Communication

Dietary Grape (*Vitis vinifera*) Seed Powder and Zn–Gly Chelate Complex for Mitigating Heat Stress in Broiler Chickens: Growth Parameters, Malondialdehyde, Paraoxonase-1, and Antibody Titer

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Abstract: A total of 300 day old broiler chicks (Hubbard) were assigned to 30 floor pens (10 birds per pen) under cyclic heat stress. Three diets including a control, as well as two levels of grape seed powder (GSP) and zinc (OZ) at the rates of 2.5 g/kg GSP + 50 mg/kg OZ and 5 g/kg GSP + 50 mg/kg OZ, were supplied to the broilers for 35 days. According to the results, broiler feed intake improved ($p < 0.05$) in GSP + OZ groups from 3–5 weeks and on an overall basis compared to the control diet. Body weight increased ($p < 0.05$) in GSP-5 + OZ-50 during weeks 2–5 and on an overall basis. The findings indicated that feed conversion ratio (FCR) decreased ($p < 0.05$) during week 5 in broilers supplemented with GSP-5 + OZ-50. The antibody titer (HI) against Newcastle disease (ND) was higher ($p < 0.05$) in GSP + OZ groups compared to control treatment. The value of malondialdehyde (MDA) decreased ($p < 0.05$) under GSP + OZ diets compared to control. Moreover, paraoxonase (PON1) was higher ($p < 0.05$) in GSP + OZ groups compared to untreated broilers. In conclusion, GSP + OZ positively supported growth traits, reduced MDA, and augmented PON1 and HI titer against ND in broilers exposed to heat stress.

Keywords: broiler; grape seeds; heat stress; zinc; redox balance

1. Introduction

The definition of heat stress is not conclusive among the scientists. Broadly speaking, when the environmental temperature exceeds the physical temperature, this may result in impaired health and performance of broilers [1]. Generally, from the poultry point of view, when the environmental temperature goes up to 25 °C, birds are considered to be heat-stressed [2]. Heat stress negatively affects the growth performance and health of broilers [3,4]. Heat stress is a major limiting factor that can cause significant economic losses every year owing to poor performance, immunity, and health [5].

Zinc (Zn) is needed for a number of physiological functions in animals [6–8]. Zinc is highly sensitive and affected by many factors, which may result in deficiency in the body [9]. Mostly inorganic Zn is used in the poultry diet in the form of oxides and sulfates; these Zn forms are known for a number of negative impacts such as less absorption, high hydrophobicity, irritation of gastrointestinal mucosa, low bioavailability, and increased

environmental excretion [7,10,11]. Recently, organic Zn chelates have become more popular in poultry nutrition. Studies have reported enhanced body weight gain and feed efficiency in broilers fed different forms of organic Zn [9,11–13]. There are continuous efforts to find a novel organic complex of Zn, which shows better bioavailability and enhanced growth traits in poultry. Recently, we found that Zn combined with glycine (Gly) improved growth, intestinal features, and redox balance in broilers under heat stress [7].

Every year in the EU, almost 2.5 million metric tons of food waste and residues are generated, yet only 40% is recycled [14]. Agro-industrial byproducts are rich in high digestible fiber, protein, and lipids and frequently reused due to the increased focus on circular economy in the agri-food system [15]. Grape (*Vitis vinifera*) is a well-known fruit grown worldwide. It is a valuable source of flavonoids and polyphenols. Most of the flavonoids of grapes are easily absorbed in the small intestines, while others are metabolized into phenolic acids in the colon by the microbiome [16]. In a previous study, Viveros et al. [17] concluded that grape seed extract decreased the count of potential pathogenic bacteria such as *Clostridium* and increased the number of beneficial bacteria such as *Enterococcus* in broilers. Furthermore, Wang et al. [18] found that grape seed extract improved the weight gain and enhanced the antioxidant capacity of broilers exposed to *Eimeria tenella* infection. Recently, Chand et al. [8] concluded that grape seed powder ameliorated the growth curve and lesion score in broilers induced by *Eimeria tenella* infection. In addition, Vlaicu et al. [19] concluded that 3% grape seed meal in combination with 9% rapeseed significantly improved the total phenolic contents and antioxidant in eggs of 50 week old Tetra SL laying hens. Romero et al. [20] found that a combination of grape seed and skin supplementation reduced lipid peroxidation (malondialdehyde) in broiler chicken preserved meat.

