The effect of the in ovo injection of some carbohydrates and antioxidants on incubating parameters, blood and immunological parameters, intestinal morphometry and post-hatching production performance in broiler chickens

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introduction

In recent decades, the growth and production of broilers have increased significantly. Despite the advances in the poultry industry, there are other factors that can affect more production in this industry. One of the factors that can affect the development of broiler production is the long-term storage of hatching eggs when there is not enough capacity to lay eggs in the incubator (Aygun et al. 2016; Ahmadi et al. 2019).

Unlike mammalian embryos, bird embryos have a certain amount of energy and nutrients (stored in eggs) for growth and development. Achieving a high incubating rate, success in embryonic development, and ultimately the health of broilers during the rearing period depends on the presence of sufficient nutrients in the fertile egg and the appropriate environmental conditions for the embryo to become a live chicken (Ahmadi et al. 2018).

Increased egg storage time may have detrimental effects on embryonic development and hatching rate, as studies have shown that keeping hatching eggs reduces the hatching rate (Araujo et al. 2017).

Long-term storage of eggs may increase embryonic mortality and decrease hatching rate through the
changes in the pH of albumin, gradual death of embryonic cells, morphological changes in the blastoderm, and disorder in embryonic metabolism. Also, long-term storage of hatching eggs reduces the rate of metabolism, reduces embryonic glycogen stores, and increases foetal dependence on carbohydrates through gluconeogenesis (Tona et al. 2003).

The development of avian embryos is associated with the accumulation of unsaturated fats in the cell membrane and a significant increase in the rate of oxidative metabolism during the hatching period. In such cases, oxidative stress may cause problems during the last days before hatching and the first living days of the chick. These cases also require the development of effective antioxidant capacities in tissues to prevent fat peroxidation (Gohari et al. 2019).

Nowadays, in ovo injection is used to prevent the reduction of glycogen stores and increase the antioxidant capacity to improve the hatching traits in the fertile eggs and growth performance (Peebles 2018).

Through in ovo injection, the nutrients are added to the embryonic environment before hatching, and those nutrients are used by the chick after hatching. These nutrients, along with yolk sac reserves, not only can help maintain the system and metabolism, but can also help chickens to continue growing, developing, and eating properly (El-Senousey et al. 2018).

Also, the in ovo injection has been investigated for the compounds that may contribute to improve the intestinal health of chicks during the rearing period (Slawinska et al. 2020). Fatemi et al. (2018) concluded, by investigating the in ovo injection of antioxidants and also antioxidant concentrations of broiler embryonic tissues, that the in ovo injection of antioxidants can positively increase the total antioxidant capacity of chickens throughout the rearing period.

Numerous results from previous research have shown that in ovo injections of carbohydrates and antioxidants have positive effects on hatching as well as improving the performance, immune system and antioxidant activity of broilers.

The increase in egg storage reduces yolk height and index (Kirunda and McKee 2000). Kirunda and McKee (2000) stated that similar to the egg white, the biochemical changes that occurred during storage are the reason for having a lower quality of the yolk and this reduction of quality increases over a longer time. The ability of the foetus to use yolk nutrients is endangered due to the limited oxygen access to aerobic metabolism until the lungs begin to breathe, causing the foetuses to gradually reduce fat metabolism and increase glycogen metabolism (Moran and Reinhart 1980). Since the storage of fertile eggs affects yolk nutrients, including carbohydrates, this lack of energy can lead to poor hatching and, in more severe conditions, can lead to foetal death (Bhattacharyya et al. 2018). On the other hand, the lack of carbohydrates in stored eggs causes the lipids metabolism in eggs to be considered as the main source of energy for growth, which leads to the production of large amounts of free radicals (Ibrahim et al. 2012). Therefore, the supply of energy in the form of carbohydrates, as well as the injection of antioxidants, is an important factor for the successful hatching of chicks from fertile eggs.

As mentioned the Storage of incubating eggs reduces the hatching rate. On the other hand, some researches showed that although the quality of eggs decreases from the 8th day of storage upwards, by controlling the temperature of the storage area, the quality of egg yolks until fourteen days changes to the extent that they can be used for incubation after enrichment (Hossieni Siyar et al. 2010).

Since a mixture of several antioxidants or a combination of several carbohydrates is rarely used in the injection of stored fertile eggs, and on the other hand, the benefits in the simultaneous use of antioxidants with carbohydrates have received less attention in the injection of stored fertile eggs. This experiment aimed to investigate the effects of in ovo injections of antioxidants and carbohydrates, separately and mixed, into the stored hatching eggs on hatching, performance, immune system, intestinal morphology, and immune system of broilers.

Materials and methods

The experimental protocol was ratified by the Animal Ethic Committee of the Science and Research Branch, Islamic Azad University, Tehran, Iran, and the experiment performed with respect to the International Guidelines for research involving animals (Directive 2010/63/EU).

To perform this experiment, 600 fertile incubating eggs with an average weight of 67.27 ± 0.7 g were selected, from a commercial broiler of Cobb 500 strain having 44 weeks of age and a hatching rate of 85%. Before the incubation period, the eggs were stored for 14 days at a temperature of 16 °C and a humidity of 65%. After the storage period, the eggs were distributed based on an average weight in 5 treatments and 5 replicates (each replicate containing 24 eggs) in an incubator (Jamesway Incubator Company, Canada) with a temperature of 37.5 °C and relative humidity of...
56%. On day 17.5 of the incubation period, first, the position of the amniotic fluid of the eggs was determined using the candling method and then half a millilitre of the experimental solutions was injected using a syringe with a 25-gauge needle and a length of 16 mm from the wide part of the egg into the amniotic fluid of the fertile eggs. The injection spot was blocked with paraffin after injection, and the eggs were placed in standard condition (a temperature of 36.8 °C and relative humidity of 70%). The experimental treatments included: (1) Negative control (NC) (without injection), (2) Positive control (PC) (injection of 0.5 ml normal saline solution), (3) Injection of 0.5 ml carbohydrates solution (CA), (4) Injection of 0.5 ml antioxidant solution (AS) and (5) injection of 0.5 ml CS + AS.

