Developmental changes in Endothelin-1 and Endothelin type A receptor expression in the lungs of chickens (Broiler versus layer)

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Abstract:

BACKGROUND: Broilers lung mechanisms that regulate endothelin (ET) in the lung are complex and poorly understood. **OBJECTIVES: METHODS:** In this experiment lung ET-1 mRNA levels and lung mRNA expression for the ET(A) receptors were determined in lung tissue weekly (term = 42 days, intervals = 7 days). Serum endothelin concentration was also measured at these ages. **RESULTS:** The study showed that expression of endothelin in lungs of layers and broilers was similar during the first three weeks and the overall trend of ET-1 expression was increasing. However, there was a significant increase of ET-1 expression which started from the fourth week and gradually increased until the end of the commercial life of the chicken (day 42). ET-A expression in the lungs of broilers was significantly higher than layers during the last three weeks of life. Overall, trends of serum ET-1 concentration increased in both layers and broilers, but serum ET-1 concentration in broilers was significantly higher than layers. **CONCLUSIONS:** The higher level of serum Endothelin and expression of ET-1 and ETA in broiler lungs may explain the higher sensitivity of broilers to the vasoconstrictions activity of endothelin and the higher sensitivity of these animals to pulmonary hypertension (PH).

Introduction

Endothelin, which was initially isolated and identified from conditioned medium of cultured porcine endothelial cells (Masaki, 2004), is a cyclic peptide of 21 amino acids which has been shown to have vasoconstrictory, hypertensive and growth regulating properties, and promotes formation of extracellular matrix, release of growth factors and adhesion factors. It is considered not only as an important regulatory peptide contributing to vascular homeostasis, but also as a pathogenic factor in atherosclerosis, hypertension and heart failure when overexpressed (Wu et al., 2003).

Endothelins are a family of peptide hormones with three members, endothelin-1(ET-1), endothelin -2 (ET-2) and endothelin-3 (ET-3) encoded by three distinct genes (Kemp et al., 1998). The effects of the ET peptides are mediated through two known distinct specific endothelin receptors (Kemp et al., 1998). Two cDNAs encoding ET receptors were cloned independently by two laboratories and the results were published in 1990 (Masaki, 2004).

In mammals, based on their binding affinities for the native and synthetic ligands, they have been classified as type A and type B receptors (ETA and

ETB) (Kemp et al., 1998), and have been shown to belong to the family of heptahelical G-protein-coupled receptors. The ETA receptor binds ET-1 and ET-2 with greater affinity than it does ET-3, whereas the ETB receptor does not discriminate between the three endothelin isoforms. These two receptors are both distributed in various tissues and cells but with different levels of expression, suggesting the presence of a multifunctional ET system (Masaki, 2004). The ETA-receptor mainly distributes in vascular smooth muscle and regulates vascular tone and cell growth and the ETB-receptor mainly distributes in vascular endothelial cells (Wu, et al., 2003).

The binding of ET-1 with the ETA receptor activates phospholipase C, leading to the formation of inositol 1, 4, 5-trisphosphate and diacylglycerol, and eventually mobilizes the release of intracellular calcium, hence mediating contraction (Bikhazi et al., 2003).

The pulmonary circulation is the primary site for both ET production and clearance in humans, and there is evidence of an increase in both ET-1 expression and ET converting enzyme (ECE) activity in lung tissue of animal models of Cardiac Heart Failure (CHF) (Sauvageau et al.,2006).

In normal humans, approximately 50% of circulating ET-1 is extracted by the pulmonary circulation within a single transit time. This clearance is exclusively mediated by the endothelial ETB receptor (Sauvageau et al., 2006).

Gomez et al found that ET-1 mRNA levels in the lungs of pulmonary hypertensive broilers (PHB) were significantly higher than the corresponding level in non-PHB ($p<0.001$). In contrast, the opposite was true for ET-1 type A receptor mRNA levels. They showed that ET-1, the connective tissue growth factor, and adrenomedullin are up-regulated in the lungs of PHB (Gomez et al., 2007).

