



## The effect of *in ovo* injection of grape seed extract and vitamin C on hatchability, antioxidant activity, yolk sac weight, performance and ileal micro flora of broiler chickens

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### Abstract

The influence of *in ovo* administration of grape seed extract (GSE) and vitamin C on hatchability, glutathione peroxidase activity (GPx), yolk sac weight, performance and ileal microflora of broiler chickens were investigated in broiler. A total of 490 fertile eggs were divided into seven groups: control group (not punctured nor treated with additive), control group (penetrated with no additive), sham group (normal saline, 0.5 ml/egg), GSE (3 mg/egg), GSE (4.5 mg/egg), GSE (6 mg/egg) and vitamin C (3 mg/egg). Experimental preparations were injected into eggs at day 18 of incubation period. Hatched chicks were raised till 10 days of age. *In ovo* injection of 4.5 mg GSE/egg significantly increased hatchability and GPx activity. However, there were no significant differences among day-old weight, yolk sac weight and mortality of chicks. *In ovo* administration of 4.5 mg GSE or vitamin C increased average daily weight gain and average daily feed intake of chickens compared to control groups. Further, *In ovo* injection of 4.5, 6 mg GSE or 3 mg vitamin C decreased ileal population of *Coliforms* and *E. Coli*. In conclusion, *in ovo* injection of 4.5 mg GSE/egg injected on 18<sup>th</sup> d of incubation has positive effect on broiler chickens and has no adverse effect on the performance of birds during starter period.

**Keywords:** *In ovo* injection; grape seed extract; GPx activity; performance

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### Introduction

The 21 day incubation period and the early post-hatch period of the chick composes about 50% of a 2 kg broiler's lifespan in the current intensive production system (Karadas et al., 2011). Therefore, anything that hinders or promotes growth and development during this neonatal period will have a marked effect on overall performance and health of poultry (Ferket, 2006).

To date, approximately 95% of broilers are vaccinated through *in ovo* technique (Maiorano et al., 2012).

Today, vaccine manufacturers desire to develop new products that can be injected *in ovo* to enhance vaccine efficacy and to improve broiler embryogenesis

and post hatch performance. Nutrients and other metabolic compounds such as amino acids, carbohydrates, vitamins and hormones are under investigation (Gore and Qureshi, 1997; Johnston et al., 1997; Henry and Burke, 1999; Tako et al., 2004; Uni et al., 2005; Foye et al., 2006; Kadam et al., 2008; Zhai et al., 2008; Keralapurath et al., 2010a&b). *In ovo* supplementation of nutrients may help late-term embryos to overcome the constraints of limited egg nutrients (Foye et al., 2006). Antioxidant protection at hatching time is considered to be an important determinant of chick viability during early post-hatch period (Surai, 2000). Given the relatively high temperature and humidity in the hatcher, the chick may be under chronic stress (Karadas et al., 2011). Furthermore, delay in food and/or water intake after

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**Table 1: Composition of the grape seed analyzed by AOAC methods and HPLC method**

	Grape seed	Grape seed extract	(mg/100 g DM)
Dry matter (%)	90.93	Catechin	1420
Gross energy (Kcal/kg)	3292	Epicatechin	1080
Crude fat (%)	24.83	Procyanidine B1	830
Crude protein (%)	10.17	Procyanidine B2	770
Nitrogen free extract (%)	17.86	Procyanidine C	530
Crude fiber (%)	35.39		
Calcium (%)	0.56		
Phosphorus (%)	0.31		
Ash (%)	2.68		

hatching as well as hatchery treatments such as vaccination, sexing and transport to the farm may result in additional stress (Geyra et al., 2001; Karadas et al., 2011). Ipek et al. (2004) reported that *in ovo* injection of 3 mg vitamin C into air sac on 13th day of incubation enhanced hatchability in broilers. Nowaczewski et al. (2012) demonstrated that *in ovo* injection of vitamin C (4, 8 mg/egg) into the air sac of duck eggs improved hatchability. Nowadays, researchers are searching for organic additives to use in poultry nutrition such as phytochemical compounds with antioxidant and antimicrobial effects.

Grape seed extract is a by-product derived from the grape seeds (*Vitis vinifera*) that is extracted, dried and purified to produce a polyphenolic compound-rich extract (Lau and King, 2003). The total extractable phenolics present in grape are about 10% or less in pulp, 60–70% in seeds, and 28–35% in skin. The phenol content of seeds may range from 5 to 8% by weight. The most abundant phenolics isolated from grape seeds are catechins (catechin, epicatechin, and procyanidins) and their polymers (Shi et al., 2003). The benefits derived from phenolic compounds in grape seeds are closely related to their antioxidant and singlet oxygen quenching ability. These phenolic compounds are able to trap and quench free radicals, and it has been shown that their antioxidant potentials is four to five fold higher than that in vitamin C or E. They are also very potent metal chelating agents (Shi et al., 2003). Grape seed extract (GSE) is a rich source of polymers of flavan-3-ols like catechin and epicatechin which have antimicrobial properties (Perumalla and Hettiarachchy, 2011). Thus, the objective of the present study was to evaluate the effects of *in ovo* administration of GSE and vitamin C on hatchability, glutathione peroxidase activity (GPx), yolk sac weight, performance and ileal microflora of broiler chickens.

