

Effect of Mash, Pellet, and Extrude Diet Form on Ascetic Gene Expression (*HIF-1 α* mRNA) and Heart Index in Broiler Chicken

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ABSTRACT

The current study was conducted to determine the effect of the feed form (mash, pellet, and extrude) on Hypoxia-Inducible Factor 1 α (*HIF-1 α* mRNA) and heart index in broiler chickens. The relative weight of hearts and lungs, the ratio of Right Ventricle heart weight to the Total Ventricle weight (RV/TV, heart index), Hematocrit value (HCT) and gene expression (*HIF-1 α* mRNA) in the hearts and lungs tissues of the broiler chickens were determined at 35 days of age. Also, mortality and performance parameters were recorded throughout the experiment. The results showed that Average Daily Weight Gain (ADWG), Average Daily Feed Intake (ADFI) ($P < 0.01$), Right Ventricle weight (RV), Ventricles Total weight (TV), hematocrit value and expression of *HIF-1 α* mRNA in the lungs tissues were higher in the pellet and extrude diet form compared to the mash diet form ($P < 0.05$). Also, a higher Feed Conversion Ratio (FCR) ($P < 0.01$) was observed in the mash diet form. The hematocrit value and expression of the *HIF-1 α* mRNA gene in the lung tissues of the broiler chickens were increased by feeding pellet and extruded diets, which was associated with increase in mortality from ascites. Our findings, therefore, revealed that the *HIF-1 α* mRNA gene might be concerned with the increase of pulmonary and ascites syndrome in the broiler chickens.

Keywords: Feed form, Hypoxia-Inducible Factor 1 α (*HIF-1 α*), Pulmonary pressure.

INTRODUCTION

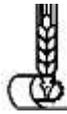
HIF refer to transcription factors including *HIF α* -1, 2, 3 and creator subunits; more than 60 genes are involved in response to hypoxia. The *HIF- α* subunit is usually activated in conditions such as anaerobic glycolysis, angiogenesis, and cell survival (Koumenis and Maxwell, 2006).

The Hypoxia-Inducible Factor (*HIF-1 α*) is activated in response to hypoxia in nucleated cells; it is also involved in increasing the pulmonary pressure in animals and humans

(Jiang *et al.*, 2007; Zhang *et al.*, 2013). Hypoxia occurs when the tissues demand for oxygen is more than the oxygen supply (Guillemin and Krasnow, 1997; Khodambashi Emami *et al.*, 2018). The Hypoxia-Inducible Factor (*HIF-1 α*) plays an important role in the pulmonary pressure increased by regulating the expression of endothelin-1 in animals. Endothelin-1 influences vasoconstrictive and cardiomyocytes (Ishikawa *et al.*, 1988), leading to the development of the pulmonary

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pressure in the broiler chickens (Ishikawa *et al.*, 1988; Julian, 1993; Yang *et al.*, 2005).

Modern poultry diets mainly include three physical forms: mash, crumble, and pellets. The form of the industrial feed, usually the pellet diet form, increases the blood volume by raising the rate of growth and metabolism of the body (Arce-Menocal *et al.*, 2009; Shabani *et al.*, 2015; Hoseini and Afshar, 2017). In this case, it activates the transcription of genes Erythropoietin (EPO) and increases the need for oxygen and production of the red blood cells (Wideman and Kirby 1995; Semenza, 2001). Increasing the red blood cells raises the lungs blood pressure (Julian, 1993), in which case the expression of the *HIF-1 α* mRNA gene may be stimulated, thereby increasing the pulmonary pressure (Rey and Semenza, 2010) and heart increases the cardiac output to provide oxygen to the body, causing dilation and right ventricle hypertrophy. These, in turn, result in ascites (Kaul and Trangadia, 2003; Wideman *et al.*, 2007; Hasani *et al.*, 2018).

According to the previous studies, the heart index (RV/TV) ratio (Julian, 1987; Silverside *et al.*, 1997; Khajali and Sharifi, 2018), and the hematocrit value (Luger *et al.*, 2001; Hasani *et al.*, 2018) have been used as the parameters for assessing the development of ascites in the broiler chickens. Little information is, however, available regarding the expression of the *HIF-1 α* gene in the development of ascites syndrome in response to the diet form in the broiler chickens. We aimed to study the possibility that *HIF-1* might be related to the development of ascites syndrome in broiler chickens. The objective of this study was, therefore, to investigate the effect of the feed form (mash, pellet and extrude) on Hypoxia-Inducible Factor 1 α (HIF-1 α mRNA) in the hearts and lungs, heart index (ratio RV/TV), the relative weight of hearts and lungs and the hematocrit value in the broiler chickens.