To date, little information is available on the effect of dietary supplementation of grape seed powder on broilers growth, immunity, and antioxidant capacity during heat stress. Most of the past studies reported an improvement in the growth performance of broilers supplemented with byproducts/waste/pomace of grapes [21–24]. Little attention has been given to the effect of grape seed powder on broiler health and production. It is speculated that grape seeds alone cannot help to alleviate the heat stress; therefore, we propose that a small quantity of Zn may work synergistically with grape seeds to decrease the negative effects of heat stress.

Therefore, the purpose of the current study was to assess the individual and combined effects of Zn–Gly chelate and grape seed powder on the growth performance, malondialdehyde, paraoxonase-1, and antibody titer against Newcastle disease in broilers under heat stress conditions.

2. Materials and Methods

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Faculty of Animal Husbandry and Veterinary Sciences, the University of Agriculture, Peshawar (14/PS/2020).

2.1. Experimental Design and Bird Management

Fresh red grape (*Vitis vinifera*) seeds were acquired from a local source and rinsed with purified water before being dried (30 °C) in the shade for 2 weeks at room temperature. After that, the seeds were crushed in an electric grinder machine into a fine powder. A total of 300 day old male broiler chicks (Hubbard hybrid), with an average weight of 43.4 ± 0.1 g, were assigned to 30 floor pens with 10 birds per pen, all of which were bedded with wood shaving. Each enclosure was 160 cm × 240 cm in size and came with circular feeders and drinkers. Throughout the research, birds had unlimited access to food and water from feeders and drinkers. Vaccinations against pathogens, such as Newcastle disease (ND), were given to the birds on a regular basis. Table 1 presents the basic dietary composition. For the first week, the birds were exposed to fluorescent lights for 23 h, followed by a 20 h/4 h light/dark cycle. This experiment was conducted in an open-sided house under

summer conditions of the local environment. The data collected were the natural status of temperature and humidity at that specific hour. As indicated in Table 2, birds were subjected to a cyclic heat stress cycle in the environment (7–35 days).

Table 1. Ingredients and chemical composition of basal diets fed to broiler chickens.

Ingredients	Starter Phase	Finisher Phase
Corn	55.5	56.0
Soybean meal	27.7	27.0
Canola meal	6.5	6.0
Sunflower meal	3.2	3.5
Vegetable oil	1.8	1.9
Molasses	1.0	1.3
Dicalcium phosphate	1.9	1.9
Limestone	1.0	1.0
Sodium chloride	1.0	1.0
Sodium bicarbonate	0.1	0.1
Vitamin mineral premix ¹	0.3	0.3
Chemical composition		
Metabolizable energy, kcal/kg	3000	3150
Crude protein, %	23.50	21.30
Methionine, %	0.55	0.44
Lysine, %Glycine, %	1.421.7	1.231.8
Sulfur amino acids, %	0.96	0.80
Threonine, %	0.95	0.85
Calcium, %	1.05	0.90
Phosphorus, %	0.50	0.45

¹ Vitamin–mineral premix containing the following per kg: vitamin A, 2,400,000 IU; vitamin D, 1,000,000 IU; vitamin E, 16,000 IU; vitamin K, 800 mg; vitamin B1, 600 mg; vitamin B2, 1600 mg; vitamin B6, 1000 mg; vitamin B12, 6 mg; niacin, 8000 mg; folic acid, 400 mg; pantothenic acid, 3000 mg; biotin 40 mg; antioxidant, 3000 mg; cobalt, 80 mg; copper, 2000 mg; iodine, 400; iron, 1200 mg; manganese, 18,000 mg; selenium, 60 mg, and zinc, 14,000 mg.

Table 2. Mean daily temperature and relative humidity of the experimental period.

Time	Temperature (°C)	Relative Humidity (%)
8:00 a.m.	34.12	72.48
12:00 p.m.	36.43	94.12
4:00 p.m.	39.63	65.51
8:00 p.m.	33.92	69.15
12:00 a.m.	34.50	76.87
4:00 a.m.	32.96	77.13

Three diets were prepared and allocated in a completely randomized design consisting of a control (basal diet) and two levels of grape seed powder (GSP) and zinc–glycine chelate (OZ) at the rates of 2.5 g/kg GSP + 50 mg/kg OZ and 5 g/kg GSP + 50 mg/kg OZ, for 35 days including 1 week as an adaptation period. Zn–glycine chelate was included in the basal diet at the expense of corn. The OZ product was Bioplex Zn[®] (Alltech Inc, Nicholasville, Kentucky, USA), a chelated Zn proteinate that contained 10% Zn. The GSP and OZ were well mixed into the diet. In addition, by adding Zn, the concentration of total Zn was greater in the treatment groups compared to the control. Feed intake, body weight gain, and feed conversion ratio (FCR) were monitored weekly per pen, with the mean of each parameter recorded over the whole time. Three birds were slaughtered by cervical dislocation in each pen on day 35, and 3 cc blood samples were collected into glass tubes. Blood samples were centrifuged for 10 min at 3000 rpm, and the serum was kept at −80 °C until analysis.