The carbohydrates solution used in this experiment contained maltose (25 mg/mL); sucrose (25 mg/mL); dextrin (100 mg/mL); insulin (1 IU/mL); chromium picolinate (2 mg/mL) and dihydrostreptomycin (1 mg/mL) as well as antioxidant solution containing vitamin E (12 mg/mL); selenium (120 μg/mL); coenzyme Q10 (2 mg/mL); vitamin C (3 mg/mL) and dihydrostreptomycin (1 mg/mL). The mixture of carbohydrates and antioxidant solution used for in ovo injection containing maltose (25 mg/mL), sucrose (25 mg/mL), dextrin (100 mg/mL), insulin (1 IU/mL), chromium picolinate (2 μg/mL), vitamin E (12 mg/mL), selenium (120 μg/mL); coenzyme Q10 (2 mg/mL), vitamin C (3 mg/mL) and dihydrostreptomycin (1 mg/mL).

Insulin was used in the solutions to have further penetration of carbohydrates into the cell. Chromium picolinate was also used to improve and enhance insulin performance in cryohydrate solution.

**Hatching characteristics and physical quality**

48 hours after hatching, when 95% of the chicks had dry cervical fluff and villi, the hatchability percentage was calculated based on the number of chicks hatched from the fertile eggs. The chickens in each treatment were weighed using a digital scale with an accuracy of 0.01 g. After measuring the weight of the chickens, the ratio of the chicken weight to the eggs weight was calculated. The scores suggested by Tona et al. (2003) were used to evaluate the physical quality of the hatched chicks.

**Blood parameters and immune system**

To evaluate blood indices and immune system, 3 chickens were randomly selected from each replicate and 1.5 cc blood samples were taken from each chicken. Half a millilitre of each sample was transferred to EDTA-containing tubes for counting red blood cells (number of red blood cells, haemoglobin, haematocrit, and mean cell volume) and white blood cell count for immunoassay. Red and white cell counts were performed by the method of Ross et al. (1976).

The remaining volume of blood samples was placed at room temperature to coagulate. After 10 minutes of centrifugation at 3000 rpm, serum was separated from blood samples and serum samples were held at −20 °C for the test of biochemical factors (glucose, triacylglycerol, and protein).

**Tissue glycogen and intestinal morphology**

After blood sampling, the chicks (three chickens selected in the blood sampling) were slaughtered with CO2 gas, and the internal components were separated from the carcass and then the liver, breast, and thigh tissues were separated and washed with physiological serum. One gram of the tissues was isolated and after removing the connective tissues, they were homogenised with 2 cc of distilled water using a homogeniser. In the next step, 2 cc of 4-normal hydrochloric acid (HCl) was added to the solution and placed in a water bath at 50–90 °C for two hours to hydrolyse glycogen to glucose. After cooling the solution, 2 cc of normal sodium hydroxide (NaOH) was added to neutralise it (brining the pH to 7–7.3). In the final step, the glucose concentration of the solution was measured using a Pars Azmun kit with the photometric method (Zhang 2012).

To study the intestinal morphology, the duodenum, jejunum, and ileum sections of the small intestine were separated and their length was measured with meters. Then one centimetre was separated from the middle part of the jejunum. The contents of the intestine were emptied from the isolated pieces and tissue blocks were prepared from jejunum tissue samples after fixation, dehydration, clearing, and placement in paraffin. After staining (Alcian blue), the slides were studied by light microscopy using Eyepiece Graticule, and the villus length, villus width, and crypt depth were measured. Then the ratio of villus length to villus width and also the ratio of villus length to crypt depth were determined (Poorghasemi et al. 2017).

**Antioxidant activity of liver tissue**

To measure the antioxidant activity, three chickens were selected from each replicate and after
slaughter with CO₂ gas and removal of the liver, one gram of the liver tissue was chopped into pieces and poured into a tube and 10 ml homogenisation buffer (Phosphate buffer, pH = 7.2) was added to it and homogenised using a homogeniser (4 minutes at 10,000 xg). The resulting suspension was centrifuged for 10 min at 4500 xg in a refrigerated centrifuge to precipitate the waste material and the pure homogeneous solution was used for the measurement of catalase, malondialdehyde, superoxide dismutase, glutathione peroxidase, free radical scavenging power of diphenyl-picrylhydrazyl (DPPH) and total antioxidant capacity (TAC) (Benzie and Strain 1996; Yagi 1998).

To measure catalase activity, 50 µL of centrifuged supernatant was taken and diluted with phosphate buffer up to 500 times. Then 2 mL of this solution was poured into the cuvette, 1 mL of 30 mM hydrogen peroxide was added to it and the changes in adsorption were monitored in front of blank at 240 nm wavelength for 1 minute, and then the enzyme activity was calculated.

The basis of tissue malondialdehyde measurement method was based on reaction with thiobarbituric acid, extraction with normal butanol, adsorption measurement by spectrophotometry and comparison of adsorption with the standard curve.

The activity of superoxide dismutase enzyme was investigated on a supernatant prepared from homogenous liver tissue using RANSOD kit (Randox Laboratories Ltd, Crumlin, UK). SOD activity was measured at a wavelength of 500 nm by the spectroscopy of supernatant solution.

The activity of glutathione peroxidase enzyme was calculated using RANSEL kit (Randox Laboratories Ltd, Crumlin, UK). The basis of the reaction was that first the reduced glutathione was oxidised by glutathione peroxidase and then the oxidised glutathione was converted to the reduced glutathione in the presence of glutathione reductase and NADPH. The decrease in uptake due to the conversion of NADPH to NADP⁺ is proportional to the concentration of glutathione peroxidase.

Free radical scavenging capacity of diphenylpicryl hydrazyl (DPPH) is a spectrophotometric technique based on the adsorption of stable colour radicals that demonstrates the ability of the obtained homogeneous solution to adsorb active radicals. DPPH radicals have the highest absorption at 517 nm and appear dark blue. But if these active radicals are neutralised, they turn yellow or become colourless. The lower value of the absorption rate means a stronger antioxidant activity in this solution.