To the best of our knowledge, the pattern of normal expression of ET receptors in the lung of broilers has not been clarified. Therefore, the aim of this study was to evaluate possible differences in ET-1 and its receptor (ETA) expression in the lungs of leghorns and broilers, and also to determine the developmental changes in serum endothelin in a corresponding period.

Material and Methods

Two hundred 1-d-old chicks (100 Ross broilers & 100 layers) were obtained from a commercial hatchery and reared in separated pens ($10.5m^2$) in Isfahan at 1570 m above sea level, using standard nutritional and management procedures for commercial operations. Feed and water were provided ad libitum , and lighting was continuous for 23hr followed by 1hr darkness. Temperature was initially 32°C and was gradually allowed to drop (1 degree in each week). All experiments were conducted with the approval of the Animal Welfare Committee of Azad University.

Tissue Preparation: Animals were decapitated and the lungs were immediately removed and washed in cold phosphate buffered saline (PBS), frozen in liquid Nitrogen and stored at -80°C.

Total RNA extraction and reverse-transcription PCR Analysis: Gene mRNA expression of the ET-1, ETA receptor and the ETB receptor in the lungs was determined by reverse-transcription PCR (RT-PCR). RNA extraction was undertaken according to Chomczynski and Sacchi (1987) with some modifications. Briefly, 100 mg of homogenized tissue was vortexed with 500 µL of denaturing solution (4 M Guanidinium Thiocyanate, 10% (w/v) N-Lauroylsarcosinate sodium salt (Sarkosyl) and 0.75 M sodium citrate). After mixing, the solution was vortexed with 500 µL saturated phenol solution (phenol in distilled water) and 200 ?l chloroform: isoamyl alcohol (49:1) mixture for 10 sec and kept at 4°C for 15 min. The whole mixture was centrifuged at 9000rpm at 4°C for 20 minutes. The upper aqueous layer was collected in a fresh tube and treated with equal volumes of pre-chilled absolute Isopropanol and kept at -20°C for 30 min. RNA was sedimented at 9000rpm at 4°C for 10 min. The supernatant was discarded and the pellet was washed with 300 µL Isopropanol. After removing the supernatant, 200 µL pre-chilled ethanol at 75% pellets was added and vortexed. After 10 min incubation at room temprature, ethanol was removed by centrifugation at 9000rpm at 25°C for 5 minutes. The pellets were dried at room temperature and re-suspended in 10 µL of DEPC-distilled water.

At the end of extraction, RNA was treated with DNase extracted RNA, incubated with DNase I at

30°C for 30min (Buffer Tris-HCl 100mM, MgCl2 2.5mM and CaCl2 0.1 mM). DNase was inactivated by 10 min incubation at 60°C in the presence of EDTA (2.5mM).

RNA concentration was determined using UV/Visible spectrophotometer (UV-120-12, Shimadzu Corp, Kyoto, Japan), and 1 µg was submitted to reverse transcription. A sample was run on 1.5% agarose gel as well.

Semi-quantitative RT-PCR: The PCR reactions were prepared with 0.8 µM of forward and reverse primers; 1 µL cDNA, 1.5 mM MgCl2 ,200 µM dNTPs; 1.25 U Taq DNA polymerase; 2.5 µL PCR buffer (10× PCR buffer, final concentrations 50 mM KCl and 10 mM Tris-HCl); and H2O for a final volume of 25 µL. PCR was carried out in a thermo cycler (BioRad Laboratories, Richmond, CA, USA), with the following amplification profile: initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 15 s, and extension at 72°C for 15 s. Negative controls were routinely included without a template.

ß-actin was added as an internal competitive standard into the PCR system to eliminate the errors from variation of exponential amplification and the plateau effect inherited in PCR, and to obtain a relatively accurate and reproducible result from a tiny sample amount.

