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Effects of *In Ovo* Injection of Ascorbic Acid and Creatine Pyruvate on Hatchability, Chick Weight and Muscular Growth in Broiler Chickens

Meray Nabil Ramsis[1], Sherif Hamed Elmosalamy[2], Ramadan Gomaa Sary[1]

[1]Anatomy and Embryology Department, Faculty of Veterinary Medicine, Cairo University, Giza, 12211 Egypt
[2]Physiology Department, Faculty of Veterinary Medicine, Cairo University, Giza, 12211 Egypt

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Corresponding author
Sherif Hamed Elmosalamy
sherifelmo@cu.edu.eg

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Abstract

The aim of the research was to determine the impact of an *in Ovo* injection of ascorbic acid and creatine pyruvate in the amnion of viable eggs on day 17 of incubation through evaluation of hatchability percentage as well as antioxidant status (Reduced glutathione (GSH) and Malondialdehyde (MDA)) and creatine kinase in pectoral muscles, blood glucose level and histopathology of liver and intestine. After the non-fertilized eggs were removed, 180 eggs that were to hatch were divided into three groups of 60. The first group was the control group, which did not receive any treatment; the positive control group received 0.6 ml of physiological saline; and the treated group received an injection of a mixture of ascorbic acid and creatine pyruvate (12 mg of creatine pyruvate and 3 mg of pure ascorbic acid were dissolved in 0.6 ml of saline). The results indicated that the treated group showed a significant increase in chick weight, hatchability percentages, creatine kinase, GSH activities in pectoral muscle tissue and blood glucose level when compared with the control and positive control groups. Moreover, the treated group showed a significant decrease in MDA concentrations in pectoral muscle tissue when compared with the control and positive control groups at $P<0.05$. Furthermore, the intestinal villi of the treated group exhibited increased villus length and width as well as enhanced muscle size relative to the control and positive control, whereas there was a notable reduction in hepatocyte cellular vacuolation in the treated group when compared with the control and positive control. These results imply that the incubation of viable chicken eggs can be optimized through the administration of ascorbic acid and creatine pyruvate injections.

Introduction

Poultry and eggs are great sources of protein, but they also include vital vitamins and minerals (FAO, 2022). Using Ovo technology for intra-ovarian injections enables the targeted delivery of bioactive substances to the growing embryo, ensuring superior life-long impacts (Siwek *et al*., 2018). Variations in hatching timings, chick handling, and transportation duration lead to a significant loss of glycogen stores by the end of the 21-day incubation period, which satisfies the increased energy requirements. This could force the embryo to use more muscle protein for gluconeogenesis, which would increase the energy deficit and cause irreversible growth and developmental defects in broilers, such as decreased body mass, delayed intestinal development, and decreased pectoral muscle volume. Since hatchling ontogeny depends on the days leading up to and soon following hatching, it stands to reason that increased energy storage during this time is necessary to support growth (Noy & Uni, 2010). The ratio of pyruvic acid to creatine is maintained at 40:60 by a specific chemical compound called creatine pyruvate (CrPyr). Pyruvate is essential for controlling energy metabolism through the Krebs cycle and the glycolytic/gluconeogenic pathways. The muscle

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energy buffering system is also closely related to creatine, which can be phosphorylated to produce phosphocreatine (Scheer *et al*., 2016). Ascorbic acid constitutes a water-soluble vitamin distinguished by its antioxidant properties, which serves as a highly efficient electron donor (Lee *et al.,* 2004). The function of antioxidant defense systems during hatching is thought to be a critical factor affecting the viability of chicks in the early post-hatch period, according to Karadas *et al*. (2011). According to previous studies, ascorbic acid (AA) may help Japanese quail's immune systems recover from the negative impacts of heat stress (Jang *et al*., 2014). Therefore, the purpose of this study was to investigate the potential benefits or drawbacks associated with the simultaneous administration of ascorbic acid and creatine pyruvate during the embryonic development phase, which may have important ramifications for the health and productivity of birds. Notably, these combined effects have not been reported in other studies.

