Pakistan J. Zool., vol. 50(1), pp 283-289, 2018. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2018.50.1.283.289

Immunolocalization and Expression of Androgen Receptor in the Oviduct of Laying Hens

Bian Xunguang[1,2], Li Jiancheng[1], Xu Chu[1] and Yang Li[2,3,4,*]

[1]School of Ocean and Biological Engineering, Yancheng Teachers University, Yancheng, Jiangsu Province, People's Republic of China
[2]Jiangsu Key Laboratory for Bioresources of Saline Soils, Yancheng Teachers University, Yancheng 224051, Jiangsu Province, People's Republic of China
[3]Jiangsu Synthetic Innovation Center for Coastal Bio-agriculture, Yancheng Teachers University, Yancheng, Jiangsu Province, People's Republic of China
[4]Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Yancheng Teachers University, Yancheng, Jiangsu Province, People's Republic of China

Article Information
Received 07 August 2017
Revised 20 September 2017
Accepted 27 September 2017
Available online 12 January 2018

Authors' Contribution
BX and YL conceived and designed the study. BX, LJ and XC collected and examined the samples, BX and LJ analyzed the data, BX, LJ, XC and YL wrote the article.

Key words
Hen, Androgen receptor, Oviduct, qPCR, Immunohistochemistry.

ABSTRACT

Androgen plays an important role in animals, and its role is mainly via androgen receptor (AR) to achieve. In this paper, the expression of AR mRNA in various parts of the oviduct was analyzed by qPCR. Oviductal structure was observed by H&E staining. The distribution of AR in the cells was detected by immunohistochemistry. The results showed that the expression of AR mRNA was found in the infundibulum, magnum, isthmus, uterus and vagina of the oviduct. The expression of the AR mRNA in infundibulum was the strongest, and the vaginal expression was weak. The immunohistochemical results was consistent with the molecular expression of AR mRNA, the positive reaction in the infundibulum, isthmus and uterus is more intense, followed by the magnum, the vaginal reaction is weak. Positive responses in the sections were mainly focused on luminal epithelium, glands and vascular epithelial cells. This study revealed the molecular expression of AR and the distribution of intracellular proteins in the oviduct of the hen, which provides an important basis for the regulation of the physiological activities of poultry oviduct and the molecular mechanism of sperm storage.

INTRODUCTION

It is widely accepted that androgens are essential for the normal development and functional maintenance of male reproductive organs. Androgens are known to exert their effects via androgen receptor (AR), which is a member of the nuclear receptor superfamily (Mangelsdorf *et al*., 1995). In contrast to studies on the role of estrogens in male development, there have been relatively few studies examining the functional significance of androgens in females.

Similar to the mammalian, the birds oviduct as the female reproductive tract that has a fundamental role in gamete transport and fertilization. It is widely accepted that functions of the oviduct are believed to be regulated by two ovarian sex steroid hormones, estrogen and progesterone (P4) (Jansen, 1984; Harper, 1994). However, in recent years, many studies have suggested that androgens also play an important role in female reproductive organs (Davis, 1999a, b, 2000). In the human, Davis and Tran (2001) have suggested that androgen insufficiency is associated with impaired sexual function.

The study of androgen receptors in mammalian ovary and oviduct has been reported extensively (Shao *et al*., 2007; Horne *et al*., 2009; Okada *et al*., 2003). However, in the birds, the research of sex hormone receptors in the oviduct is mainly focused on the estrogen receptor alpha and beta (Hansen *et al*., 2003; Das *et al*., 2006; Hrabia *et al*., 2008), and the research on AR in oviduct is rarely reported.

Although AR or high levels of circulating plasma androgens have been documented in females in a wide array of taxa (Iela *et al*., 1986; Yalcinkaya *et al*., 1993; Yoshimura *et al*., 1993), the molecular mechanism of androgen action and the role of AR in the female birds reproductive tract have not yet been demonstrated. In this study, mRNA expression and protein localization of AR were determined in the laying hens oviduct using real-time RT-PCR and immunohistochemistry.

* Corresponding author: bxguang8311@126.com
0030-9923/2018/0001-0283 $ 9.00/0

MATERIAL AND METHODS

Animals and samples

Total of 20 hens were used in this study. Animals were rendered comatose using intraperitoneal administration of sodium pentobarbital (20 mg/kg) and killed by cervical dislocation. The oviducts were removed and segmented five regions in accordance with the anatomical structures.