## Materials and Methods

### Animals, diets and management

The experiment was conducted according to the protocols of Animal Care Committee of the Ferdowsi

University of Mashhad, Iran. Fertile eggs of broiler breeder hens were obtained from a commercial farm (Pouya Iujeh Company). The vitamin C (L-Ascorbic acid, 99%) was purchased from Sigma Aldrich Company. The basal diet was fed in mash form and prepared with the same batch of ingredients for starter period and formulated to meet the nutrient requirements according to Cobb-500 rearing guidelines (Cobb-Vantress, 2012). All birds had free access to feed and water during the whole rearing period. The ingredients and chemical composition of the basal diets are shown in Table 1. Temperature was initially set at 34°C on d 1 and decreased linearly by 0.5°C per day up to 28 d. During the study, the birds received a lighting regimen of 23L: 1D from 1 to d 10.

### Preparation of GSE

Grape seeds were ground, and extracted with acetone: methanol: water (60:30:10 v/v/v) solution for 12h with shaker incubator. Solvents were removed by rotary evaporator. Then, the extract was dried in vacuumed oven and kept in freezer at -20°C (Salari et al., 2009). The composition of grape seeds was measured by AOAC procedures (AOAC, 1990).

### GSE analysis

The chromatographic analysis was carried out on a Knauer HPLC system (Berlin, Germany) equipped with a Triathlon auto sampler, a K-1001 pump and a UV-visdetector (K-2600). A reversed-phase C18 Nucleosil 100 (12.5 cm × 5.0mm × 5.0 μm) column was used for the separation of sample components. Analysis of catechin, epicatechin, procyanidin B1, B2, C1 performed according to the method of Iacopini et al. (2008) with little modification. Standards of catechin, epicatechin, procyanidin B1, B2, C1 were purchased from Sigma-Aldrich (St. Louis, USA).

### *In ovo* injection

Four hundred and ninety fertile eggs were divided into seven groups: control group (not punctured), control group (punctured with no additive), sham group (normal saline, 0.5 ml/egg), GSE at the rate of 3, 4.5, 6 mg/egg and vitamin C (3 mg/egg). Eggs were sanitized and prepared for incubation in a commercial automatic hatchery. Experimental preparations were filtered with syringe, then injected into air sac of the eggs at d 18 of incubation period. Injection site on eggs were cleaned with 70% ethylic alcohol, and then bored with a needle and treatments were injected into the air sac from broad end of the eggs by insulin syringe and then sealed by melted paraffin.

### Hatchability, day old weight, mortality and yolk sac weight

Hatchability was determined as the percentage of fertile eggs. The hatched chickens after hatching were

weighed immediately. Mortality was determined after two days of hatching and expressed in percentage.

### GPx activity

Blood hemolysate of chicken was prepared on d 1. The methodology of Paglia and Valentine (1967) was used for measurements of GPx activity in blood hemolysate. Samples were assayed with commercially available GPx kits (Randox, Crumlin, UK).

### Performance traits

A total of 280 hatched chicks were raised till d 10 of age in 35 floor pens (8 chicks per pen). On d 10, birds were pen weighed, and feed consumption was recorded in order to calculate feed conversion ratio.

### Ileal microflora

The ileum was excised and contents were collected by gently pressing the fingers to move the content into tubes at 10 d of age. Digesta of two birds within a replicate were pooled, put on ice until they were transported to the laboratory for enumeration of microbial population. One g of ileal content was homogenized in 9 ml sterile water. Each sample was serially diluted. Using these diluted samples, Lactobacillus was counted on De Man-Rogosa-Sharpe (MRS) agar after incubation at 37°C in an anaerobic chamber for 48 h (Guban et al., 2006), and Coliforms and *E. coli* were counted on CHROM agar ECC (EF322- Paris France) after incubation at 37°C in an aerobic chamber for 48 h (Sallam, 2007).

### Statistical analysis

Statistical analysis was conducted using the General Linear Models procedure of SAS. Data of the experiment were statistically analyzed using randomized complete block design (SAS Institute, 2002). Means were compared using Duncan's new multiple range test (Duncan, 1955). The level of significance was reported at  $P < 0.05$ .