Materials and methods

Birds and experimental design

This study utilized 200 viable broiler eggs (Ross 308), with individual egg weights ranging from 49.2 to 58.9 grams. The eggs were placed in an automatic incubator following standard procedures, which specified a temperature of 37.5°C and a relative humidity of 60%. On the 16th day of incubation, candling was performed, resulting in the removal of 20 unfertilized eggs. The remaining eggs were then divided into three groups, each consisting of 60 eggs, and were randomly injected on the same day. For the injections, 12 mg of powdered creatine pyruvate and 3 mg of pure ascorbic acid from DSM were dissolved in 0.6 ml of 0.9% physiological saline (Zhang *et al*., 2019). These solutions were subsequently filtered through a 0.22-micron filter paper and allowed to incubate for two hours. The experimental design included three groups: the first served as a control, the second received an injection of 0.6 mL of physiological isotonic saline (0.9%), served as a positive control, and the third was administered an injection containing both ascorbic acid and creatine pyruvate serving as the treated group. Prior to the injection of fluid using 21-gauge needles into each group, the surface of the egg was sanitized with 70% ethanol. The amniotic fluid was examined through candling. Following the injection, the perforations were promptly sealed with paraffin wax.

Hatchability

To ascertain the number of hatchlings in each group, a count was performed after the hatchlings emerged. Using the formula (number of hatchlings/number of viable eggs) times 100, the hatchability % was calculated. The average weight of each group was noted, and the chicks were housed in cages with three tiers, guaranteeing them unlimited access to food and water. A total of 36 healthy chicks from each of the 3 groups with similar weights close to the average BW of their pooled group were randomly assigned into 3 replicates with 12 birds each replicate. They were initially housed in a temperature-controlled setting that ranged from 32°C to 34°C for 4 days. In alignment with the acclimatization protocols established by the Institutional Animal Care and Use Committee, the birds were allowed a period of acclimatization. The ethical committee of Cairo University, Egypt approved the experiment (Vet CU110520251132).

Biochemical parameters

Blood Glucose Level

Blood was obtained from the common carotid artery on a sodium fluoride tube from 15 chicks that were four days old, and centrifuged to separate the plasma to obtain the blood glucose level using kit purchased from a Spectrum company, Egypt. Blood glucose measurement was carried out following the manufacturer's protocol.

Creatine Kinase, GSH and MDA in pectoral muscle tissue

Prior to dissection, the pectoral muscle tissue was perfused with a phosphate-buffered saline (PBS) solution at pH 7.4 and 0.16 mg/mL of heparin to eliminate blood cells and clots. The tissue was homogenized in 5-10 ml of cold buffer, specifically a 50 mM potassium phosphate solution at pH 7.5 per gram tissue using a tissue homogenizer. Lastly, the supernatant was carefully and stored on ice for further testing after centrifugation at 4000 rpm for 15 minutes at 4°C. Reduced glutathione (GSH) and Malondialdehyde (MDA) were purchased from a Biodiagnostic company, Egypt, whereas creatine kinase was purchased from a Spectrum company, Egypt. All measurements were carried out following the manufacturer's protocol.

Histological examination of colon, liver and pectoral muscles

The steps involved in histological preparations are described in detail by Bancroft *et al*. (2013). After being cut to a thickness of 3-4 mm, tissues from the colon, liver, and pectoral muscles were preserved in 10% neutral buffered formalin (10% NBF). After being preserved, the samples were washed with xylene, dehydrated using a range of ethanol concentrations, and then embedded in paraffin blocks. According to Bancroft & Gamble (2008), the paraffin blocks were sectioned using a Leica microtome to a thickness of 4-6 µm to enable the application of hematoxylin and eosin (H&E) staining, which made it easier to examine the general tissue architecture. A

Leica microscope (LEICA DM500) was then used to examine the stained slides at magnifications of x10 and x40. After that, images were taken using the microscope's Leica ICC50 HD camera and evaluated with image analysis software. Computerized morphometrics was used to perform the measurements. The villus's height was measured from the tip to the base, and its width was measured in millimeters at three different points: the apex, middle, and base. The villus width to height ratio was computed using the methods developed by Özel *et al*. (2018).

Additionally, the absorption surface area (ASA) was calculated using a modified methodology that was inspired by the research of Ferreira *et al*. (2016) and Kisielinski *et al*. (2002). Using Image J software, the percentage of cellular vacuolation was assessed following the acquisition of images of the liver parenchyma, as explained by Abràmoff *et al*. (2004). The terminology used in this study followed the requirements set forth by the *Nomina Anatomica Avium* (Baumel *et al*., 1993).

Statistical analysis

Statistical presentation and analysis of the present study were conducted, using the mean, standard error, and analysis of variance [ANOVA] test by SPSS V20.) was used to identify the significantly different groups at (P <0.05) by one-way anova

Result

The hatchability percentage in the treated group showed a remarkable increase of approximately 90%, in contrast to the control group, which experienced a significant decline of about 83.87 % (Table 1). The average weight of the chicks that hatched was approximately 43.97 g in the treated group, compared to 40.58 g in the control group. Notably, the group that received the combination injection hatched roughly one day earlier than both the control and saline groups (Table 1).

