7.4. Assays were done for serum and liver. Concentrations of serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) were measured using commercial kits (Asan Pharamaceuticals Co., Ltd., Seoul, Korea) following the manufacture's specification.

2.10. Measurement of IgM Concentration in Serum

Serum samples (4μl) were (dilute samples 1:60,000 into 1X Diluent N) collected from three hatched chicks of each replicate group per treatment chicks to determine serum immunoglobulin (Ig) M levels using chicken IgM ELISA kit (Abcam, Suite B2304, Cambridge, MA, USA) following the manufacture's specification. IgM levels were analyzed based on absorbance values measured at 450 nm.

2.11. Analysis of Heat-Shock Proteins (HSPs) and Muscle related markers by Western Blot

Proteins were extracted from 100 mg of muscle samples using radioimmunoprecipitation assay (RIPA) buffer to determine protein expression levels of HSP-60, HSP-70, myoD, and myogenin in experimental groups. Protein concentrations were determined using a BIO-RAD protein assay

kit (BIO-RAD). Extract samples containing 50 µg of protein were solubilized in *Laemmli buffer*, separated by 12% acrylamide gel, and then transferred to Hybond-P PVDF membranes (GE Healthcare Inc., Amersham, UK) for 60 min at 200 mA. These PVDF membranes were blocked with 5% skimmed milk powder in 0.5 M of Tris-buffered saline (pH 7.4) with 0.05% Tween 20 (TBST) at room temperature for 2 h. Western immunoblotting with HSP-60, HSP-70, Myo-D, and myogenin primary antibodies (1:2500 dilution) was performed at 4°C overnight. After washing three times with TBST, these membranes were incubated with HRP-conjugated secondary antibodies (1:5000 dilutions) at room temperature for 60 min and then washed three times with TBST (10 min each wash). Protein bands were visualized using a Chemiluminescent assay kit from Thermo Scientific for 1–5 min. Bands were imaged with an iBright™ CL1000 Imaging System (Invitrogen in Thermo Fisher Scientific, Life Technologies Korea LLC, Jeonju-si, Jeollabuk-do, Korea) and quantified using Image J Software. The relative density of the band was normalized to that of β-actin as an internal control.

3. Statistical Analysis

Data for biochemical analyses are expressed as mean ± SD (n=12). Statistical evaluation was carried out by Tukey's HSD test post hoc following ANOVAs followed by Duncan's multiple range test (DMRT) with the SAS® software, version 9.4 (Institute of INC, North Carolina, USA).

4. Results and Discussion

4.1. Characteristics of Ag NPs

The aqueous BOL extract is pale pink in color. Addition of AgNO$_3$ has changed the color of the solution to reddish brown, within 2 h. UV–visible spectra of the solution exhibit absorption

maxima at 430 nm for Ag NPs, which indicates the formation of Ag NPs by chemical and BOL

extract (Fig. 1). The HR-TEM images (Fig. 2) of the Ag NPs reveal that they are well dispersed

without much agglomeration. The Ag NPs are spherical in shape with an average size ranging from

5 to 40 nm (Fig. 1). ICP-MS analysis reveals that the concentration of BOL-Ag NPs and C-Ag

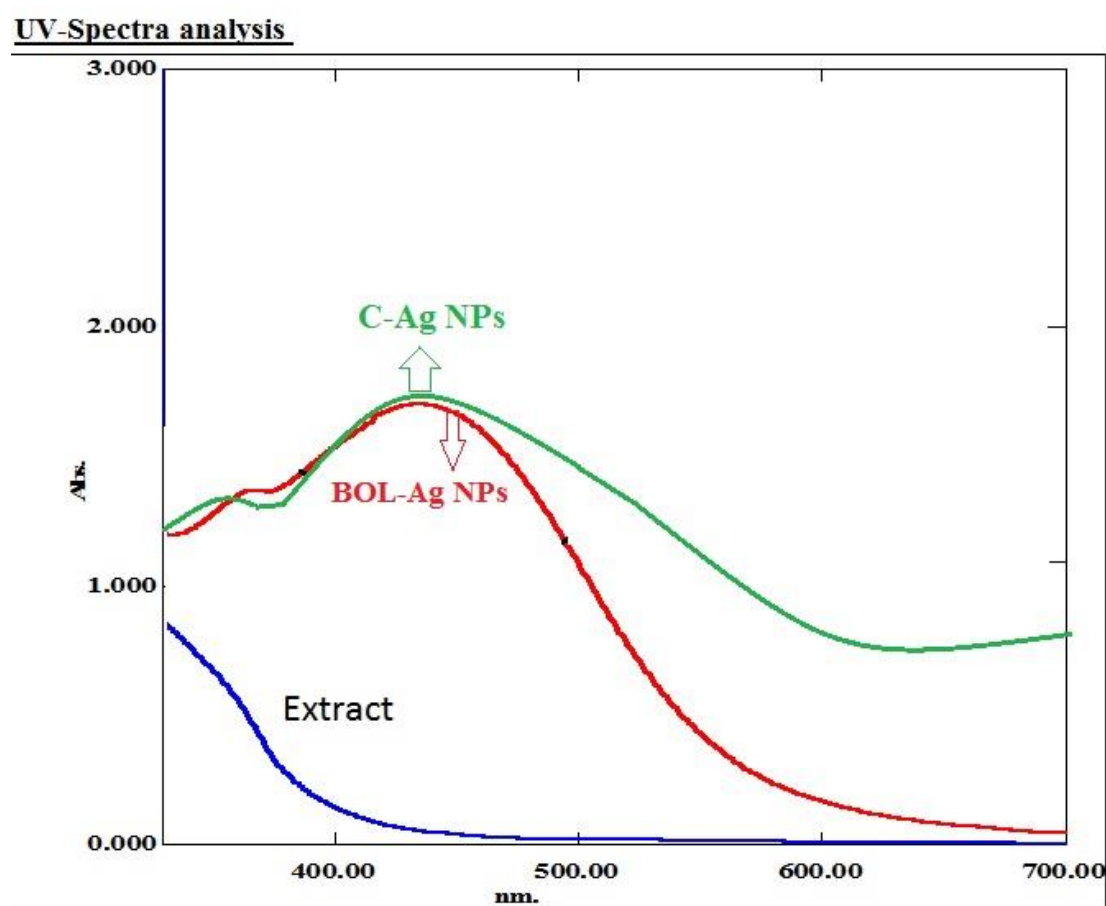NPs is 514 and 628 μg/mg of powder, respectively.

