

Research Article

The Protective Effect of Pomegranate peel/Powder on Pulmonary Hypertension in Broiler Chickens

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• Chicken; Growth; *Punicagranatum*; Antihypertensive activity; Gene expression

Abstract

This study investigated the effects of dietary pomegranate peel powder on growth performance, hematological parameters, cardiac indices and expression endothelin 1 (ET-1) in the liver in the broilers reared at high altitude (2100 m above sea level). A total number of 250 day-old male broilers (Cobb 500) were randomly assigned to five treatments including different levels of *Punicagranatum* (0%, 0.25%, 0.5%, 0.75% and 1%) in a 40-day trial. Feed intake and feed conversion ratio (FCR) responses of broiler chickens were significantly ($P<0.05$) improved when *Punicagranatum* administered at 0.75 and 1%. The birds received *Punicagranatum* had significantly higher plasma nitric oxide concentration whereas the control birds had significantly ($P<0.05$) higher malondialdehyde concentration. A significantly suppressed the expression of ET-1 in liver of chickens fed *Punicagranatum*. In addition, inclusion of *Punicagranatum* in broiler diets at 0.75% and 1% significantly ($P<0.05$) declined the RV: TV ratio. In conclusion, *Punicagranatum* supplementation at 0.75 and 1% had a potential to improve growth performance and could prevent Pulmonary Hypertension Syndrome.

ABBREVIATIONS

ET-1: Endothelin 1; PHS: Pulmonary Hypertension Syndrome; PAH: Pulmonary Arterial Hypertension; ROS: Reactive Oxygen Species; FCR: Feed Conversion Ratio; BWG: Body Weight Gain; FI: Feed Intake; NO: Nitric Oxide; MDA: Malondialdehyde; RV: Right Ventricle; TV: Total Ventricles; ACE: Angiotensin Converting Enzyme

INTRODUCTION

Hypoxia is defined as reduced availability of atmospheric oxygen (reduced partial pressure of oxygen) that occurs as the altitude increases. The partial pressure of oxygen drops approximately 7mmHg for each 1,000 m elevation in altitude, which is equal to a drop of approximately 2.5% in the air oxygen for each 1,000 m increase in altitude [1]. In broiler chickens that are raised at high altitude, pulmonary hypertension syndrome (PHS) is a common problem. 'Pulmonary Hypertension Syndrome' (PHS), 'Pulmonary Arterial Hypertension' (PAH), and 'Ascites Syndrome' commonly are used synonymously [2]. Pulmonary hypertension syndrome (PHS, as ascites in the clinical form) is a metabolic defect associated with hypoxemia, heart/lung overload, venous and heart congestion, right ventricular hypertrophy, a flaccid heart, cirrhosis of the liver, and accumulation of ascitic fluid into the abdominal cavity [2,31]. The susceptibility of broilers to PAH exacerbates under conditions of limited oxygen availability (such as hypobaric hypoxia) or with increased oxygen demands at the tissues (e.g., thermoregulation in cold temperatures) [3]. As a consequence, a mismatch between oxygen demanding organs (i.e. muscles) and oxygen-

supplying organs (i.e. heart and lungs) has emerged and resulted in increased blood pressure within the pulmonary arteries, which subsequently can lead to the progressive development of pulmonary arterial hypertension syndrome [4]. Physiological responses to sustained hypoxia imposed by high altitude include excessive production of reactive oxygen species (ROS) and vascular remodeling in the lung characterized by hypertrophy and hyperplasia of the smooth muscle layer of arterioles [5]. Oxidative stress occurs when tissue depleted of antioxidants [6]. Increases in the pulmonary arterial pressure theoretically can be attributed to increases in cardiac output as well as to increases in the resistance to blood flow through the pulmonary vasculature [7]. Anything that increases the pulmonary vascular Resistance (vasoconstriction) can initiate or accelerate the pathophysiological progression leading to pulmonary arterial hypertension (PAH). Factors known to increase the resistance to blood flow through broiler lungs include hypoxia, adrenergic neurotransmitters, eicosanoids, methylglyoxal, endothelin-1 (ET-1), serotonin, respiratory damage or disease, and endotoxin [2]. Endothelin-1 is a peptide mainly produced by endothelial cells with increased expression in pulmonary hypertension. Its action is first by a powerful vasoconstrictor effect and second as a potent mitogen that can induce proliferation in multiple cell types, including vascular smooth muscle cells, leading to remodeling of pulmonary arteries leading to remodeling of pulmonary arteries [8]. In broilers, ET-1 elicits dose-dependent constriction of pulmonary arteries that can be modulated by nitric oxide [9]. Broilers developing PAH had higher serum levels of ET-1 than non-hypertensive control broilers [10]. In recent