Animals were treated in accordance with the Care and Use of Wild Animals in The People's Republic of China, all efforts were taken to minimize pain and discomfort to the animal while conducting these experiments. All protocols were approved through the Science and Technology Agency of Jiangsu Province (SYXK (SU) 2010-0005).

Light microscopy

The samples were fixed in neutral buffered formalin, embedded in paraffin, and serial sectioned (at 5 µm). These sections were stained with Harris' haematoxylin and eosin for light microscopic observation following a previous report (Lebelo and Horst, 2017). Slides were assessed using an Olympus microscope (BX53), camera (Olympus DP73) and the AnalySIS image-analyzing system (AnalySIS).

Quantitative real-time reverse transcription polymerase chain reaction

Total RNA was extracted from 50 mg of tissue using TRIzol Reagent (Invitrogen, USA) as described by the manufacturer. RNase-free DNase I was used to remove contaminating genomic DNA (Promega, USA). RNA integrity and DNA contamination were assessed on a 1% formaldehyde gel, and then analyzed for quantity and quality via a NanoDrop spectrophotometer (Thermo Scientific, USA). RNA was reverse transcribed and qPCR was performed as the below, 1.5 µg of RNA in 41.5 µL ddH_20 was reverse transcribed using Promega reagents; 0.5 µL AMV reverse transcriptase, 16 µL 5× RT buffer, 1 µL RNasin, 16 µL $MgCl_2$, 4 µL deoxynucleotides, and 1 µL Oligo (dT) primer (Promega, Madison, WI, USA). Samples were incubated at 42°C for 60 min followed by 95°C for 5 min.

Complementary DNA (cDNA) was diluted in the ratio 1:1 with ddH_20, and the qPCR reactions were run on an ABI 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, USA) with the SYBR Premix Ex Taq Kit (TaKaRa, Japan). Reaction mixtures (20 µL) contained10 µL 2×SYBR Premix, 1 µL forward and reverse primers, 1 µL cDNA, and 7 µL RNase-free H_2O. The PCR procedure was as follows: 95 °C for 10 s, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s. At the end of the reaction, a melting curve was produced by monitoring the fluorescence continuously while slowly heating the sample from 60 to 95 °C. Each independent experiment was conducted in triplicate and the relative expression level of the gene of interest was determined using the $2^{-\Delta\Delta Ct}$ method as previous reported (Livak and Schmittgen, 2001).

The primers of the AR gene used in qPCR were designed using Primer Premier 5.0 software based on the GenBank (No. AB193190.1), the β-actin gene (GenBank No. L08165.1) was used as an internal reference, their sequences are shown in Table I. Negative control (water in place of cDNA) samples were included in each PCR run along with the RT negative and RT H_2O control samples described above.

Table I.- Primers and annealing temperature for real-time PCR.

Genes	Primer sequence (5'-3')	Annealing (°C)	Length (bp)
AR	F:TGTGATGGACTCTGGTGATG	57°C	161 bp
	R:CGGCTGTGGTGGTGAAG		
β-actin	F:TATTGTGATGGACTCTGGT	57°C	165 bp
	R:TCGGCTGTGGTGGTGAAG		

Immunocytochemistry

Paraffin sections (5 µm) were deparaffinized, hydrated and then treated with 3% H_2O_2 in phosphate-buffered saline (pH 7.6) for 30 min. These steps were followed by heating the sections in a microwave oven for antigen retrieval using citrate buffer (pH 5.5) as previously described (Tacha and Chen, 1994). The sections were then incubated overnight at 4°C with AR antibody (see below for details) at a concentration of 1 µg/ml. Sections were then washed in Tris-saline (pH 7.6) and incubated at room temperature for 4 h with peroxidase-labelled goat anti-rabbit-IgG diluted at 1:200. The ABC universal staining kit (Wuhan Boster Biological Technology Co., Ltd., China) was employed for detection. The peroxidase activity was monitored using 0.03% 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Wuhan Boster Biological Technology Co., Ltd., China) in 0.05 M Tris, pH 7.6, and 0.1% H_2O_2 for 5 min. Immunohistochemical controls included omission of the primary antibodies and staining with non-immunized rabbit serum and mouse IgG. No positive staining was observed in these slides (data not shown).