Figure 1

Figure 1. UV–Vis spectra of biosynthesized Ag NPs (1 mM AgNO$_3$ in aqueous BOL extract) and chemical synthesized Ag NPs observed at 430 nm.

Figure 2

Figure 2. TEM images of Ag NPs (BOL-Ag NPs and C-Ag NPs), L-Arg, and bio-complex of L-Arg with BOL-Ag NPs and L-Arg with C-Ag NPs.

4.2. FT-IR

Prior to Fourier-transform infrared (FT-IR) analysis, the samples were mixed with potassium bromide with appropriate ratio and was compressed in semi-transparent disk. Such samples were exploited for analysis. The FT-IR analysis was performed in the range between 4000-400 cm^{-1}. L-Arg exhibits the peaks at 3426 cm^{-1} is related to stretching vibrations of O-H. The combination of peaks positioned at 2913 and 2845 cm^{-1} are attributed to NH_3^+/C-H (asymmetrical bending) and NH_3^+ (torsional oscillation). The absorption band at 1650 cm^{-1} is ascribed to symmetric C=O bond. Amide stretching of L-Arg found around 1318 cm^{-1}. The stretching vibrations of C-C in backbone structure of L-Arg can be seen at 1225 and 1057 cm^{-1}. Besides, the NH peak of L-Arg appears at 770 cm^{-1} FTIR spectrum of Ag nanoparticles obtained from BOL-extract synthesis is shown that

the peaks at 3550, 2926, 1635 and 797 are related to O-H, C-H, C-N, and N-H, respectively. These

all peaks indicate that still some green extract present in Ag nanoparticles. L-arginine+Ag (BOL)

shows the peaks of both L arginine and Ag NPs indicating the successful combination of Ag and
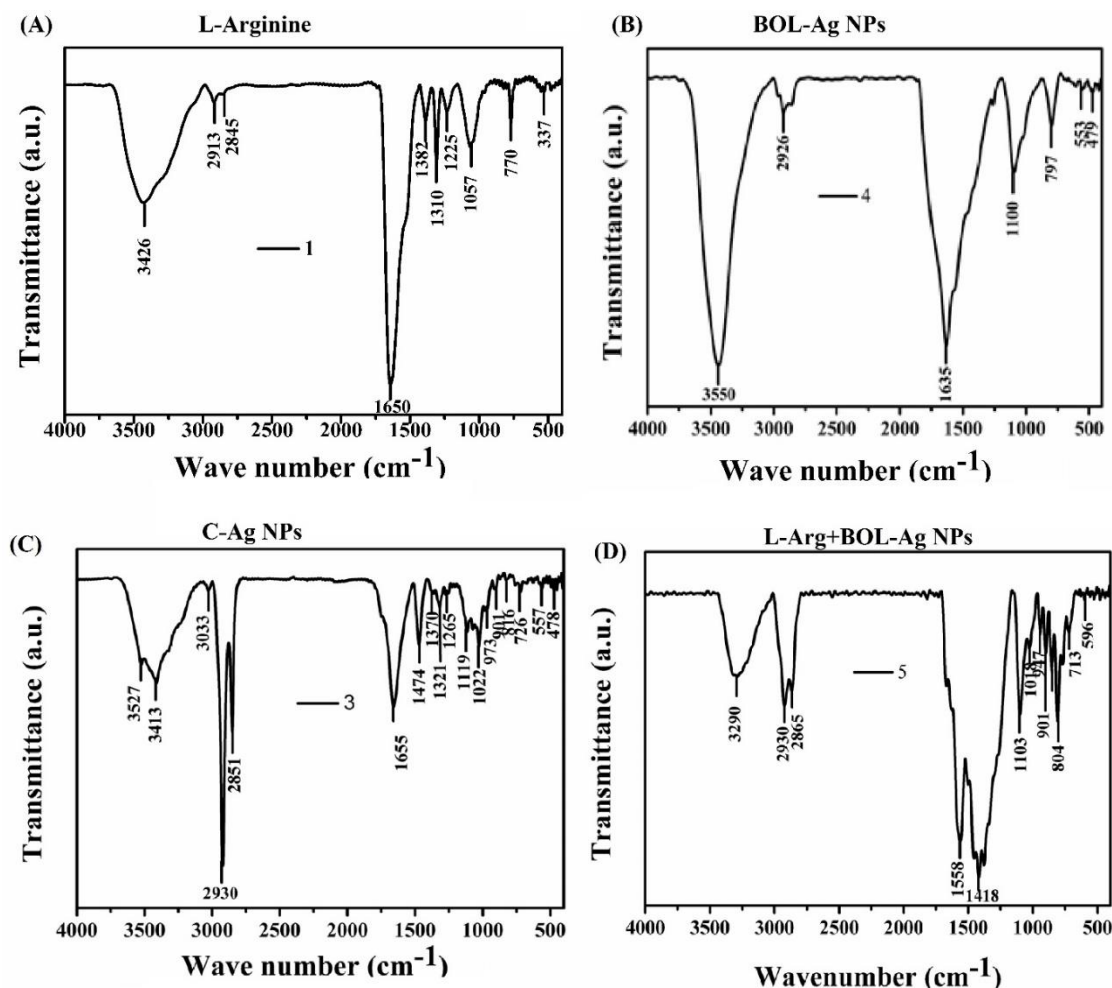
L-Arg.