In comparison to the saline and negative control groups, the group receiving the injected eggs with the combination treatment demonstrated a marked increase in CK activity in the pectoral muscle, as illustrated in Table 2, with a significance level of P < 0.05. Furthermore, the blood glucose level of the chicks in the combination group were significantly elevated compared to those in the control negative and saline groups shown in (Figure 1). Additionally, the MDA levels in the pectoral muscles of chicks that received the combination treatments were significantly lower than those observed in both the control and saline groups, as indicated by (Figure 2). While the GSH levels in the pectoral muscles of chicks that received the combination treatments were significantly higher than those observed in both the control and saline groups, as indicated by as shown in Figure 3.

Table 1: Effect of the combination of ascorbic acid with creatine pyruvate upon the chick weight and hatchability percent

Groups	Hatching weight	hatchability %
Control	40.58 ^{a} ± 0.31	83.87 %
Positive Control	41.09 ^{a} ± 0.27	85.00 %
Treated group	43.97 ^{b} ± 0.38	90.00 %
P-Value	< 0.05	

Data presented as means ± S.E., N per group = 36.

Means having the different superscripts in the same columns are significantly different at P< 0.05.

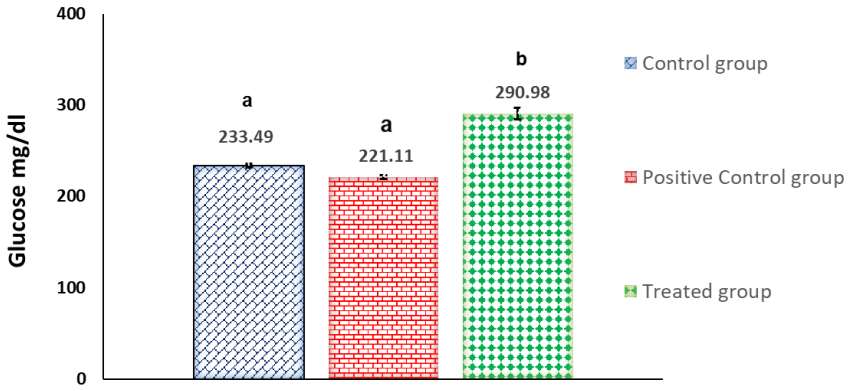

Figure 1. Glucose level in different groups (n=5); data are represented as Mean ± SEM., columns having the different superscript are significantly different at P<0.05.

Table 2: Effect of the combination of ascorbic acid with creatine pyruvate on muscle creatine kinase activity (U/L)

Groups	Creatine kinase activity
Control	$24.88^{a} \pm 0.67$
Positive Control	$28.97^{a} \pm 1.68$
Treated group	$52.51^{b} \pm 3.72$
P-Value	< 0.05

Data presented as means ± S.E., N per group = 5.
Means having different superscripts in the same columns are significantly different at $P<0.05$.

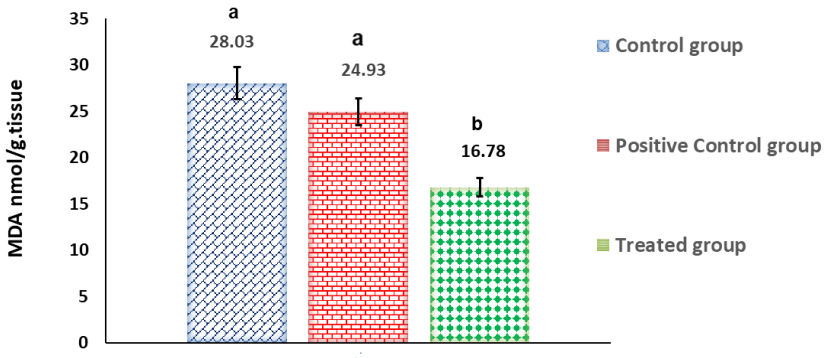

Figure 2. MDA level in pectoral muscle of different groups (n=5); data are represented as Mean ± SEM., columns having the different superscript are significantly different at $P<0.05$.

