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Nano-nutrition to chicken at different embryonic development stages: Using conjugated L-Arginine with Ag NPs to improve muscle growth and immune related proteins

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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) with 1000 µg (BOL-Ag NPs) and L-Arg (100 µg) with 100 µg (C-Ag NPs) were injected into fertile eggs at 8, 14 and 18 of incubation. Survival and hatched chicks were calculated. Survivability and hatchability were unaffected 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 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 14-day incubation period in serum at the same concentration. Collectively, these results indicate that injecting L-Arg (100 µg) with 1000 µg of BOL-Ag NPs or L-Arg (100 µg) with 100 µg of C-Ag NPs to eggs at day 14 after incubation could improve their hatchability, survival rate, and muscle growth marker.

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

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1. Introduction

Chickens have been genetically improved for increased body weight gain, growth rate, and breast muscle mass to meet demands of consumers [1]. In 1953, chicken producers required more than 70 days to rear chickens to reach final body weights of ~1.5 kg. By 2001, chicken producers could rear chickens within 42 days to reach final body weights of ~2.5 kg by increasing breast muscle mass [2,3]. Muscle mass is close related to body weight [4]. Muscle growth and development consist of two periods: hyperplasia (increase the number of muscle cells) and hypertrophy (increase in the volume of muscle cells). During embryogenesis, both hyperplasia and hypertrophy are increased. During the embryonic period of muscle development, there are three distinct stages: (i) formation of myoblasts, (ii) fusion of myoblasts to form myotubes, and (iii) conversion of myotubes to form myofibers [5].

Immediate growth performance can be improved by in-ovo feeding, a technique that can add exogenous nutrition [6] (Kornasio et al., 2011). Similarly, the survival, hatchability, and growth performance of chickens can be increased by *in ovo* injection [7]. 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 [8,9]. 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 [10,11]. 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

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84 embryo during embryogenesis. However, chicken embryo development occurs inside the egg.
85 Thus, we need a right method to add exogenous nutrients into the egg.

86 Nanoparticles (NPs) are of interest due to their use in the prevention of infectious avian
87 influenza viruses which can spread rapidly to poultry flocks. They need further research,
88 particularly with regard to their effects in biological systems. Recent studies have shown that plant
89 mediated synthesized Ag NPs are superior choices to other amino acids or proteins with the aim
90 to produce a new method of *in ovo* nano-nutrition that avoids the toxicity of chemically synthesized
91 Ag NPs [12]. *In ovo* supplementation of Ag NPs conjugated with glutamine can improve the
92 growth and immune status of embryos and chicks. Furthermore, Ag NPs conjugated with
93 hydroxyproline can enhance the development of blood vessels [13]. *In ovo* injection of Ag NPs
94 and amino acid complexes can enhance both innate and adaptive immunity in chicken [14].

95 Modern broiler lines are intensively selected for a higher growth rate with increased size
96 of muscles, including pectoral muscles [15]. This leads to an enhanced requirement of chicken
97 embryos for energy and protein, consequently leading to imbalance between requirement and
98 reserves of nutrients stored within eggs. They may limit maximal (according to genotype) growth
99 and development of chicken embryos. Some authors have indicated that concentrations of certain
100 amino acids in eggs are insufficient to fully support embryonic development [16, 17]. Furthermore,
101 because of limited carbohydrate storage in eggs, amino acids are important substrates for glycogen
102 synthesis which may limit the availability of amino acids for protein synthesis [18]. It has been
103 demonstrated that supplying embryos with extra nutrients and energy could enhance *in ovo*
104 nutrition [19,20]. Recently, it has been shown that *in ovo* administration of L-glutamine to chicken
105 embryos can increase mRNA and protein levels of vascular endothelial growth factor (VEGF-A)

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(Sawosz et al., 2012) which is responsible for endothelial cell proliferation. In addition, VEGF-A can stimulate vasculogenesis and angiogenesisand affect pectoral muscles morphology [13].

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 [21].

Muscle growth and development consist of two periods: hyperplasia and hypertrophy. Hyperplasia (an increase in the number of muscle cells) mostly occurs during embryogenesis. Hypertrophy (an increase in the volume of muscle cells) takes place during the post hatching growth period. During the embryonic period of muscle development, there are three distinct stages: the formation of myoblasts, the fusion of myoblasts to form myotubes, and the conversion of myotubes to form myofibers. 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 [19, 20, 22]. 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 [12].

Muscle development is mainly determined during embryogenesis. Consequently, the final number of muscle fibers is accomplished in prenatal and early post hatch periods [5]. 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

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127 status of muscle maturation during embryogenesis by affecting myoblast determination protein 1
128 (MyoD1) and paired box protein 7 (Pax7) [23].

129 L-arginine is an amino acid classified as a semi-essential amino acid [24]. Foye. [25] has
130 reported that the administration of L-Arg in the embryonic phase could increase the growth rate of
131 turkey after hatching. [26] have suggested that L-arginine is an important stimulator of growth
132 hormone release. Another study report has shown that L-arginine can increase muscle mass [27].
133 L-arginine is also a basic amino acid which has a major role as a stimulator of other amino acids
134 such as proline, ornithine, glutamine, and creatine [24]. It is also a compound that plays important
135 roles in physiological activities of chicken such as nitric oxide, polyamines, and
136 dimethylargininesa [4].