Figure 3

Figure 3. Nature of functional groups and structure of the BOL extract and Ag NPs and conjugation

of L-Arg with BOL-Ag NPs assessed by FT-IR spectra.

4.3. XRD

XRD patterns of both chemical synthesized Ag and BOL synthesized Ag reveals the peaks at 2θ=39.1, 43.2, 65.1 and 78.1 are found at (111), (200), (220) and (311) reflections of metallic Ag nanoparticles (JCDPS File No. 04-0783) (file:///C:/Users/User/Downloads/Room_temperature_sintering_of_printer_silver_nanop.pdf). In the case of green synthesized Ag, the additional peaks of green reducing agent were also observed. For the spectrum of L-arginine+Ag (BOL), both L arginine and Ag peaks were found, demonstrating the combination of Ag with L-arginine.
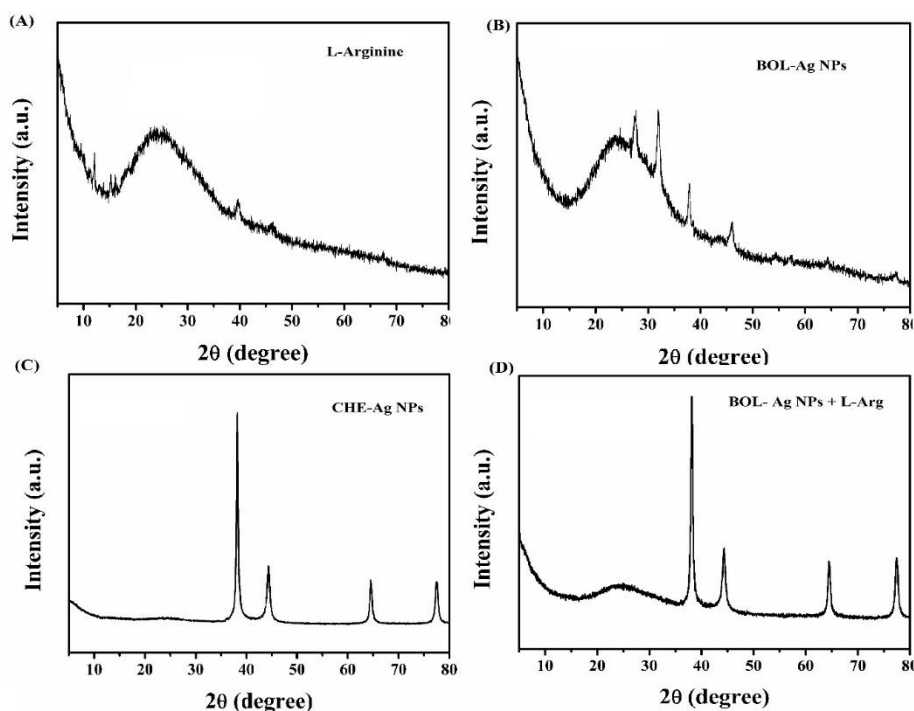

Figure 4

Figure 4. (A) Energy-dispersive X-ray spectroscopy profile of L-arginine; (B) biosynthesized (BOL) Ag NPs; (C) CHE-Ag NPs; (D Conjugation of L-Arg with BOL-Ag NPs).