years, antioxidants derived from natural resources, mainly from medicinal plants, have been extensively used to prevent oxidative damage. Medicinal herbs are excellent source of wide array of natural and active components such as flavonoids, polyphenols, tannins, and alkaloids, which can explain their pharmacological activities in the treatment of various human and animal diseases, and they offer alternative remedies for different health problems [11]. Pomegranate (*Punicagranatum*) is one of the oldest edible fruits and is widely grown in many tropical and subtropical countries such as United States, Iran and South East Asian countries [12]. The edible part and the peel part comprise 52 and 48%, respectively, of the total weight of the pomegranate fruit [13]. The pomegranate peel is an inedible part obtained during processing of pomegranate juice [14]. The pomegranate peel and its extract contain antioxidants compounds such as flavonoids (flavonols, flavanols and anthocyanins), condensed tannins (proanthocyanidins) and hydrolysable tannins (ellagitannins and gallotannins) [15]. Previous studies established that administration of regular pomegranate juice to animals and humans afflicted with atherosclerosis produced a significant protective effect. In addition, pomegranate juice administered to hypertensive patients caused a significant drop in blood pressure [16]. The objective of the present study was to evaluate the effect of different levels of *pomegranate peel* in the diet, whether this herbal medicine is able to modulate the development of PHS and enhance performance in broiler chickens reared at high altitude.

MATERIALS AND METHODS

Birds and experimental facility

The experiment was carried out in the experimental facility of Shahrekord University, Shahrekord, Iran (an altitude of 2100 m) according to the Institutional Animal Care and Use Committee. A total of 250 day-old male broiler chicks (Cobb 500) were allocated randomly to 5 treatments groups with 5 replicates (10 birds per pen). Each pen was supplied with a bell drinker and a feed trough. One-day old chicks were assigned to each pen in a way that all pens had equal initial body weights (420 ± 10 g). Birds were allowed to 23 hrs light and 1 hr dark throughout the trial with free access to mash feed and water. The house temperature was set at 32 ± 1 C on day one, and declined to 25 ± 1 °C on day seven, 20 ± 1 °C on day 14, and 15 ± 1 °C on day 21 onward (until 40 days of age) as previously described [17].

Treatments

A commercial broiler diet was prepared according to the broiler performance and nutrition supplement guide Cobb-Vantress for the starter (3050 Kcal/kg ME and 214 g/kg crude protein), grower (3050 Kcal/kg ME and 194 g/kg crude protein) and finisher (3000 Kcal/kg ME and 185 g/kg crude protein) and considered as control [18]. Four additional diets were prepared by substituting 0.25%, 0.50%, 0.75% and 1% *pomegranate peel* for wheat bran in the control diet. *pomegranate* was collected from Ardal region located in Chahrmahal-Va-Bakhtiari province, Iran. The shell of pomegranate was separated, air-dried, and ground for use in the experimental diets.

Measurements

Body weight gain and feed intake were calculated for the 1-

to 40-day periods. Feed conversion ratio, corrected for mortality body weights, was also calculated for the mentioned periods. At 40 days of age, 10 birds per treatment (2 birds per pen) were selected for blood collection and processing. The selected birds had body weights within approximately 5% of the average pen body weight. Blood samples (3mL) were collected from the brachial vein and centrifuged at 2500g for 10 min to obtain sera. Serum samples were used for the determination of nitric oxide (NO) and malondialdehyde (MDA). Serum NO was measured according to the method described by Ahmadipour et al, [4]. Serum MDA concentration as biomarker of oxidative stress was assayed by the method of Nair and Turner [19]. Data obtained at processing included liver and heart. The atria of heart were removed from the plane of the atrial-ventricular valves, and then the total ventricles (TV) were weighed. The right ventricle (RV) wall was then dissected free of the left ventricle (LV) and septum [20]. The RV was weighed and the RV/TV ratio was calculated. Chickens were included in the PHS morbidity when RV/TV ratio was above 0.25 [21] or when ascetic fluid accumulation/hydropericardium was evident. All birds that died after day 7 were necropsied during the experiment to identify all PHS-related morbidity