## Results and Discussion

### Hatchability

*In ovo* injection of 4.5 mg GSE/egg increased hatchability significantly compared to control groups (Table 2). It is well known that chick viability is an important factor in determining profitability and factors such as egg quality, egg storage conditions, incubation and post-hatch environment affect chick quality (Decuyper et al., 2001). It seems that polyphenolic compounds (4.5 mg) in GSE could help the chick to overcome oxidative stress at hatch time. Higher amount of GSE failed to have positive effect on hatchability that may be due to change in pH and osmolality of egg environment or detrimental effects of tannins. The lower levels were not efficient which may relate to their low bioavailability. Moreover, the dose of ascorbic acid was not as effective as to 4.5 mg/egg GSE. In contrast to our finding, Tag El-Din et al. (2004) reported that *in vivo* treatment of ascorbic acid at the rate of 3.0 mg/egg resulted in improved hatchability. In contrast, Selim et al. (2012) reported that *in ovo* injection of 3.0 mg vitamin C on the 12<sup>th</sup> day of incubation had no effect on ducks hatchability. However, Ghonim et al. (2009) indicated that hatchability and embryonic mortality percentages were significantly improved by ascorbic acid dipping and spraying methods in ducklings as compared to the control.

### GPx activity, yolk sac weight and mortality

*In ovo* administration of different levels of GSE or vitamin C increased GPx activity. However, there were no significant differences among day-old weight, yolk sac weight and mortality of chicks. Oxidative protection against reactive oxygen species (ROS) originated during body physiological processes during embryonic development and egg contents can be a key factor in the extension of ROS generation causing deleterious effects in poultry metabolism. Because antioxidant status of hatching eggs is variable and unpredictable, *in ovo* supplementation of antioxidants may prove to yield

**Table 2: Effect of grape seed extract and vitamin C on hatchability, day-old weight, GPx activity, yolk sac weight and mortality of broiler chickens**

Treatment	Hatchability (%)	Day old weight (g)	GPx activity (U/l of hemolysate)	Yolk sac weight	Mortality (%)
Intact control	85.71 <sup>bc</sup>	41.72	186.3 <sup>cd</sup>	1.40	0.00
Punctured control	87.14 <sup>b</sup>	39.36	178.4 <sup>de</sup>	1.67	1.53
Sham control	80.00 <sup>c</sup>	40.91	171.2 <sup>e</sup>	1.60	3.07
GSE (3 mg/egg)	84.28 <sup>bc</sup>	40.54	197.2 <sup>bc</sup>	1.69	0.00
GSE (4.5 mg/egg)	92.85 <sup>a</sup>	41.05	220.0 <sup>a</sup>	1.73	0.00
GSE (6 mg/egg)	75.71 <sup>c</sup>	40.27	207.6 <sup>ab</sup>	1.85	1.53
Vitamin C (3 mg/egg)	90.00 <sup>ab</sup>	42.11	211.8 <sup>ab</sup>	1.47	3.07
SEM	0.479	0.195	0.560	0.121	0.216
P value	0.0038	0.1115	<0.0001	0.5903	0.3391

Means within columns with different superscripts differ significantly ( $P < 0.05$ ); GSE, grape seed extract.

**Table 3: Ingredients and nutrient composition of experimental diets**

Ingredient (%)	1-10 d
Corn, ground	56.2
Soybean meal	37.11
Soybean oil	2.26
Dicalcium phosphate	1.92
Oyster shell	1.16
Common salt	0.3
Minerals mix <sup>1</sup>	0.25
Vitamins mix <sup>2</sup>	0.25
DL-Methionine	0.31
L-Lysine hydrochloride	0.24
Calculated composition	
ME (kcal/kg)	3000
CP (%)	21.23
Ca (%)	1
AP (%)	0.50
Methionin (%)	0.63
Lysine (%)	1.32
Methionine+Cystine	0.98

<sup>1</sup>Mineral mix supplied the following per kg of diet: Cu, 20 mg; Fe, 100 mg; Mn, 100 mg; Se, 0.4; Zn, 169.4 mg;

<sup>2</sup>Vitamins mix supplied the following per kg of diet: vitamin A, 18,000 IU; vitamin D3, 4,000 IU; vitamin E, 36mg; vitamin K<sub>3</sub>, 4 mg; vitamin B<sub>12</sub>, 0.03 mg; thiamine, 1.8 mg; riboflavin, 13.2 mg; pyridoxine, 6 mg; niacin, 60 mg; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 0.2 mg; choline chloride, 500 mg.

significant benefits (Malheiros et al., 2012). In agreement to our result, Brenes et al. (2010) reported an increase in antioxidant activity in diet and excreta of broilers fed diet containing GSE.