AR was determined using a rabbit polyclonal antibody (PG21, Upstate, Lake Placid, NY, USA), which had been used in detecting AR in the chick embryos by immunohistochemistry (Gould *et al.*, 1999) and had been demonstrated to react with chicken AR in the product manual (PG21, Upstate, Lake Placid, NY, USA).

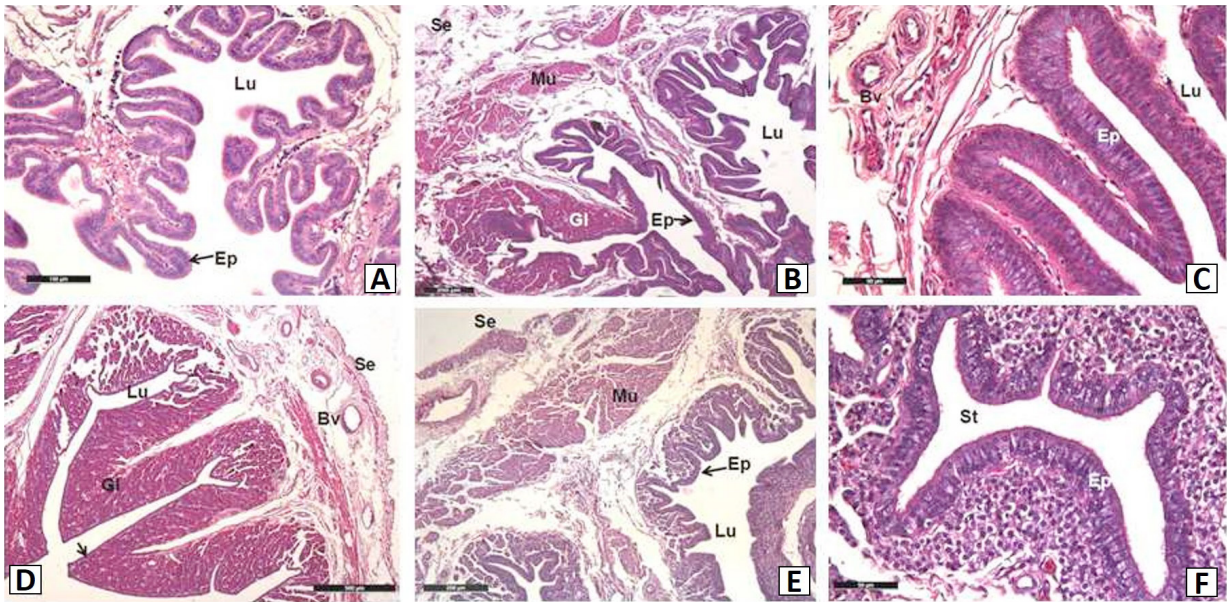

Fig. 1. Light micrographs in different regions of the hens oviduct. A, infundibulum; B, magnum; C, isthmus; D, uterus; E, vagina; F, the SST in the anterior vagina. Ep, epithelium; Gl, gland; Lu, lumen; St, sperm-storage tubul; Mu, muscle; Bv, blood vessel; Se, serous. Bar=100 μm (A), bar=500 μm (D), bar=200 μm (B, E), bar=50 μm (C, F).

Immunohistochemical evaluation and statistical analysis

Sections were examined and photographed using a light-microscope (BX53; Olympus Optical Co., Ltd, Tokyo, Japan) attached to a digital camera (DP73; Olympus). Staining intensity was graded as negative, weak, moderate or strong marked for AR immunohistochemistries. At least ten specimens from each of five ovidutcal regions were examined for all investigations.

The qPCR data are presented as the means ± standard error of the mean, and the statistical analysis was performed using SPSS 17.0 program (SPSS, USA). The data were subjected to a one-way analysis of variance (ANOVA), followed by Duncan's test. The differences were considered to be significant when the P-value was <0.05.

RESULTS

General microstructure of the hens oviduct

The hen has only one functional oviduct, the left one. The oviduct of the hen consisted of five distinct morphological regions: infundibulum, magnum, isthmus, uterus and vagina. The oviductal mucosa forms longitudinal folds running along the axis of the tube. Infundibulum was only a few gland and showed larger blood vessels in the lamina propria (Fig. 1A). The glands in the magnum were extensive and filled most of the lamina propria (Fig. 1B). The epithelium of isthmus consisted of high columnar secretory cells and ciliated cells, the gland in this region is fewer than the magnum (Fig. 1C). The uterus also showed various gland and middle thickness of smooth muscle with the circular layer lying internal to the longitudinal layer (Fig. 1D). The histomorphology of vagina was similar to uterus, but vagina showed less glands and the thickest smooth muscle (Fig. 1E). In addition, sperm storage tubules (SST) at the utero-vaginal junction are often shown, which consists of columnar ciliated cells (Fig. 1F). Obviously, the tissues in five regions are differ in structure.