4.4. In ovo study

In the present work, we studied the effect of the conjugation of BOL-Ag NPs with L-Arginine or the conjugation of C-Ag NPs with L-Arginine by *in ovo* injecting them to three different embryonic stages on survival and hatching rates. Results are shown in Fig. 5 and Fig 6. The survival rate was significantly increased in 2Tb and 2Tc (mentioned in Table 1) groups compared to other treatment groups. Results showed that the survival rate was different depending on the injection time of embryogenesis (8th, 14th, or 18th day injection) with the same concentration (1000 µg for BOL-Ag NPs and 100 µg for C-Ag NPs). Ag NPs, glutamine (2.5mg), and the complex of Ag NPs/Glu were non-toxic and did not affect the growth or the development of chicken embryo. Furthermore, Ag NPs showed no harmful effects on the growth or development of embryos when these nanoparticles were used at concentrations below 100 μg/ml [24]. In addition, existing evidence demonstrated that on 14d injection of L-carnitine results the hatchability was increased than 16d and 18d of injection. No significant were observed in the *in ovo* L-carnitine injection (up to 8 mg dissolved in 100 μL of a commercial diluent) at the 18th day of incubation on hatchability of fertilized eggs in a young broiler breeder [25]. Moreover, the injection of chicken eggs with 100 μg /egg pyridoxine at 14 days of incubation period resulted in apparently higher hatchability than in un-injected control [26]. The 14th day incubation can be an appropriate time for improving the embryonic viability through the development of vessel network (embryo until day 15) [27]. The same mechanism might have occurred in our current experiment. In our experiment, the survival rate in the group injected on the 14th day of embryogenesis was not decreased after the injection. Instead, it was increased compared to that in the group injected on the 8th day or the 18th day of embryogenesis with the same dose of BOL-Ag NPs or C-Ag NPs.
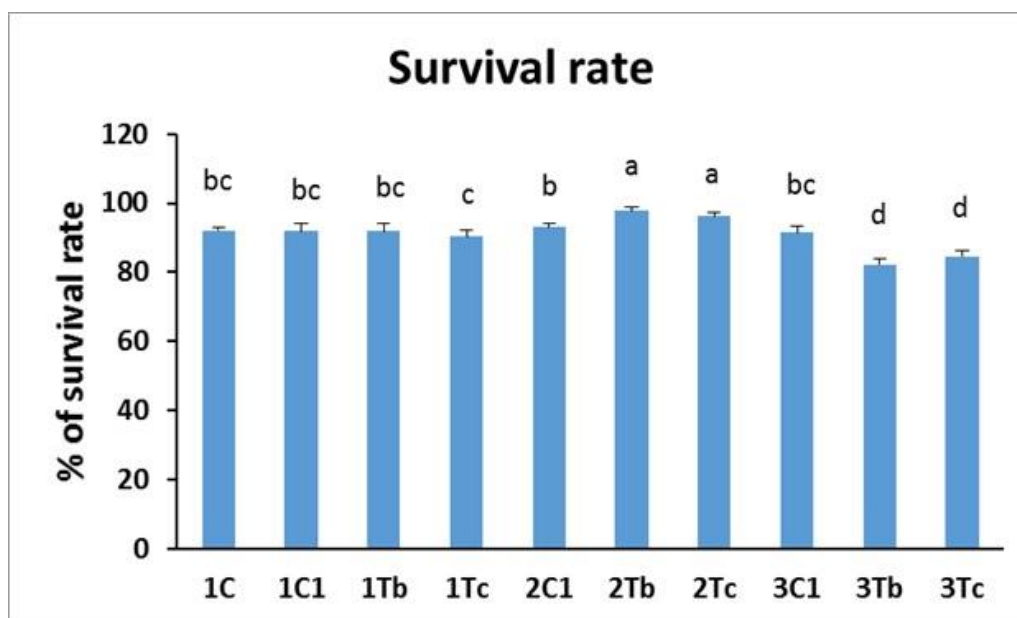
Figure 5

Figure 5. Effects of *in ovo* injections of L-Arg (100 µg) with 1000 µg (BOL-Ag NPs) or L-Arg (100 µg) with 100 µg (C-Ag NPs) at different developmental embryonic stages on survival rate. Small characters indicate significant differences among experimental groups at $p < 0.05$. Values are presented as mean ± SD from 12 determinations.

Subsequently, we measured the hatchability rate. Results indicated that hatching rates were significantly different among groups. Significantly ($p < 0.05$) higher hatchability was recorded when both conjugated amino acids were injected on the 14th day than other groups. *In ovo* administration of nanoparticles acting as bioactive agents and carries of nutrients may be seen as a new method of nano-nutrition [10]. Recent studies have shown that *in ovo* supplementation of Ag NPs [28], either alone or in combination with glutamine (25 mg/ml), can improve the growth and immunity status of embryos and chicks. The higher hatchability in the high immunity group after *in ovo* amino acid injection might be due to the availability of free amino acid through *in ovo* injection [29]. Such free amino acid might have stimulated embryonic gluconeogenesis which in turn helps hatching activities. Previous results showed that the hatchability of fertile eggs is not affected by *in ovo* L-carnitine administration (500 or 1000 µmol) at 18 days of incubation [30]. On the other hand, the dietary supplementation with L-carnitine (50, 100, or 150 mg) caused