Benzie and Strain method was used to measure the total antioxidant capacity of liver tissue homogenate by Ferric Reducing Antioxidant Power (FRAP) test. In the FRAP experiment, the changes in absorbance at 593 nm due to the production of a blue dye induced by the reaction of Fe³⁺ with 2,4,6-tripyridyl-s-triazine (TPTZ) were measured. The compounds that have stronger electron donating properties can reduce the Fe³⁺ in the FRAP reagent to Fe²⁺. In this case, Fe²⁺ binds to TPTZ and produces a blue colour whose intensity can be measured at the wavelength of 593 nm.

**Performance**

48 hours after hatching, ten male chicks were randomly selected from the chicks of each replicate and transferred to the breeding hall. Breeding conditions were the same for all chicks in terms of temperature changes and vaccination time during the period and free access to feed and water was provided. The diets used in this experiment were the same for all experimental groups and were adjusted according to the recommendations of the Cobb 500 breeding guide (Table 1). The feeds of each replicate were weighed before consumption and by specifying the weight of the remaining feeds at the end of each week, finally, the amount of feed intake for each treatment was calculated for each period and also the entire period.

| Ingredients (% on dry matter)       | Starter (1–21 days old) | Grower (22–42 days old) |
|-------------------------------------|-------------------------|-------------------------|
| Corn                                | 61.5                    | 63.00                   |
| Soybean meal (CP 44 %)              | 31.00                   | 30.20                   |
| Soybean oil                         | 3.00                    | 3.00                    |
| Calcium bicarbonate                 | 1.40                    | 1.50                    |
| Dicalcium phosphate                 | 1.5                     | 1.00                    |
| Salt                                | 0.35                    | 0.20                    |
| Mineral premix<sup>a</sup>          | 0.50                    | 0.50                    |
| Vitamin premix<sup>b</sup>          | 0.50                    | 0.50                    |
| DL-methionine                       | 0.25                    | 0.10                    |
| Chemical analyses                   |                         |                         |
| Metabolizable energy (kcal/kg)      | 3027                    | 3036                    |
| Crude protein (DM)                  | 21.07                   | 19.02                   |
| Lysine (%)                          | 1.10                    | 1.00                    |
| Methionine + Cysteine (%)           | 0.90                    | 0.72                    |
| Arginine                            | 1.19                    | 0.31                    |
| Methionine (%)                      | 0.48                    | 0.34                    |
| Threonine (%)                       | 0.80                    | 0.74                    |
| Ca (%)                              | 0.94                    | 0.86                    |
| Available phosphorus (%)            | 0.41                    | 0.38                    |
| CI (%)                              | 0.18                    | 0.17                    |

<sup>a</sup>Mineral mixture per kg of diet: Mn: 64 mg; Zn: 75 mg; Fe: 40 mg; Cu: 10 mg; Se: 0.3 mg, and I: 1.85 mg.

<sup>b</sup>Vitamin premix per kg of diet: vitamin A: 11013 U; vitamin D₃: 3525 U; vitamin E: 33 U; vitamin K₃: 2.75 mg; vitamin B₆: 5 mg; vitamin B₁₂: 0.028 mg; Thiamine: 2.2 mg; Riboflavin: 7.7 mg; Pantothenate: 17.6 mg; Nicotinic acid: 55.1 mg; Folic acid: 1.1 mg; vitamin H₂: 0.22 mg and Choline chloride: 378 mg. Ca: calcium; Cl: chlorine

Table 1. The ingredients of the basal diet.
The chickens were weighed weekly and weight gain and conversion factor were determined at the end of each period as well as the entire period.

**Statistical analysis**

This experiment was performed in a completely randomised design and the results were analysed by ANOVA using the GLM procedures of SAS software and the mean of treatments were compared using Duncan’s test at a probability level of 5% \((p < .05)\). The statistical model of the design was \(Y_{ij} = \mu + A_i + e_{ij}\). In this model, \(Y_{ij}\) is the value of each observation for the studied trait, \(\mu\) is the average of the observations, \(A_i\) is the effect of experimental treatments and \(e_{ij}\) is the effect of experimental error.

**Results**

**Features of hatching**

The results associated with the effect of the *in ovo* injection of carbohydrates and antioxidants alone or in combination on hatching parameters are presented in Table 2. The hatching percentage and the losses percentage in the treatments that used a mixture of carbohydrates and antioxidants had a significant increase and decrease compared to the control (NC and PC), respectively \((p < .05)\). Also, the difference in weight of the newly hatched chicks and the ratio of body weight to eggs in the treatments of carbohydrates and a mixture of carbohydrates and antioxidants showed a significant increase compared to the control \((p < .001)\).

**Physical quality of chickens**

Table 3 shows the results associated with the effect of the *in ovo* injection of carbohydrates and antioxidants in the stored hatching eggs on the physical quality of the newly hatched chicks.

According to the results, the activity of chickens, appearance and eye condition in all experimental treatments were significantly different from the control. This difference was more in the eggs that were injected with a mixture of carbohydrates and antioxidants compared to the other treatments \((p < .05)\). The condition of the legs of chickens in groups treated with carbohydrates and antioxidants had higher values \((p < .005)\) compared to control groups (NC and PC). Umbilical status, residual membrane status, yolk residual size and total Tona score were significantly different from the control in all the experimental treatments, but this difference was more in the eggs injected with carbohydrates and a mixture of carbohydrates and antioxidants \((p < .001)\).

**Table 2.** The effect of the *in ovo* injection of carbohydrates and antioxidants into the stored incubating eggs on hatching parameters.

| Items                   | Treatments | SEM | \(p\)-Value |
|-------------------------|------------|-----|-------------|
| Hatchability (%)        | NC         | 76.72 \(^b\) | 76.41 \(^b\) | 78.89 \(^{ab}\) | 74.46 \(^{ab}\) | 89.66 \(^a\) | 4.068 | .0340 |
| BW of hatched chicks (g) | PC         | 46.18 \(^d\) | 46.30 \(^{bc}\) | 47.21 \(^*\) | 47.11 \(^{ab}\) | 47.75 \(^*\) | 0.299 | .0008 |
| BW of hatched chicks / Egg Weight | CS         | 69.30 \(^b\) | 69.11 \(^b\) | 70.42 \(^*\) | 67.69 \(^{ab}\) | 72.69 \(^*\) | 2.076 | <.0010 |
| Losses (%)              | AS         | 5.64 \(^{d}\) | 5.65 \(^*\) | 4.96 \(^{ab}\) | 4.52 \(^{ab}\) | 1.82 \(^*\) | 1.255 | .0212 |

NC: negative control; PC: positive control; CS: carbohydrates solution; AS: antioxidants solution and CS + AS: carbohydrates solution + antioxidants solution.