MATERIALS AND METHODS

This study was conducted in Karaj, Iran (1,400 m) and 468 broiler chickens (Ross, 308) were assigned randomly into 3 treatments: mash, pellet, extrude feed form. Each treatment included 6 replicates and 26 broiler chickens in each, which were reared from 1 to 42 days of age on the litter. The composition of the diets was starter diet (CP= 23.5%, ME= 3,000 kcal kg⁻¹, from 1 to 14 days), grower diet (CP= 20.8%, ME= 3,070 kcal kg⁻¹, from 14 to 28 days), and finisher diet (CP=18.8%, ME= 3,110 kcal kg⁻¹, from 28-42 day). Diets were supplied by Beyza Feed Mill. Nutritional requirements were provided based on the standard recommendations (Aviagen, 2014, 2014). The extrude diet form was done by application of moisture content < 15%, temperature 85-95°C with pressure 5-7 bar for 30 seconds, and moisture content < 15%, temperature 70-75°C, and pressure 1-1.5 bar for one minute was used for pellet diet form. The average diameter of pellet and extrude diet form was 1-1.8 mm, 1.8-3 mm, and 3-4 mm for the periods of starter, grower, and finisher, respectively. Access to feed and water was ad libitum and 23 hours with daily light. The hypothesis of the development of ascites was based on the feed form.

In order to determine broiler chickens susceptible to ascites at 35 days of the experiment, two chickens in each replication were selected to assess the hematocrit value; so, blood samples were collected in tubes including heparinized microhematocrit and determined by a microhematocrit method. Also, the relative weight of hearts and lungs (Bradley *et al.*, 1994), the ratio of the Right Ventricle Weight to the Total Ventricles Weight (Heart index: RVW/TVW \times 100, Ascites > 0.25) (Julian, 1987; Silversides *et al.*, 1997) and the expression of the gene *HIF-1 α* mRNA in the hearts and lungs tissues of broiler chickens were determined (Zhang *et al.*, 2013). Feed intake, body weight, and feed conversion ratio were also measured and mortality was recorded daily. All chickens were necropsied to determine the cause of their death. Clinical signs of

Table 1. Primers used for Real-time PCR analysis.

Gene	Accession number	5' Primer ^a	Product size (Base pairs)
HIF-1 α	NM204297	F: TGAGAGAAATGCTTACACACAG R: TGATGGGTGAGGAATTGGTTCAC	184
β -Actin	NM205518	F: CAGTGCCAGCCTCGTCTCAT R: AGGGGCCATCCACAGTCTTC	341

^a F: Forward, R: Reverse.

ascites included cyanosis of the head (comb and wattles) and body skin, yellow liquid accumulation in the abdominal cavity or blood clots in the surface liver, and fluid around heart and lung (Khodambashi Emami *et al.*, 2018; Julian, 1987).

Gene Expression

Extraction of RNA and RT- PCR

At 35 days of age, 100 mg of the hearts and lungs tissues was placed in a phosphate buffer containing 10% formaldehyde with neutral acidity (pH= 7.4) and liquid nitrogen; then, it was stored at -80°C to be used for the extraction of RNA. In general, the procedure can be described as follows:

Extraction of the total RNA from the hearts and lungs tissues.

Formation of cDNA from mRNA samples.

Implementation of PCR to obtain the binding temperature of the primer to the *HIF-1 α* gene (Zhang *et al.*, 2013).

Execution of the real-time PCR reactions by using specific primers.

Quantitative measurement of the mRNA content (level of the expression of the gene) by comparing the resulting curves with the standard curve (housekeeping β -Actin gene).

Total RNA was extracted using the manufacturer's instructions (Gene JET PCR Purification Kit, Fermentas, Qiagen). Then, cDNA was the reverse transcribed using the product (Revert Aid First Strand cDNA Synthesis Kit, Fermentas, Qiagen) and cDNA PCR was performed by employing the SYBER Green master mix through the

Real-Time PCR technique (Zhang *et al.*, 2013). Details of the primers specifically designed for the *HIF-1 α* gene are described in Table 1. The PCR thermocycling was as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, the annealing and extension temperature at 60°C for 30 seconds, and a final extension step of 72°C for 30 seconds.