Quantitative real time PCR Analysis

At the end of experiment (40 days of age), 10 chickens from each experimental group were randomly selected and slaughtered. The livers were harvested and immediately stored in liquid nitrogen at -70 °C for subsequent RNA analysis. Total RNA from the harvested tissues was extracted using RNX-Plus reagent (Sinaclon Bioscience, Tehran, Iran). The homogenate was mixed with chloroform and centrifuged. Total RNA was separated in the upper aqueous phase of the mixture. Following precipitation with isopropanol, the RNA pellet was rinsed with ethanol (75%). The samples of RNA were re-suspended in DEPC-treated water. To remove residual DNA, the RNA was also treated by DNase (Sinaclon Bioscience, Tehran, Iran). The RNA was then measured and qualified spectrophotometrically. Only RNA with an absorbance ratio (A260/A280) greater than 1.9 was used for synthesis of cDNA. Total RNA was reverse transcribed into cDNA using Prime Script™ RT Reagent Kit (Takara Bio Inc., Japan). The reverse transcription mix was heated to 85°C for 5s to inactivate reverse transcriptase and denature the RNA and then stored at -20 °C.

The level of endothelin-1 (ET-1) and β -actin transcripts were determined by real-time reverse transcriptase (RT)-PCR using SYBR® Premix Ex Taq™ II (TliRnase H Plus) (Takara Bio Inc., Japan). To normalize the input load of cDNA among samples, β -actin was used as an endogenous standard. Specific primers ET-1 and β -actin were designed with Primer-Blast ([www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC = Blast Home](http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome)). Details of the primers are listed in Table (1). PCRs were carried out in a real-time PCR cycler (Rotor Gene Q 6000, Qiagen, USA) in three replicates for each sample of ventricles. One microliter cDNA was added to the 10 μ l of SYBR® Premix Ex Taq II Mix and 0.5 μ M of each specific primer in a total volume of 20 μ l. The thermal profile was 95°C for 30s, 40 cycles of 94°C for 40 s, 64°C for 35 s and 72°C for 30 s. At the end of each phase, the measurement of fluorescence was done and used for quantitative

Table 1: Details of the primers used for quantitative real time PCR analysis of chicken mRNAs.

Target	Primers	PCR product (bp)	Accession no.
β-Actin	5'-AGCGAAGCCCCCAAAGTTCT-3' 5'-AGCTGGGCTGTTGCCTTCACA-3'	139	NM_205518.1
ET-1	5'-GGACGAGGAGTGCCTGTATT-3' 5'-GCT CCAGCAAGCATCTCTG-3'	141	XM418943

ET-1: endothelin 1; bp: base pair

objectives. Gene expression data were normalized to β-actin. Data were analyzed using LinRegPCR software version 2012.0 (Amsterdam, Netherland), to give the threshold cycle number and reaction efficiency [22]. Relative transcript levels and fold changes in transcript abundance were calculated using efficiency adjusted Paffl methodology [23].

Statistical analysis

Results were analyzed by GLM procedure of SAS [24] software in a completely randomized design. Means were separated by Duncan's multiple range test.

RESULTS AND DISCUSSION

Table (2) represents growth performance of broilers fed with different levels of *Pomegranate peel*. Feed intake and feed conversion ratio were significantly ($P < 0.05$) decreased when *Pomegranate peel* included in broiler diets at 0.75 and 1%. However, body weight gain did not significantly change among the treatments. Improved feed intake and feed conversion ratio by feeding *Pomegranate peel* can be explained by beneficial effects of phenolic compounds such as oleuropein, hydroxytyrosol and tannins have been reported to inhibit various pathogenic microorganisms found in *Pomegranate peel*. Sarica and Urkmez [25] estimated the amount of Proanthocyanidin, Oleuropein and total phenolics in the Pomegranate peel extract of 8.4 mg/g, 196 mg/g and 191 mg/g, respectively. In broiler chickens which are fast growing tend to have pulmonary hypertension and ascites, body weight is reduced, but feed intake and feed conversion ratio factor increases [27]. Sarica and Urkmez [25] Showed that pomegranate peel-extract supplementation to diets of broilers increased BWG and improved FCR of broilers during the period of 0 to 6 weeks compared to the control group. Research has indicated that proanthocyanidin in *Pomegranate peel* extract might have increased the growth of potentially beneficial gut bacteria, the activity of the endogen digestive enzymes in the pancreas and small intestine and the digestibility and absorption of nutrients [28]. The antimicrobial activity of tannins includes inhibition of extracellular microbial enzymes, deprivation of substrates required for microbial growth or direct action on microbial metabolism through prevention of oxidative phosphorylation [29]. Pomegranate has nutritional and health assiting attributes of antioxidative, antimicrobial and disease prevention actions, which facilitate the improvement of growth performance [30].