The means within the same row with at least one common letter do not have significant difference \((p > .05)\).

SEM: standard error of the means.

**Table 3.** The effect of the *in ovo* injection of carbohydrates and antioxidants into the stored incubating eggs on the physical quality of the newly hatched chickens.

| Items                  | Treatments | SEM | \(p\)-Value |
|------------------------|------------|-----|-------------|
| Chicken activity       | NC         | 4.71 \(^{b}\) | 4.56 \(^b\) | 5.35 \(^*\) | 5.62 \(^*\) | 5.72 \(^*\) | 0.154 | <.001 |
| Appearance             | PC         | 9.33 \(^{b}\) | 9.32 \(^{b}\) | 9.73 \(^*\) | 9.78 \(^*\) | 9.85 \(^*\) | 0.137 | <.001 |
| Status of eyes         | CS         | 13.93 \(^{b}\) | 13.45 \(^{b}\) | 15.30 \(^*\) | 15.57 \(^*\) | 15.90 \(^*\) | 0.337 | <.001 |
| Status of legs         | AS         | 12.56 \(^{b}\) | 12.82 \(^b\) | 15.27 \(^*\) | 15.17 \(^*\) | 15.20 \(^*\) | 0.421 | <.001 |
| Status of navel        | CS         | 8.85 \(^{b}\) | 8.08 \(^b\) | 11.08 \(^*\) | 10.93 \(^*\) | 11.12 \(^*\) | 0.299 | <.001 |
| Status of residual membrane | PC       | 9.29 \(^{b}\) | 9.26 \(^{b}\) | 11.22 \(^*\) | 10.93 \(^*\) | 11.25 \(^*\) | 0.278 | <.001 |
| Amount of residual yolk | CS         | 12.44 \(^{b}\) | 12.78 \(^b\) | 14.82 \(^*\) | 14.40 \(^*\) | 15.02 \(^*\) | 0.368 | <.001 |
| Status of yolk stretching | AS      | 8.74 \(^{b}\) | 8.65 \(^*\) | 11.18 \(^{ab}\) | 10.83 \(^{ab}\) | 11.58 \(^{ab}\) | 0.252 | <.001 |
| Total Tona score       | CS         | 79.67 \(^{d}\) | 79.69 \(^{d}\) | 94.20 \(^*\) | 91.32 \(^{ab}\) | 95.60 \(^*\) | 1.663 | <.001 |

NC: negative control; PC: positive control; CS: carbohydrates solution; AS: antioxidants solution and CS + AS: carbohydrates solution + antioxidants solution.

The means within the same row with at least one common letter do not have significant difference \((p > .05)\).

SEM: standard error of the means.
**Blood parameters**

The results associated with the effect of the *in ovo* injection of carbohydrates and antioxidants on the number of red blood cells in the newly hatched chicks are presented in Table 4. The number of red blood cells of the chickens in the eggs that were injected with a mixture of carbohydrates and antioxidants was significantly increased compared to the control (*p* < .05). Haemoglobin concentration in all the experimental treatments was significantly increased compared to the control (*p* < .05).

Haematocrit concentration and mean corpuscle volume were not significantly different in any of the experimental treatments compared to the control (*p* > .05). Numerically, the highest concentrations of haematocrit and mean corpuscle volume were related to the injected mixtures of carbohydrates and antioxidants.

**Immunity system**

The results associated with the effect of the *in ovo* injection of carbohydrates and antioxidants on the immune system of newly hatched chicks are presented in Table 6. The lymphocyte percentage in the chickens

| Items                              | Treatments      | SEM | p-Value |
|------------------------------------|-----------------|-----|---------|
| Red blood cells count (10⁶/mm³)    | NC  | PC  | CS  | AS  | CS + AS | NC  | PC  | CS  | AS  | CS + AS | NC  | PC  | CS  | AS  | CS + AS |
| Haemoglobin (g/dL)                | 3.17b          | 3.10b| 3.36ab | 3.55ab | 3.76a    | 0.135 | .0138 |
| Haematocrit (%)                   | 10.11b         | 10.03b| 11.18a | 12.21a | 12.55a    | 0.059 | .0200 |
| Mean corpuscle volume (µm³)       | 34.11           | 34.24  | 34.78  | 34.36  | 34.63     | 0.561 | .9006 |
|                                  | 130.78          | 130.93  | 130.50  | 129.98  | 130.87     | 10.53 | .509  |

NC: negative control; PC: positive control; CS: carbohydrates solution; AS: antioxidants solution and CS + AS: carbohydrates solution + antioxidants solution.

The means within the same row with at least one common letter do not have significant difference (*p* > .05).

SEM: standard error of the means.

| Items                              | Treatments      | SEM | p-Value |
|------------------------------------|-----------------|-----|---------|
| White blood cells count (10³/mm³)  | NC  | PC  | CS  | AS  | CS + AS | NC  | PC  | CS  | AS  | CS + AS |
| Neutrophil (%)                     | 42.60           | 43.88  | 45.13  | 45.69  | 45.94     | 1.629 | .6354 |
| Lymphocyte (%)                     | 45.25b          | 45.27b | 47.29ab | 48.88ab | 50.10a    | 1.458 | .0418 |
| Monocyte (%)                       | 2.94            | 3.00   | 3.00   | 3.12   | 3.13      | 0.316 | .9893 |
| Eosinophil (%)                     | 2.70            | 3.71   | 3.94   | 3.93   | 4.13      | 0.467 | .3491 |
| Basophil (%)                       | 0.53            | 0.53   | 0.60   | 0.60   | 0.75      | 0.191 | .9104 |

NC: negative control; PC: positive control; CS: carbohydrates solution; AS: antioxidants solution and CS + AS: carbohydrates solution + antioxidants solution.