2.2. Determination of Malondialdehyde (MDA), Paraoxonase (PON1), and HI Titer against ND

The thiobarbituric acid (TBA) reaction, originally reported by Ohkawa et al. [25], was used to determine lipid peroxidation in blood samples. TBA's reaction color was spectrophotometrically quantified at 532 nm. The technique of Mackness et al. [26], using phenol as a substrate, was used to detect blood paraoxonase (PON-1). In this case, 1 mM phenylacetate, calcium chloride, and 20 mM Tris HCl buffer were used to make the working reagent. After diluting the sample 1:3 with buffer before mixing with the substrate, the change in absorbance at 270 nm was measured for 5 min. The hemagglutination inhibition (HI) assay was used to evaluate the immune response to the ND virus.

2.3. Statistical Analysis

A completely randomized design was used to analyze the data in Statistix 8.1 Software. Tukey's test was used to calculate significant differences between experimental groups at a probability threshold of 5%.

3. Results

The findings of feed intake in broilers exposed to heat stress in response to different levels of GSP are given in Table 3. At the end of week 2, feed intake increased significantly ($p < 0.05$) only in GSP-5 + OZ-50 compared to the control. The feed intake improved significantly ($p < 0.05$) in GSP + OZ groups from week 3 to 5 and on an overall basis compared to the control. The findings of body weight gain in response to dietary supplementation of GSP and OZ are given in Table 4, where this parameter increased significantly ($p < 0.05$) in GSP-5 + OZ-50 compared to the control during weeks 2 to 5 and on an overall basis. The results of FCR in broilers exposed to heat stress and dietary supplementation of OZ and GSP are given in Table 5. The FCR decreased significantly ($p < 0.05$) during week 5 in broilers exposed to heat stress and supplemented with GSP + OZ groups. No significant change was observed during the other weeks and on an overall basis, indicating that the feed efficiency was improved in broilers supplemented with OZ and GSP during the finisher phase when exposed to heat stress.

The effects of OZ and GSP on MDA, PON1, and ND titer on broilers under heat stress conditions are given in Table 6. The antibody titer against ND was significantly ($p < 0.05$) higher in GSP + OZ groups compared to the control. The value of MDA decreased significantly ($p < 0.05$) in GSP-2.5 + OZ groups compared to the control. The blood concentration of PON1 was significantly ($p < 0.05$) higher in GSP + OZ groups compared to the control.

Table 3. Cumulative feed intake ($g \pm SE$) in broiler chicks fed different levels of zinc–glycine chelate and grape seed powder under summer stress conditions.

Item	Week 2	Week 3	Week 4	Week 5	Overall
Control	364.70 ^b $\pm$ 3.57	573.95 ^b $\pm$ 7.56	691.33 ^b $\pm$ 5.63	889.07 ^c $\pm$ 7.35	2519.1 ^c $\pm$ 17.73
GSP-2.5 + OZ-50	370.38 ^{ab} $\pm$ 7.38	600.20 ^a $\pm$ 4.24	712.33 ^a $\pm$ 10.73	909.08 ^b $\pm$ 11.4	2588.3 ^b $\pm$ 16.95
GSP-5 + OZ-50	376.45 ^a $\pm$ 7.34	604.48 ^a $\pm$ 8.54	720.55 ^a $\pm$ 6.21	927.73 ^a $\pm$ 10.44	2629.2 ^a $\pm$ 16.16
<i>p</i> -value	0.001	<0.001	0.001	0.001	<0.001

Means within the same column with different superscripts are statistically different ($p < 0.05$). GSP-2.5 + OZ-50: grape seed powder and zinc–glycine chelate at the rates of 2.5 g/kg and 50 mg/kg, respectively; GSP-5 + OZ-50: grape seed powder and zinc–glycine chelate at the rates of 5 g/kg and 50 mg/kg, respectively.

Table 4. Body weight gain (g/bird) ($g \pm SE$) in broiler chicks fed different levels of zinc–glycine chelate and grape seed powder under summer stress conditions.

