Short Communication

Expression and Localization of Growth Hormone Receptor in the Oviduct of the Laying Hen (*Gallus domesticus*)*

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The purpose of the present study was to examine growth hormone receptor (GHR) gene expression by real-time PCR and demonstrate immunocytochemically the localization of GHR in four chicken oviductal parts, i.e. infundibulum, magnum, isthmus and shell gland. Experiments were carried out on Hy-Line laying hens decapitated 2 h after oviposition. GHR mRNA was expressed in all examined oviductal segments with a significantly lower level in the infundibulum in comparison to other parts of the oviduct. Specific GHR immunoreactivity was also detected in the wall of the oviduct. The intensity of the staining was as follows: infundibulum<isthmus≤shell gland≤magnum. In all oviductal parts, a positive reaction for GHR was observed in the mucosa whereas a very weak or no reaction was observed in the stroma. Within the mucosa a strong reaction for GHR was observed in the epithelium of the infundibulum and in the tubular gland of the magnum, isthmus and shell gland. Immunoreactivity for GHR was very weak in the mucosal epithelium of the magnum, isthmus and shell gland. In conclusion, the results point to the possibility of an important role of GH in oviduct functions in domestic hens.

Key words: Growth hormone receptor, RT-PCR, immunocytochemistry, oviduct, chicken.

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During formation of the egg in the bird laying cycle, the oviduct undergoes dynamic hormonal, biochemical and cellular changes (CHOUSALKAR & ROBERTS 2008) and is characterised by a high metabolic activity. One of the key regulators of metabolism and growth processes is growth hormone (GH) produced mainly by the pituitary gland, and also by numerous extra-pituitary tissues including reproductive ones (HARVEY 2010; HRABIA *et al*. 2008; AHUMADA-SOLÓRZANO *et al*. 2012).

GH is involved in a wide array of reproductive functions such as sexual differentiation, pubertal maturation, gonadal steroidogenesis, gametogenesis and ovulation as well as pregnancy and lactation (see review HULL & HUMADA 2001; CODNER & CASSORLA 2002; SHIMIZU *et al*. 2008; HARVEY 2010; HRABIA *et al*. 2011, 2012; AHUMADA--SOLÓRZANO *et al*. 2012). The pleiotropic functions of GH in vertebrates are the results of its direct effect via GH receptors (GHR) localised in the target cells, belonging to the class I cytokine receptor family, or indirectly by insulin-like growth factors (IGFs) produced in a variety of tissues and in the liver in response to GH action (HOSSNER 2005). Widespread distribution of GHR in the reproductive system, including the mammalian oviduct and uterus, indicates that GH exerts a direct action on reproductive functions (see review HULL & HARVEY 2001; HARVEY 2010). The responses of tissues to GH are cell-type specific and include carbohydrate and lipid metabolism, immune response, cell migration, proliferation, prevention of cell death and gene transcription (see review PILECKA *et al*. 2007; HARVEY 2010).

Previously, DONOGHUE *et al.* (1990) observed increased shell thickness of eggs laid by hens injected with exogenous GH near the end of the reproductive period suggesting that the chicken oviduct, especially the shell gland, is also a target site for GH action. This suggestion was supported by a study carried out by NI *et al.* (2007) who showed GHR mRNA expression in the shell gland of chicken. To our knowledge there is no study identifying GHR in all parts of the chicken oviduct. Therefore, the aim of current study was to fill this gap by examining the expression of GHR in four segments of laying hen oviduct at the mRNA level as well as to localize GHR in the oviductal wall at the protein level.

Material and Methods

Birds

The experiment was carried out in accordance with a research protocol approved by the Local Animal Ethics Committee (No. 30/2010). Hy-Line laying hens (n=5) at the age of 19 weeks purchased from commercial farm Drobeco (Palowice, Poland) were caged individually under a photoperiod of 14L:10D. They had free access to commercial food and water. Birds were killed by decapitation 2 h after oviposition, and the oviduct was quickly removed, placed on ice, and the following oviductal parts were isolated: the infundibulum, magnum, isthmus and shell gland. Fragments of the middle part of each oviductal segment were fixed for imunocytochemical localization of GHR and other ones were frozen in liquid nitrogen for total RNA extraction.