The means within the same row with at least one common letter do not have significant difference (*p* > .05).

SEM: standard error of the means.

The results associated with the effect of the *in ovo* injection of carbohydrates and antioxidants on the biochemical parameters of the blood of newly hatched chicks are presented in Table 5. Blood glucose concentrations in the chickens hatched from the eggs in which only the injection of carbohydrate solution was used had a significant increase compared to the control and treatments under injected antioxidants (*p* < .05). The difference in protein and triacylglycerol concentrations was not significant in any of the treatments (*p* > .05).

| Items                              | Treatments      | SEM | p-Value |
|------------------------------------|-----------------|-----|---------|
| Glucose (mg/dL)                    | 192.00b         | 192.70b | 218.00a | 193.88a | 204.80a  | 5.9618 | .0345 |
| Total Protein (mg/dL)              | 1.93            | 1.90   | 1.98   | 2.04   | 2.04      | 0.0991 | .8123 |
| Triacylglycerol (mg/dL)            | 72.85           | 69.90  | 66.30  | 69.15  | 69.50     | 3.315  | .7398 |

NC: negative control; PC: positive control; CS: carbohydrates solution; AS: antioxidants solution and CS + AS: carbohydrates solution + antioxidants solution.

The means within the same row with at least one common letter do not have significant difference (*p* > .05).

SEM: standard error of the means.
hatched from in ovo injection of a mixture of carbohydrates and antioxidants showed a significant increase compared to the control ($p<.05$). Although the number of white cells, as well as the percentages of neutrophils, monocytes, eosinophils, and basophils were significant in none of the experimental treatments compared to the control, the chickens hatched from the eggs produced by injecting a mixture of carbohydrates and antioxidants had higher levels ($p>.05$).

**Tissue glycogen**

The results associated with the effect of in ovo injection of carbohydrates and antioxidants on the glycogen store of liver and muscle tissue of the newly hatched chickens are presented in Table 7. The amount of glycogen stored by the liver in the chickens produced by injecting carbohydrates, antioxidants, and their mixtures into the stored eggs increased significantly compared to the control ($p<.05$). The amount of glycogen stored in muscle tissue (breast and thigh) of chickens born from injections of carbohydrates and antioxidants had a significant increase compared to the control and also compared to AS group for glycogen stored in thigh muscle ($p<.05$).

| Items                        | Treatments                  | NC        | PC        | CS         | AS         | CS + AS    | SEM        | $p$-Value |
|------------------------------|------------------------------|-----------|-----------|------------|------------|------------|------------|-----------|
| Liver glycogen (mg/g)        |                              | 44.45c    | 47.19c    | 62.95a     | 52.14b     | 59.22ab    | 5.100      | .0459     |
| Glycogen of breast muscle (mg/g) |                             | 8.19b     | 8.21b     | 10.48a     | 9.27b      | 9.90a      | 0.444      | <.0010    |
| Glycogen of thigh muscle (mg/g) |                             | 5.48b     | 5.39b     | 9.35a      | 5.67b      | 9.23b      | 0.400      | <.0010    |

NC: negative control; PC: positive control; CS: carbohydrates solution; AS: antioxidants solution and CS + AS: carbohydrates solution + antioxidants solution. The means within the same row with at least one common letter do not have significant difference ($p > .05$). SEM: standard error of the means.

The ratio of duodenal length to the total length of the small intestine in the treatment under the injection of a mixture of carbohydrates and antioxidants was significantly increased under the injection of antioxidants ($p<.05$).

The length of villi in the jejunum in the newly hatched chicks from all the three treatments under the injection of carbohydrates and antioxidants increased significantly compared to the control ($p<.05$). The difference in the crypt depth, as well as the ratio of villus length to crypt depth of the jejunum in the treatment under the injection of a mixture of carbohydrates and antioxidants, decreased and increased significantly compared to the control, respectively ($p<.05$). The difference between villus width and the ratio of villus width to villus length in the jejunum was not significant in any of the treatments compared to the control ($p>.05$).

**Intestinal morphology**

The results associated with the effect of the in ovo injection of carbohydrates and antioxidants into the stored incubating eggs on the intestinal morphology of the newly hatched chickens are presented in Table 8. The difference in the jejunum length, as well as the ratio of jejunum length to the total length of the small intestine in the newly hatched chickens from all the three treatments under the injection of carbohydrates and antioxidants, had a significant increase compared to the control ($p<.05$). The differences in the length of the duodenum and ileum as well as the ratio of ileum length to the total length of the small intestine were not significant in any of the treatments compared to the control ($p>.05$).

**Antioxidant activity**

The results associated with the effect of the in ovo injection of carbohydrates and antioxidants into the stored incubating eggs on the antioxidant activity in the liver tissue of the newly hatched chickens are presented in Table 9.

Catalase concentration in the chickens hatched from the in ovo injection of antioxidants had a significant increase compared to the treatments under the injection of carbohydrates and a mixture of carbohydrates and antioxidants and also with the control ($p<.05$).

Superoxide dismutase concentration, DPPH free radical scavenging ability, and total antioxidant capacity in the chickens hatched from in ovo injection of antioxidants were significantly increased compared to the control ($p<.05$). The malondialdehyde concentration in the chickens hatched from the same treatment (under antioxidant injection) also had a significant decrease compared to the control ($p<.05$). The difference in the superoxide dismutase concentrations was not significant in any of the treatments ($p<.05$).
Performance

The results associated with the effect of in ovo injection of carbohydrates and antioxidants into the stored incubating eggs on the growth performance of broilers are presented in Table 10. Weight gain, feed intake, and conversion ratio in the starter period (1–21 days) as well as in the entire rearing period (1–42 days) in the chickens hatched from the eggs injected with a solution of carbohydrates, antioxidants and their mixtures had a significant improvement compared to the control (p < 0.05). During the grower period (22–42 days), the increase in the body weight
of broilers in all the experimental treatments was significantly different compared to the control \((p<.05)\). Feed intake during the grower period in the chickens hatched from the in ovo injection of a mixture of carbohydrates and antioxidants had a significant increase compared to the control \((p<.05)\). The difference in the conversion ratio wasn’t significant in any of the treatments in the grower period \((p<.05)\).