137 Administration of L-arginine at a dose of 0.7% in turkeys [25] and 1.0% in quail [24] by
138 *in-ovo* feeding could increase body weight and post-hatch performance. Up to date, there have
139 been no attempts to address obstacles that impede the manipulation of stages of chick embryos *in*
140 *ovo* by treatment with conjugated silver nanoparticles (Ag NPs)s with L-Arginine (L-Arg).
141 Therefore, the aim of the present study was to investigate the effect of *in ovo* injection of
142 conjugated L-Arg to eggs after different incubation time on hatchability, survivability, and muscle
143 growth markers of 1d old chicks.

144 **2. Results and Discussion**

145 *2.1. Characteristics of Ag NPs*

146 The aqueous BOL extract was pale pink in color. Addition of $AgNO_3$ changed the color of
147 the solution to reddish brown within 2 h. UV–visible spectra of the solution exhibited absorption

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maxima at 430 nm for Ag NPs, indicating the formation of Ag NPs from BOL extract; and chemical synthesis (Fig. 4). HR-TEM images of the Ag NPs revealed that they are well dispersed without much agglomeration.

2.2.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.

2.3.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 AgNPs 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

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indicate that still some green extract present in Ag nanoparticles. L-Arg+Ag (BOL) shows the peaks of both L arginine and Ag NPs indicating the successful combination of Ag and L-Arg.

2.4.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-Arg+Ag (BOL), both L arginine and Ag peaks were found, demonstrating the combination of Ag with L-arginine.

2.5. In ovo study

2.5.1. Survival rate and hatchability

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 (8^{th}, 14^{th}, or 18^{th} day injection) with the same concentration (1000 µg for BOL-Ag NPs and 100 µg for C-Ag NPs). Ag NPs, glutamine (Glu), 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

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these nanoparticles were used at concentrations below 100 µg/ml [28]. In addition, hatchability of fertile eggs was significantly ($p < 0.01$) increased when eggs were injected with L-carnitine on day 14 of incubation compared to that of eggs injected on days 16 or 18 of incubation. Keralapurath et al. [29] observed no significant effect for 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. Bhanja et al. [30] reported that 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. The 14th day incubation can be an appropriate time for improving the embryonic viability through the development of vessel network (embryo until day 15) [31]. The same mechanism might have occurred in our current experiment. In our experiment, the survival rate in the group injected on the 14^{th} day of embryogenesis was not decreased after the injection. Instead, it was increased compared to that in the group injected on the 8^{th} day or the 18^{th} day of embryogenesis with the same dose of BOL-Ag NPs or C-Ag NPs.

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 [13]. Recent studies have shown that *in ovo* supplementation of Ag NPs [32], either alone or in combination with glutamine, can improve the growth and immunity status of embryos and chicks. Vilchez et al. [33] have reported that 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. Such free amino acid might have stimulated embryonic

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gluconeogenesis which in turn helps hatching activities. Previous results of Nouboukpo et al. [34] have shown that the hatchability of fertile eggs is not affected by *in ovo* L-carnitine administration (500 or 1000 µmol) at 18 days of incubation. On the other hand, 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 [35]. Ohta et al. [16] have reported that injection on the 14th day of incubation could utilize amino acids. 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 [13]. 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 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 [36].

2.5.2. 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 [37, 38]. *In ovo* administration of nanoparticles acting as bioactive agents and carries of nutrients may be seen as a new method of nano-nutrition [13]. Recent studies have shown that *in ovo* supplementation of

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Ag NPs [32], either alone or in combination with glutamine [39], 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 [40]. It has also been reported that Ag NPs, glutamine, 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 [39]. Similarly, *in ovo* injection of Gly and Pro might have resulted in higher body weights of chicks in the 14^{th} day injection group. Injection on the 14^{th} day of embryonic stage can utilize the amino acids and promote protein synthesis [9]. 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.

2.5.3. 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 [41]. 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

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257 blood which can lead to liver function damage. In fact, free radicals from Ag NPs can attack
258 hepatocytes and release stored SGOT and SGPT to re-enter the blood serum [42]. Injection of Ag
259 NPs into chicken embryo did not result in any negative changes in SGOT or SGPT levels, in
260 agreement with previous results from experiments carried out *in ovo* [43]. However, the toxicity
261 of silver nanoparticles remains controversial. It is far from completely understood [44]. These
262 inconsistent results appearing in the literature concerning the responsiveness to *in ovo* injection of
263 L-carnitine might have been resulted from many factors such as differences in strains and age of
264 breeder hens, injection technique, site of *in ovo* injection, timing of injection (incubational age),
265 dose, and so on [45].

266 2.5.4. Measurement of IgM concentration in serum

267 At the 8^{th}, the 14^{th}, or the 18^{th} day of embryo stage (Fig. 9), *in ovo* injection of BOL-Ag
268 NPs (1000 µg) or C-Ag NPs (100 µg) was performed. At the 14^{th} day injection, both BOL-Ag
269 NPs and C-Ag NPs significantly increased the immune response measured by levels of IgM
270 compared to the control and other stages (8^{th} or 18^{th} day). There was no significant difference in
271 IgM level between the control and the 18^{th} day injection groups (BOL-Ag NPs or C-Ag NPs treated
272 groups). Up to date, only a few studies have been conducted on poultry to evaluate the effect of
273 nanosilver on immune and redox responses and lipid status of chicken blood [46, 32, 14] (Ahmadi
274 2012; Pineda et al. 2012; Bhanja et al. 2015). *In ovo* feeding of amino acids can enhance growth-
275 related genes and modulate the expression of immune genes in broilers [14]. Moreover, *in ovo*
276 administration of Ag NPs (15 mg) in combination with amino acids (threonine and cysteine at 15
277 mg) could improve the immune status of embryos. Thus, Ag NPs in combination with amino acid
278 can act as a potential agent to enhance both innate immunity and adaptive immunity in chickens

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279 [47]. In our results, injection of BOL-Ag NPs (1000 µg) conjugated with L-Arg or C-Ag NPs (100
280 µg) conjugated with L-Arg (100 µg) on the 14th day improved the immunity by increasing IgM
281 levels compared to other treatment groups.

