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Evaluation of expression of endothelin A receptor gene in the follicular granulosa cells of broiler hens as related to variation of plasma leptin*

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This study was designed to investigate levels of ET_AR gene expression in the granulosa layer of broiler hens with different levels of plasma leptin and lipids (cholesterol and triacyglycerol). To induce different plasma leptin and lipid levels, the hens were fed high (20 and 40% more than recommended) and low (20% less than recommended) feed rations for 30 days. Variations of plasma leptin and lipids followed those found in the levels of feed intake and body weight in individual groups while the relative amount of ET_AR mRNA increased in all groups. The effect, however, was significant ($P<0.05$) only for T+20% group. It is concluded that ET_AR gene expression in follicular granulosa cells could be influenced by leptin in the broiler hens.

KEY WORDS: broiler hen / endothelin / follicular granulosa

Broiler hens are prone to several metabolic disorders and reproductive anomalies. In females, the capacity for rapid early growth coupled with free access to feed leads

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to enhanced adult fatness [Havenstein *et al.* 2003] and poor reproductive performance [Heck *et al.* 2004]. These undesirable outcomes appear to arise from increase in feed intake that occurred concomitantly with genetic selection for rapid early growth [Barbato 1994, Renema and Robinson 2004]. Hens fed *ad libitum* exhibit a dramatic drop in egg production at an early age and consequently produce fewer eggs overall [Yu *et al.* 1992].

Endothelin-1 (ET-1) is the predominant isoform of a group of three 21 amino-acid peptides including ET-2 and ET-3 which show different profiles of activity in various tissues. Both ET-1 and ET-3 are vasoactive peptides, although ET-1 is more than 100-fold potent than ET-3 as a vasoconstrictor. There are two subtypes of ET receptors: ET_A (ET_AR) and ET_B (ET_BR) – Burton and Lusher [1999]. ET-1 has the highest affinity to ET_AR, followed by ET-2 and ET-3, with all ETs exhibiting equal affinity to ET_BR. There is an ongoing debate on the exact roles of ET_A and ET_B receptors, but important functions include eliciting vasoconstriction and vasodilatation (*via* release of NO), ET clearance, salt balance, cell proliferation and extracellular matrix production. A general feature of ET receptors is that they are abnormally expressed in various diseases associated with vasoconstriction, vasospasm and vascular as well as ventricular hypertrophy. The ETs play significant roles in controlling vascular tone by acting on vascular smooth muscle cells and it has been suggested that ETs are involved in the pathophysiology of various cardiovascular diseases in mammals and birds [Abraham and Dashwood 2008, Hassanpour *et al*. 2010ab]. Some authors have observed the direct ET-1 action on gonadal cells of several mammalian species. For example, ET-1 stimulated steroidogenesis in perfused ovarian follicles from immature rats treated with eCG [Meidan and Levy 2007]. Gonadotropin-stimulated steroidogenesis of cultured porcine and rat granulosa cells was inhibited by ET-1. Endothelin-1 has been reported to have several direct effects on bovine follicular and luteal cells [Meidan and Levy 2007]. On the basis of these observations, the hypothesis of a gonadal role for ET-1 has been raised.

Recent studies have suggested that leptin, a 167-amino acid peptide hormone produced by white adipose tissue, is related to the pathogenesis of obesity-related hypertension [Chao *et al.* 2007] Support has been provided for the role of leptin and the ET system in the pathogenesis of obesity-associated hypertension [Juan *et al.* 2008]. In the present study, we tested the hypothesis that different intakes of feed as well as varying plasma leptin levels alter gene expression of the endothelin type A receptor (ET_AR) in the follicular granulosa of broiler hens.

Material and methods

Birds, management and sampling

Used were 108 commercially reared Arbor Acres Plus Fast Feathering broiler hens aged 30 weeks. Hens were maintained according to the Arbor Acres Plus nutritional recommendations and fed the soybean- and corn-based mesh that provided 11.5 MJ

metabolizable energy (ME) and 155g crude protein (CP) per kg. Diet composition was calculated from published values for feed ingredients [National Research Council 1994]. Feed was offered between 0700 to 0730 h a.m. within a 15L: 9D photoperiod in which lights were turned on at 0600 h. Hens had free access to water. Feed intake were recorded daily. Hens were allocated to four levels (groups) of feeding for 30 days: control (163 g mesh /day) per bird, T-20% (20% less than control, i.e. 130.4 g/day), T+20% (20% more than control, i.e. 195.6 g/day) and T+40% (40% more than control, i.e. 228.2 g/day). Each group included twenty seven birds with three replicates.

