Pulmonary hypertension syndrome in fast-growing broilers

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This study investigated variations of plasma angiotensin II and gene expression of renin, angiotensin converting enzyme (ACE) and angiotensin II type 1 receptor (AT_1R) in the heart of pulmonary hypertensive chickens. Plasma angiotensin II was significantly increased at 28 days of age ($P < 0.05$). Transcripts of renin and ACE in the right ventricle of the treated groups were increased at 12 days and decreased at 42 days of age compared to controls while AT_1R was increased at 12 and 42 days ($P <0.05$). AT_1R and ACE transcripts in the left ventricle of the treated group were increased at days 12 and 42 respectively compared to controls ($P <0.05$). It is concluded that renin, ACE and AT_1R mRNAs were relatively upregulated in heart of chickens developing pulmonary hypertension while considerable reduction of renin/ACE and elevation of AT_1R in right ventricle of heart may involve in dilated cardiomyopathy.

Key words: Renin-angiotensin system, Pulmonary hypertension, Broiler chicken.

Introduction

Pulmonary hypertension syndrome (PHS, ascites) in fast-growing broilers is an elevated blood pressure within the pulmonary circulation (Baghbanzadeh and Decuypere, 2008). Pulmonary hypertension initiates the sequential development of hypoxemia, right-sided congestive heart failure, central venous congestion, cirrhosis of the liver, and accumulation of ascitic fluid into the abdominal cavity (Balog, 2003). It is commonly accepted that PHS in fast-growing broilers is a direct effect of right atrioventricular valve insufficiency, ventricular volume overload and right ventricular dilation and failure (Baghbanzadeh and Decuypere, 2008). In PHS, a high vascular

resistance due to an anatomically inadequate pulmonary vascular capacity and excessive vascular tone reflecting an imbalance between pulmonary vasoconstrictors and vasodilators has been demonstrated (Wideman *et al.* , 2013). Many vasoactive elements are involved in the pathophysiology of PHS such as norepinephrine, thromboxane, endothelin, serotonin, nitric oxide, prostacyclin and angiotensin II (Teshfam *et al.* , 2006, Hassanpour *et al.* , 2009, Hassanpour *et al.* , 2011, Wideman *et al.* , 2013). Pathophysiologic alterations in the cellular and molecular levels of this syndrome have been noted (Kim and Iwao, 2000, Sato *et al.* , 2012, Hassanpour *et al.* , 2013a, Hassanpour *et al.* , 2013b).

Angiotensin II (Ang II) is the central active component of the renin–angiotensin system (RAS) that plays a major role in regulating the cardiovascular system, and disorders of the RAS contribute largely to the pathophysiology of hypertension, renal disease and chronic heart failure (Dostal and Baker, 1999). This system is an ever-evolving endocrine system with considerable checks and balances on the production and catabolism of angiotensin peptides most likely due to the manifold effects of angiotensin (Putnam *et al.* , 2012). In the RAS, a precursor peptide, angiotensinogen, is cleaved by renin to form the decapeptide angiotensin I. The dipeptidase angiotensin-converting enzyme (ACE) cleaves angiotensin I to form the octapeptide angiotensin II (Levy, 2004). Ang II, through the activation of specific Ang II types 1 and 2 receptors (AT_1R; AT_2R), regulates cardiac contractility, cell communication, and impulse propagation. In addition, Ang II is involved in cardiac remodeling, growth, and apoptosis (Paul *et al.* , 2006, Ferreira *et al.* , 2008).

The concept of a local RAS located in the heart with autocrine and paracrine roles has been confirmed in mammals by many studies, particularly with the demonstration that elements of the RAS and Ang receptors are present in cardiac tissue (De Mello and Danser, 2000). The objective of this study was to determine plasma angiotensin and the relative amounts of renin, ACE and AT_1 R mRNA expression in the heart ventricles (right and left) of broiler chickens with pulmonary hypertension experimentally induced by 3, 5, 3′-l-triiodothyronine (T_3). This hormone increases metabolism via increasing number and size of mitochondria and stimulation of the cell membrane enzyme Na^+-K^+ ATPase), thus, increases oxygen consumption and requirement (Griffin and Ojeda, 2000). The increased body demand for oxygen prompts an increase in cardiac output. High cardiac output triggers an increase in lung arterial pressure, presumably because of the low compliance of the pulmonary vasculature (Balog, 2003).