282 2.5.5. Protein analysis by western blot

283 Western blot was performed for muscles to determine effects of L-Arg conjugated with
284 BOL-Ag NPs (1000 µg) and C-Ag NPs (100µg) injected at three different days of incubation
285 period (8th day, 14th day, and 18th day). Treatment altered protein levels of HSPs (such as HSP-60
286 and HSP-70) as well as Myogenin and Myo-D. As shown in Fig. 10, protein expression levels of
287 HSP-60 and HSP-70 were significantly ($p < 0.01$) down-regulated in the following order: 3Tb <
288 2TC < 2Tb < 2C1 < 1Tc < 1Tb < 1C1 (Fig. 1A and 1B) whereas expression levels of myogenin
289 and Myo-D were significantly up-regulated in the order of 2Tb > 2Tc > 1Tb > 1Tc > 3Tb > 3Tc
290 (Fig..1D and Fig. 1E). Injection on the 17th day up-regulated HSP-60 and HSP-70 when compared
291 to 15-day incubation [48].

292 HSP-70 is a reliable index of stress in chickens while "3-hydroxyl-3-methyl-glutaryl coenzyme
293 A reductase" has been used as an indicator of stress [49]. Pretreatment with L-Arg markedly
294 reduced the dramatic down-regulation of HSP-60 and HSP-70 in a hypoxic rat model. The
295 increased expression of HSP-60 and HSP-70 might be related to their leakages from tissue which
296 may cause tissue injury due to free radical production [50]. Tissue injury can be caused by nitric
297 oxide, a free radical, through stimulation of endothelial cells and neutrophils caused by a high dose
298 of L-Arg. High dose of L-Arg after injection on the 18th day could increase the expression of HSP-
299 60 and HSP-70 which can be involved in tissue injury due to free radical production. 3Tb and 3Tc
300 groups might have shown the induction of tissue injury via the up-regulation of HSP-60 and HSP-

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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.

3. Materials and Methods

3.1.Ethics Statement

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Jeonbuk National University with the project number 2017R1D1A1B03032217. Animal care and handling are in compliance with the regulations of the IAEC Guidelines for the Euthanasia of Animals: 2015 Edition. Sampling procedures complied with the "Guidelines on Ethical Treatment of Experimental Animals" (2015) No. CBNU 2015048 set by the Ministry of Science and Technology, Korea.

3.2.Chemicals

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

3.3.Synthesis of Ag NPs using BOL extracts

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Ten grams of fresh and healthy 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. The resulting extract was filtered through a Whatman filter paper. Two different sets of reaction mixtures (6 ml of extract + 44 ml of $D.H_2O$) were prepared. Ag NO_3 in the amount of 1 mM was added to them. Mixtures were then kept in the dark at room temperature. A control setup with only leaves' extract was also maintained throughout the experiment.

3.4.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 5 s. 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 [51].

3.5.Characterization of Ag NPs

An aliquot of the reaction mixture was analyzed using UV-Vis spectroscopy (UV-1800, Shimazdu Corp., Kyoto, Japan) in wavelength range of 200 to 800 nm. The reaction mixture was centrifuged at 15,000 rpm for 20 min and the pellet was collected. One portion of the pellet was dispersed in 0.1 ml of twice-distilled water for further characterization. In electron microscopy, 25

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343 µl of the reaction mixture was coated on a copper grid and Ag images were examined with a JEM-
344 2010, a high-resolution transmission electron microscope (HR-TEM). Energy-dispersive X-ray
345 analysis (EDS) (XPERT-MRD, Philips, Amsterdam, the Netherlands) was performed to determine
346 compositions and phase contents. Another portion of the pellet was freeze-dried with a
347 concentrator (Model MCF D8512, Ilshin Biobase Co., Ltd., Dongducheon, South Korea) and the
348 resulting powder was used for further examinations.

349 *3.6.Experimental Design and Incubation*

350 Ross 880 broiler chicken eggs were obtained from Samhwa-Won Jong, South Korea. On the
351 first day of incubation, eggs were weighed (60 ± 1.36 g) and randomly divided into 10 groups (4
352 × 20 × 3 = replication × eggs × injection) as described in Table 1. L-Arg (100 µg/100 µL/egg) and
353 conjugation of L-Arg with BOL extract synthesized Ag NPs [100 µg (L-Arg) +1000 µg (BOL-Ag
354 NPs)/100 µL/egg] as well chemical synthesis Ag NPs [100 µg (L-Arg) +100 µg (C-Ag NPs)/100
355 µL/egg] were injected at three different incubation periods (eighth, 14^{th}, and 18^{th} day, respectively).
356 Immediately after the injection, the hole was sealed with liquid paraffin. Eggs were then placed in
357 an incubator for 20 days under standard conditions (temperature of 37.8°C; humidity of 60%). On
358 the 18th day, eggs were transferred to hatching boxes and promptly placed in a hatcher incubator
359 with humidity maintained at 60% and temperature set at 37°C to hatch chicks.

