

*Short communication*

## Effect of long-term oral administration of extra thyroxine on oviductal expression of carbonic anhydrase and avidin-related protein-2 genes in broiler breeder hens

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**Abstract** Avian sperm are stored in the sperm storage tubules (SSTs) of the hen oviduct for a prolonged period. The impact of avidin-related protein-2 (AVRP<sub>2</sub>) and carbonic anhydrase II (CA II) in sperm viability in the SSTs has been suggested. The aim of the present study was to investigate the effect of oral administration of a high dose of thyroxine on the oviductal expression of AVRP<sub>2</sub> and CA II genes in broiler breeder hens. The birds (n=70), housed in separate cages, were randomly allotted to two treatment groups to either zero (CON) or 0.30 mg thyroxine per day (T<sub>4</sub> group) for 12 weeks. Feed and water were supplied according to the Cobb 500 standards (metabolizable energy: 2700 kcal/kg and crude protein: 14%). Blood samples were prepared seven times for determination of plasma triiodothyronine (T<sub>3</sub>) and T<sub>4</sub> concentration. At the end of the treatment period, 20 hens were randomly selected and killed to determine the expression of AVRP<sub>2</sub> and CA II in the SSTs using the real-time PCR procedures. Expressions of AVRP<sub>2</sub> and CA II genes were influenced by T<sub>4</sub> treatment where an increased expression of CA II was recorded for T<sub>4</sub>-exposed hens ( $P < 0.05$ ). However, expression of AVRP<sub>2</sub> was not significantly different between the treatment groups ( $P > 0.05$ ).

**Keywords:** avidin-related protein-2, breeder hen, carbonic anhydrase, oviduct, thyroxine

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## Introduction

The avian oviduct consists of 5 sections: the infundibulum, magnum, isthmus, uterus, and vagina. Among these sections, the utero-vaginal junction (UVJ) is an important area, because of having more than 3000 sperm storage tubules (SST; Bakst et al., 2010). The period of sperm storage among various birds is different [e.g. 6 d in pigeons, over 2 months in some seabirds (Hemmings et al., 2015) or about 3 months in the domestic turkey (Christensen and Bagley, 1989)]. On the other hand, there is a positive correlation between the duration of fertility and the number of the SSTs (Pierson et al., 1988).

Therefore, modification of the SST microenvironment may be instrumental in prolonged storage of sperm within the sperm SST. In birds, in addition to a complex systemic effect associated with the maintenance of an adequate basal metabolic rate (Sechman, 2013) and their impact on the processes of growth and development (McNabb, 2007), thyroid hormones are necessary

for the normal functioning of the reproductive system (Sechman et al., 2000). Thyroid inhibition in adult hens has led to a decline or even a cessation of egg production in laying hens (Akhlaghi et al., 2012).

Avidin is a member of a gene family that encoding 7 avidin-related proteins (AVRP<sub>1</sub> to AVRP<sub>7</sub>) with a 91 to 96% structural similarity (Daryabari et al., 2015). Avidin-related proteins bind to biotin with high affinity (Laitinen et al., 2002). Avidin is a four-component protein with 128 amino acids in each section that forms strong non-covalent bond with a biotin molecule (Kuramitz et al., 2003). In the oviduct of laying hens, the synthesis of avidin is influenced by the progesterone (P<sub>4</sub>) and protects the growing embryo against microbial infections (Tuohimaa et al., 2003).

Carbonic anhydrase (CA) catalyzes the reaction  $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-$ , consists of seven isozymes (CA I – CA VII), and carbonic anhydrase may be soluble (CA I, II, III and V), membrane bound (CA IV) or secr-

eted (CA VI), while many cells may contain more than one isoenzyme (Dobyan and Bulger, 1982). Kuryl (1981) suggested the presence of CA activity infundibular mucosa of birds, and Holm et al. (1996) localized CA histochemically in the infundibulum and the UVJ and provided the first evidence for the role of CA in rapid pH changes in these regions.

The objective of the present study was to determine the effects of oral administration of a high dose of T<sub>4</sub> on the oviductal expression of AVRP<sub>2</sub> and CA II genes in broiler breeder hens.