Materials and methods

Birds and treatments

A total of 60 One-day old male Ross 308 broiler chickens were assigned to a control or treatment group (30 birds per group). Each group was randomly divided into three equal replicates of 10 chickens per pen. The chickens were reared for seven weeks on wood shavings under standard conditions and provided ad libitum access to water and a standard ration (Starter: 12. 6 MJ metabolisable energy (ME)/kg of diet, 230 g/kg crude protein (CP), Grower: 13. 2 MJ ME/kg of diet, 210 g/kg CP, Finisher: 13. 4 MJ ME/kg of diet, 190 g/kg CP formulated) to meet requirements for broilers . In the treatment group, T_3 was included in the basal diets at a concentration of 1. 5 mg T_3 /kg after

day 6 of rearing period (Hassanpour *et al.* , 2013a). Throughout the study mortality was recorded daily. Those broilers that died during the experimental period were examined for lesions of heart failure and ascites.

The Institutional Animal Care and Use Committee of Ahvaz and Shahrekord Universities approved all procedures used in this study.

Assessment of right ventricular hypertrophy

At 12 and 42 d of age, six chickens from each group were selected at random, weighed and killed by decapitation. The heart was resected and right ventricle hypertrophy was estimated as described by Teshfam et al. (2006). The ratio of right ventricle to total ventricle (RV/TV) was calculated as an index of pulmonary hypertension. Chickens with an RV/TV ratio > 0. 28 were classified as pulmonary hypertensive chickens (Wideman, 2001). The right and left ventricles of the heart were immediately frozen in liquid nitrogen and stored at -70°C for subsequent RNA analysis.

RNA extraction and cDNA synthesis

In this experiment, frozen ventricular tissues which had been prepared in the aseptic condition, were homogenized in a liquid nitrogen bath. Total RNA from right (six samples from each group at 12 and 42 days) and left ventricles (six samples from each group at 12 and 42 days) was extracted by a guanidine / phenol solution (RNx plus, Sinaclon Bioscience, Karaj, Iran). 100 mg of homogenized tissue was prepared in this solution. The homogenate was then mixed with chloroform. The resulting mixture was centrifuged (9000 rpm, 4°C, 15 min), yielding an upper aqueous phase containing total RNA. Following 100% isopropanol precipitation, the RNA pellet was washed

with 75% ethanol. The RNA samples were resuspended in DEPC-treated water. Total RNA was treated with RNase-free DNase (Sinaclon Bioscience, Karaj, Iran) to avoid amplification of contaminating genomic DNA. RNA was evaluated by agarose gel (1. 5%) electrophoresis to determine extracted RNA quality as indicated by discrete 18S and 28S rRNA bands. The amount and quality of RNA were determined by spectrophotometry. Only RNA of sufficient purity, having an absorbance ratio (A260/280) greater than 1. 9, was considered for synthesis of cDNA.

Total RNA was reverse transcribed into cDNA in a short time after extraction (less than 6 hours) using M-MLV reverse transcriptase (Sinaclon Bioscience, Karaj, Iran) as described by Hassanpour et al. (2010). The reverse-transcription (RT) was done in a 20 µl volume containing 2 µg of extracted RNA, 200 ng random hexamer, 0. 5 mM dNTP. This mixture was heated to 65°C for 5 min, and 40 u of RNase inhibitor, RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl2), 10 mM DTT and 200 u M-MLV reverse transcriptase were added. This mixture was incubated for 5 min at 25°C, followed by 50 min at 38°C. The reverse transcription mix was heated to 75°C for 15 min to denature the RNA and then stored at -20°C.

Quantitative real time PCR Analysis

In this study, relative quantification of real time PCR was used to measure changes in a gene expression in response to T_3 treatment. The levels of renin, AT_1R, ACE and β-actin transcripts were determined in the six samples of right and left ventricles from each group at 12 and 42 days by real-time reverse transcriptase (RT)-PCR using Eva-Green chemistry (Sinaclon Bioscience, Karaj, Iran). This method requires a suitable internal standard to

control for variability between samples and to normalise the input load of cDNA. β-actin was used as an internal standard. Specific primers of Renin, AT $_1$ R, ACE and β-actin were designed with Primer-Blast (www. ncbi. nlm. nih. gov/tools/primerblast/index. cgi? LINK_LOC= blastHome). The expected products of primers in PCR were checked in Nucleotide-Blast (www. blast. ncbi. nlm. nih. gov/Blast. cgi? PROGRAM= blastn&PAGE_TYPE= BlastSearch&LINK_LOC= blasthome)which found no similarity with other chicken genes. 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. 1 µl cDNA was added to 4 µl Titan Hot Taq Eva-Green Ready Mix (Sinaclon Bioscience, Iran), 0. 5 µM of each specific primer in a total volume of 20 µl. The thermal profile was 95°C for 5 min, 35 cycles of 95°C for 40 s, 60°C for 35 s and 72°C for 30 s. At the end of each phase, fluorescence was assessed by the real-time PCR cycler and used for quantitative objectives. The no-template control and no-reverse transcriptase control were used to check contamination in the PCR reagents. Gene expression data were normalized to β-actin. Data were analyzed using Rotor Gene-software, version 2. 0. 2 (build 4) (Qiagen, USA) and LinRegPCR software version 2012. 0 (Amsterdam, Netherland), to give the threshold cycle number and reaction efficiency (Ruijter et al., 2009). Relative transcript levels and fold changes in transcript abundance were calculated using efficiency adjusted Livak methodology (âˆ†âˆ†C $_T$ method) (Livak and Schmittgen, (2001).