Chemicals

The chemicals for RT-PCR were purchased from the following companies: TRI-reagent (MRC, Inc., Cincinnati, USA), RevertAid M-MuLV Reverse Transcriptase, Ribonuclease inhibitor, dNTP mix, buffers, (Fermentas, Vilnius, Lithuania), primers, oligo-dT$_{18}$ (IBB, Warszawa, Poland), SYBR Green Master Mix, Eukariotic 18S rRNA Endogenous Control (Applied Biosystems, Foster City, USA). The reagents for immunocytochemistry including biotinylated goat anti-rabbit immunoglobulin, normal goat serum and Vectastain ABC kit were obtained from Vector Laboratories (Burlingame, USA). All other reagents were obtained from ICN Biomedicals (Aurora, USA), Sigma (St. Louis, USA) or POCH (Gliwice, Poland).

Total RNA isolation, cDNA synthesis and real time qPCR analysis

Total RNA was extracted from the tissues using TRI-reagent according to the manufacturer's recommendation. Two μg of total RNA from each tissue were reverse-transcribed with RevertAid M-MuLV reverse transcriptase (200 U) and oligo-dT$_{18}$ primers (0.5 μg). Non-transcribed tissue RNA (reverse transcriptase omitted) was used as a negative control. Two μl of cDNA (10x diluted samples after the RT) were amplified in a 96-well thermocycler (StepOne Plus; Applied Biosystems, USA) according to the recommended cycling program: 10 min at 95^{0}C initial denaturation, 95^{0}C/15 s; 60^{0}C/60 s (40 cycles). The singleplex real time qPCR reactions were performed in 10 μl of volume containing 5 μl of SYBR Green Master Mix, 0.16 μmol of sense and antisense primers and water. All samples were run in duplicates. Negative control (water) was included in all runs. PCR amplification of cDNA samples was carried out using the following primer pairs: GHR sense 5'-CAGA-CAGCACTGACTCAGCTA-3' (1342-1362) and GHR antisense 5'-TGGTAAGGCTTTCTGTGGTGA-3' (1643-1662) designed to amplify a fragment of the chicken GHR cDNA located in the coding sequence of the intracellular domain (GeneBank No. 47604939). The relative expression (RQ) of GHR mRNA was calculated after normalization with a 18S rRNA transcript and the expression in the infundibulum as the calibrator using the $2^{-\Delta\Delta Ct}$ method. Results were analyzed statistically using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significant at P<0.05. The data are presented as means ± SEM.

Immunocytochemical localization of GHR

The fragments of the oviductal segments were fixed in freshly prepared 4% (v/v) buffered paraformaldehyde, processed and embedded in paraffin wax. Immunocytochemical staining was performed according to HRABIA *et al.* (2008) with small modification. Briefly, microtome sections (6 μm thick) were deparafinized in xylene, rehydrated by passing through graded alcohols, rinsed in water and heated in citric buffer (pH=6.0, 75°C, 20 min). After washing in TBST buffer (Tris buffer saline + 0.1% Tween 20) slices were treated with 3% H$_2$O$_2$ to block endogenous peroxidase activity. Nonspecific binding of the secondary antibody was blocked by incubation with 5% (v/v) normal goat serum in TBST (RT, 30 min). Sections were then incubated for 3 h (37°C) with specific rabbit polyclonal antibody against chicken GHR (HARVEY *et al.* 2000; HRABIA *et al.*, 2008) (dilution 1:400) followed by washing with TBST and incubation with biotinylated goat anti-rabbit antibody (1.5 h, dilution 1:300, 37°C) and with Vectastain ABC kit (30 min, 37°C). The colour reaction was developed by incubation with diaminobenzidine (DAB) and H$_2$O$_2$ solution. The specificity of staining for GHR was determined previously by HULL *et al.* (1996)
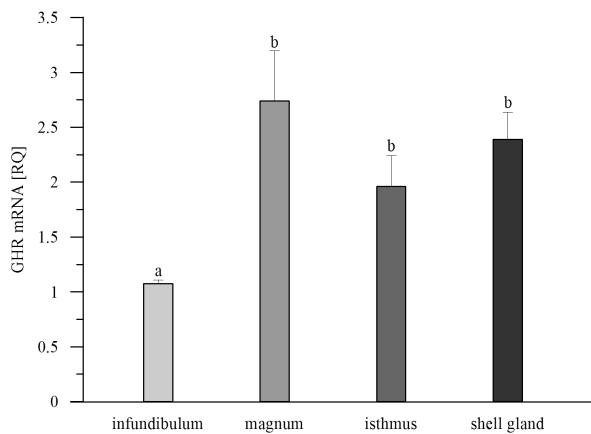
Fig. 1. Expression of growth hormone receptor (GHR) mRNA in the oviduct of the laying chicken. Data represent the mean of relative quantity (RQ) ± SEM from 5 birds standardized to the infundibulum. Values with different superscripts (a, b) differ significantly at P<0.05.