## Materials and methods

### *Birds and experimental treatments*

Cobb 500 broiler breeder hens (n=70) were randomly selected at 47 wk of age and assigned to two treatment groups of either zero (CON) or 0.30 mg T<sub>4</sub> per day (T<sub>4</sub> group) for 12 consecutive weeks in a completely randomized design. Each treatment consisted of 5 replicates of 7 birds that were kept in separate cages. A total of 20 Cobb 500 broiler breeder roosters was also used for artificial insemination. The roosters were habituated to abdominal massage for semen collection for 10 days. Semen samples were prepared, pooled, and diluted in homogenized-pasteurized low-fat milk (Akhlaghi et al., 2013). Each hen was inseminated with 200 × 10<sup>6</sup> sperm to eliminate the effect of sperm number on gene expression (Foye-Jackson et al., 2011). The hens were inseminated on two successive days for three separate phases. The birds were fed on a corn-soybean meal-based diet (Table 1), and a 16L:8D artificial lighting schedule was provided throughout the trial.

### *Blood samples*

During the treatment period, blood samples were prepared from all hens once every two weeks (7 times in total), centrifuged at 1800 × g for 15 min, and plasma was separated for determination of T<sub>3</sub> and T<sub>4</sub> using commercially available ELISA kits (Pars Azmoon Co., Tehran, Iran).

### *Oviductal gene expression*

*Tissue sampling:* At the end of the treatment period, SST tissue specimens were prepared according to the procedure described by Foye-Jackson et al. (2011). Twenty hens in each group, 2 hens/replicate (10 hens/treatment) were randomly selected and killed by cervical dislocation. The UVJ was dissected and rinsed with phosphate buffered solution (0.15 M at pH 7.4). The UVJ mucosa containing the SSTs was removed, im-

**Table 1.** Ingredients and chemical composition of the experimental diets

Ingredient (DM basis) %	
Corn grain	0.366
Wheat grain	25
Barley grain	0.134
Soybean meal (44%)	0.1576
Oyster shell	0.0706
Vitamin premix <sup>1</sup>	0.001
Mineral premix <sup>2</sup>	0.001
Sodium chloride	0.0018
Sodium bicarbonate	0.0016
DL-methionine	0.00095
Dicalcium phosphate	0.0148
L-threonine	0.00025
L-Lysine	0.0004
Dietary composition	
Metabolizable energy (kcal/kg)	2700
Crude protein (%)	14.00
Ca (%)	2.99
P (%)	0.36

<sup>1</sup>Supplied per kg diet: vitamin A, 14,000 IU; vitamin D<sub>3</sub>, 3000 IU; niacin, 50 mg; vitamin E, 35 mg; calcium pantothenate, 20 mg; vitamin K<sub>3</sub>, 4 mg; riboflavin, 7.0 mg; pyridoxine, 5.7 mg; vitamin B<sub>12</sub>, 25 µg, and biotin, 50 µg.

<sup>2</sup>Supplied per kg diet: Fe (FeSO<sub>4</sub>·H<sub>2</sub>O), 85 mg; Mn (MnSO<sub>4</sub>·H<sub>2</sub>O), 90 mg; Zn (ZnO), 67.3 mg; Cu (CuSO<sub>4</sub>·5H<sub>2</sub>O), 11.1 mg, and Se (Na<sub>2</sub>SeO<sub>3</sub>), 0.19 mg.

mediately transferred into liquid nitrogen and subsequently stored at -80°C for RNA isolation.

*Total RNA extraction and cDNA synthesis:* Total RNA isolation from the SST tissue samples was performed by using RNX-Plus (CinnaGen) and the real-time PCR reactions were carried out by Master SYBR Green I Mix kits according to the manufacturer's instructions. DNase was used for removing genomic DNA contamination.

*Real-Time PCR analysis:* The real-time PCR analysis was used to compare the expression of AVRP<sub>2</sub> and CA II in the oviductal SST tissues. Table 2 shows the gene-specific primers used in the present study. The reference gene, β-actin (Li et al., 2010), was used as endogenous control to normalize the expression of AVRP<sub>2</sub> and CA II genes.