The relative levels of the AR mRNAs in the five regions of the oviduct are shown in Figure 2. The result shows that AR mRNA was expressed at higher levels in the infundibulum and uterus, and there was no significant difference between them, but which is significantly higher than other three regions. The relative expression of AR in the vagina is the lowest.

Localization of AR in the hens oviduct

Our immunohistochemical study showed intense immunostaining of AR in the cytoplasm of luminal epithelial cells of all five oviductal regions (Fig. 3). A very strong immunostaining of AR was also observed in the apical cytoplasm of the luminal epithelial cells and blood vessels of the infundibulum and uterus (Fig. 3A, B, E). There was a distinct decrease in staining intensity for AR in the isthmus compared with the infundibulum and uterus (Fig. 3D). A faint immunostaining of AR was

observed in the magnum and vagina, and the glands of the infundibulum (Fig. 3B, C, F). However, in the magnum, the apical of some epithelial cells showed AR immune activity medium to high intensity (Fig. 3C).

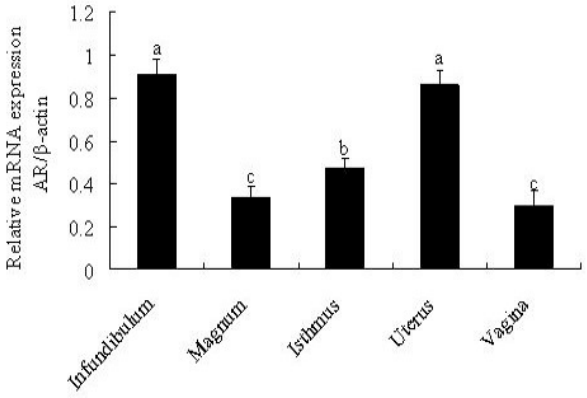

Fig. 2. Relative mRNA expression levels of AR in different regions of oviduct, determined by qPCR. The *β-actin* gene was used as an internal standard. Different letters indicate significant differences between regions ($p<0.05$). Values represent means ± SE (n=5).

All positive reactivity was noted as predominantly luminal epithelium and blood vessel nuclear staining and there was no nonspecific staining when tissues were treated with normal serum instead of primary antibodies in control sections (not shown). Scoring of staining intensity was classified as negative (no staining), weak (+), moderate (++), or strong (+++). The staining intensity details of the each oviductal regions showed in Table II.

DISCUSSION

Androgens primarily exert their functions via the AR through genomic or transcriptional pathway that ultimately modulates the expression of their target gene. The expression of AR gene in the ovary has been found in a variety of mammals (Gelmann, 2002; Rosenfeld *et al*., 2006; Sen and Hammes, 2010), while the expression of AR gene in the birds oviduct has not been reported. Kawashima *et al*. (1999) using radio-labeled [17α-methyl-^{3}H]-methyltrienolone ([^{3}H]R1881) uptake demonstrated the presence of AR in the hen uterus. However, to our knowledge, this study firstly reported the expression of AR mRNA and related immune distribution in various parts of the hens oviduct.

Table II.- Expression intensity of AR in five regions of hens oviduct.

	Infun.	Mag.	Isth.	Uterus	Vagina
Epithelium	+++	+/-	++	+++	+
Gland	+	+/-	+	++	-
Blood vessel	+++	-	++	+++	+

Infun., Infundibulum; Mag., Magnum; Isth., Isthmus.