Table (3) shows blood and serum variables of broiler chickens fed different levels of *Pomegranate peel*. Although dietary supplementation with *Pomegranate peel* (0.75 and 1%) significantly ($P < 0.05$) increased serum NO concentration, it reduced the MDA level compared to the control group. Feeding

Pomegranate peel from 0.5 to 1% caused a significant ($P < 0.05$) reduction in hematocrit when compared to the control. Increased serum NO concentration in *Pomegranate peel* groups presumably counterbalanced right ventricular hypertrophy, as appeared in lower RV/TV ratio. Nitric oxide is a potent vasodilator that directly reduces the pulmonary vascular resistance by causing vascular smooth muscle to relax, and inhibits the production and release of vasoconstrictors such as serotonin and endothelin-1 [7]. It has been suggested that NO insufficiency is associated with the pathophysiology of right ventricular hypertrophy in broilers with pulmonary hypertension [31]. The results of Nigris et al [32] experiments obese Zucker rats indicated that supplementation with Pomegranate fruit extract (PFE) or pomegranate juice (PJ) significantly decreased the expression of vascular inflammation markers. Furthermore they found that PFE and PJ were the most effective in increasing vascular eNOS expression and plasma nitric oxide (NOx) levels.

Ascites syndrome induced a large number of reactive oxygen species (ROS) and MDA production in many tissues, causing lipid peroxidation in mitochondrial membrane, leading to over-consumption of antioxidant enzymes and inadequately synthesizes [33]. Results from this study revealed *Pomegranate peel* as a powerful free radical scavenger, So that this medicinal herb with antioxidant compounds polyphenols, tannins, punicalagins, ellagitannin, gallotannins and anthocyanins reduces oxidative stress and pulmonary hypertension. The extracts of pomegranate peel exhibited higher antioxidant activity *in vitro* compared to the seed extracts [34]. Heart and right ventricular hypertrophy index (RV/TV) up to 40 d of age in broilers fed with *Pomegranate peel* at 0.75 and 1% were significantly ($P < 0.05$) lower than the control, But there is no significant difference between groups 0.25, 0.5% and control (Table 4). High hematocrit increases the viscosity of the blood. Increased blood viscosity is the primary or main secondary factor causing pulmonary hypertension at altitude. Hypoxia stimulates the production of erythropoietin, which induces polycythaemia, increasing blood viscosity [1]. It seems that phenolic compounds in the *Pomegranate peel* have an inhibitory effect on the secretion of erythropoietin from the kidneys. Several studies have shown that the use of medicinal plants in chickens susceptible to pulmonary hypertension reduces the ratio of RV/TV [21,35].

Wang et al [36] indicated that liver and heart of ascites broilers was significantly hypertrophic with the lower body weight, which increased the susceptibility to ascites and aggravated the occurrence of ascites syndrome. The pomegranate shell seems to have a potent antioxidant effect on reducing liver tissue damage in chickens that are involved with pulmonary hypertension and preventing weight of liver. Hepatoprotection is the ability to prevent damage to the liver, prevent the liver affections

Table 2: Effects of different levels of *Pomegranate peel* on performance of broiler chickens (40 d).

	Dietary levels of <i>Pomegranate peel</i> (%)						SEM	P-value
	Control(0)	0.25	0.5	0.75	1			
Weight gain (g/bird):								
1- 40 days of age	1807 ^a	1842 ^a	1817 ^a	1842 ^a	1768 ^a	22.7	0.16	
Feed intake (g/bird):								
1- 40 days of age	3421 ^a	3389 ^{ab}	3305 ^b	3268 ^c	3236 ^c	33.6	0.003	
Feed conversion ratio:								
1- 40 days of age	1.89 ^a	1.84 ^{ab}	1.82 ^{ab}	1.77 ^b	1.83 ^{ab}	0.024	0.06	

^{ab}Means in the same raw with different letters are significantly different (P<0.05).

Table 3: Effect of *Pomegranate peel* on blood and serum variables in broiler chickens (40 d).