and HRABIA *et al.* (2008). Non-specific staining was demonstrated by replacement of the primary antibody with TBST. Another control included the omission of the secondary antibody. Slides were examined under a light microscope (Jena Zeiss, Germany). The intensity of immunoreactivity was estimated subjectively as strong (+++), weak (++), very weak (+) and no reaction (-).

Results

Expression of GHR

Real-time PCR analysis showed the presence of GHR mRNA in all examined segments of the chicken oviduct, i.e. the infundibulum, magnum, isthmus and shell gland (Fig. 1). Relative expression of GHR mRNA was significantly lower (1.07 ± 0.03) in the infundibulum than 2.74 ± 0.46, 1.96 ± 0.28 and 2.39 ± 0.25 in magnum, isthmus and shell gland, respectively (P<0.05).

Immunocytochemistry for GHR

Specific immunostaining for GHR was found in the wall of all examined oviductal parts (Fig. 2). The intensity of the reaction was as follows: the infundibulum<isthmus≤shell gland≤magnum. In all oviductal parts immunoreactivity for GHR was observed in the mucosa (epithelium and tubular glands) whereas in the stroma (muscles and connective tissue) there was a very weak or no reaction. Within the mucosa, a strong reaction for GHR was observed in the epithelium of the infundibulum and in the tubular gland of the magnum, isth-

mus and shell gland. Immunoreactivity for GHR was very weak in the mucosal epithelium of the magnum, isthmus and shell gland. The distribution of GHR immunoreactivity in the chicken oviduct is summarised in Table 1. Replacement of the primary antibody with TBST abolished staining (Fig. 2E).

Discussion

To our knowledge, the current study is the first to demonstrate the presence of GHR in four parts of the oviduct of the laying hen, i.e. the infundibulum, magnum, isthmus and shell gland. The expression of GHR mRNA in the infundibulum was lower than in the other segments of the chicken oviduct. Similarly, GHR immunoreactivity was lower in the infundibulum. Our observations indicate that the magnum, isthmus and shell gland are more responsive to GH. As these parts of the oviduct are characterised by higher secretory activity than the infundibulum, and GH is a well known regulator of metabolic processes, GH may have an influence on the synthesis of egg components in these 3 parts. Egg formation during its passage through the oviduct is very energy-consuming, and different metabolic pathways participate in it. It should be noted that during each ovulatory cycle the hen lays an egg weighing 50-70 g which is composed of 25-35 g white and 5-6 g shell formed in the oviduct. In the present study GHR expression was examined 2 h after oviposition, i.e. when the the next egg in the sequence was present in the middle part of the magnum. Hence, the elevated expression of GHR mRNA in the magnum, isthmus and shell gland may be related to egg position in the oviduct. Further studies could examine GHR expression according to the stage of the egg formation cycle.

Within the oviductal wall, GHR immunoreactivity was localized mainly in the mucosa while in the stroma no staining was detected except for the infundibulum in which a very weak GHR-positive reaction was found. This finding indicates that in the chicken oviduct the mucosa is a target site for GH. Since components of egg white, shell membranes and egg shell are produced and secreted by the mucosa of the magnum, isthmus and shell gland, respectively, a possible role of GH in the mucosa may be associated with the production of egg constituents.