One day before the hens were killed, blood samples were withdrawn into anticoagulant tubes from each bird and centrifuged at 500 g to collect blood plasma. Plasma samples were stored at - 20°C until assayed for leptin.

At the end of 30 days-period, 24 birds from each group were randomly selected, weighted, anesthesized and killed. Abdominal fat pad was measured. Ovarian follicles were separated from the ovary and measured in order to identify the largest one (F_1) in each ovary based on hierarchical follicles weight. The granulosa layer of F_1 follicle was separated [Gilbert *et al.* 1977], immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

RNA samples and semiquantitative reverse-transcription PCR

Single-step, acid guanidinium thiocyanate/phenol/chloroform extraction was used for total RNA extraction of granulosa cells [Chomczynski and Sacchi 2006]. Amount and quality of RNA were determined spectrophotometrically. Only RNA of sufficient purity, having an absorbance ratio (A260/280) greater than 1.9, was considered for synthesis of cDNA. It was analysed electrophoretically on a 1.5% agarose gel, stained with 0.5 mg/ml ethidium bromide.The extracted RNA was reverse-transcribed to cDNA in a 20 µl volume containing 1 µg of extracted RNA, 200 ng random hexamer and 0.5 mM dNTP. The 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 $MgCl_2$), 10 mM DTT and 200 u M-MLV reverse transcriptase (FERMENTASE, Germany) were added. The mixture was incubated for 10 min at 25°C, followed by 50 min at 37°C. The prepared cDNAs were heated at 75°C for 15 min to denature the MMLV reverse transcriptase and then stored at -20°C.

The following primer sequences for PCR reactions were included: ET_AR primers (accession number NM204119), sense: 5′-GTGGCCTTTTGGAGATTCTG-3′, antisense: 5′-GATTCCGATTCCCTGAACAC-3′ and β-actin primers (accession number: L08165), sense: 5′-ACTGGATTTCGAGCAGGAGAT-3′, antisense: 5′-TTAGAAGCATTTGCGGTGGACAA-3′ (used as a housekeeping gene) – Hassanpour *et al.* [2010b]. Normalization of the samples was accomplished using RT-PCR for the housekeeping gene β-actin to control the efficacy of the RNA extraction, integrity and amount of ET_AR mRNA present in the samples. PCR reaction conditions were optimized for each of the primer pairs to obtain a linear relationship between

input RNA and final PCR product. The PCR was performed in a total volume of 25 µl, containing 5 mM Tris-HCl, 10 mM NaCl, 0.01 mM EDTA, 1 mM $MgCl_2$, 0.1 mM of each dNTP, 0.1 mM of each primer, 2 µl cDNA and 2.5 u Taq polymerase (FERMENTASE, Germany).

PCR amplification of the generated cDNA was carried out in 25 µl of 1× PCR buffer in the presence of 1 u *Taq*-DNA polymerase (PROMEGA, Germany). The amplification profile for ET_AR and β-actin genes was as follows: denaturation at 96°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The final step was extension at 72°C for 10 min. Forty (ET_AR) and twenty (β-actin) PCR cycles were chosen for analysis of these genes in the individual groups. An aliquot of each reaction mixture was subjected to electrophoresis in 2% agarose gel and stained with 0.5 µg/ml ethidium bromide. Density of bands was determined using Photo-Capt V.99 Image software, and relative densities were expressed as ET_AR/β-actin density.

Plasma cholesterol, triacylglycerol (TAG) and leptin determination

Total cholesterol and TAG plasma concentrations were calculated by "Cholestrol, Enzymatic Photometric, Parsazmun, Tehran, Iran" and "Triglycerides, Colorimetric Enzymatic, Parsazmun, Tehran, Iran." kits, respectively, according to the manufacturer's recommendations. Plasma level of leptin was determined by a multi-species leptin RIA (DRG Research Inc, Germany) according to the recommendations given by the manufacturer.