### Growth performance

*in ovo* administration of vitamin C increased average daily weight gain of chickens compared to penetrated control and sham control groups followed by 4.5 GSE at day 10 (Table 4). This is in agreement with the findings of Zakaria et al. (1998) and Zakaria (2001) who recorded that *in ovo* injection of 3 mg ascorbic acid at the 15th day of incubation resulted in greater

body weight of male broiler chickens. Also, Ghonim et al. (2012) reported that body weight gain of ducklings during the first two weeks of age after hatch were non-significantly increased due to ascorbic acid addition in fertile eggs during incubation period. In present study, *in ovo* injection of vitamin C increased average daily feed intake of broiler chickens. However, there were no significant different in FCR or mortality. In agreement with our result, Selim et al. (2012) reported that *in ovo* administration of ascorbic acid increased feed intake of Muscovy ducklings. Bhanja et al. (2007) stated that vitamin A and vitamin C may influence the embryonic development, whereas vitamin E and vitamin B1 may be required for early post hatch growth.

### Ileal microflora

*In ovo* injection of 4.5, 6 mg GSE or 3 mg vitamin C decreased ileal population of *Coliforms* and *E. Coli* (Table 4). However, there was no significant difference in ileal population of *Lactobacillus*. Perumalla and Hettiarachchy (2011) stated that phenolic compounds of grape have inhibitory effect on bacteria. In agreement to our results, Jamroz and Kamel (2002) reported that the dietary herbal treatment results in lower *E. coli* counts compared to the control group. The outer cell membrane or cytoplasmic membrane of a bacterium is essentially composed of a phospholipid bilayer and proteins and is the major site of interaction with antimicrobial compounds. Damage to this vital membrane can result in death of the bacterium (Perumalla and Hettiarachchy, 2011). Functional hydroxyl groups and conjugated double bonds in the reactive groups of natural plant extracts may be involved in their binding to the cell wall components. Catechins have deteriorating effect on the lipid bilayer membrane that results in the loss of cell structure and function eventually leading to cell death (Cox and Markham, 2001). Presence of gallic acid esters in Epicatechin and Epicatechin galat are responsible for their high affinity for lipid bilayers, and affect the membrane structure. Also, major phenolic constituents

**Table 4: Effect of grape seed extract and vitamin C on performance, mortality and ileal microflora of chickens from 1- 10 days of age**

Treatment	ADWG (g/b/d)	FI (g/b/d)	FCR (g/g)	Mortality (%)	Coliforms	<i>E. Coli</i>	<i>Lactobacillus</i>
Intact control	20.76 <sup>abcd</sup>	28.77 <sup>bc</sup>	1.38	5.00	5.89 <sup>a</sup>	5.83 <sup>a</sup>	5.86
Punctured control	20.19 <sup>dc</sup>	28.41 <sup>bcd</sup>	1.41	7.50	5.91 <sup>a</sup>	5.84 <sup>a</sup>	5.78
Sham control	19.74 <sup>d</sup>	27.42 <sup>d</sup>	1.39	10.00	5.93 <sup>a</sup>	5.81 <sup>a</sup>	5.92
GSE (3 mg/egg)	21.46 <sup>abc</sup>	29.03 <sup>bc</sup>	1.35	0.00	5.91 <sup>a</sup>	4.97 <sup>b</sup>	5.95
GSE (4.5 mg/egg)	21.71 <sup>ab</sup>	29.52 <sup>b</sup>	1.35	0.00	5.54 <sup>b</sup>	3.92 <sup>c</sup>	6.14
GSE (6 mg/egg)	20.45 <sup>bcd</sup>	28.15 <sup>cd</sup>	1.37	5.00	5.47 <sup>b</sup>	4.78 <sup>b</sup>	6.17
Vitamin C (3 mg/egg)	22.12 <sup>a</sup>	30.94 <sup>a</sup>	1.39	7.50	5.45 <sup>b</sup>	5.17 <sup>b</sup>	6.10
SEM	0.527	0.515	0.042	0.364	0.06	0.093	0.085
P value	0.0053	<0.0001	0.7799	0.3739	<0.0001	<0.0001	0.1615

ADWG, average daily weight gain; FI, feed intake; FCR, feed to gain ratio

like epicatechin may alter the cell morphology by influencing the osmotic pressure of the cell, thus disrupting the cytoplasmic membrane and causing leakage of cell constituents (Sivaroban et al., 2008). In contrast to our results, Viveros et al. (2011) reported that in the ileal content, birds fed control and GSE diets had the highest populations of *Lactobacillus*.

### Conclusion

*In ovo* injection of 4.5 mg GSE/egg on 18<sup>th</sup> d of incubation increased hatchability of broiler chickens and it had no adverse effect on the broiler chickens performance during starter period. Furthermore, its effect was almost similar to the effect of ascorbic acid. It seems that grape seed extract can be used as an effective anti-stress additive during incubation period to improve broilers hatchability and performance.

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