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

Group	Dosage	No. of Eggs x No. of Replication	Eggs

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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

3.7.Survival Rate Measurement

Embryos' survival rates during the incubation period were measured on the eighth day. Treated eggs were checked to determine the number of live and dead eggs as well as fertilized and non-fertilized ones among the total number of eggs. At the 18th day of incubation, after injection, live eggs were moved to another hatching incubator with their respective experimental group. The survival rate was calculated with the following equation (1):

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

3.8. 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. These hatched chicks were kept without food or water at 32°C. They were then weighed to record their live body weights. The hatching rate was calculated with the following equation (2):

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

3.9.Biochemical Indices

At the end of the experimental period, hatched chicks were sacrificed under anesthesia (diethyl ether). 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).

3.10. Measurement of IgM Concentration in Serum

Serum samples were collected from individual experimental animals 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.

386 *3.11. Analysis of Heat-Shock Proteins (HSPs) and Muscle related markers by Western Blot*

387 Proteins were extracted from 100 mg of muscle samples using radioimmunoprecipitation assay
388 (RIPA) buffer to determine protein expression levels of HSP-60, HSP-70, myoD, and myogenin
389 in experimental groups. Protein concentrations were determined using a BIO-RAD protein assay
390 kit (BIO-RAD). Extract samples containing 50 µg of protein were solubilized in *Laemmli buffer,*
391 separated by 12% acrylamide gel, and then transferred to Hybond-P PVDF membranes (GE
392 Healthcare Inc., Amersham, UK) for 60 min at 200 mA. These PVDF membranes were blocked
393 with 5% skimmed milk powder in 0.5 M of Tris-buffered saline (pH 7.4) with 0.05% Tween 20
394 (TBST) at room temperature for 2 h. Western immunoblotting with HSP-60, HSP-70, Myo-D, and
395 myogenin primary antibodies (1:2500 dilution) was performed at 4°C overnight. After washing
396 three times with TBST, these membranes were incubated with HRP-conjugated secondary
397 antibodies (1:5000 dilutions) at room temperature for 60 min and then washed three times with
398 TBST (10 min each wash). Protein bands were visualized using a Chemiluminescent assay kit
399 from Thermo Scientific for 1–5 min. Bands were imaged with an iBright™ CL1000 Imaging
400 System (Invitrogen in Thermo Fisher Scientific, Life Technologies Korea LLC, Jeonju-si,
401 Jeollabuk-do, Korea) and quantified using Image J Software. The relative density of the band was
402 normalized to that of β-actin as an internal control.

403 **4. Conclusion**

404 *In ovo* injection of L-Arg with BOL and C-Ag NPs on the 14 day could promote the
405 hatching rate, survival rate, and immune response. Based on obtained results, it can be concluded
406 that *in ovo* injection of L-Arg (100 µg) with 1000 µg (BOL-Ag NPs) or L-Arg (100 µg) with 100
407 µg (C-Ag NPs) is beneficial for hatchability when applied on day 14 of incubation. In addition, L-

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carnitine injection within fertile eggs at a level of 8.0 mg/100 µL on day 16 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.

Figure Legends

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. 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.

Figure 3. (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).

Figure 4. 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.

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.

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.

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.

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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 $p < 0.05$.

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.

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 $p < 0.01$. (B) 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.