This suggestion is additionally supported by another observation. Namely, within the mucosa strong immunoreactivity for GHR was present in the tubular glands of the magnum, isthmus and shell gland, whereas a very weak reaction was noted in the epithelium of these segments of the
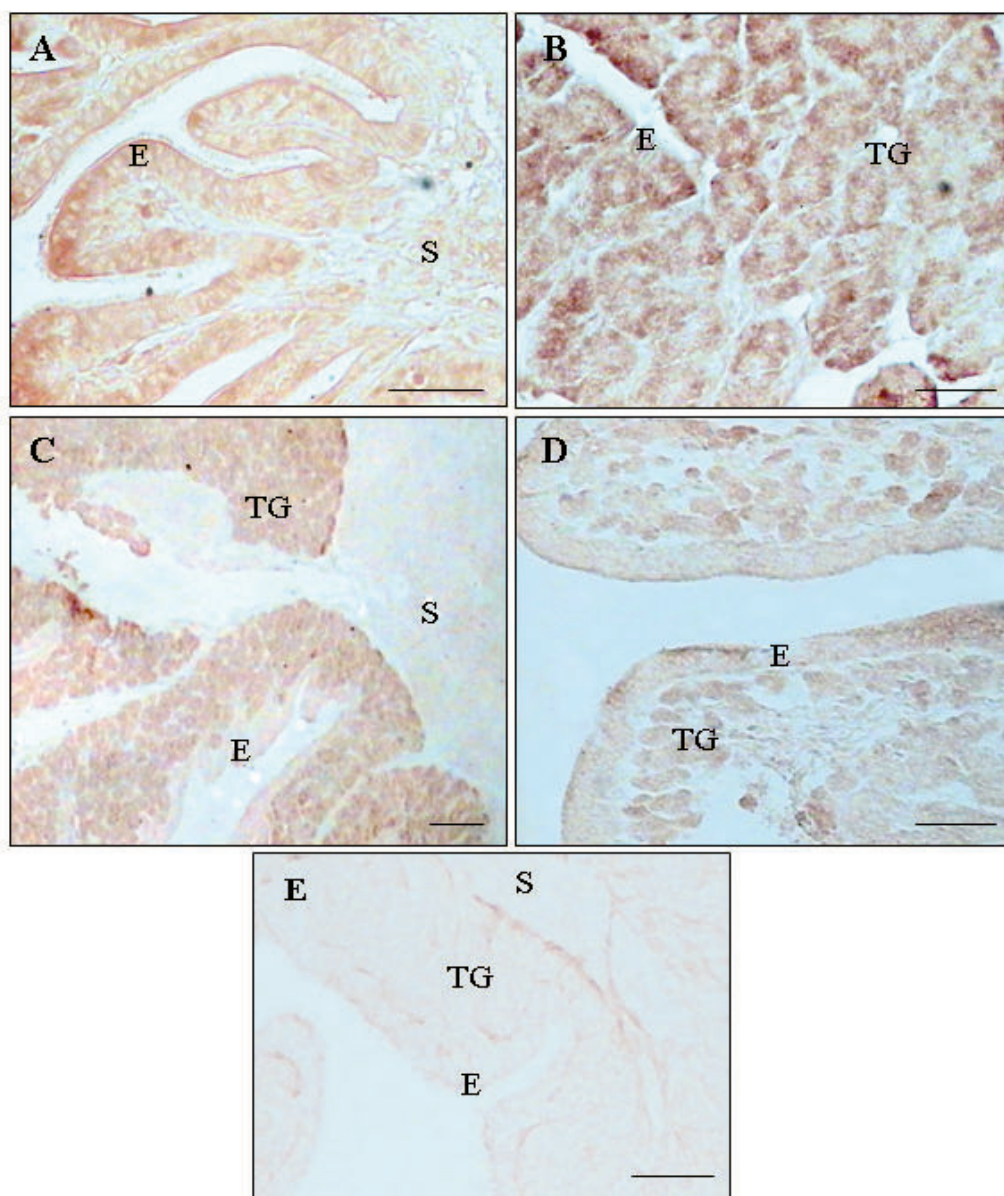
Fig. 2. Immunocytochemical localization of GHR in the oviduct of the laying chicken. A – Section of the infundibulum. Mucosal epithelium shows strong GHR staining, while staining of stroma is very weak. B – Mucosa of the magnum with strong GHR immunoreactivity in the tubular glands. C – Localization of immunoreactive GHR in the isthmus. Strong GHR immunoreactivity in tubular glands, very weak or no reaction in epithelium and stroma. D – Localization of GHR in the shell gland. Immunoreactivity comparable to that in the isthmus. E – Control treatment for immunocytochemical assay for the presence of GHR; fragment of oviductal isthmus. Primary antibody to GHR omitted. No staining. E – epithelium, TG – tubular glands, S – stroma (muscles + connective tissue). Bar = 50 μm.