significant increases in egg fertility and hatchability with significant decrease of embryonic mortality on the 5th day of incubation [31]. *In ovo* injection on the 14th day of incubation could utilize amino acids [5]. These injected amino acids might have stimulated higher protein synthesis with lower protein degradation. Furthermore, Ag NPs conjugated with hydroxyproline can enhance the development of blood vessels [10]. Injecting the above two things on 14th day of incubated embryo might have led to the development of blood vessels. The conjugated BOL-Ag NPs and C-Ag NPs might have travelled through these developed blood vessels to promote protein synthesis and improve hatching rate and survival rate. *In ovo* administration of Ag NPs nanoparticles up-regulates the expression of fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGF) that are needed for satellite cell proliferation, differentiation, vasculogenesis, and angiogenesis in tissues [32].
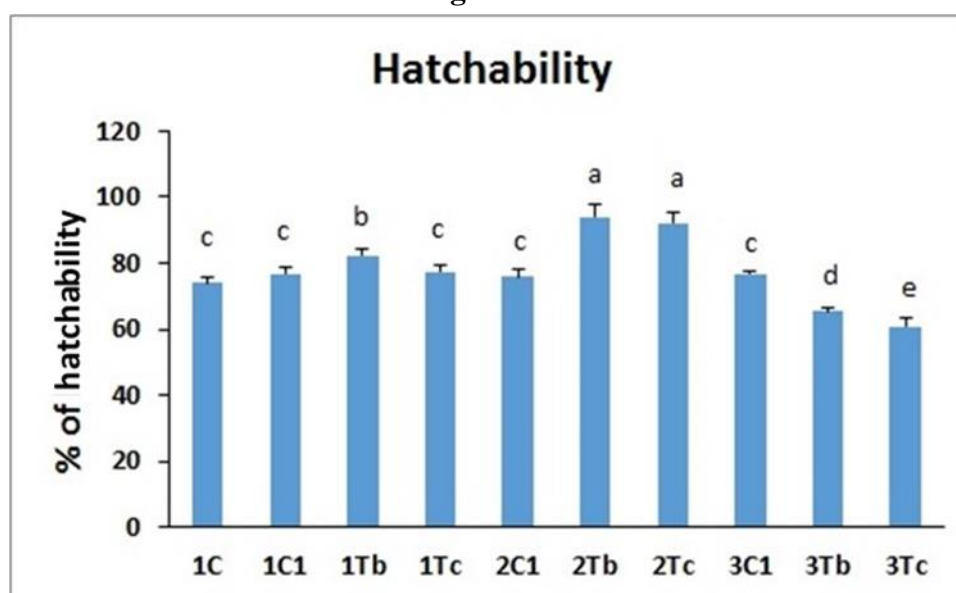
Figure 6

Figure 6. Effects of *in ovo* injections of L-Arg (100 μg) with 1000 μg (BOL-Ag NPs) or L-Arg (100 μg) with 100 μg (C-Ag NPs) at different developmental embryonic stages on hatching rate. Small characters indicate significant differences among experimental groups at *p* < 0.05. Values are presented as mean ± SD from 12 determinations.

4.5. Body weight

The concentration of BOL-Ag NPs and C-Ag NPs and were injected at three different time periods of embryogenesis. Body weights of hatched chicks in 2Tb and 2Tc groups were significantly ($p <$ 0.05) higher than those of other groups (Fig. 7). Body weights were low when conjugated amino acids were injected on the 18th day of embryonic stage. Ag NPs with size less than 10 nm can penetrate into tissues and cells and localise inside cells [33,34]. *In ovo* administration of nanoparticles acting as bioactive agents and carries of nutrients may be seen as a new method of nano-nutrition [10]. Recent studies have shown that *in ovo* supplementation of Ag NPs [28], either alone or in combination with glutamine (25 mg/ml), can improve the body weight and immunity status of embryos and chicks. Hence, *in ovo* injection of Ag NPs with L-Arginine might have increased the body weight through Ag NPs that might have carried L-Arginine into the tissue and inside the cell to increase the body weight in 2Tb and 2Tc groups. Some investigations have demonstrated that Ag NPs do not have toxicity or affect the immune responses [35]. It has also been reported that Ag NPs, glutamine (25 mg/ml), and a complex of Ag NPs and glutamine do not affect embryos. However, the muscle percentage in the group treated with Ag NPs+glutamine is significantly increased compared to that in treated with Ag NPs alone [36]. Similarly, *in ovo* injection of Gly and Pro might have resulted in higher body weights of chicks in the 14th day injection group.
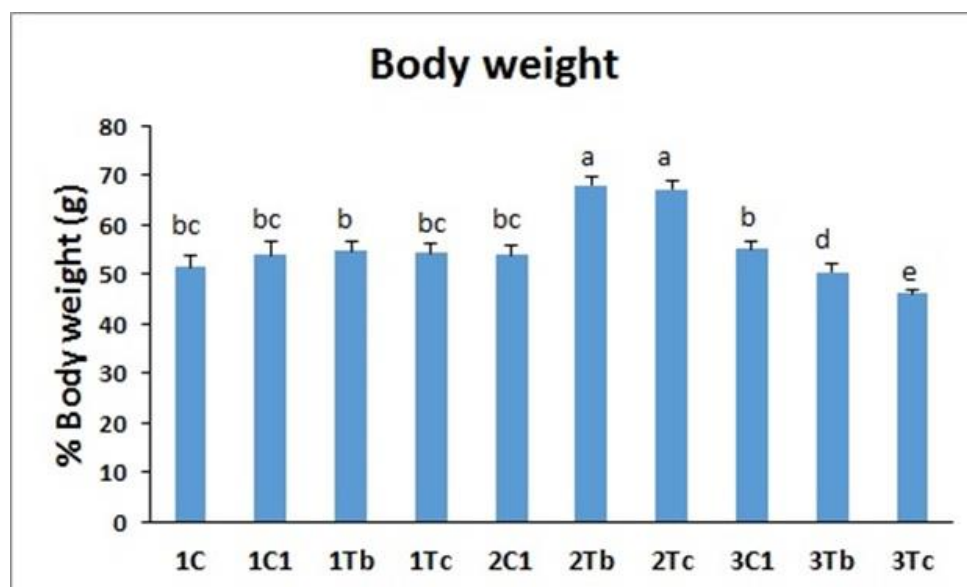
Figure 7