Measurement of angiotensin II in plasma

The six chickens per group at 12, 21, 28, 35 and 42 days were selected for blood collection. Blood samples were collected from the brachial vein in heparinized syringes and centrifuged at 2, 500g for 10 min to obtain plasma. The total amount of Ang II plasma level was quantified by using a commercially available Ang II-EIA kit (catalog No.: S-1133, Bachem Chemical Company, Germany) following the manufacturer's instructions. Plasma proteins was precipitated as follows before the use of this kit. Briefly, 1 mL plasma was mixed with 2 mL acetone and centrifuged (10000 rpm, 4ºC, 10 min). The supernatant was extracted with 4 mL petroleum benzine and left at room for 30 min. After discarding the ether phase, the aqueous, lower phase containing the angiotensin was evaporated to dryness at 40ºC. The dried extracts were redissolved in 0. 25 mL assay buffer (0. 1 M Tris-HCl, (pH= 7. 4), 3 mg/mL bovine serum albumin and 2 mg/mL neomycin sulfate) and stored at -20ºC prior to assay (Gray and Simon, 1985). The materials for protein precipitation were purchased from Sigma-Aldrich Chemical Co.

The Ang II-EIA kit is an in vitro quantitative assay for detecting the angiotensin II peptide based on the principle of a competitive enzyme immunoassay (competitive binding to the Ang II antibody between biotinylated Ang II peptide and peptides in samples). This kit has intra-assay variation < 5% and inter-assay variation < 14%. The assay limit of this EIA kit is 40 pg/mL. In this experiment, because of low amounts of plasma Ang II per milliliter of plasma (less than the detection limit of the kit), about 2-3 mL of plasma were precipitated and finally resolved in the half of assay buffer suggested by Gray and Simon, 1985 (i. e., 0. 25 mL instead of 0. 5 mL / 1 mL

plasma), then Ang II was measured while it was offered in one milliliter of plasma. The samples run as duplicate in the Ang II-EIA kit.

Statistical analysis

Data are represented as mean ± SE. Comparisons were made using an independent sample t-test between each treatment and its control. Statistical analysis was done using SPSS-16 software (SPSS Institute Inc.). All data were checked to have a normal distribution and log transformed if necessary. Any data requiring log transformation were back-transformed for presentation of data. P values less than 0. 05 were considered significant.

Results

Estimation of right ventricular hypertrophy

The RV/TV ratio was greater in the treated groups at 42 days of age (0. 303 ± 0. 021) than controls (0. 215 ± 0. 017) (P= 0. 004), while this ratio was not significant at 12 days (control: 0. 154 ± 0. 014; treatment: 0. 171 ± 0. 012) (P= 0. 091). The increase of RV/TV ratio was 29% at 42 days. The clinical signs of ascites was observable in the most treated chickens at the end of rearing period.

Expression of renin, AT_1R and ACE genes in the right and left ventricles

Real-time PCR results of renin, ACE and AT_1R genes are shown in Figs. 1–3. The expression of β-actin was detected in all samples. The renin, AT_1R and ACE genes were expressed in the right and left ventricles of control and T_3-treated broilers at 12 and 42 days of age. The relative amount of renin mRNA expression in the right ventricle of the treated groups was significantly

increased at 12 days (15. 5 fold) (P= 0. 009) and decreased at 42 days (4 fold) of age compared to controls (P= 0. 012; Fig. 1).

The relative amount of ACE mRNA expression in the left ventricle of the treated group was significantly increased (9 fold) at 42 days of age compared to controls (P= 0. 008), but did not differ at 12 days of age (Fig. 2). In the right ventricle, the expression of this gene was increased (2. 9 fold) at 12 days (P= 0. 031) while decreased (3 fold) at 42 days of age in the treated group compared to control (P= 0. 024).

The relative amount of AT_1 R mRNA expression in the right ventricle of the treated group was significantly increased at 12 (5. 9 fold) (P= 0. 036) and 42 (3. 7 fold) (P= 0. 044) days of age compared to control. In the left ventricle of the treated group, the mRNA amount of this gene was only higher (3. 9 fold) at 12 days of age than control (P= 0. 043; Fig. 3).