One µM cDNA template was used in a 15 µL RT-PCR reaction mixture containing 1 µM gene specific primers, 7.50 µL QuantiFast SYBR Green Master Mix, and 4.50 µL RNase-free water, under the following conditions: initial denaturation 95°C for 300 s, 50 cycles each of denaturation (95°C, 10 s) as well as annealing and extension (60°C, 30 s). To prepare the standard curve for each gene, pooled cDNA was serially diluted in distilled H<sub>2</sub>O and amplified by the gene-specific primer pairs. The relative expression of the desired genes

**Table 2.** Primer sequences for the real-time PCR amplifications of oviductal target genes

Gene <sup>1</sup>	Primer sequence (5'-3')	Base pair	Reference
AVRP <sub>2</sub>	Forward: ATCATGACCATCGGAGCTGT	155	Foye-Jackson <i>et al.</i> , 2011
	Reverse: CAATGGACAGTGAAGCCAAA		
CA II	Forward: TGACCCTACTGGACTGCTGC	199	Holm and Ridderstrale, 1998
	Reverse: TGACAGTGATGGGCTCCTTC		
β- actin	Forward: TGCGTGACATCAAGGAGAAG	300	Li <i>et al.</i> , 2010
	Reverse: TGCCAGGGTACATTGGTGGTA		

<sup>1</sup>AVRP<sub>2</sub>: Avidin-related protein-2, CA II: Carbonic anhydrase II, β-actin: Beta actin.

was measured for each sample by using the 2<sup>(-ΔΔCt)</sup> method (Livak and Schmittgen, 2001), where the average ΔCt values of the control treatment.

### Statistical analysis

The expression levels of AVRP<sub>2</sub> and CA II mRNA measured by RT-PCR were analyzed, using the GLM procedure (SAS, 2003). Differences between means were determined by the least squares means contrasts.

### Results and discussion

Plasma T<sub>4</sub> level, but not T<sub>3</sub> concentration, was higher in T<sub>4</sub>-treated hens (Table 3), which may be the consequence of quick and almost complete conversion of T<sub>4</sub> to the metabolically inactive reverse-T<sub>3</sub> (Akhlaghi *et al.*, 2012).

Because thyroid disorders often lead to abnormalities in the reproductive system, thyroid hormones have long been implicated to have a role in the avian reproduction (Sechman, 2013). Administration of exogenous T<sub>4</sub> in drinking water for 4 weeks decreased the incidence of ascites in the cold-exposed broilers (Akhlaghi *et al.*, 2012). The UVJ, containing the SST, is an important region affecting the fertility in birds (Bakst *et al.*, 2010). It is sensitive to a changing hormonal environment that provides the oviduct for development of the ovulated egg and promotes the most efficient use of sperm to fertilize as many eggs as possible (Foye-Jackson *et al.*, 2011). Other studies have shown the presence of several UVJ genes that would be needed to optimize the fertilization being affected by E<sub>2</sub> (Das *et al.*, 2006a) and P<sub>4</sub> (Yoshimura *et al.*, 2000) signals to control tissue development and maturation, avidin expression, and response

to transforming growth factor β isoforms (Das *et al.*, 2006b). Since avidin and possibly avidin analogs (Foye-Jackson *et al.*, 2011) and carbonic anhydrases (Holm and Ridderstrale, 1998) are involved in maintaining spermatozoa in the SSTs, and because of the well-established role of thyroid hormones in the normal functioning of the reproductive system (Sechman *et al.*, 2000; Sechman, 2013), we hypothesized that expressions of AVRP<sub>2</sub> and CA II genes might be affected by THs.

In previous studies, the expression of avidin was P<sub>4</sub>-dependent or independent, according to the region from which the tissues were obtained (Elo, 1980). The expression of avidin in the oviduct was dependent on P<sub>4</sub> (Gope *et al.*, 1987; Kunnas *et al.*, 1992) but not in other tissues (Board and Fuller, 1974; Kunnas *et al.*, 1993). In the chick oviduct, injections of E<sub>2</sub> and P<sub>4</sub> led to the production of ovalbumin and avidin, respectively (Chan *et al.*, 1973). Estradiol stimulates ovalbumin gene activity in the oviduct, which parallels the *in vivo* ovalbumin accumulation (Comstock *et al.*, 1972), but the absence of E<sub>2</sub> results in the disappearance of ovalbumin gene activity (Means *et al.*, 1972). Carbonic anhydrase II level is also influenced by the steroid hormones (especially P<sub>4</sub> and E<sub>2</sub>). Carbonic anhydrase is an important index for study of hormone actions on the reproductive system (Miyake and Pincus, 1959). Miyake and Pincus (1959) showed that injection of of E<sub>2</sub> to ovariectomized mature rats led to a reduction in uterine CA activity. On the other hand, Bialy and Pincus (1962) reported that uterine CA level in sexually immature rats is almost comparable to that of mature rats. In rabbits, CA activity was increased by P<sub>4</sub> but decreased by E<sub>2</sub> (Hodgen and Falk, 1971); however, E<sub>2</sub> and P<sub>4</sub> showed opposite effects in the mice (Maren, 1967). Carbonic anhydrase activity