The size of the PCR products was verified in 1.2% agarose gel in Tris acetate buffer (TAE) stained with 1% ethidium bromide. The band corresponding to the expected size (compared to DNA ladder) was compared against an internal standard. Photo-capt v.99 Image software was used to obtain the density of bands and the relative amount of RNA was expressed as:

Relative amount= Density of each band / Density of internal standard

The primer sequences were copied from the Gomez report (Gomez et al., 2007). Sequences and expected products sizes are shown in Table 1.

Determination of serum ET-1 level: At days 7, 21 and 42 the blood was collected from brachial wing vein using a 23-gauge needle in a polypropylene tube, containing sodium citrate, and immediately centrifuged at 1800g for 15 min at 4°C. Plasma was then stored at -80°C until assayed. Serum ET-1 level was measured by enzyme immunoassay (Cyman Chemical Company Eliza Kit, Cat no. 583141). Values were reported as pg/mL.

Results

Expression of ET-1 and ETA mRNA in the lung: Representative images of ethidium bromide-stained gel electrophoresis of the specific amplicons of ET-1 and ETA are presented in Figure 1.

ET-1 (lung expression): The graph 2 shows ET-1 expression in the broilers' lungs. The amount of ET-1 mRNA in the lungs of broilers was relatively constant in the first three weeks. Expression in the last three weeks was significantly higher than the first three weeks (0.732 ± 0.024, 0.717 ± 0.013, 0.740 ± 0.015, 0.895 ± 0.023, 0.937 ± 0.036 and 0.950 ± 0.0, .71 was recorded for the first to sixth week, $p<0.05$), but values were not significantly different between the three last weeks ($p>0.05$).

The relative amount of ET-1 mRNA in the lung of layers from the first to the sixth week was 0.669 ± 0.022, 0.684 ± 0.025, 0.691 ± 0.024, 0.771 ± 0.021, 0.772 ± 0.039 and 0.840 ± 0.017, respectively. The overall trend of ET-1 mRNA in the lung of layers was increasing, but significant increases started from the fourth week and continued until the end of the period (the sixth week); and, at the sixth week was significantly higher than the fourth and fifth week of the rearing period.

ET-1 mRNA level in the lungs of broilers was significantly higher than the corresponding lungs in layers during the last three weeks of the experiment ($p<0.05$), but lung expression of ET-1 did not show significant differences among groups in the first three weeks.

ETA (Lung Expression): We found that the ETA expression in broilers' lungs was constant for three weeks after birth, but our data showed a progressive and significant concomitant increase from the fourth to sixth week.

ETA expression in the lungs of layer chickens (Figure 3) was relatively constant in the first four weeks (0.742 ± 0.012, 0.733 ± 0.017, 0.724 ± 0.030 and 0.762 ± 0.023 respectively), while in the last two weeks of the experiment it increased significantly (0.822 ± 0.032and 0.857 ± 0.018 respectively, $p>0.05$).

ETA expression in the lungs of layers and broilers

Table 1. Primer pairs sequence used for reverse-transcription PCR (RT-PCR) and real-time PCR analysis of endothelin 1 (ET-1) in lung samples reported by Gomez et. al., 2007.

Target	Forward and reverse primers (5'-3')	Length (pb)	GenInfo Identifier (gi)	Sequence
ET-1	GGA CGA GGA GTG CGT GTA TT GTG GCC TTT TGG AGA TTC TG	141	50733669	ggacgaggag tgcgtgtatt tctgccacct ggatatcatc tggatcaaca cccccgagaa gactgttccc tatggtcttg gaggcccttc tcggtccaga agatcactga aggacataat gccagagatg cttgctggag c
ETA	GCT CCA GCA AGC ATC TCT G GAT TCC GAT TCC CTG AAC AC	160	45383867	gtggcctttt ggagattctg aatttgggca gtttctttgc aaattccttc cctttataca gaaggcatca gtgggaatca cagtccttaa tctctgtgca cttagtgtgg acaggtatag agcagttgcc tcctggagcc gtgttcaggg aatcggaatc

had the same pattern in the first three weeks (Figure 4), but ETA expression in broilers' lungs during the second three weeks of the experiment was significantly higher than layers.