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Figure 1

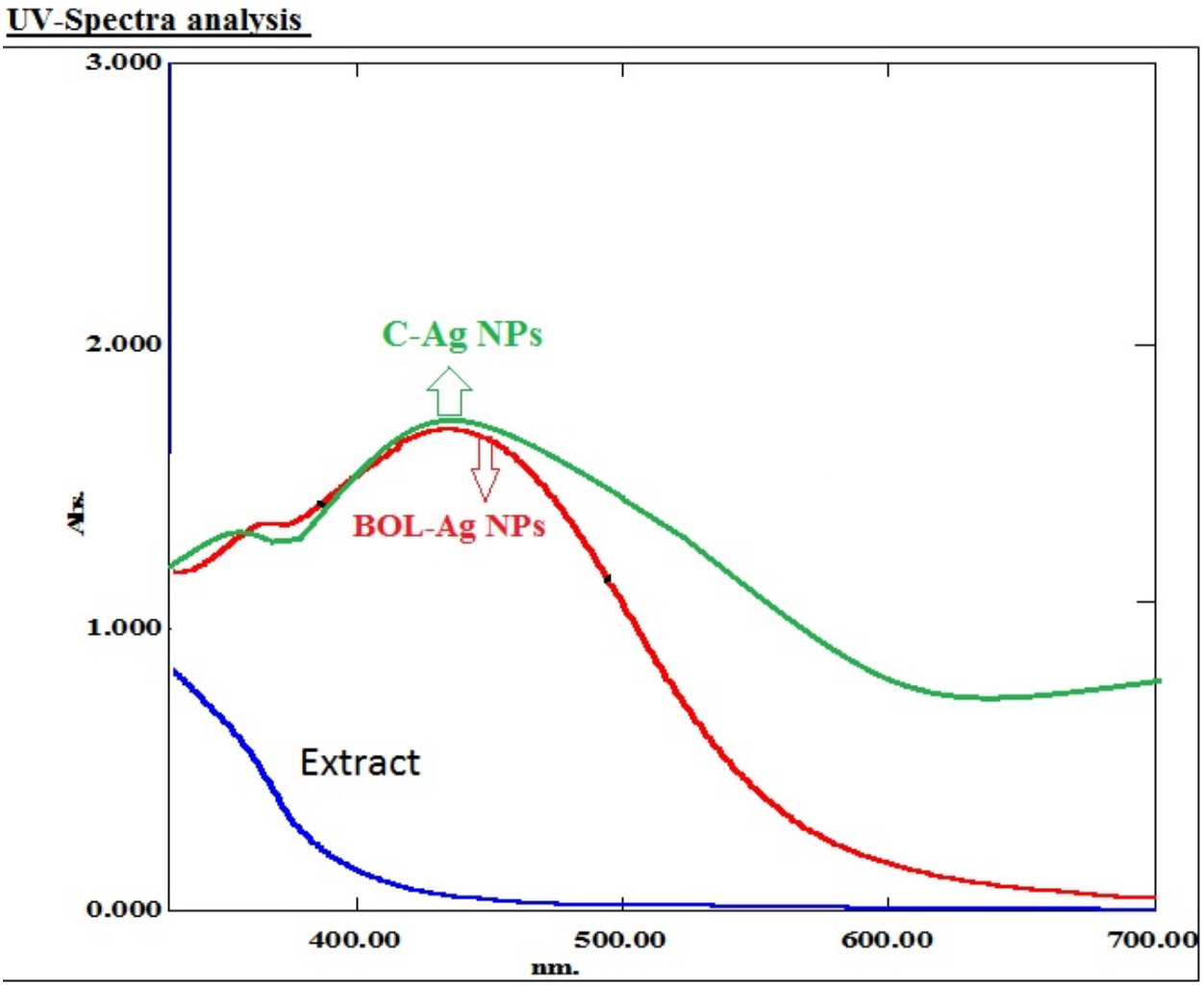

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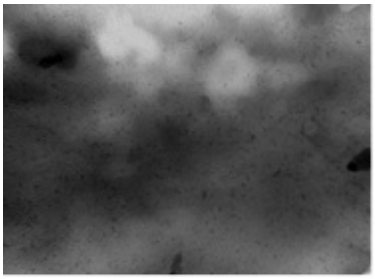
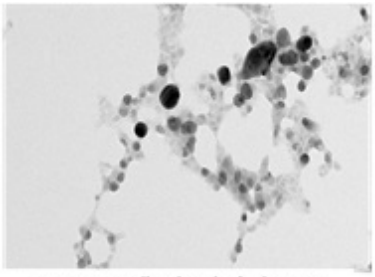
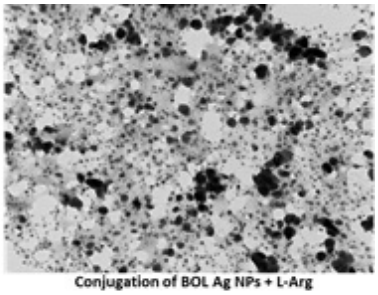
Figure 2



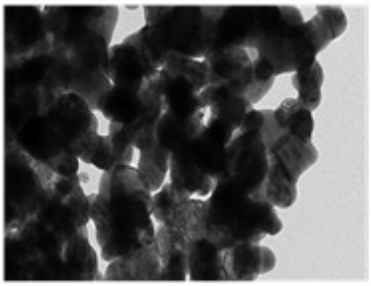
L-Arginine