**Discussion**

In this experiment, the injection of carbohydrates, as well as a mixture of carbohydrates and antioxidants into the incubated eggs, increased the weight of hatched chickens and the ratio of the weight of hatched chick to the weight of the incubated egg, which is consistent with the results of Uni et al. (2005) and El-Senousey et al. (2018).

As glucose is necessary for better embryonic development in the final stages of hatching, when more glycogen is stored in the liver, the glycolysis process can better provide the energy needed for the hatching stage, thus preventing the breakdown of muscle protein and the weight gain in the hatched chicks. Thus, embryonic nutrition of carbohydrates increases the glycogen stores of the liver and can ultimately increase the weight of the hatched chicks and reduce losses (Uni et al. 2005). Tangara et al. (2010) and Surai et al. (2016) also stated, that the in ovo injection of carbohydrates and antioxidants increases hatching and reduces embryonic losses.

But in the present experiment, the injection of carbohydrates could not individually have a significant effect on the hatchability.

However, in another study (Zhai et al. 2011), the injection of 0.4 mL of saline containing various carbohydrates into embryonated broiler chickens eggs was found to reduce hatchability. The introduction of a 0.4-mL volume of external water may itself have reduced hatchability. Eggs having low rates of water loss commonly exhibit delayed hatches or even fail to hatch (Romanoff 1930), and to hatch successfully, eggs must lose approximately 12–15% of initial egg weights up to the point of pipping. In the current study, the detrimental effects of injection volume 0.5 mL for certain carbohydrates further suggests that the injection volume should be considered and possibly limited to prevent excessive hydration of the embryo and a subsequent decrease in hatchability.

In the present study, the injection of antioxidants increased the weight of newly hatched chicks compared to the control (NC). Urso et al. (2015) in their study on the effect of antioxidants on hatching rate and the weight of newly hatched chicks, observed that the hatching percentage increased 6.54 percent by in ovo injection of antioxidants, and body/egg weight after hatching increased 4.74% compared to the control, which is consistent with the results of the present study.

They reported that high concentrations of unsaturated fatty acids in cell membranes increase the sensitivity of embryonic tissues to the destruction by free radicals or reactive oxygen species and that cells may be destroyed in these processes (Urso et al. 2015). At these times, during and immediately after hatching, their metabolic rate and oxygen consumption for endothermic needs and the demand of physiological activity increase (Urso et al. 2015; Surai et al. 2016). Pulmonary respiration is a natural and essential process for broilers, but it increases circulating oxygen rates and thus increases the production of free radicals due to oxidative processes (Surai et al. 2016). According to the theory of Surai et al. (2016), yolk antioxidants control oxidation by reducing and inactivating the free radicals before they influence the tissue of organs, which can increase the hatching rate and the weight of the newly hatched chicks and also reduce losses.

In the present experiment, injecting a mixture of carbohydrates and antioxidants into the hatching eggs significantly increased the hatchability of the chickens.

Previous studies have shown that the enrichment of fertile eggs on one hand had better foetal oxygen consumption in the last days of hatching, on the other hand, can increase the hatching capability of chicks (Lourens et al. 2011). It seems that in the present experiment, providing a carbohydrate source and preventing the reduction of glycogen stores, as well as increasing antioxidant capacity and protecting unsaturated lipids against hatching stress in the stored fertile eggs, significantly improved hatching ability in the treatments injected with carbohydrates and antioxidants.

As can be seen in the results, from all the stored eggs that were injected with carbohydrates and antioxidants during the incubation period, chickens with higher physical quality were produced compared to the control, which is consistent with the results of Khaligh et al. (2017) and Bhanja et al. (2008).

Excessive depletion of glycogen stores during hatching has a negative effect on growth. Near the end of the incubation, the embryo uses its energy reserves for the large amounts of glucose required for hatching (the energy required for muscle activity).
Although glucose can be made from fat and protein, due to the limited oxygen in the last quarter of incubation, glucose is mostly produced from gluconeogenesis and glycolysis from protein and glycogen, respectively (Bhanja et al. 2008).

On the other hand, the quality of yolk and albumin may decrease in the storage of hatching eggs and as a result, it reduces glycogen stores. Therefore, carbohydrate injection can also be a good solution for the embryo to easily use the energy source, because carbohydrates can reduce the consumption of muscle protein, which is used as an energy source, by providing the necessary energy for the embryo, and thus produce chickens with better quality (Retes et al. 2018).

Also, Shafey et al. (2012) reported that antioxidants, along with glycogen stores, not only help maintain embryonic metabolism but also lead to better yolk sac absorption and the production of high-quality chickens. Rafiee et al. (2016) stated that the in ovo antioxidant compounds are among the various nutrients that can significantly affect the development of chicken embryos and their survival in the early stages of life after hatching. High levels of antioxidants in the embryonic egg and tissues can act as a mechanism consistent to protect tissues during normal oxidative stress at hatching and produce high-quality chicks (Zhang et al. 2018).

According to the results, the number of red blood cells and the haemoglobin concentration of the chickens hatched from the eggs that were injected with a mixture of carbohydrates and antioxidants had a significant increase compared to the control. Consistent with the results of the present experiment, Panda and Cherian (2014) stated in their experimental results that the increase in the haemoglobin concentration of the newly hatched broilers is directly related to the embryo’s nutrition during the incubation period. They stated that the in ovo injections of vitamin E and carbohydrates increased the haemoglobin concentration of the newly hatched chicks, which may have been due to an increase in the red blood cells in the process of improving haematopoiesis. They also stated that carbohydrates and antioxidants improve the circulation of nutrients and oxygen in the embryo, which can enhance the haematopoietic process (Panda and Cherian 2014). According to some studies, dehydration and nutrient deficiency can adversely affect the haematopoietic process in broiler embryos. Therefore, the decrease in blood cells in the control group can be attributed to the loss of humidity and insufficiency of nutrients in the stored eggs (Campbell, 1994).