Serum Endothelin: Significantly, the lowest serum ET-1 was in the first week, rather than the third and sixth week of the experiment when comparing both groups (layers: 1.476 ± 0.029, 1.747 ± 0.092 and1.968 ± 0.064 pg/mL; broilers: 1.77 ± 0.131, 2.352 ± 0.210 and 3.138 ± 0.124 pg/mL respectively). Broilers serum ET-1 was significantly higher than layers in each point of the experiment. The higher the age, the higher the serum ET-1 concentration, in both groups.

Statistics: Results are expressed as mean ± S.D. The comparison between the two groups was done by t test. One-way ANOVA, followed by the Tukey test for significance, was carried out among three or more groups. Differences were considered significant at $p<0.05$.

Discussion

Researchers have indicated that the primary cause of ascites in broiler chickens is pulmonary hypertension (PH) which will develop into pulmonary hypertension syndrome (PHS, ascites). The surveys have shown that PHS accounts for over 25% of annual broiler losses in the United States at an annual cost to the industry of about US $1 billion. A global incidence was reported of 4% to 5% PHS. The incidence of PHS is usually higher in broiler males than in broiler females and Leghorn males (Zhou et al., 2008).

Two of the major factors produced by the endothelium that play important roles in pulmonary arterial vasoactivity are Nitric Oxide (NO) and ET-1. Alterations in the activity of these factors may be partly responsible for maintaining an abnormally elevated pulmonary arterial pressure by increasing the constriction ability and reducing the relaxation capacity of the pulmonary vasculature (Martinez-Lemus et al., 2003).

Numerous studies have shown the important role of the endothelium in modulating vascular reactivity in systemic and pulmonary circulations (Martinez-Lemus et al., 2003).

Evidence in mammals indicates that dramatic changes in the function of the pulmonary vascular endothelium occur in pulmonary hypertension and during maturation. Unfortunately, little is known about these changes in chicken (Martinez-Lemus et al., 2003). Although vascular endothelial cells are the major source of endothelin, it is also produced by a wide variety of cell types including renal tubular endothelium, glomerular mesangium, cardiac myocytes, glia, the pituitary, macrophages, and mast cells (Jain et al., 2002).

Although ET-1 is believed to play a minor role in the changes of pulmonary circulation during the transition between placental and pulmonary respiration, ET-1 plasma concentrations and receptor characteristics exhibit significant changes at birth. Plasma concentrations of ET-1 sharply increase at birth and decline during early life. Production and release of ET-1 has been primarily associated with cellular stretch and ischemia.

With the onset of pulmonary respiration, blood flow through the lungs is increased, endothelial cells are stretched, and blood oxygen tension increases. These and other changes in hemodynamic and vascular mechanics are believed to be responsible for the maturational changes observed in vascular

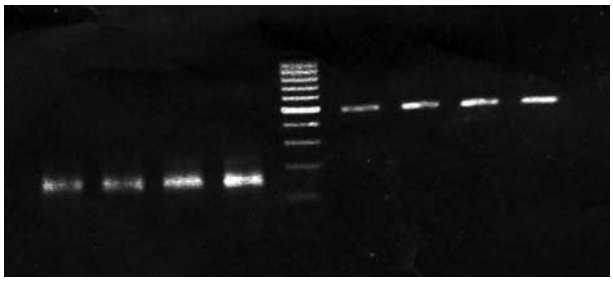

Figure1. Detection of ETA and ETB receptor-coding genes in the lungs. Bands of the expected length for ETA (141 bp) and ETB receptors (161 bp) were detected from agarose gel electrophoresis of PCR-amplified products of cDNA.