Figure 7. Effects of *in ovo* injections L-Arg (100 µg) with 1000 µg (BOL-Ag NPs) or L-Arg (100 µg) with 100 µg (C-Ag NPs) at different developmental embryonic stages on chick weight. Small characters indicate significant differences among experimental groups at *p* < 0.05. Values are presented as mean ± SD from 12 determinations.

Ag NPs and the complex with glutamine improve nucleic acid synthesis and metabolic programing within cell, increasing their fibre area and consequently muscle mass [10]. The same mechanism might be occurred in our current study that the conjugated L-Arg could be increase the number of nuclei per cell and by increasing fiber area to increase the growth performance. Injection on the 14th day of embryonic stage can utilize the amino acids and promote protein synthesis[14]. These results suggest that the 14th day of injection could be a good time to promote the growth factor and increase the body weight of chicks.

4.6. Biochemical indices (SGOT and SGPT)

Subsequently, we measured activities of hepatic enzymes (SGOT and SGPT) in blood serum as markers of functional and morphological states of the liver. Our results indicated that levels of

SGOT and SGPT were significantly influenced by embryonic stages of injection for both BOL-AG NPs and C-Ag NPs. Biochemical indices (SGOT and SGPT) were significantly ($p < 0.05$) decreased in 2Tb and 2Tc groups than in other groups which did not show significant effects of treatments (Fig. 8). Previously, we have shown that treatment with BOL-Ag NPs (1000µg) or C-Ag NPs (100µg) did not have any toxic effect on the liver. Consequently, only SGPT levels were significantly increased when embryos were treated with C-Ag NPs (5000 µg). Thus, increasing the concentration of Ag NPs could increase levels of SGOT and SGPT in the blood which can lead to liver function damage. In fact, free radicals from Ag NPs can attack hepatocytes and release stored SGOT and SGPT to re-enter the blood serum [37].
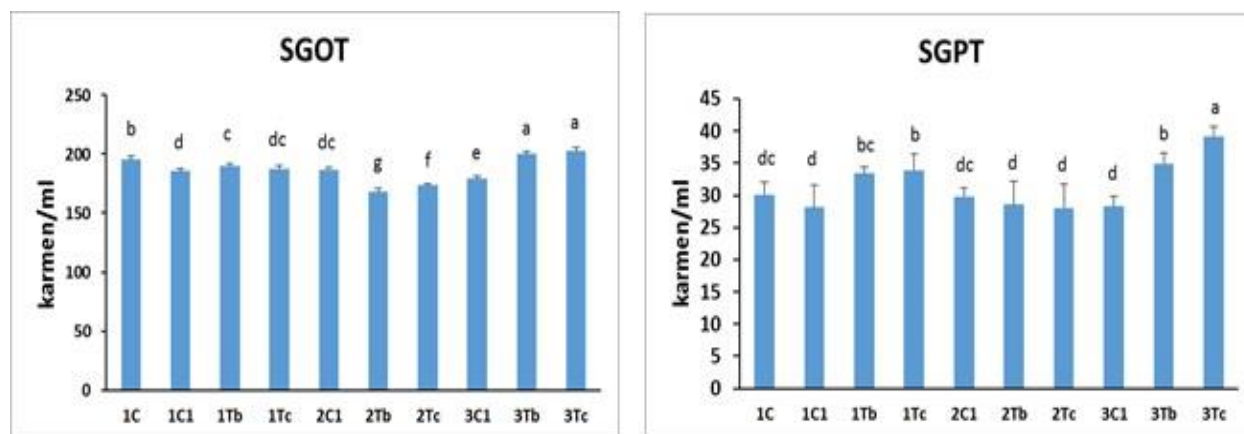
Figure 8

Figure 8. and 4B. Effects of *in ovo* injections of L-Arg (100 µg) with 1000 µg (BOL-Ag NPs) or L-Arg (100 µg) with 100 µg (C-Ag NPs) at different developmental embryonic stages on SGOT and SGPT concentrations in serum. Small characters indicate significant differences among experimental groups at $p < 0.05$.

Injection of Ag NPs into chicken embryo did not result in any negative changes in SGOT or SGPT levels, in agreement with previous results from experiments carried out *in ovo* [38]. Some studies have reported that the size, superficial coating effect, concentration of particles, surface charge,

Zeta potential, composition, and crystal form of the Ag NPs influence toxicity in embryos [39]. However, the toxicity of silver nanoparticles remains controversial. It is far from completely understood [40]. These inconsistent results appearing in the literature concerning the responsiveness to *in ovo* injection of L-carnitine might have been resulted from many factors such as differences in strains and age of breeder hens, injection technique, site of *in ovo* injection, timing of injection (incubational age), dose, and so on [41].