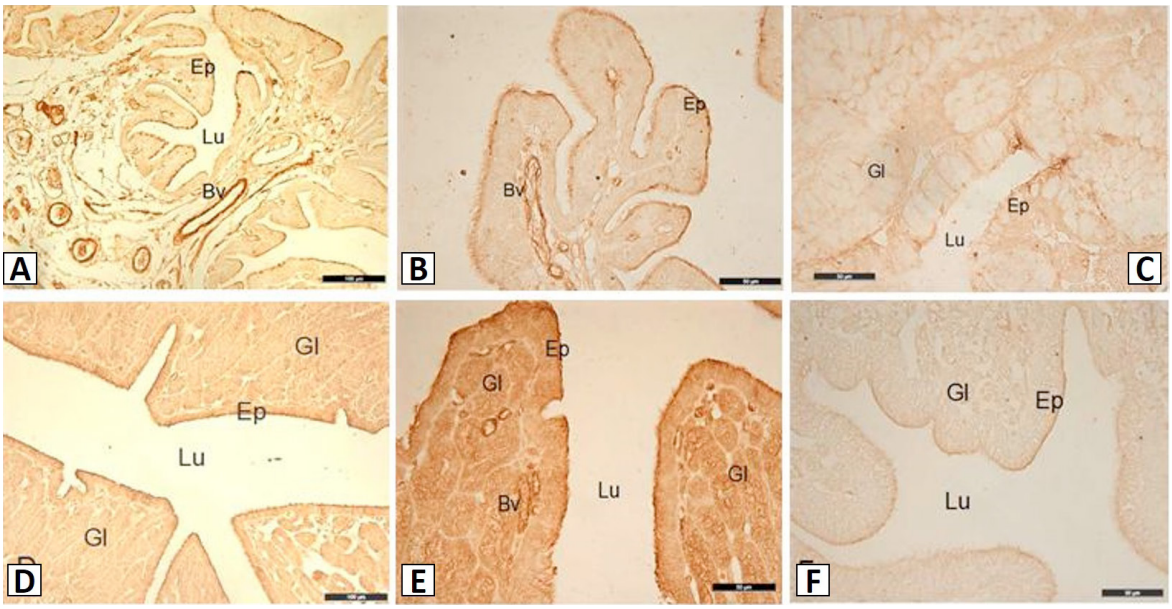

Fig. 3. Immunostaining of AR in five regions of the hens oviduct. A and B, infundibulum; C, magnum; D, isthmus; E, uterus; F, vagina. Ep, epithelium; Gl, gland; Lu, lumen; Bv, blood vessel. Bar=100 μm (A, D); bar=50 μm (B, C, E, F).

The important role of androgens in the periovulatory time was demonstrated by Hu *et al*. (2004). In knockout studies, female mice lacking functional AR ($AR^{-/-}$) exhibited markedly reduced fertility, an impaired expression of ovulatory genes.

Our results showed that AR immunoreactivity was present in nuclei of epithelial cells, which agrees well with previous reports indicating similar localization in both the human and rat oviduct (Kimura *et al*., 1993; Pelletier *et al*., 2000). In the prostate of the rat and the human, nuclear staining for AR was found in epithelial cells and stromal cells as well as endothelial cells in capillaries and larger blood vessels (El-Alfy *et al*., 1999). Our results also showed that strongly immunstaining of AR observed in endothelial cells in most of the blood vessels. These results indicate that androgen cannot play a role only in the development and function of the epithelial and stromal cells but may also influence blood vessel development and function.

In the rat, it has been shown, by in situ hybridization, that AR mRNA could be detected in the endometrium and endometrial glands as well as in the myometrium (Hirai *et al*., 1994). The presence of AR in uterine epithelial, stromal and myometrial cells suggests that androgens may exert a direct influence on the development and function of uterus.

Sperm enter the vagina and are stored in the sperm host gland at the utero-vaginal junction and subsequently move to the infundibulum. After ovulation, the ovum enters the infundibulum where fertilization takes place at the white spot of ooplasm on the yolk (Ivarie, 2003).

CONCLUSION

The present study found that mRNA expression and immune response of the AR in the infundibulum and uterus were highest. Combined tissue functions, we speculated that the high expression of AR in this two regions is associated with physiological activity of spermatozoa in the oviduct.

ACKNOWLEDGMENTS

This work was supported by the National University Students' Innovation and Entrepreneurship Training Program (201410324018); Natural Science Fund for Colleges and Universities in Jiangsu Province of China (15KJB230003 and 16KJB180030); the Special Guide Fund Project of Agricultural Science and Technology Innovation of Yancheng city (YKN2013013 and YKN2014019), the Open Project of Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection (JLCBE14006), the Jiangsu Provincial Key Laboratory for Bioresources of Saline Soils (JKLBS2014010).

Statement of conflict of interest

The authors disclose no conflicts of interest for the present research.

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