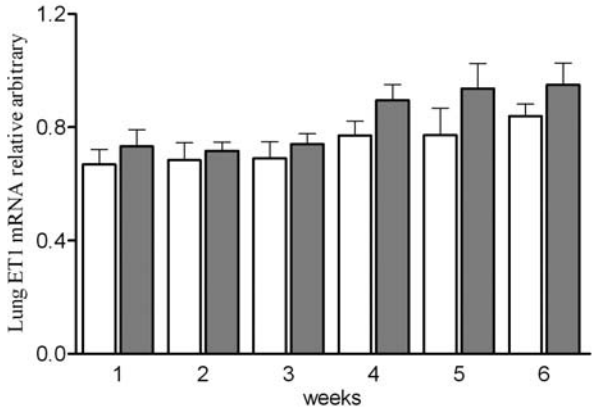

Figure 2. Relative amount of endothelin mRNA in the lung of broiler and leghorn chickens (ET-1/ß-actin). Endothelin mRNA in lung was determined by competitive quantitative RT-PCR. ☐ Layer ■ Broiler

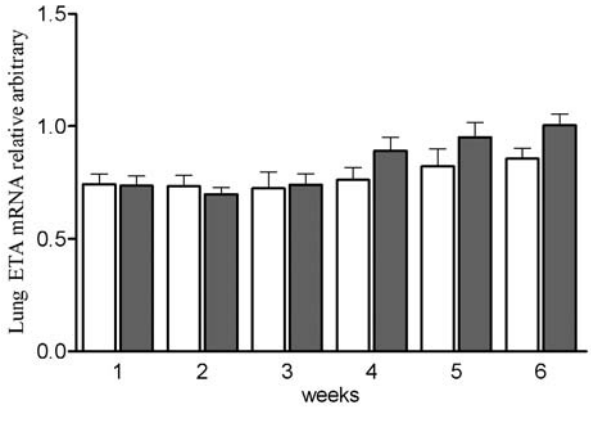

Figure 3. Relative amounts of ETA receptor mRNA in the lungs of leghorn and broiler chickens (ETA/ß-actin). Endothelin mRNA in lung was determined by competitive quantitative RT-PCR. ☐ Leghorn ■ Broiler

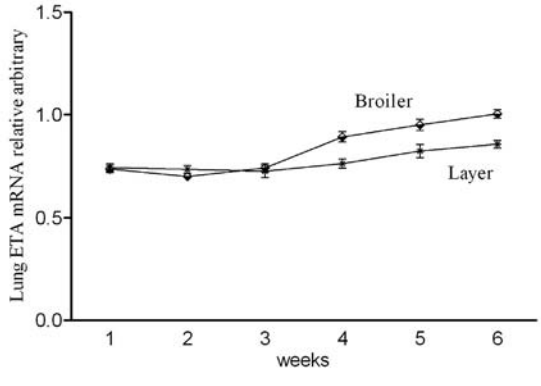

Figure 4. Pattern of ETA receptor expression in the lungs of chickens (Broiler and Leghorn). Endothelin mRNA in lung was determined by competitive quantitative RT-PCR.

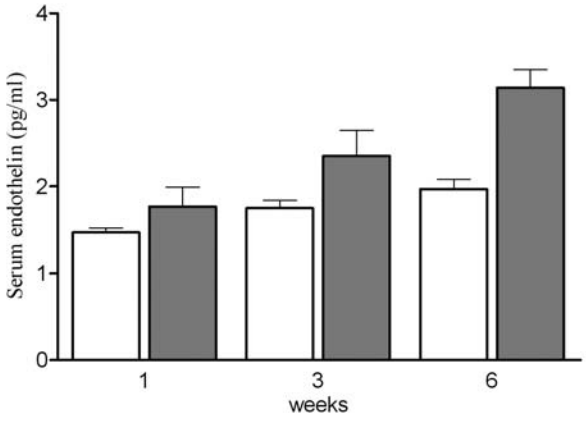

Figure 5. Serum ET-1 in leghorn and broilers (rearing period 42 days, interval 7 days). ☐ Layer ■ Broiler

endothelium reactivity at birth and during early life (Martinez-Lemus et al., 2003). It has been recognized that the vascular endothelium plays a crucial part in local regulation of the function of smooth-muscle cells (Giaid et al., 1993). Our findings showed that serum endothelin concentration increased gradually from the first week to the last week of the chickens' life, and serum endothelin concentration in the broilers is significantly higher than that of the layers.