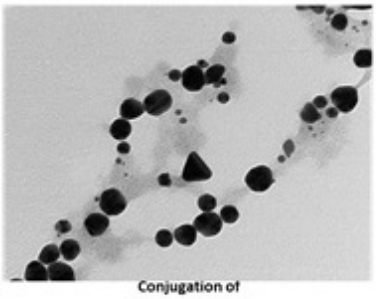
BOL extract mediated synthesized Ag NPs (BOL-Ag NPs)



Conjugation of BOL Ag NPs + L-Arg



Chemical synthesized Ag NPs (C-Ag NPs)



Conjugation of C-Ag NPs + L-Arg

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678 **Figure 3**

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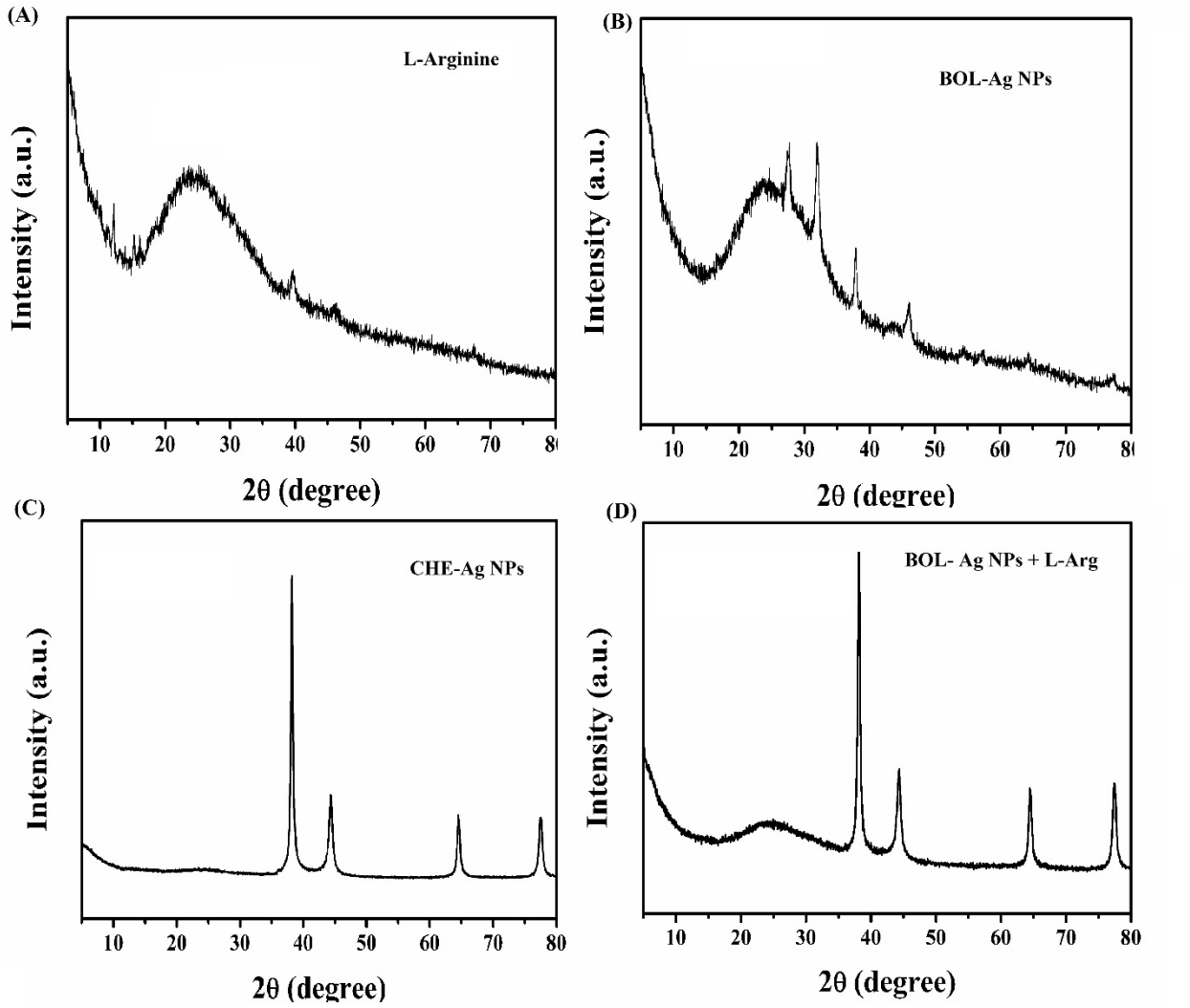