Statistical

All results are presented as means ± standard errrors (SEM). Comparisons were made between control and experimental groups using Independent-Sample t-test (SPSS 14.0 software, SPSS Inc., New York, USA)., with $P < 0.05$ accepted as significant.

Results and discussion

Lipid-index parameters of hens are shown in Table 1. Compared to controls, intensive and very intensive feeding significantly ($P<0.05$) increased body weight in T+20% and T+40% hens, respectively. Restricted feeding applied in T-20% group led to significantly lower weight of the body and of abdominal fat in hens as compared to controls. The two parameters, however, occurred significantly higher in the other two groups as compared to the control.

After 30 days of experimental feeding, plasma levels of TAG and leptin increased in T+20% and T+40% hens compared to controls ($P<0.05$). The levels of these parameters were lower in T-20% group than in the control, but the differences were not found significant. Cholesterol level of plasma was also significantly higher in T+40% group than in the control hens while lower ($P<0.05$) in T-20% group. The change of plasma cholesterol level that took place in T+20% group was not found significant.

Table 1. Lipid-index parameters in control and experimental broiler hens on day 30

Parameter	Feeding							
	Control group		T-20% group		T+20% group		T+40% group	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
Body weight (g)	3514	47.5	3107*	50.0	3692*	46.1	4071*	51.0
Abdominal fat weight (g)	90.5	7.0	55.2*	7.1	109.4	6.0	129.0*	7.2
Cholesterol (mg/dl)	142.3	5.2	116.9*	4.4	143.3	5.4	173.1*	3.9
TAG (mg/dl)	573.8	21.3	464.2	19.5	885.7*	27.8	1023.5*	30.1
Leptin (ng/dl	0.63	0.05	0.51	0.06	0.79*	0.07	1.20*	0.09
Number of birds	24		24		24		24	

T(-20%) – 130.4 g feed consumed per hen daily, T(+20%) – 195.6 g feed consumed per hen daily, T(+40%) – 228.2 g feed consumed per hen daily.
SEM – standard error of mean; *different from control at P≤0.05.

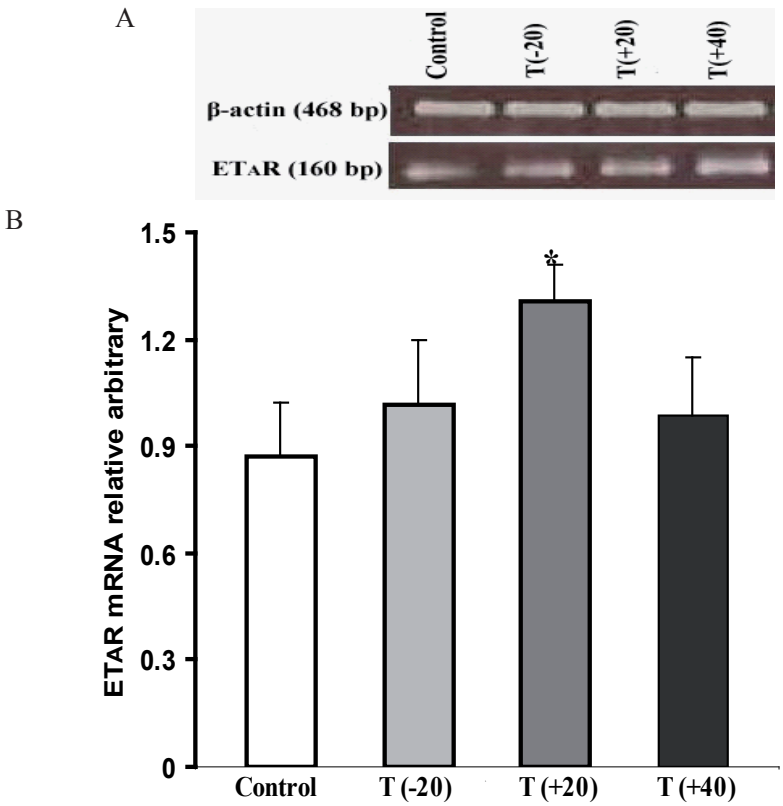

Fig.1. A. Gel electrophoresis of semiquantitative RT-PCR for determination of β-actin and ETAR mRNA levels. B. Comparison of relative density of ET_AR/β-actin PCR products in the follicular granulosa between control and treated groups of chickens. T(-20%), treated group with food intake of 130.4 g/day; T(+20%), treated group with food intake of 195.6 g/day; T(+40%), treated group with food intake of 228.2 g/day. Values are means ± SE. *P<0.05 from corresponding control.