Assessment of plasma angiotensin II

The level of Ang II was measured in plasma samples of chickens at 12, 21, 28, 35 and 42 days of age. The amount of Ang II was significantly increased in T_3 -treated chickens only at 28 days of age when compared with control (P= 0. 041; Fig. 4).

In this study, total mortality was 23. 3% for treatment group and 3. 3% for control group.

Discussion

In the present study, the effect of T_3 hormone was observed at 42 days of age which increased cardiac index (i. e., RV/TV > 0. 28). According to

Wideman (2001), this high cardiac index is associated with sustained pulmonary hypertension (significant high blood pressure of pulmonary artery and right ventricle). In T_3 -treated chickens of our experiment, cardiac index was not critically high to be noticed as pulmonary hypertension at 12 days of age. Thus, any alterations in cardiac RAS gene expression at this age were not related to this syndrome (Klein and Danzi, 2007, Vargas *et al.* , 2012). Ang II, apart from its effect of elevating arterial pressure, exerts mitogenic and growth promoting effects on cardiac myocytes; both of these effects contribute to the development congestive heart failure (Varagic and Frohlich, 2002). In our experiment, the amount of plasma Ang II considerably was higher at 28 days of age and so at this time of rearing period could be critical in the incidence of PHS, as previously suggested by Hassanpour et al. (2011). However, our data showed that Ang II may be involved as an important factor in the induction of PHS, but its role in the development of this syndrome and heart dilation is not predominant, versus PHS in mammals (Wollert and Drexler, 1999). It must be noticed that hyperthyroidism increases degradation of proteins far exceed synthesis (Decuypere *et al.* , 2005). Thus, variation of Ang II amount during rearing period of chickens could be affected by excess T_3 .

At 42 days of age, cardiac index was considerably high to cause heart failure and PHS. It is noticed that this stage could be associated with heart dilation, which may differ cardiomyocytes structurally and functionally from hypertrophic stage (Lowes *et al.* , 2002, Hassanpour *et al.* , 2013a). Thus, alternations in the expression of mentioned genes in the heart ventricles,

particularly in the right ventricle, which was more affected by PHS than the left ventricle, could be due to dilated cardiomyopathy.

Renin mRNA has been detected in the heart of various species (Paul *et al.* , 2006). Pieruzzi et al. (1995) described that volume overload of heart was able to increase renin mRNA in the rat heart. In contrast, Iwai et al. (1995) were unable to confirm these findings. In the present study, mRNA variations of this gene were not considerable in the left ventricle of the treated chickens while in the right ventricle, conspicuous increase (12 days) and decrease (42 days) were observed. The initial increase of renin mRNA may be influenced by volume overload of heart due to thyroid hormone while consequent decrease of this gene could be due to occurrence of the PHS. As previously mentioned, the end stage of PHS could be associated with dilated cardiomyopathy of the right ventricle in which cardiomyocytes are unable to contract properly. Apparently, this disability occurs in the expression of many genes (Ladenson *et al.* , 1992, Lowes *et al.* , 2002, Teshfam *et al.* , 2006, Hassanpour *et al.* , 2013b). A reduction of renin mRNA in the right ventricle may be due to negative compensatory feedback of cardiomycytes against high activation of systemic RAS (high plasma Ang II). It may be also noticed that T_3 initially stimulates expression of genes (such as renin) and then, suppresses transcription in long time, similar to its effect on protein (Ruckebusch et al., 1991). Further, the elevation of ACE mRNA might be influenced by thyroid hormone and initial induction of hypertrophy in the heart ventricles, while the reduction of this transcript occurred in the dilated right ventricle at the end stage of PHS. Hao et al. (2013) reported an increase of ACE mRNA and concentration of Ang II in the right ventricular

tissue of cold stress-chickens at 42 days of age. This apparent discrepancy between our results and study of Hao et al. (2013) could be due to different routes in the induction of PHS. Comparison of cardiac index in these two studies confirms that induction of PHS with T_3 was more severe than cold stress. Probably, the right ventricular remodeling in the cold stress-chickens was not completely progressed. Thus, it could be logical reason for increasing of ACE mRNA and Ang II in the hypertrophic right ventricle.

The increasing of AT_1R in the heart hypertrophy and heart failure has been confirmed (Barlucchi *et al.* , 2001, Diniz *et al.* , 2007) which is in relative agreement with our findings. Wollert and Drexler (1999) reviewed that AT-receptors-dependent signaling cascades potently modulate cardiac myocyte function and growth. They also reported that cardiac hypertrophy in response to haemodynamic overload can occur independently of the AT-receptors.

In conclusion, the gene expression of renin, ACE and AT_1R was relatively upregulated in the heart of chickens developing PHS. The right ventricle of hearts from pulmonary hypertensive chickens showed considerable reduction of renin, ACE and elevation of AT_1R which may be involved in dilated cardiomyopathy.