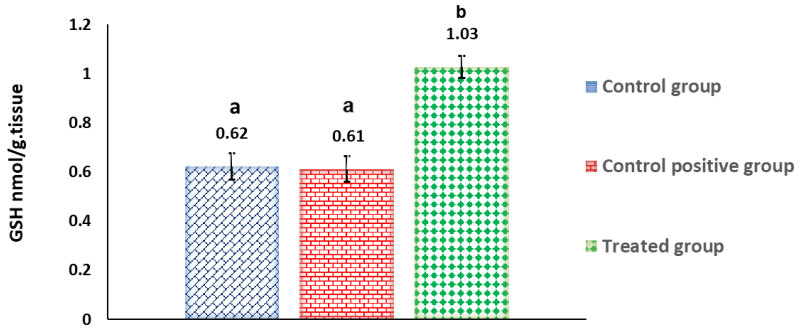

Figure 3. GSH level in pectoral muscle of different groups (n=5); data are represented as Mean ± SEM., Columns having different superscripts are significantly different at $P<0.05$.

Histological findings:

Upon histological examination of the intestinal villi, a normal histoarchitecture was noted, with the villus length and width in the treated group being significantly greater than those observed in both the control and positive control groups, as illustrated in Figure 4. Additionally, a histological evaluation of muscle size indicated a marked increase in the treated group relative to the control and positive control groups, as shown in Figure 5. Moreover, when compared to the combination group, the hepatocytes in the control and positive control groups exhibited a higher degree of cellular vacuolation, as depicted in Figures 6 and 7.

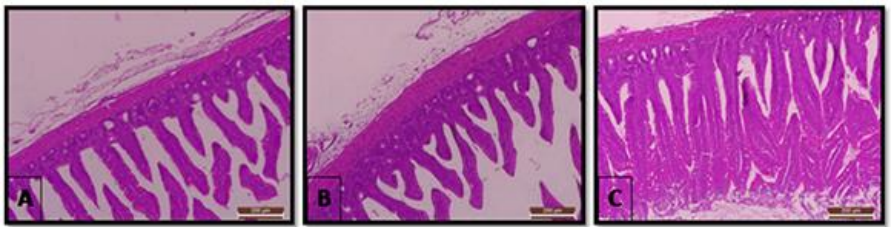

Figure 4. Photomicrographs of the intestine of chicks stained with H&E showing: (A) control, (B) positive control, (C) treated group: normal histological architecture of intestine with variable length and width of intestinal villi (H&E, 4X, scale bar 200 µm).

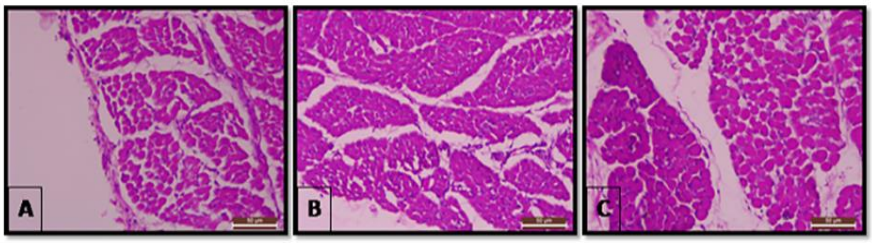

Figure 5. Photomicrographs of pectoral muscle of chicks stained with H&E showing: (A) control, (B) positive control, (C) treated group: normal histological architecture of muscle with variable muscle size. (H&E,40 X, scale bar 50 µm).

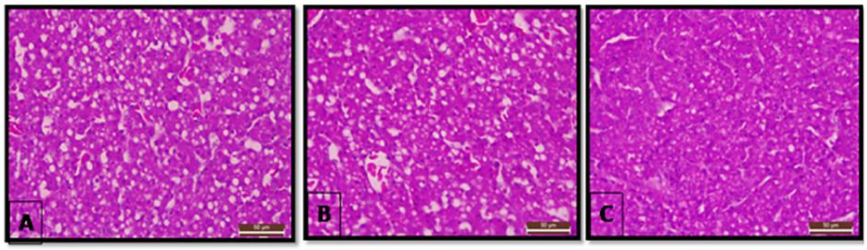

Figure 6. photomicrographs of the liver of chicks stained with H&E showing various degrees of cellular vacuolation: (A) control, (B) positive control (C) treated group. (H&E, 40X, scale bar 50 µm).