The target gene of (HIF1 α) and housekeeping reference gene (β -Actin) were calculated by the ABI I7300 (ABI Applied Biosystems) sequence detector. Relative expression of the *HIF-1 α* gene in the hearts and lungs tissues was calculated by applying the Cycle Threshold (CT) equation (Areiza *et al.*, 2010; Zhang *et al.*, 2013):

$$\text{CT}(\beta\text{-Actin}) - \text{CT}(\text{HIF-1}\alpha) = \text{CT}\Delta$$

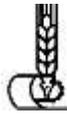
Where, ΔCT : Cycle Threshold; *HIF-1 α* : Target gene; β -Actin: Standard gene.

Statistical Analysis

A Completely Randomized Design (CRD) and the GLM procedure of SAS 9.1 software (2009) were used for statistical analysis of the data. Differences between treatments means were compared by using one-way ANOVA and Duncan's test. All significance was based on a P-value equal to 0.05. Mortality analyses were performed based on a Chi-Square test.

RESULTS

The effects of a diet physical form on performance in broiler chickens are shown in Table 2. Increase in ADWG and ADFI

**Table 2.** Average performance of broiler chicken by treatments at different weeks.

Variable/ Week ^A	Dietary treatments			SEM	P-value
	Mash	Pellet	Extrud		
ADWG (g d ⁻¹)					
1	13.81 ^c	17.05 ^b	17.71 ^a	0.115	0.0001
2	31.04 ^b	38.76 ^a	41.03 ^a	0.959	0.0018
3	52.77 ^b	74.20 ^a	75.66 ^a	1.094	0.0001
4	70.60 ^b	95.49 ^a	89.11 ^a	1.342	0.0001
5	85.80 ^b	95.55 ^a	93.64 ^a	1.168	0.0091
6	76.19 ^a	63.12 ^b	78.01 ^a	1.726	0.0059
ADFI (g d ⁻¹)					
1	18.88	19.57	19.52	0.317	0.6171
2	44.04 ^b	53.16 ^a	51.48 ^a	0.719	0.0003
3	80.30 ^b	96.70 ^a	99.34 ^a	0.738	0.0001
4	116.52 ^c	144.92 ^a	136.97 ^b	1.341	0.0001
5	164.52 ^b	176.60 ^a	170.44 ^{ab}	1.567	0.0231
6	189.81	185.44	190.54	1.778	0.4676
FCR					
1	1.37 ^a	1.15 ^b	1.12 ^b	0.021	0.0004
2	1.24	1.18	1.11	0.021	0.0849
3	1.37 ^a	1.24 ^b	1.206 ^b	0.007	0.0001
4	1.48 ^a	1.36 ^b	1.33 ^b	0.008	0.0001
5	1.63 ^a	1.51 ^b	1.48 ^b	0.010	0.0001
6	1.84 ^a	1.82 ^a	1.70 ^b	0.018	0.0153

^{a-c} Means followed by different superscripts are significantly different ($P < 0.01$), ($P < 0.05$).

^A ADWG= Average Daily Weight Gain; ADFI= Average Daily Feed Intake, FCR= Feed Conversion Ratio.

($P < 0.01$) and better FCR at 1-6 weeks of age ($P < 0.01$) was shown by P and E diet form in broiler chicken. Average daily feed intake was increased by the P diet form at 2-5 weeks of age ($P < 0.01$). This indicated that the performance of the broiler chickens was strongly influenced by the physical form of the diet. No significant differences were observed in ADWG at 2-5 weeks of age between the P and E diet form in the broiler chickens (Table 2). There were significant differences in mortality among treatments ($P < 0.05$).

A lower mortality percentage was observed in the mash treatment during the experiment. The rate of mortality was 5.45% during the experimental period. The higher mortality percentage of ascites was found in broiler chickens that had received the extrude and pellet diet form than the mash diet one (Table 3). The ascetic broilers exhibited the lesions of ascites and fluid accumulation in the abdominal cavity and

oedematous lungs, liver, and the right ventricular hypertrophy of the heart.

The results also showed that the physical form of feed had a significant effect on the right ventricular weight and total ventricle weight, and hematocrit value at 35 days of their age, and these parameters in the pellet and extrude diet form were significantly higher than those in the mash diet ($P < 0.05$). However, no significant differences were found in terms of heart index (RV/TV), relative weight of hearts and lungs among treatments ($P > 0.05$) (Table 4). While there was no significant difference, feeding the mash form caused the highest relative weight of hearts and lungs at 35 days of age (Table 4).