**Table 3.** Effect of long-term oral administration of a high dose of thyroxine on thyroid hormone concentration in broiler breeder hens (LS means ± SE)<sup>1</sup>

Treatment	Control (no thyroxine)	Thyroxine
Triiodothyronine (T <sub>3</sub> )	1.59±0.26	1.64±0.24
Thyroxine (T <sub>4</sub> )	10.24±0.93	27.08±0.89*
T <sub>3</sub> :T <sub>4</sub> ratio	0.152±0.007	0.059±0.007*

<sup>1</sup>Thyroxine was orally administered at 0.30 mg/bird/day for 100 days and the control group received drinking water (n=35 hens/group).

\*Significantly different from control (P≤0.05)

**Table 4.** Oviductal expression of AVRP<sub>2</sub> and CA II in broiler breeder hens orally administered with thyroxine<sup>1</sup>

Gene	Control (no thyroxine)	Thyroxine	P-value
Avidin-related protein-2 (AVRP <sub>2</sub> )	1.01±0.08	1.54±0.42	NS
Carbonic anhydrase II (CA II)	1.14±0.25	4.50±1.23*	0.0287

<sup>1</sup>Thyroxine was orally administered at 0.3 mg/bird/day for 100 days and the control group received drinking water (n=35 hens/group). \*Significantly different from control. NS: Non-significant.

was shown in the uterus of estrogen-treated immature pullets; however, CA activity was not as high as in mature birds (Nys et al., 1986). Estradiol seems to be involved in the regulation of the avian oviductal CA (Holm et al., 2000). Sechman et al. (2009) reported that E<sub>2</sub> secretion was decreased by T<sub>3</sub> in all of ovarian follicles in a dose-dependent manner, whereas P<sub>4</sub> secretion was increased by T<sub>3</sub> in the granulosa layer of the pre-ovulatory F<sub>1</sub> and F<sub>2</sub> follicles. In the present work, expression of CA II, but not CA I, was increased in T<sub>4</sub>-supplemented hens (Table 4). Based on the published data on the effect of steroids on the expression of AVRP<sub>2</sub> and CA II in the oviduct (Kunnas et al., 1992; Miyake and Pincus, 1959), it may be concluded that long-term administration of thyroid hormones on the expression of these gene in the present study could have been affected through changes in the secretion of ovarian steroids, although blood levels of steroid hormones were not measured in this study.

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## تأثیر دراز مدت تجویز خوراکی تیروکسین اضافی بر بیان ژن‌های کربنیک آنهیدراز و پروتین وابسته به آویدین-۲ در اویداکت مرغ‌های مادر گوشتی

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**چکیده** اسپرم پرندگان در لوله‌های انباشت اسپرم (SSTs) اویداکت پرنده، برای مدت طولانی ذخیره می‌شود. تأثیر پروتین وابسته به آویدین-۲ (AVRP<sub>2</sub>) و کربنیک آنهیدراز II (CA II) بر زنده‌مانی اسپرم در SST مرغ‌ها پیشنهاد شده بود. مطالعه حاضر با هدف تعیین تأثیر تجویز خوراکی یک دوز بالای تیروکسین بر بیان اویداکتی ژن‌های AVRP<sub>2</sub> و CA II در مرغ‌های مادر گوشتی انجام شد. پرنده‌ها (n=70) در قفس‌های جداگانه نگهداری شدند. تیمارها به طور تصادفی تیروکسین به مقدار صفر (CON) و ۰/۳۰ (T<sub>4</sub> group) میلی‌گرم به شیوه دهانی هر روز به مدت ۱۲ هفته دریافت کردند. نمونه‌های خون برای هفت بار برای سنجش غلظت‌های T<sub>3</sub> و T<sub>4</sub> پلازما تهیه شدند. در پایان، شمار ۲۰ مرغ به طور تصادفی انتخاب و برای اندازه‌گیری بیان نسبی ژن‌های AVRP<sub>2</sub> و CA II در SST کشته شدند. داده‌های Real-time PCR نشان دادند که بیان نسبی ژن‌های AVRP<sub>2</sub> و CA II در پرنده‌هایی که ۰/۳۰ میلی‌گرم تیروکسین دریافت کرده بودند، افزایش یافتند؛ همچنین بیان نسبی ژن CA II اختلاف معنی داری را نشان داد ( $P < 0.05$ ).