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Figure 4

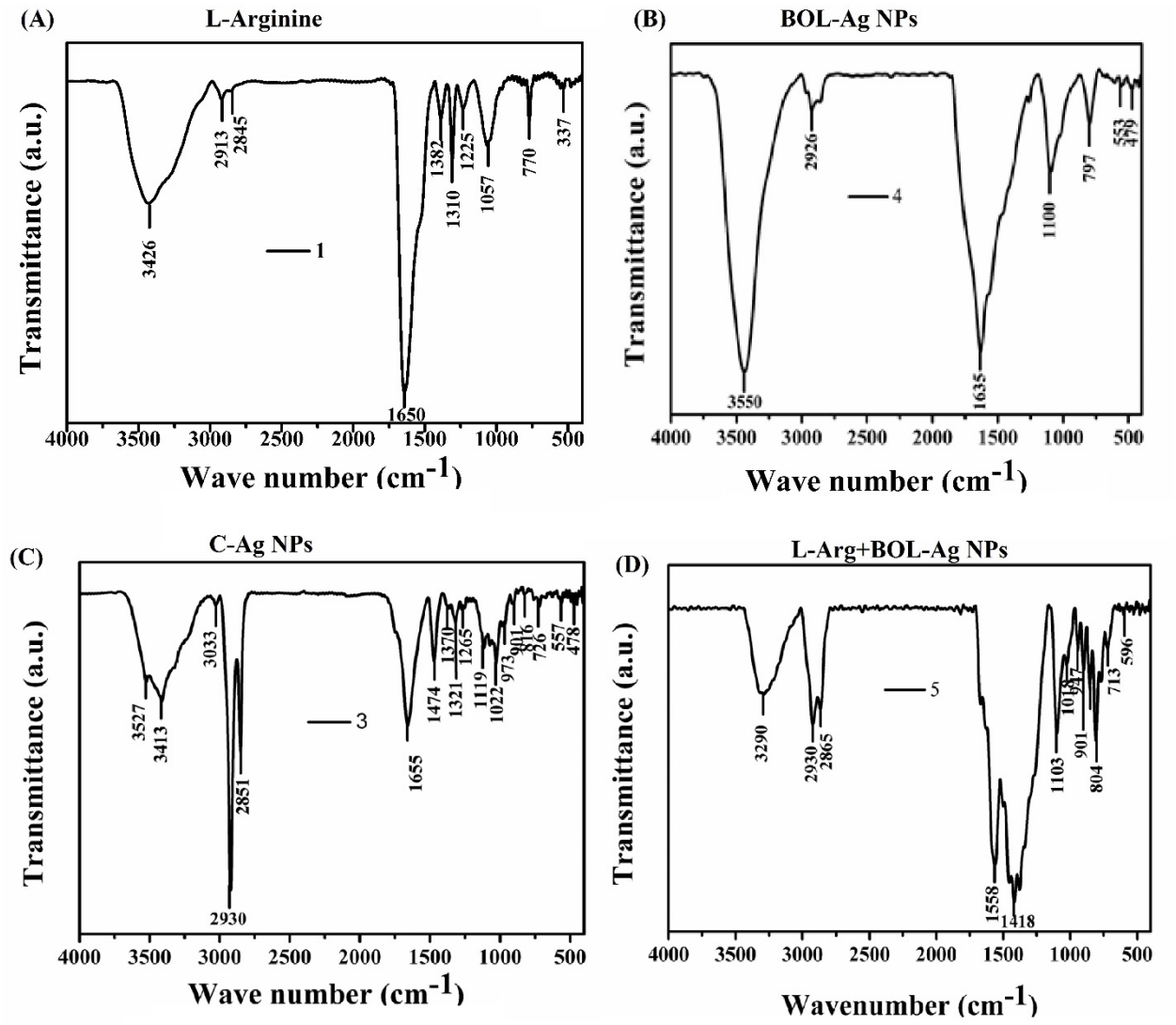

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Figure 5

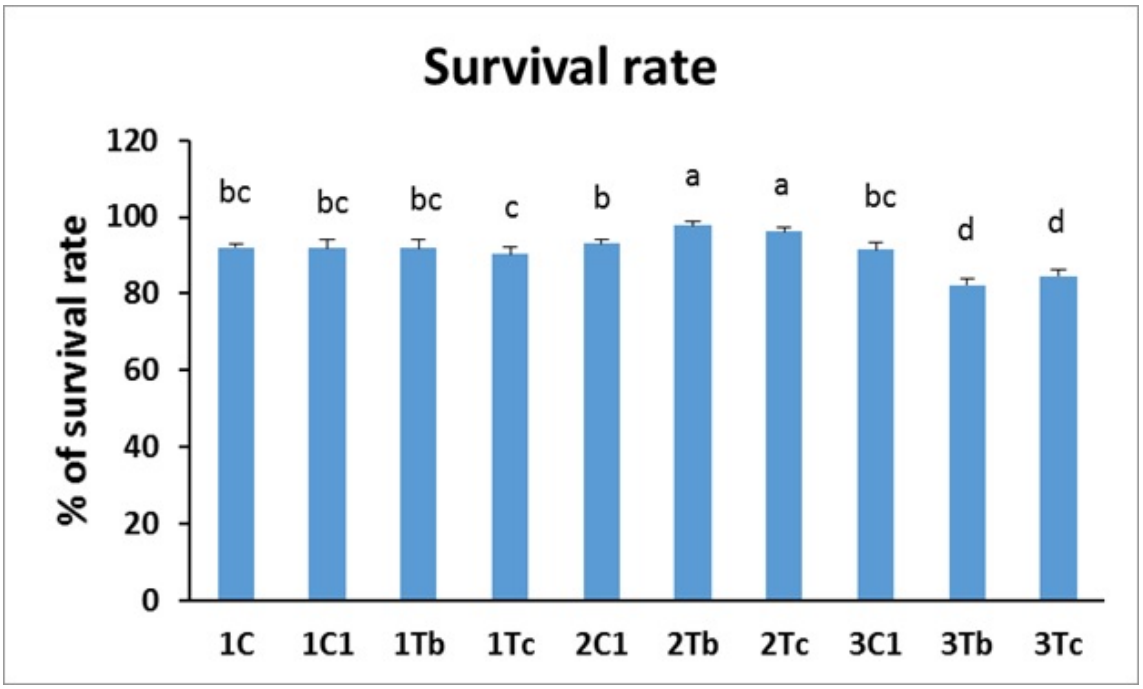

Figure 6

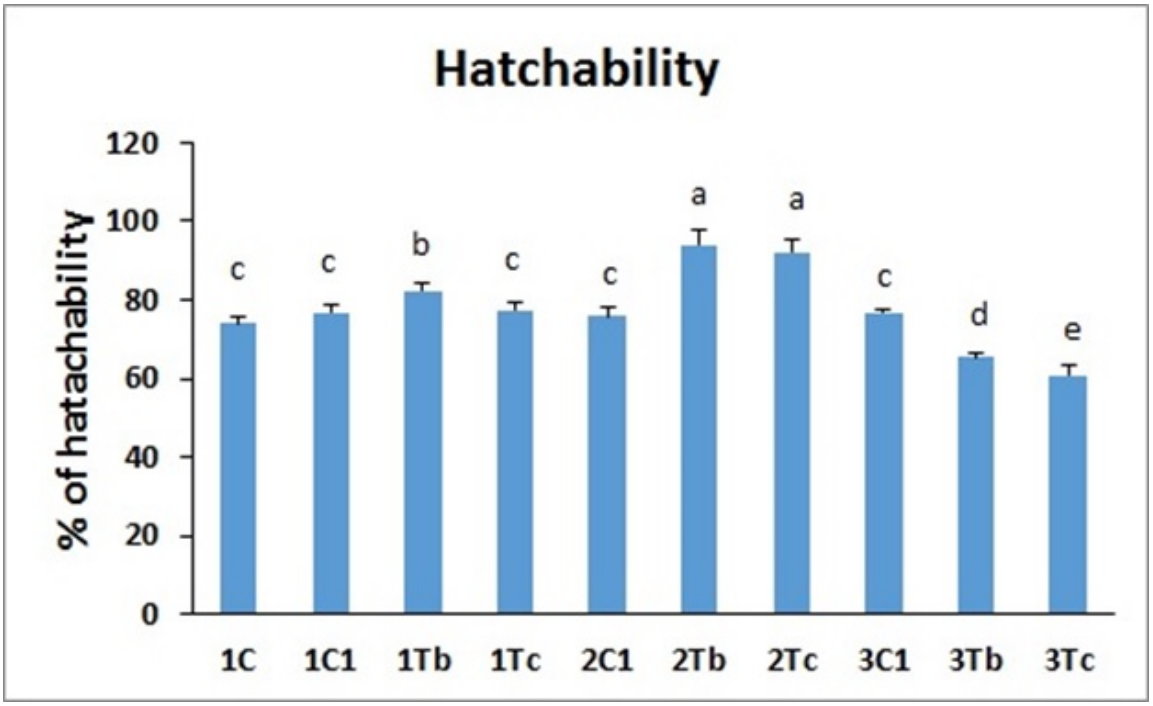