The effects of the feed form on the expression of the gene *HIF-1 α* mRNA in the hearts and lungs tissues of broilers at 35 days of age are shown in Table 5. The expression of the gene *HIF-1 α* mRNA in the lung tissue was significantly different among

Table 3. Number and rate of mortality of ascites in broiler chicken by different dietary treatments.^a

Mortality	Mash		Pellet		Extrude		Total	
	(No)	(%)	(No)	(%)	(No)	(%)	(No)	(%)
Ascites	0	0	12	1.28	9	0.96	21	2.24
Others	10	1.07	5	0.53	15	1.61	30	3.2
Total	10	1.07	17	1.82	24	2.56	51	5.45
P-value	0.0471							

^a Number (No), Percent (%) (χ^2 test, $P < 0.05$).

Table 4. Relative weights of heart, lung and cardiac index (RV/TV), hematocrit value of the broilers by different dietary treatments at 35 day of age.

Tissue ^A	Dietary treatments			SEM	P-value
	Mash	Pellet	Extrude		
Lung (%BW)	0.46	0.36	0.44	0.022	0.177
Heart (%BW)	0.45	0.48	0.49	0.022	0.746
RVW (g)	1.137 ^b	1.340 ^a	1.368 ^a	0.028	0.0089
TVW (g)	6.000 ^b	6.579 ^a	6.812 ^a	0.100	0.0135
RV/TV (%)	19.000	20.341	20.133	0.367	0.303
Hematocrit (%)	32.450 ^b	36.400 ^a	34.625 ^a	0.457	0.0107

^a RVW: Right Ventricle Weight; TVW: Total Ventricle Weight, RV/TV: Right Ventricle weight/Total Ventricle weight $\times 100$. ^{a-b} Means followed by different superscript are significantly different levels ($P < 0.05$).

treatments ($P < 0.05$) and the expression of this gene was significantly higher in comparison to the pellet and extrude form, as compared with the mash diet, however, the expression of this gene was not significant in the hearts tissue among treatments ($P > 0.05$) (Table 5). It should be noted that an increase in the expression of this gene in the hearts was observed in the birds fed by the pellet and extrude diet form.

DISCUSSION

Broilers in the mash treatment had a significantly lower mortality rate due to ascites. Ascites caused high mortality in the

broilers that were in their 3 to 5 weeks of age (Wideman, 2000).

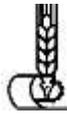
Genetic, metabolic and dietary factors could influence health (Khodambashi Emami *et al.*, 2018). Feed form is one of the effective factors in the performance and growth rate of the broiler chickens. Feeding by pellet diet form, as compared with the mash diet, increased body metabolism, rapid growth, nutrient efficiency and oxygen demand. As a result, the incidence of ascites and mortality in commercial broiler chickens was increased (Kaul and Trangadia, 2003; Arce-Menocal *et al.*, 2009; Sing *et al.*, 2011; Shabani *et al.*, 2015).

In this experiment, no significant differences were shown in the relative

Table 5. mRNA expression (*HIF-1 α*) in the heart and lung of the broilers by different dietary treatments at 35 day of age.

Tissue	Dietary treatments			SEM	P-value
	Mash	Pellet	Extrude		
Lung	10.587 ^b	17.356 ^a	14.978 ^a	0.843	0.0159
heart	13.510	17.244	20.079	1.867	0.3783

^{a-b} Means followed by different superscript are significantly different.



weight of hearts and lungs and heart index in the treatments, but the right ventricular weight and the total ventricle weight and hematocrit value were increased by the extrude and pellet form, indicating the increase of the pulmonary pressure. Although there was no significant difference in the RV/TV ratio, the right ventricle hypertrophy of the heart was observed.

A positive genetic link has been reported between the right ventricular hypertrophy and the mortality of ascites in the broiler chickens (Arce-Menocal *et al.*, 2009). The researchers have reported that the RV/TV ratio is higher in the pellet diet form compared to the mash diet in ascetics' broiler chicks (Julian, 1987; Julian, 1993; Arce-Menocal *et al.*, 2009; Hasani *et al.*, 2018). Also, the changes in the production of blood red cell could increase the erythrocyte fragility, and blood viscosity, well as raising pulmonary hypertension or ascites syndrome (Julian, 1993; Luger *et al.*, 2001).