The results of the present experiment showed that chickens with higher glucose concentrations were hatched from the eggs that were injected with a carbohydrate solution. Consistent with the results of the present experiment, Ebrahimnezhad et al. (2011) reported that the injection of 0.5 ml 20% and 25% glucose at the 7th day of incubation into embryonic albumin increased the blood glucose of one-day-old chicks compared to the control (without injection). Injecting carbohydrates into the eggs increases the amount of the stored glycogen (Taghipour Shahbandi et al. 2019). As at the end of the incubation period, the activity of the glycolysis cycle than the lipid oxidation in the period of oxygen shortage is more essential for embryonic respiration than pulmonary respiration, the amount of glucagon and the glycolysis process increase, which leads the embryos with higher glycogen store to have increased glucose concentration in the newly hatched chicks (Nazem et al. 2019).

The results of the present experiment showed that the injection of a mixture of carbohydrates and antioxidants can increase the percentage of lymphocytes in the newly hatched chickens, which indicates the strengthening of the immune system.

Yi et al. (2005) showed in their research that primary nutrition leads to better development of the immune system (primary and secondary immune system maturation). The greater advantage of glycogen stores in the post-hatching period probably supports the development of vital systems, including the immune system (Zhai et al. 2011).

The oxidation of cellular unsaturated fatty acids alters the composition, structure, and cellular membrane properties of the embryo (fluidity, permeability, etc.) and the function of membrane-bound enzymes. The corresponding damages to biological molecules ultimately affect the efficiency of the immune system (Sgavioli et al. 2016).

Salary et al. (2014) stated in their experimental results that the injection of vitamin E into the fertile eggs, not only helps integrate the lipoprotein parts of the cell membrane, but also reduces oxidative changes and develops cellular immune responses. Vitamin E can increase immunity as an antioxidant by improving the proliferation and increase of lymphocytes (Salary et al. 2014). Studies have also shown that vitamin E and selenium play an important role in the production of blastocyst lymphocytes (Xiao et al. 2016). Some researchers have stated in their experiments that the in ovo injection of vitamin C as an antioxidant plays a role in strengthening the leukocyte membranes and that the presence of appropriate
amounts of this vitamin increases the phagocytic activity of neutrophils (Givisiez et al. 2020). Therefore, in the present experiment, due to the increase in the number of lymphocytes in the hatched chickens, it can be stated that the injection of carbohydrates and various antioxidants into the stored eggs increases the quality of the eggs and thus produces chickens with the appropriate immune system.

Uni et al. (2005) observed similar results of carbohydrate injection on liver glycogen levels on days 19 and 20 of incubation period for both Cobb and Ross strains.

Abousaad et al. (2017) in their study on the expression of gluconeogenic genes and enzymes during the development of chick embryos concluded that liver glycogen synthesis is the main source of embryonic growth, and injection of carbohydrates into the amniotic sac leads to an increase of about 1 to 60 mg glycogen per gram of liver in the day-old chicks. They also stated that glucose is the most important stimulant of insulin secretion. Insulin increases the transport of glucose and amino acids into the cell, and then glucose is stored as glycogen. Since insulin was present along with the carbohydrate solution in the present experiment to inject into the eggs, it may have increased the rate of glucose transport to the tissue compared to the control, which in turn increased the tissue glycogen. On the other hand, the availability of required energy during the embryonic period prevents the excessive depletion of glycogen in liver and muscle tissues (Bottje et al. 2010).

It has been also stated that in ovo feeding of a solution containing 20% dextrin and 3% maltose increased the tissue glycogen index. Adequate levels of glucose metabolism are preserved in the final embryonic stage by glucose uptake from liver glycogen and gluconeogenesis of albumin in the amniotic fluid and muscle (Foye et al. 2006).

Araújo et al. (2019) stated in their research that the use of antioxidants in the nutrition of broiler embryos may be effective by inactivating the effect of free radicals on glycogen stored in the liver and muscle tissue of the newly hatched chickens.

As soon as the radicals and hydroperoxides produced by the oxidation of the unsaturated lipids of the embryonic membrane in the incubation exceed the antioxidant capacity of the proteins and other recovering compounds in the growth medium, the proteins, lipids, and other sensitive molecules will be exposed to oxidative damage and as a result, can attack the side chains of sensitive amino acids and prevent the store of glycogen in liver and muscle tissue (Urso et al. 2015).

As seen in the results, increasing the length of the jejunum, increasing the length of the villi, and also decreasing the crypt depth in the jejunum of the chickens hatched from the eggs injected with carbohydrates and antioxidants compared to the control indicate better development and growth of intestinal tissue during the embryonic period.

Maintaining a stable and optimal state of glucose supply is very important for the final stages of embryonic development, hatching process and post-hatching growth until the beginning of feed consumption in poultry. In practical conditions, most of the chickens do not have access to water and feed for 48 hours after hatching or more, and feeding pre-hatching embryos by injecting the needed nutrients into eggs, given that early access to feed causes the growth and development in the newly hatched chicks, can have positive effects on the growth and development of the gastrointestinal tract (Uni and Ferket 2004).

Smirnov et al. (2006) showed that the in ovo injection of carbohydrates increased the level of villi in jejunum on the hatching day and 3 days after hatching. They stated that the in ovo injection of carbohydrates into eggs increased the ratio of acidic mucin-producing goblet cells and the expression of mucin mRNA in the intestine goblet cells of broilers.

Tako et al. (2004) reported that the injection of dextrose and a mixture of sucrose and dextrose into the amniotic sac at the end of the broiler embryonic period increased the length of intestinal villi, which is consistent with the results of this study. Injecting a mixture of carbohydrates and antioxidants resulted in a significant difference in crypt depth and the villus-length to crypt depth ratio in the newly hatched chicks, which is consistent with the results of Ostażewski and Nissen (1988) and Rajkumar et al. (2015).

In this experiment, carbohydrate injection probably caused a significant increase in the length of intestinal villi, probably due to the increased proliferation of intestinal cells (Ostażewski and Nissen 1988). Embryonic nutrition of carbohydrates can also increase the growth and development of the digestive system by increasing the proliferation and differentiation of enterocytes and/or reducing the breakdown of protein (Tako et al. 2004).