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Figure 7

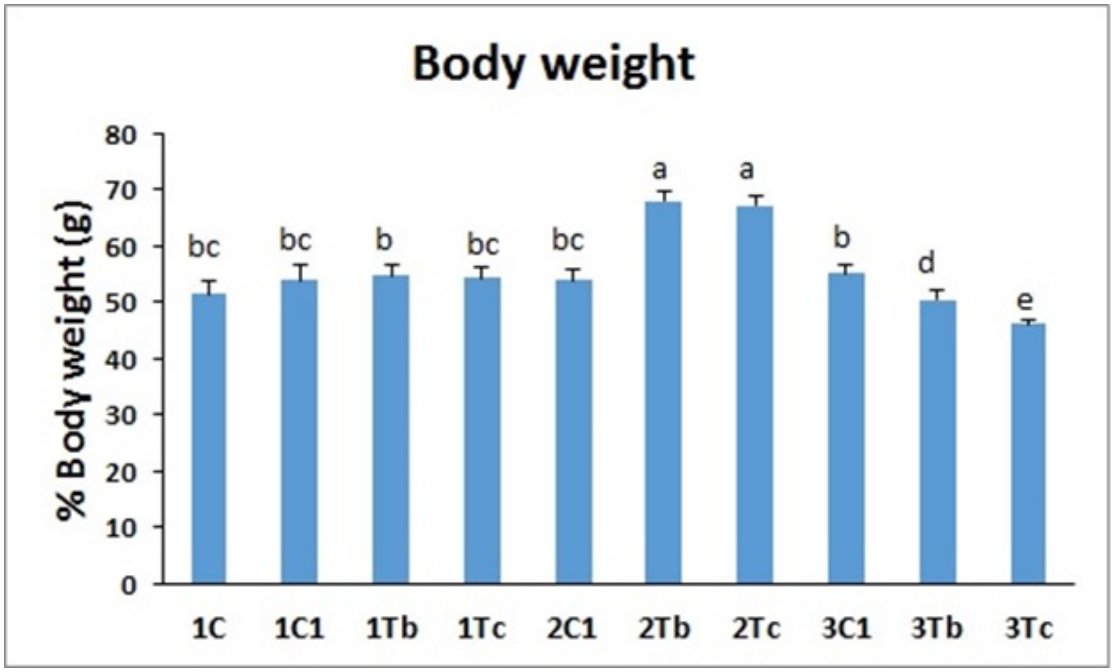
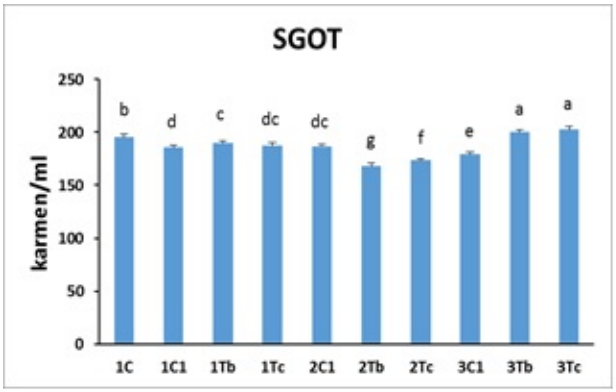


Figure 8



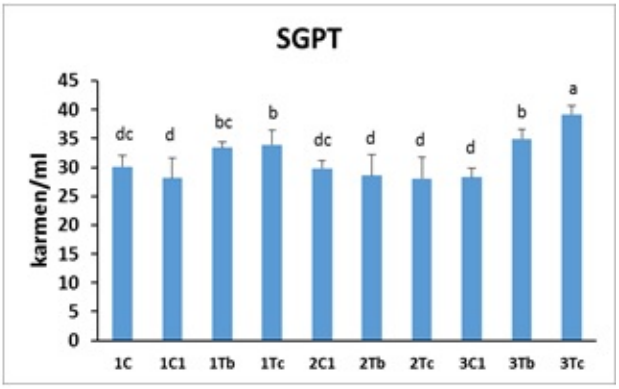


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Figure 9

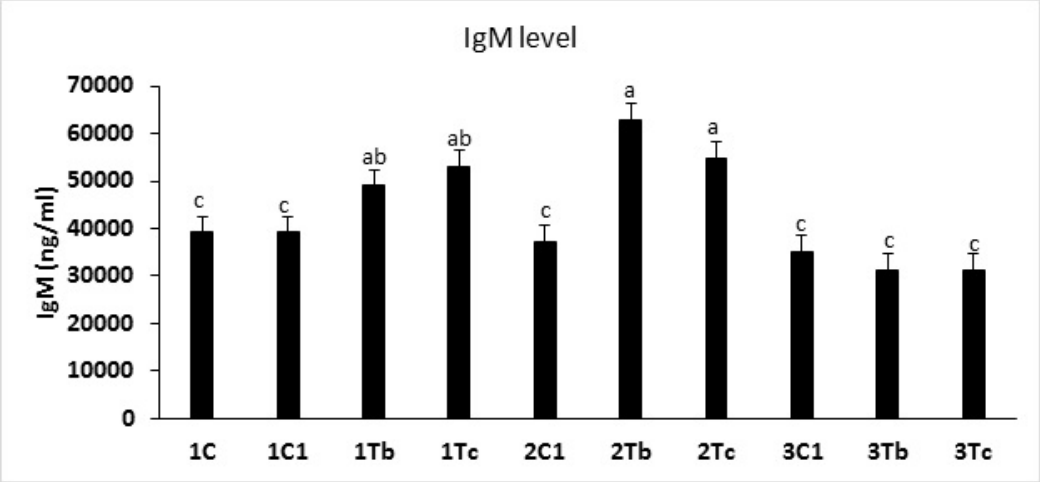

Figure 10