The results showed that the expression of *HIF-1 α mRNA* was increased by the pellet and extrude diet form in lungs at 35 days of age. The mash diet form showed the lower expression levels of this gene in hearts and lungs; probably, the expression of *HIF-1 α mRNA* was enhanced with the increase of the pulmonary arterial pressure, which might be related to the mortality of ascites. A significant increase was also found in the expression of *HIF-1 α mRNA* in the lung tissues of ascites chicks. Zhang *et al.* (2013) investigated the effect of the form of the feed on the expression of the *HIF-1 α mRNA* gene in the hearts and lungs tissues at 36 and 43 days of age. Also, the increase in the expression of *HIF-1 α mRNA* gene in ascetic birds has been reported by Catron *et al.* (2001) and Areiza *et al.* (2010). High levels of *HIF-1 α* were found in the brain, lung, and kidney tissues when exposed to the oxygen deficiency of the environment in livestock (Wang *et al.*, 2006). The *HIF-1* gene has been reported to increase the pulmonary pressure in mice (Jiang *et al.*, 2007). Peng *et al.* (2013) also stated that birds fed with a

high-energy and high protein diet, as compared to the control group, did not show a significant difference in the expression of *HIF-1 α mRNA*. Also, Song *et al.* (2010) found that the *HIF-1 α* gene did not affect the incidence of ascites in the broiler chickens.

Hypoxia-Inducible Factor 1 (*HIF-1 α mRNA*) plays an important role in response to hypoxia (Powell and Fu, 2008).

Generally, using the pellet and extrude diet form increases the relative weight of the body without increasing the lung and heart capacity, leading to the inadequate supply of oxygen (hypoxia) to the body. As a result, enhancing the cardiac output in response to the oxygen demand and the reduced oxygen content of blood (Julian, 1987; Wideman and Tackett, 2000; Hasani *et al.*, 2018) may simulate the *HIF-1 α mRNA* gene expression. This could help the expression of EPO, which increases blood volume and production of red blood cells. In this case, blood resistance was limited in the lung (Julian, 1993). This increase of pulmonary hypertension and right ventricular hypertrophy, valvular insufficiency, right ventricular dilation, and right ventricular failure were observed (Julian, 1993; Wideman *et al.*, 1995; Hasani *et al.*, 2018; Khajali and Sharifi, 2018). The results related to ascites were in agreement with those found in the current study (Decuyper *et al.*, 2005; Olkowski *et al.*, 2007).

CONCLUSIONS

The results showed that the pellet and extrude diet form increased the pulmonary hypertension in comparison with the mash diet in the broiler chickens. This increased pulmonary hypertension might be related to the increase of performance, the expression of *HIF-1 α mRNA* in the lung tissue, hematocrit value, and the right ventricle weight and mortality of ascites in broiler chickens. However, there were no significant differences in the relative weight of hearts and lungs, the heart index (RV/TV

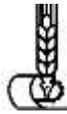
ratio), as well as the expression of *HIF-1a* mRNA in the heart tissue.

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شکل خوراک آردی، پلت و اکستروود بر بیان ژن آسیتی (*HIF-1 α mRNA*) و شاخص قلب در جوجه های گوشتی

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چکیده

مطالعه حاضر با هدف تعیین اثر شکل خوراک (آردی، پلت و اکستروود) بر فاکتور ایجادکننده هیپوکسی *HIF-1 α mRNA* و شاخص قلب در جوجه های گوشتی انجام شد. وزن نسبی قلب و ریه، نسبت وزن بطن راست به وزن کل بطن ها (RV / TV شاخص قلب)، مقدار هماتوکریت خون (HCT) و بیان ژن (*HIF-1 α mRNA*) در قلب و ریه های جوجه های گوشتی در سن ۳۵ روزگی تعیین شد. همچنین مرگ و میر و پارامترهای عملکرد در کل دوره آزمایش ثبت شد. نتایج نشان داد که وزن بطن راست (RV)، وزن کل بطن ها (TV)، میانگین افزایش وزن و دریافت خوراک روزانه، مقدار هماتوکریت خون، بیان ژن *HIF-1 α mRNA* در بافت های ریه در شکل خوراک پلت و اکستروود بالاتر از شکل خوراک آردی بود ($P < 0.05$). همچنین در شکل خوراک پلت و اکستروود با افزایش مقدار هماتوکریت خون و بیان ژن *HIF-1 α mRNA* در ریه های جوجه های گوشتی و بهبود ضریب تبدیل غذایی، فشار ریوی و مرگ و میر آسیت افزایش پیدا کرد. یافته های ما نشان داد که ژن *HIF-1 α mRNA* ممکن است با افزایش فشار ریوی و سندرم آسیت در جوجه های گوشتی مرتبط باشد.