4.7. Measurement of IgM concentration in serum

At the 8th, the 14th, or the 18th day of embryo stage (Fig. 9), *in ovo* injection of BOL-Ag NPs (1000 µg) or C-Ag NPs (100 µg) was performed. At the 14th day injection, both BOL-Ag NPs and C-Ag NPs significantly increased the immune response measured by levels of IgM compared to the control and other stages (8th or 18th day). There was no significant difference in IgM level between the control and the 18th day injection groups (BOL-Ag NPs or C-Ag NPs treated groups).
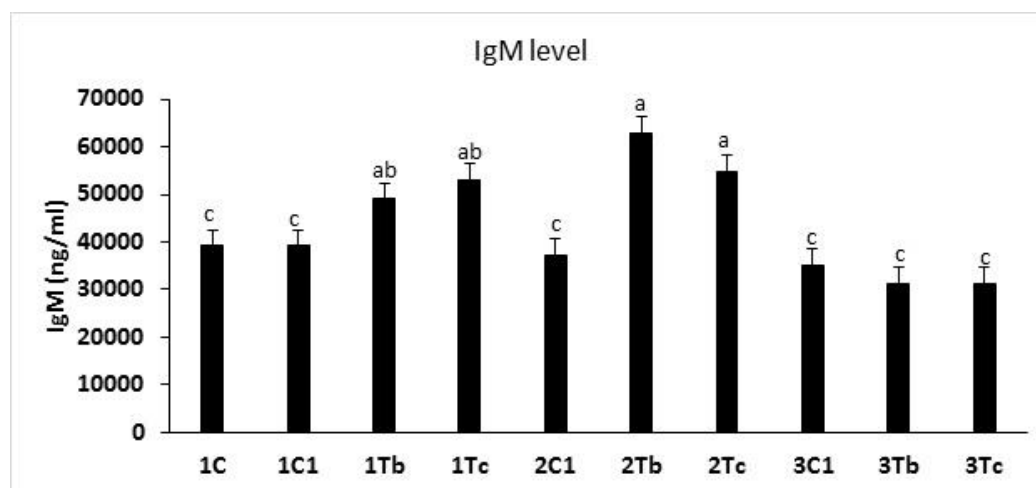
Figure 9

Figure 9. L-Arg induces IgM levels in different stages of chicken embryos. Small characters indicate significant differences among experimental groups at $p < 0.05$. Values are presented as mean ± SD from 12 determinations.

Up to date, only a few studies have been conducted on poultry to evaluate the effect of nanosilver on immune and redox responses and lipid status of chicken blood [42,28,11]. *In ovo* feeding of amino acids can enhance growth-related genes and modulate the expression of immune genes in broilers. Moreover, *in ovo* administration of Ag NPs (15 mg) in combination with amino acids (threonine and cysteine at 15 mg) could improve the immune status of embryos. Thus, Ag NPs in combination with amino acid can act as a potential agent to enhance both innate immunity and adaptive immunity in chickens [43]. In our results, injection of BOL-Ag NPs (1000 µg) conjugated with L-Arg or C-Ag NPs (100 µg) conjugated with L-Arg (100 µg) on the 14th day improved the immunity by increasing IgM levels compared to other treatment groups.

4.8. Protein analysis by western blot

Western blot was performed for muscles to determine effects of L-Arg conjugated with BOL-Ag NPs (1000 µg) and C-Ag NPs (100µg) injected at three different days of incubation period (8th day, 14th day, and 18th day). Treatment altered protein levels of HSPs (such as HSP-60 and HSP-70) as well as Myogenin and Myo-D. As shown in Fig. 10, protein expression levels of HSP-60 and HSP-70 were significantly ($p < 0.01$) down-regulated in the following order: 3Tb < 2TC < 2Tb < 2C1 < 1Tc < 1Tb < 1C1 (Fig. 1A and 1B) whereas expression levels of myogenin and Myo-D were significantly up-regulated in the order of 2Tb > 2Tc > 1Tb > 1Tc > 3Tb > 3Tc (Fig..10 C and Fig. 10 D). Injection on the 17th day up-regulated HSP-60 and HSP-70 when compared to 15-day incubation [44].