Item	Dietary levels of <i>Pomegranate peel</i> (%)					SEM	P-value
	Control(0)	0.25	0.5	0.75	1		
Malonedialdehyde (μmol/l)	3.68 ^a	3.3 ^{ab}	2.9 ^{ab}	2.7 ^b	2.5 ^b	0.3	0.06
Plasma nitric oxide (μmol/l)	11.9 ^b	13.2 ^{ab}	14.3 ^{ab}	16 ^a	16.5 ^a	1.05	0.02
Hematocri (%)	46.5 ^a	44.7 ^a	43.2 ^b	42.3 ^b	42.2 ^b	1.07	0.03

^{ab}Superscripts in the same row with different letters are significantly different (P<0.05).
Each mean represents values from 10 replicates

Table 4: Effect of *Pomegranate peel* on liver and cardiac indices in broiler chickens (40 d).

Item (% unless noted)	Dietary levels of <i>Pomegranate peel</i> (%)					SEM	P-value
	Control(0)	0.25	0.5	0.75	1		
Liver/BW	2.53 ^a	2.37 ^{ab}	2.31 ^{ab}	2.1 ^b	2.2 ^{ab}	0.1	0.19
Heart/BW	0.611 ^a	0.585 ^{ab}	0.564 ^{ab}	0.538 ^b	0.548 ^b	0.02	0.044
RV:TV ratio (g/g)	0.30 ^a	0.29 ^a	0.25 ^{ab}	0.24 ^b	0.24 ^b	0.01	0.017

^{ab}Superscripts in the same row with different letters are significantly different (P<0.05).
Each mean represents values from 10 replicates. RV:TV – right ventricle to total ventricle weight ratio.

Table 5: Effect of *Pomegranate peel* on expression of ET-1 genes in the liver of broiler chickens (40 d).

Item	Gene	Dietary levels of <i>Pomegranate peel</i> (%)					SEM	P-value
		Control (0)	0.25	0.5	0.75	1		
Liver	ET-1	2.36 ^a	1.07 ^{ab}	0.188 ^{ab}	0.058 ^b	0.052 ^b	0.72	0.13

^{ab}Superscripts in the same column with different letters are significantly different (P<0.05).
ET-1:endothelin1
Number of observation=8

prophylactically and maintains balance in liver enzymes [37]. Results from this study revealed *Pomegranate peel* as a powerful free radical scavenger, So that this medicinal herb with antioxidant compounds reduces oxidative stress and pulmonary hypertension.

The production of angiotensin II from angiotensin I is inhibited by angiotensin converting enzyme (ACE) inhibitors. Angiotensin converting enzyme is a glycoprotein peptidyl dipeptide hydrolase that cleaves histidylleucine dipeptide from angiotensin I (a relatively inactive decapeptide), forming the potent vasoconstrictor angiotensin II [38]. Aviram and Dornfeld [39] *Pomegranate* juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. We thus conclude that the significant inhibitory effect of *pomegranate* juice on serum ACE activity and the minor attenuation in blood pressure in hypertensive patients, in addition to its potent inhibitory effect on lipid peroxidation.

Hepatic expression of ET-1 gene in broiler chickens fed with

Pomegranate peel at 0.75 and 1% was significantly (P<0.05) decreased compared to their control counterparts (Table 5). Studies have shown that the use of *Kelussia odoratissima* Mozzaf reduces the expression of ET-1 gene in the heart and lungs compared to the control group in chickens reared under cold and high altitude conditions, which is agree with the results of this experiment [35]. ET-1 is the most potent vasoconstrictor substance produced by the cardiovascular system. Therefore, a pathophysiological role for this peptide has been proposed in arterial hypertension. Several lines of evidence have also demonstrated a strong relationship between ET system dysfunction and pulmonary arterial hypertension [40]. Gomez et al. [41] demonstrated that ET and its receptor (ETAR) could affect the pathophysiology of ascites. They showed that lungs from broilers with pulmonary hypertension expressed higher levels of ET-1 compared with lungs from non-hypertensive broilers. In addition, Hassanpour et al. [10], confirmed that in broilers developing pulmonary arterial hypertension had higher serum levels of ET-1 than non-hypertensive broilers.

Regulation of ET-1 is multifactorial. Interactions between ET system and several factors including hypoxia, nitric oxide, angiotensin II, catecholamines, cytokines and growth factors determine gene expression and circulatory level of ET-1 [42]. Apparently, adding *Pomegranate peel* to diet of chickens would divert these interactions (via NO production) to reduce ET-1 gene expression and decreased vascular resistance and pressure on the heart due to vasodilator effect.

CONCLUSION

In conclusion, supplemental 0.75 and 1% *pomegranate peel* to diets of broilers played complementary roles to increase nitric oxide bioavailability and decrease ET-1 gene expression, thus improving cardio-pulmonary performance and prevent the development of PHS in broiler chickens reared under cold and high altitude.

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