Endothelins appear to act mainly as local paracrine/autocrine peptides, but circulating levels of endothelins also have great biological significance, especially in the pathological states of increased serum concentration. Jain et al., (2002) and Giadi et al, (1993) have demonstrated low levels of the expression of ET-1 in the normal adult as compared

with fetal lung tissue. Indeed, the normal lung may function to clear ET-1 from the circulation (de Nucci et al., 1988), a concept supported by studies examining the gradient of plasma levels across the lung (Stewart et al, 1991). However, Stewart has shown that in patients with pulmonary hypertension (particularly primary pulmonary hypertension) there may be a net production and release of ET-1 into the pulmonary circulation (Giaid et al., (1993); Stewart et al, (1991). In normal humans, approximately 50% of circulating ET-1 is extracted by the pulmonary circulation within a single transit time. This clearance is exclusively mediated by the endothelial ETB receptor (Sauvageau et al., 2006).

It has been observed that, based on the status of the vasomotor tone, the endothelin isopeptides can cause either pulmonary vasodilatation or vasoconstriction. Under basal conditions all isopeptides of endothelins contract pulmonary vascular rings and increase pulmonary vascular resistance. However, when vascular tone is already high these peptides induce a dose- related pulmonary vasodilatation. The constrictorresponse is mediated by the ETA receptor, whereas the dilator response is ETB mediated.ET-1 which also stimulates DNA synthesis and proliferation of pulmonary artery smooth muscle cells (Jain et al., 2002).

Our results show that the expression of Endothelin in the lung of layers and broilers was constant during the first three weeks. The overall trend of ET-1 was increasing, but a significant increase was noted from the fourth week, and gradually increased until the end of the commercial life of the chicken (day 42). The ET-1 expression in broilers' lungs was significantly higher than that of the lungs of layers during the last three weeks of life. These findings are good evidence for explaining the higher incidence of PH in the fast growing broilers.

Both ET receptors exert their effects through guanine nucleotide-binding proteins (G-proteins) leading to the activation of phospholipase C, protein Kinase C (PKC) and other second messenger systems (Brehm et al., 2002). Hirai et al., (2004) showed that age, creatinine and smoking were significantly correlated to plasma ET in humans. Epinephrine, thrombin, angiotensin II (Ang II), vasopressin, cytokines, insulin and growth factors (e.g.: TGF-h1), and hypoxia and leptin all stimulate ET-1 generation. Leptin-induced stimulation of ET-1 production (Schiffrin, 2005) although varied in different ages and stress situations, can directly or indirectly change the sensitivity of the animals to the vasoconstrictory effect of endothelin and PH.

The effects of the ET peptides are mediated through two known distinct specific endothelin receptors. In mammals, based on their binding affinities for the native and synthetic ligands, they have been classified as type A and type B receptors (ETA and ETB). The ETA receptor has a high affinity for ET-1 and ET-2, and a low affinity for ET-3; whereas, the ETB receptor does not discriminate between the three endothelin isoforms (Kemp et al., 1998).

In mammalian species, both receptors have been cloned and characterized. These receptors belong to the G-protein coupled receptor family and activate the phospholipase C pathway, leading to an inositol phosphate accumulation and subsequent Ca2+ release. In non-mammalian classes of vertebrates, an ETA receptor and another atypical receptor, called ETC, have been cloned in Xenopus, whereas in birds two type B related sequences have been identified and published so far (Kemp et al., 1998).

ETB receptors are also localized on endothelial cells and act through the production of NO and prostacyclin to exert a vasodilator effect. It has been reported that in coronary arteries there are few endothelial vasodilator ETB receptors. As a result, ET-1 acts on coronary vessels mainly as a vasoconstrictor. In other vascular beds, however, ET-1 may function as a vasodilator under certain physiological conditions (Schiffrin, 2005).