Consistent with the results of the present experiment, Murakami et al. (2007) stated that in ovo injection of vitamin E protects enterocytes, thereby increasing cell proliferation and increasing villus growth. Enterocytes were inactive during embryonic development and have irregular shapes and play no
role in digestion and absorption of nutrients (Rajkumar et al. 2015). Once the inner membrane of the egg ruptures and oxygen is available, although the chicks are still inside the egg, they begin to use enterocytes for digestion (Rajkumar et al. 2015). Therefore, the development of the chicken intestines after hatching is very dependent on the content of nutrients in the yolk. The injection of antioxidants can protect these nutrients in the eggs and develop the digestive system of chickens.

Increased CAT and SOD enzymes, as well as increased TAC in the liver cells of the newly hatched chicks from the in ovo injection of antioxidants, indicate oxidative improvement in the chicks.

Yalcın et al. (2017) in their research reported that increasing the concentration of CAT and SOD in the birds hatched from the in ovo injection of vitamin E showed a higher defense potential against protein and lipid oxidation.

Geng et al. (2004) reported that the use of 0.2 mg coenzyme Q10 in incubated eggs had a significant effect on the body’s antioxidant capacity, increase in the amount of SOD enzyme and decrease in the MDA of the newly hatched chicks. They stated that the presence of antioxidants in the growth environment of avian embryos is the major determinant of the development of the antioxidant system during the embryonic and early post-embryonic periods.

Since vitamin E, carotenoids, coenzyme Q, and minerals (selenium) are transported from the egg yolk to the tissues of the embryonic body, and on the other hand, the storage time of incubating eggs may decrease the quality of the material in the eggs, the injections of antioxidant supplements during the incubation period of the present experiment may have been able to increase the level of antioxidants in the contents of the injected eggs, thereby strengthening the activity of the antioxidant system of chickens hatched from these eggs compared to the control.

Previous studies have shown that the in ovo injection of antioxidants (Q10 and vitamin E) during incubation is effective on post hatch growth (Eslami et al. 2014; Kalantar et al. 2019). Higher body weight in this study may be due to the effect of antioxidants on energy efficiency in nutrient metabolism during incubation or post hatch growing period, as well as their antioxidant properties.

Vlaicu et al. (2020) showed that increasing the level of immunity in the newly hatched chicks improves the performance of broilers during the rearing period. In the present experiment, the increase in lymphocyte levels of the chickens hatched from the eggs injected with carbohydrates and antioxidants indicates a greater immune response in the embryonic period, and therefore the improved growth of chicks during the grower and finisher stages can be due to the good health of birds.

Bottje et al. (2010) reported, consistent with the present study, that elevated blood glucose levels in the chicks hatched from the in ovo injection of carbohydrates stimulated the digestive system by increasing the secretion of pancreatic digestive enzymes, which could increase feed intake, and have a positive effect on increasing the growth rate and body weight.

The disaccharides (maltase and sucrase) present in the membrane of intestinal micro-villi in broiler chickens on the twelfth day of embryonic were in low concentration and a few days before hatching simultaneous with the increase of the number and length of intestinal villi, a significant increase in their activity were observed so that from the 19 to 21 embryonic days, maltase and sucrase activity increased rapidly and continued until 21 days after continuous hatching (Iji et al. 2001). In the present experiment, the activity of disaccharides increased by injecting carbohydrates on day 17.5 of incubation. This high activity of disaccharides in the intestines of the hatched chickens allows the rapid digestion of dietary carbohydrates in the form of grains and starches, which can lead to weight gain and improved growth performance.

Recent studies consistent with the present results have shown that the high antioxidant activity in the newly hatched chickens can have a positive effect on the growth performance of chickens (Min et al. 2018). During the transfer of newly hatched chicks to the rearing hall, an oxidative stress condition will be created among the chickens. If the chickens have higher antioxidant activity in these conditions, their body tissues will have higher protection against this stress, which can improve the initial growth and also the final performance (Min et al. 2018).

The results of studies that showed an increase in the performance and an improvement in the conversion ratio of broilers through the in ovo injection of carbohydrates showed that the injection of 1 ml dextrin solution significantly accelerated intestinal development (Nazem et al. 2019).

Similar performance-enhancing effect was found by Maiorano et al. (2017) in a study carried out under field conditions on Ross 308 broiler chickens injected in ovo (12 day of incubation) with different prebiotics (raffinose family oligosaccharides, a commercial extract of beta-glucans and commercial trans-galactooligosaccharides).
In the present study, the effect of injecting carbohydrates into eggs on the development of intestinal morphology was observed after hatching. Increasing the level of intestinal absorption due to its effect on digestion and absorption has always been an important factor in improving the conversion ratio in broilers (Sklan, 2000). According to the results of the present experiment, this effect can be seen in the injected carbohydrates solution used in this experiment, so that the conversion ratio in the entire breeding period is significantly reduced due to the injection of the nutrients.

Therefore, the sooner the digestive system obtains its functional capacity, the faster the chickens will be able to use the nutrients in the diet, which may increase the performance of broilers compared to the control in the present experiment.

**Conclusion**

According to the results, the injections of carbohydrates, antioxidants and their mixtures in the stored fertile eggs had a significant improvement in the hatching characteristics, physical quality, blood indices, immune system, liver glycogen stores, development of small intestine and growing performance of chickens. The antioxidant effects in the chickens showed better results among the treatments by injecting an antioxidant solution into the eggs. In general, the results show that injecting a mixture of carbohydrates and antioxidants in the stored fertile eggs, further improves the incubation performance and the overall quality of the newly hatched chicks, and the growth performance. Therefore, due to the increase in hatching and improvement of performance indices in born chickens, it is concluded that the injection of a mixture of carbohydrates and antioxidants in the pre-hatching period in stored fertile eggs, is effective.

**Ethical approval**

The experimental protocol was ratified by the Animal Ethics Committee of the Science and Research Branch, Islamic Azad University, Tehran, Iran.

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