Item	Week 2	Week 3	Week 4	Week 5	Overall
Control	251.75 ^b $\pm$ 6.65	360.50 ^b $\pm$ 9.46	411.75 ^b $\pm$ 16.31	453.25 ^c $\pm$ 6.34	1487.3 ^b $\pm$ 11.32
GSP-2.5 + OZ-50	253.50 ^b $\pm$ 5.56	364.25 ^{ab} $\pm$ 4.03	429.50 ^b $\pm$ 10.40	476.00 ^b $\pm$ 4.69	1508.3 ^{ab} $\pm$ 17.66
GSP-5 + OZ-50	262.75 ^a $\pm$ 4.78	370.50 ^a $\pm$ 3.10	444.75 ^a $\pm$ 12.03	469.50 ^a $\pm$ 4.65	1576.0 ^a $\pm$ 21.08
<i>p</i> -value	0.049	0.0124	0.013	<0.001	0.032

Means within the same column with different superscripts are statistically different ($p < 0.05$). GSP-2.5 + OZ-50: grape seed powder and zinc–glycine chelate at the rates of 2.5 g/kg and 50 mg/kg, respectively; GSP-5 + OZ-50: grape seeds powder and zinc–glycine chelate at the rates of 5 g/kg and 50 mg/kg, respectively.

Table 5. Feed conversion ratio (FCR) ($g/g \pm SE$) in broiler chicks fed different levels of zinc–glycine chelate and grape seed powder under summer stress condition.

Item	Week 2	Week 3	Week 4	Week 5	Overall
Control	1.44 $\pm$ 0.037	1.59 $\pm$ 0.57	1.67 $\pm$ 0.066	1.95 ^a $\pm$ 0.026	1.69 $\pm$ 0.074
GSP-2.5 + OZ-50	1.45 $\pm$ 0.040	1.64 $\pm$ 0.02	1.61 $\pm$ 0.047	1.90 ^b $\pm$ 0.031	1.75 $\pm$ 0.073
GSP-5 + OZ-50	1.43 $\pm$ 0.037	1.62 $\pm$ 0.017	1.61 $\pm$ 0.033	1.85 ^c $\pm$ 0.275	1.66 $\pm$ 0.017
<i>p</i> -value	0.662	0.166	0.178	0.003	0.535

Means within the same column with different superscripts are statistically different ($p < 0.05$). GSP-2.5 + OZ-50: grape seed powder and zinc–glycine chelate at the rates of 2.5 g/kg and 50 mg/kg, respectively; GSP-5 + OZ-50: grape seed powder and zinc–glycine chelate at the rates of 5 g/kg and 50 mg/kg, respectively.

Table 6. Effect of dietary grape seed powder and zinc–glycine chelate on antibody titer against Newcastle disease (ND), malondialdehyde (MDA), and paraoxonase (PON1) in broiler chicks under summer stress conditions.

Groups	ND Titer	MDA (nmol/mL)	PON1 (μ mL)
Control	4.27 ^c $\pm$ 0.039	11.09 ^a $\pm$ 0.88	8.31 ^b $\pm$ 0.80
GSP-2.5 + OZ-50	5.74 ^b $\pm$ 0.311	6.42 ^b $\pm$ 0.55	12.15 ^a $\pm$ 0.78
GSP-5 + OZ-50	6.78 ^a $\pm$ 0.428	6.91 ^b $\pm$ 0.52	12.89 ^a $\pm$ 0.77
<i>p</i> -value	<0.001	<0.001	<0.001

Means within the same column with different superscripts are statistically different ($p < 0.05$). GSP-2.5 + OZ-50: grape seed powder and zinc–glycine chelate at the rates of 2.5 g/kg and 50 mg/kg, respectively; GSP-5 + OZ-50: grape seed powder and zinc–glycine chelate at the rates of 5 g/kg and 50 mg/kg, respectively.

4. Discussion

In the current study, it was found that feed intake and body weight gain improved significantly in GSP + OZ-supplemented birds under heat stress. On the other hand, it is evident that the feed intake and corresponding weight gain in birds exposed to heat stress were significantly decreased in the control group. In heat-stressed birds, energy is diverted from production system toward the protective strategy against heat stress [27,28]. Organic Zn supplementation has been known to ameliorate and prevent the negative impacts of heat stress in broilers [28–30].