Our finding shows the expression pattern of ETA is similar in physiologically healthy leghorns and broilers. Expression of ETA in the broilers' lungs was constant for three weeks after birth, but a progressive and significant concomitant increase was seen from the fourth to sixth week of life. ETA expression in broilers' lungs during the second three weeks of the experiment was significantly higher than layers.

It has been reported that ETA mRNA levels in the lungs of hypoxic rats, and in cells from the pulmonary artery of PH sheep, increased. But Gomez et al., (2007) reported a decrease in ETA mRNA level in the lungs of PHB chickens.

We did not evaluate the affinity of the receptors

during the growth of chickens. Noguchi et al found that the binding density of ET-1 for both receptor types ETA and ETB, located primarily in vascular smooth muscle and endothelial cells, respectively, rapidly reduced after birth (Martinez-Lemus et al., 2003).

In summary, for the first time it is shown that there are differences in the expression of ET-1 and ETA expression in the lungs of broilers and layers. Expression of both genes increased during the rearing period. Serum endothelial in broilers and layers increased during their lives and a higher level of serum endothelin and expression of ET-1 and ETA in broiler lungs was seen in this study, which may explain the higher sensitivity of broilers to the vasoconstrictor activity of endothelin and the higher sensitivity of these animals to pulmonary hypertention.

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تغییرات تکاملی بیان ژن اندوتلین ۱ و رسپتور نوع آ در ریه جوجه‌های گوشتی و تخمگذار

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چکیده

زمینه مطالعه: شناخت مکانیسم‌های کنترل اندوتلین در ریه جوجه‌های گوشتی و تخمگذار پیچیده بوده و دانش اندکی در مورد آن وجود دارد. **هدف:** هدف از این تحقیق بررسی تغییرات سرمی اندوتلین ، بیان ژن اندوتلین و بیان گیرنده اندوتلین نوع آ (ETA) در ریه جوجه‌های گوشتی و تخمگذار در طول دوره پرورش (۶ هفته) می باشد. **روش کار:** در پژوهش حاضر میزان mRNA اندوتلین-۱ و بیان mRNA گیرنده اندوتلین- آ در ریه جوجه‌های گوشتی و تخمگذار در طول مدت پرورش (۶ هفته - با فواصل هفت روزه) تعیین شد. همچنین غلظت سرمی اندوتلین در این جوجه‌ها در این دوره زمانی اندازه گیری شد. **نتایج:** نتایج حاصله نشان داد که بیان اندوتلین در سه هفته اول زندگی در جوجه‌های گوشتی و تخمگذار یکسان بود البته الگوی کلی تغییرات بیان اندوتلین به صورت افزایشی بود . از هفته چهارم افزایش معنی‌داری در بیان اندوتلین-۱ مشاهده شد که این روند افزایشی تا پایان دوره پرورش جوجه‌ها حفظ شد. بیان ژن گیرنده اندوتلین- A در ریه جوجه‌های گوشتی در سه هفته آخر دوره پرورش به صورت معنی داری از جوجه‌های تخمگذار بیشتر بود. الگوی تغییرات اندوتلین سرم در دوره پرورش افزایشی بود اما در تمام طول دوره غلظت سرمی آن در جوجه‌های گوشتی بالاتر بود. **نتیجه گیری نهایی:** بالاتر بودن غلظت سرمی اندوتلین و بالاتر بودن بیان ژن اندوتلیت-۱ و گیرنده اندوتلین A می تواند توضیح هنده حساسیت بالای جوجه‌های گوشتی به فعالیت وازوکانستراکسیون اندوتلین و حساس تر بودن این جوجه ها به افزایش فشارخون ریوی باشد.

واژه‌های کلیدی: آندوتلین، هیپرتانسیون ریوی، بیان ژن، گیرنده اندوتلین.

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