Expression of ET_AR gene was studied using semi-quantitative RT-PCR in the granulosa layer of F_1 follicles in hens after 30 days of experimental feeding. Reverse transcription-PCR results are shown in Figure 1. The expression of β-actin as housekeeping gene and ET_AR gene were detected in all samples of granulosa layer of F_1 follicles in control and T-20%, T+20% and T+40% hens.

The relative amount of ET_AR mRNA expressed in the granulosa layer of F_1 follicles was, in all experimental groups, higher than in controls. However, the increase was significant ($P<0.05$) in T+20% group only (Fig.1).

Variations in plasma leptin and plasma lipids followed those occurring in the levels of feed intake and body weight.

It has been confirmed that obesity-associated disorders commonly occurred in broiler hens fed on high level of their defined nutritive requirements [Hocking *et al.* 1989, Yu *et al.* 1992]. In addition, nutritionally satisfied hens with abnormal ovaries showed significantly higher plasma leptin concentration [Chen *et al.* 2006]. Several studies conducted on theca and granulosa cells have shown that leptin may have direct negative effect on ovarian steroidogenesis in various mammalian species [Spicer 2003]. Leptin inhibits insulin-induced progesterone and 17β-estradiol production by isolated bovine granulosa cells and impairs the hormonally-stimulated *in vitro* release of 17β-estradiol by rat granulosa cells [Cassy *et al.* 2004]. In chickens, leptin attenuates the negative effects of fasting on ovarian function. Injections of leptin during fasting delay cessation of egg laying, attenuate regression of yellow hierarchical follicles, alter ovarian steroidogenesis and limit apoptosis [Cassy *et al.* 2004].

It has been confirmed that ET-1 stimulates leptin production and secretion in murine adipocytes [Xiong *et al.* 2001] and that circulating ET-1 levels are significantly higher in obese than in the control patients [Ferri *et al.* 1997]. The elevated ET-1 levels enhance leptin production and the elevated levels of cellular or plasma leptin stimulate vascular ET-1 and ET_AR expression, creating a vicious circle between the two hormones [Juan *et al.* 2008].

Several studies indicated that ETs play crucial role in ovarian physiology and participate in the reproductive processes. For example, ET-1 inhibits premature luteinization of granulosa cells and promotes *corpus luteum* regression in mammals. The presence of the ET receptors that mediate the diverse effects of ETs has been reported in different ovarian compartments [Meidan and Levy 2007].

In this study we measured the relative amount of ET_AR mRNA in broiler hens with different levels of feed intake, body weight and leptin and lipids of plasma. Plasma leptin and lipid levels were altered by increasing or decreasing of feed intake and body weight while the relative amount of ET_AR mRNA increased significantly only in one group of high feed intake (T+20%). Earlier Juan *et al.* [2008] reported that leptin increased ET_AR levels in the vascular smooth muscle cells. Chao *et al.* [2007] reported that leptin induced endothelin-1 expression in the vascular system and suggested that it may cause partial hypertension *via* endothelin-1 release. It is possible that high production of endothelin-1 due to high level of leptin in the obese hens

(T+40% group) downregulate**s** the expression of ET_AR gene. Ahmed *et al.* [2000] have also shown that over-expression of endothelin-1 in the cancer downregulates ET_AR (compensatory role). Reduction of ET_AR mRNA in the group of low feed intake is unclear, species-specific differences in the interaction of leptin and ET system in reproduction are not surprising [Meidan and Levy 2007]. However, the effects of leptin on the reproduction in hens could in part be due to its influence on ET_AR gene expression in follicular granulose layer.

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