Under heat stress, the demand for energy increases, which results in increased glyco-genesis, suppression of appetite, gastric lesions, and disruption of immunological mechanisms. In addition, higher temperature with relative humidity causes airway desiccation and dehydration in birds. Such factors may directly or indirectly influence feed consumption, weight gain, and feed efficiency in birds. Zinc plays a vital role in several metabolic pathways. The absorption of Zn is influenced by metallothioneine in the enterocytes. It has been suggested that the organic form of Zn increases the absorptive and utilization capacity of feed [7]. In addition, organic Zn provides higher bioavailability and increases the digestibility of nutrients. Furthermore, organic Zn remains for longer period in the gut and is more ecofriendly. There are conflicting results regarding the use of different byproducts of grapes on the performance of broilers under heat stress with limited or no improvement [21–23]. However, recently, Chand et al. [8] reported improved feed

intake, weight gain, and feed efficiency in response to Zn–Gly and GSP in birds exposed to *Eimeria tenella* challenge. It seems from the findings of Chand et al. (2021) and the current study that organic Zn works best in combination with GSP. Grape seed contains flavanol monomers (epicatechin 3-O-gallate, epicatechin, and catechin), flavanol dimers (procyanidin gallate, procyanidin B3, procyanidin B2, and procyanidin B1), flavanol trimers (procyanidin trimer and procyanidin C1), flavanol tetramers (procyanidin tetramer and procyanidin cinnamtannin A2), and phenolic acids (coumaric acid, ferulic acid, caffeic acid, and gallic acid) (Romero et al. [20]). Recently, Romero et al. (2021) reported that grape seed at the rate of 30 g/kg improved the weight gain and feed efficiency in broilers under thermoneutral conditions. However, detrimental effects of grape seed supplementation have also been reported on the growth performance in broilers [17,31], depending upon the type and dose of polyphenols present in the grape seeds [20]. The improvement in growth traits of broilers could also be due to the extensive intestinal utilization and microbial metabolism of grape polyphenols as reported in poultry [31].

Heat stress is well known to reduce immune response in broilers through decreasing the weight of lymphoid organs and antibody production [32,33]. The Zn–glycine complex has a beneficial impact on the immune system [9]. Zn–glycine at the rate of 50 mg/kg improved the antibody titer against ND in broilers under thermoneutral conditions [7]. This again justifies our conclusion that combined doses of OZ and GSP work best in improving of immune status of birds during heat stress. In the study of Hosseini-Vashon et al. [23], the antibody titer against ND was not affected by different levels of grape pomace in broilers during heat stress, justifying our findings that GSP works best when combined with Zn.

Heat stress directly affects the oxidant/antioxidant status of the birds. Heat stress directly reduces the activity of antioxidant enzymes and molecules [1,5], but enhances the plasma concentration of MDA [34]. In the current study, supplementation of GSP and OZ reduced serum MDA in heat stress broilers. Grapes are a rich source of polyphenols, which exhibit strong antioxidant effects by neutralizing oxidative molecules [23,35]. Polyphenolic chemicals and other plant-derived secondary metabolites can be found in a variety of foods, including fruits, leaves, roots, and seeds, and they constitute a significant component of both human and animal diets [36]. They have antioxidant properties, they can scavenge free radicals, and their consumption has been associated with a lower risk of diseases such as cancer, cardiovascular disease, and chronic inflammation [37]. In broilers, supplementation of both grape seeds and pomace resulted in improved antioxidant status in the blood and meat, which has been associated with a high concentration of alpha- and gamma-tocopherol [20,31]. Alpha-tocopherol oxidizes itself during the process of lipid peroxidation, and it is thought to be recycled by grape procyanidins [38].

In the current study, serum PON1 rose significantly in GSP + OZ groups compared to the control. PON1 is considered an antioxidant-based enzyme, and its level was found to be improved as a result of some feed additives in broilers under heat stress [7,39,40]. Zn is known for its important role in the antioxidant system by suppressing free radicals [41]. It is also an essential part of the antioxidant enzymes in blood. Therefore, we speculate that Zn also has a beneficial impact on the status of PON1. Similarly, in the current study, GSP doses improved the PON1 concentration. This is the first study reporting PON1 concentration to be influenced by GSP. It could not be confirmed whether the effect was due to polyphenols, and it is speculated that polyphenols in grape are responsible for such a beneficial effect in broilers during heat stress.

5. Conclusions

According to the present findings, it can be concluded that GSP + OZ positively supported body weight gain, reduced MDA, and augmented PON1 and HI titer against ND in broiler chickens exposed to heat stress.

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