HSP-70 is a reliable index of stress in chickens while "3-hydroxyl-3-methyl-glutaryl coenzyme A reductase" has been used as an indicator of stress [45]. Pretreatment with L-Arg markedly reduced the dramatic down-regulation of HSP-60 and HSP-70 in a hypoxic rat model.
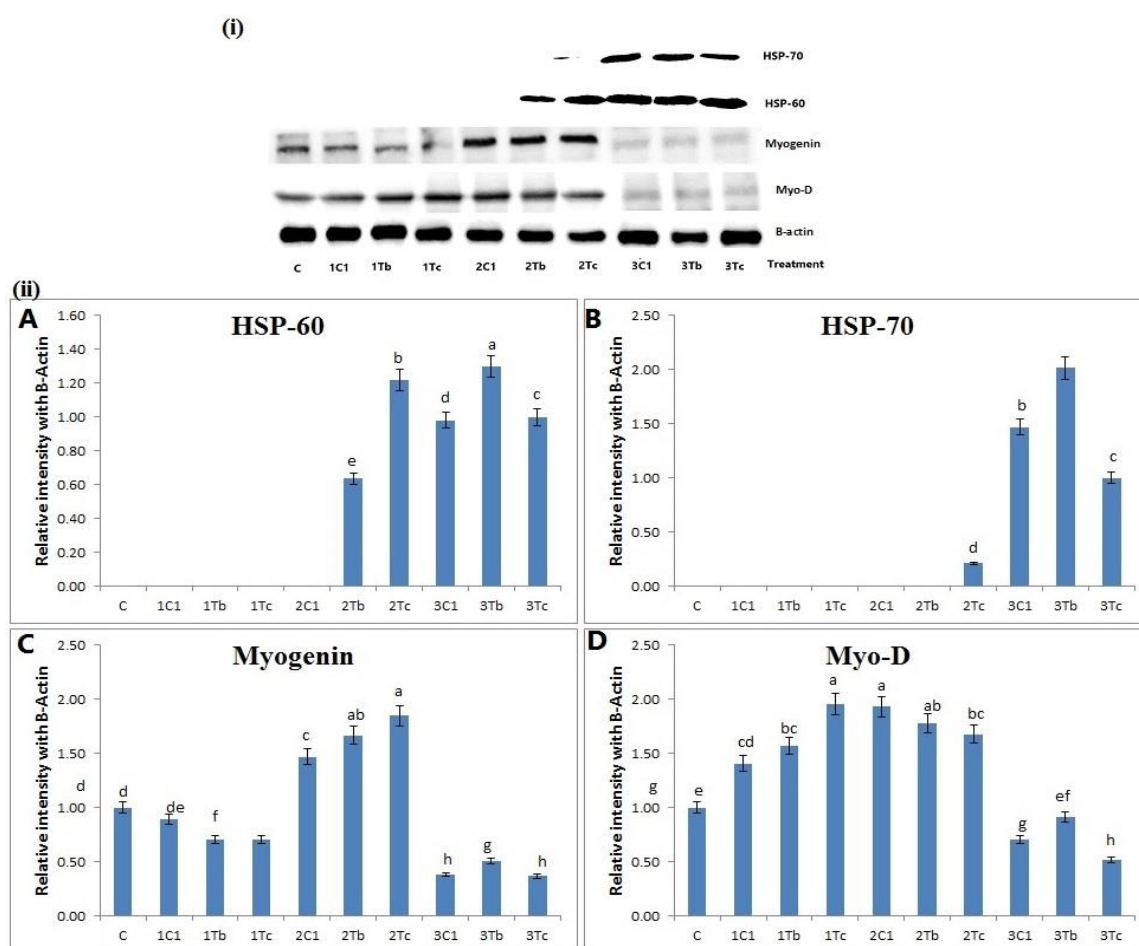
Figure 10

Figure 10. Expression levels of L-Arg, HSP-60, and HSP-70 as well Myogenin and MyoD protein expression levels in different stages of chicken embryos after injection at different doses. Small characters indicate significant differences among experimental groups at $p < 0.01$. Figure 10 (A, B, C, &D) Bar graph represents quantitative expression of different proteins in all groups. Data are expressed as the ratio of relative intensity to the level of β-actin.

The increased expression of HSP-60 and HSP-70 might be related to their leakages from tissue which may cause tissue injury due to free radical production [46]. Tissue injury can be caused by nitric oxide, a free radical, through stimulation of endothelial cells and neutrophils caused by a high dose of L-Arg. High dose of L-Arg after injection on the 18th day could increase the expression of HSP-60 and HSP-70 which can be involved in tissue injury due to free radical production. 3Tb and 3Tc groups might have shown the induction of tissue injury via the up-

regulation of HSP-60 and HSP-70, whereas the expression of HSP-60 and HSP-70 was down-regulated in groups 2Tc and 2Tb in the order of 2Tc < 2Tb. Moreover, protein expression levels of myogenin and MyoD were significantly up-regulated in 2C1, 2Tb, and 2Tc groups, whereas they were down-regulated in 3Tb and 3Tc groups compared to other experimental groups. Hence, the 14th day of injection of L-Arg (100 µg) + BOL-Ag NPs (1000 µg) could promote muscle growth better than 8th or 18th day of injection.

5. Conclusion

In ovo injection of L-Arg with BOL and C-Ag NPs on the 14 day could promote the hatching rate, survival rate, and immune response. Based on obtained results, it can be concluded that *in ovo* injection of L-Arg (100 µg) with 1000 µg (BOL-Ag NPs) or L-Arg (100 µg) with 100 µg (C-Ag NPs) is beneficial for hatchability when applied on day 14 of incubation. In addition, conjugation of L-Arg with 1000µg of BOL-Ag NPs and 100µg of C-Ag NPs injection within fertile eggs on day 14 of incubation is advantageous for subsequent performance. Additional studies on the effect of L-Arg (100 µg) with 1000 µg (BOL-Ag NPs) or L-Arg (100 µg) with 100 µg (C-Ag NPs) on the growth performance are currently underway in our laboratory.

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