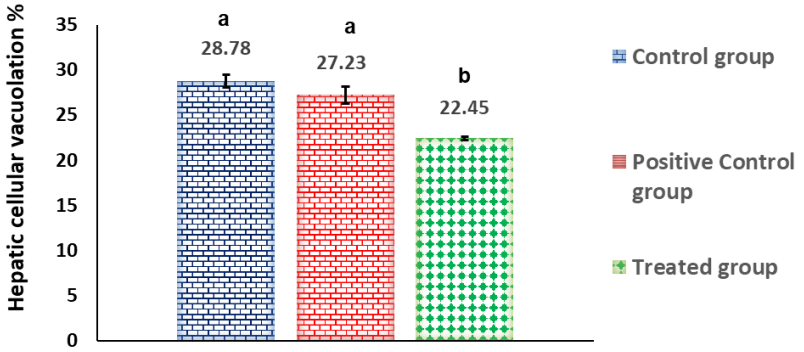

Figure 7. Hepatic cellular vacuolation % in different groups (n=5); data are represented as Mean ± SEM., Columns having different superscripts are significantly different at $P<0.05$.

Discussion

This study indicated that Ovo injection of a combination of ascorbic acid with creatinine pyruvate into the amniotic fluid on the seventeenth day showed a statistically significant increase in both chick weight and hatchability percentages when compared with other groups. In addition, the intestinal villi of the combination group had larger muscles and longer and wider villus lengths than the control and saline groups, whereas hepatocyte cellular vacuolation was much lower. The main storage of glucose in embryos is glycogen present in liver and muscles, which utilized as energy instead of lipids and proteins (Moran, 2007). During late incubation period, the source of energy obtained through hepatic gluconeogenesis of amino acids from amnion and muscles in avian embryos, so Ovo injection creatine pyruvate improve hatching percent, chick weight and muscular weight through raising glucose and glycogen of liver and maintain energy homeostasis by buffering ADP and ATP ratios via reversible reaction catalyzed by creatine kinase in muscles (Zhao *et al*., 2017). This agrees with Firman *et al*. (2023) observed that the administration of creatine pyruvate in the amnion during *in ovo* feeding can enhance the energy status of embryos, as well as increase both hatching weight and pectoral muscle weight. As reported by Keralapurath *et al*. (2010), the administration of l-carnitine using a commercial diluent at 18 days into the incubation period of broiler hatching eggs resulted in a decrease in hepatic glucose levels

The excessive metabolism during late incubation period evolve excessive heat and increase oxidative stress which lead to death of embryo during late embryonic development, Ovo injection of ascorbic acid increase hatching percent, chick and muscle weight through relieving the oxidative stress and enhance immunity (Mousstaaid *et al*.,2022), and

improve liver functions which enhance metabolism provide a good source of energy during late incubation period (Du *et al*., 2025). This agrees with Zhao *et al*. (2018), who stated that the optimal hatchability outcomes were achieved when chickens were administered an injection of 3 mg of vitamin C for each egg on the 17th day of the incubation period. Furthermore, the hatchability of broiler chickens, along with their intestinal morphology and bone characteristics, may be positively influenced by the *in ovo* injection of ascorbic acid into the air cell on the fifteenth day of incubation within the amnion (Zhang *et al*., 2019). In agreement with Du *et al*. (2012) vitamin C, functioning as a cofactor for hydroxylase, has the potential to enhance gluconeogenesis and support embryos by providing a greater nutrient supply, thereby improving hatchability and facilitating adaptation during the later stages of incubation, which may lead to a decrease in the incidence of dead embryos or the culling of chicks.

The results of the applied study show that the combination group's blood glucose, creatine kinase (CK), and glutathione (GSH) levels are significantly higher than those of the other groups, while the combination group's malondialdehyde (MDA) levels are significantly lower, indicating a reduction in oxidative stress. These results align with earlier studies conducted by (Soltani *et al*., 2019; El-Senousey *et al*., 2018; Hajati *et al*., 2014 and Ayala *et al*., 2014). Additionally, we found that the combination group's intestinal villus length and width were noticeably larger than those of the other groups, corroborating the results of Ali *et al*. (2023), the hepatocytes in this group showed decreased cellular vacuolation while pectoral muscle size showed marked increase.

Conclusion

In conclusion, introducing ascorbic acid and creatinine pyruvate into the amnion on the seventeenth day of incubation showed a significant increase in chick weight, hatchability percentages, creatine kinase, GSH activities in pectoral muscle tissue and blood glucose. Moreover, combination group showed a significant decrease in MDA concentrations in pectoral muscle tissue indicating improving hatchability percentages, antioxidants status and energy status of chicks. This is confirmed by the histology of muscle, intestine, and liver tissues. A combination of creatine pyruvate and ascorbic acid on day 17 of incubation may recommend encouraging the growth and development of chicks.

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