Table 1

Intensity of immunocytochemical reaction for GHR in the wall of the oviduct in the laying chicken. Staining intensity: (-) – no staining, (+) – very weak staining, (+++) – strong staining; none – lack of tubular glands.

oviductal part	mucosa		stroma	
	epithelium	tubular glands	muscles	connective tissue
infundibulum	+++	none	-	+/-
magnum	+	+++	-	-
isthmus	+/-	+++	-	-
shell gland	+	+++	-	-

oviduct. The most probable role of GH in the tubular glands is the regulation of gene expression of egg proteins synthesized therein. The tubular glands of the magnum synthesize most of the egg white proteins. Tubular glands of the isthmus are a source of protein that compose the inner and outer shell membranes, whereas the tubular glands of the shell gland produce the mineral components of the egg shell. In the infundibulum lacking tubular glands, a strong reaction for GHR was observed in the mucosal epithelium which produces a small amount of the first layer of egg white. However, very weak GHR immunoreactivity, present in the epithelium of the magnum, isthmus and shell gland, may be related to the regulation of gene expression of specific proteins produced by epithelial cells such as organic components of the egg shell in the shell gland.

It is well established that GH is an important regulator of bone growth and remodeling, and tooth development (see review HARVEY 2010). On the other hand, very rapid calcium absorption and storage occurs in the shell gland of the avian oviduct. During calcification of the egg shell 2-2.5 g of calcium is introduced into the shell. It is thus tempting to speculate that GH in the tubular glands of the chicken shell gland may be involved in the process of mineralization, the fastest one known in biology (HINCKE *et al.* 2010).

It is also possible that the presence of GHR in the hen oviduct may be associated with regulation of cell proliferation and/or apoptosis by GH. The involvement of GH in the regulation of these processes in the mammalian reproductive system is well documented (see HARVEY 2010 for a review), and has recently been extended to the ovary of the growing chicken (HRABIA *et al.* 2011). Our preliminary experiment in which recombinant chicken GH was injected into chickens during maturation revealed an inhibitory action of GH on apoptosis in the oviduct (unpublished data).

The avian oviduct has also been found to express IGF-I, IGF receptor and IGF binding protein-2 genes (KIDA *et al.* 1994; FU *et al.* 2001; NI *et al.* 2007), and autocrine and /or paracrine action of IGF-I in the quail oviduct during its development was suggested. Subsequently, in primary cultures of quail oviduct cells, enhancement of ovalbumin synthesis by IGF-I in cooperation with estrogen was shown (KIDA *et al.*1995). Some oviductal actions of GH in chicken may thus be mediated by IGFs. Of pertinence, therefore, is a study indicating complex and tissue-specific regulation of the uterine IGF system components by exogenous GH in cows (PERSHING *et al.* 2002).

In conclusion, the results of this study demonstrate the differential expression of GHR in four parts of the laying chicken oviduct and differential localization in the oviductal wall, and point to the possibility of an important role of GH in oviduct function in domestic hens. Additional experiments are necessary to clarify the role of GH in the chicken oviduct.

Acknowledgements

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