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Research paper

Expression of gonadotropin and sex steroid hormone receptor mRNA in the utero-vaginal junction containing sperm storage tubules of oviduct during sexual maturation in Japanese quail

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ABSTRACT

Sex steroid hormones play an important role in reproductive tissue development of avian species. However, their role in Japanese quail is yet to be established. To understand the physiological role of hormones involved in the development of sperm storage tubules (SSTs) in quail, we investigated expression profiles of gonadotropin (LH-R and FSH-R) and sex steroid hormone (PR-R, ER-α and ER-β) receptors in the uterovaginal junction (UVJ) containing SSTs before and during sexual maturation i.e. four to eight weeks. Every week four birds were sacrificed to collect blood and UVJ for sex steroid hormone (progesterone and estrogen) estimation and gene expression profiling of sex steroid hormone (PR-R, ER-α and ER-β) and gonadotropin receptors (LH-R and FSH-R) using qRT-PCR. Receptor expression results showed that the expression of sex steroid receptor (PR-R, ER-α and ER-β) genes were upregulated significantly ($P < .05$) in SSTs with the advancement of age. The expression of gonadotropin receptors (LH-R and FSH-R) was only high at week 5 and 6 respectively. Serum hormone analysis indicated a significant ($P < .05$) rise in estradiol till 7th week and progesterone from 7th week onwards. These results suggest that the gonadotropin and sex steroid hormone receptors may have the role in the development and maintenance of UVJ that contains predominantly SSTs during sexual maturation.

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1. Introduction

Sperm storage tubules (SSTs), a specialized sperm reservoir structure in birds are characterized by a single layer of non-ciliated cells that are formed by the invagination and differentiation of mucosal surface epithelium (Tingari and Lake, 1973; Brillard, 1993). These SSTs are primarily present at the uterovaginal junction (UVJ) of the hen's oviduct, and store sperm for prolonged time. Although infundibulum possesses SSTs, UVJ is the primary site for sperm residence (Fujii and Tamura, 1963; Bobr et al., 1964) with the series of events involved within. In all avian species, SST's are thought to perform similar functions of sperm uptake, maintenance and its controlled release at the fertilization site with the duration of storage that varies between 1 to 15 weeks (Birkhead and Moller, 1992; Bakst et al., 1994, 2011). Therefore, the fertility success of any hen is an obvious reflection of sperm stored in its SSTs. The presence of SSTs has been well established in different avian species such as chicken (Brillard, 1992; Holm et al., 1996) turkey (Brillard and Bakst, 1990; Bakst, 1992, 1994; Bakst et al., 2010) and quail (Frieb et al., 1978; Holm and Ridderstrale, 2002; Sasanami et al., 2013; Birkhead and Fletcher, 1994; Matsuzaki et al., 2015). In addition, several studies have examined the features of sperm survivability in SST and suggested that quiescence of spermatozoa during the storage (Holm et al., 2000), the interaction of SST fluid outflow from SST cells and sperm mobility (Froman, 2003; Zaniboni and Bakst, 2004) and close contact of sperm with SST epithelium (Ashizawa and Nishiyama, 1983) may be important events in this process. Unlike mammals, resident sperm in quail's oviduct remain separated from SSTs epithelial cells (Sasanami et al., 2013). In SSTs, they are reported to be packed as bundles of agglutination in head to head manner (Ito et al., 2011). Reports have been made on SSTs in poultry regarding their development, distribution, number, sperm uptake, maintenance, release etc. However, the physiological mechanisms regulating their development are unknown (Khillare et al., 2018).

Gonadotropins regulate the majority of reproductive processes and its biological action in gonadal tissue is mediated by their

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respective receptors, i.e. luteinizing hormone receptor (LH-R) and follicle-stimulating hormone receptor (FSH-R) (Ascoli et al., 2002). These receptors are transmembrane domain G-protein associated receptors (Puett et al., 2007; Panga and Rajkovic, 2015). FSH is thought to stimulate estrogen synthesis and serves as a key factor in the selection of dominant follicles (Bousfield et al., 2007; Tran et al., 2008). Accordingly, FSH receptors are responsible for maintaining the viability of prehierarchical follicles and in initiating granulosa cell differentiation (You et al., 1996). Ovarian progesterone and estrogen (Tilly et al., 1991) are reported to stimulate the development of SSTs by acting through their receptors (Yoshimura and Bahr, 1991; Yoshimura et al., 2000). Recently Agarwal et al. (2013) observed the expression of LH-R in the chicken oviduct. To the best of our knowledge, there is no evidence indicating the physiological basis of LH and FSH regulating SST development in quail. Therefore, to answer this biological question, we proceeded with an objective of investigating the expression profile of gonadotropin and sex steroid hormone receptors in SSTs and to elucidate their role in relation to sexual maturity in Japanese quail.

2. Materials and methods

2.1. Experimental birds and study design

All the procedures followed in the present study are in accordance with the Institute Animal Ethics Committee, ICAR-Central Avian Research Institute, Izatnagar, India.

Twenty female Japanese quail (4 weeks old) birds randomly selected from the same hatch were used for this experiment. The experimental birds were housed in multi-tier individual cages and reared under uniform husbandry conditions. The experimental birds were provided *ad libitum* water and quail layer ration with a constant 14 hr light per day throughout the experiment. From 4 weeks of age onwards 4 birds were sacrificed every week up to 8 weeks of age. The UVJ of the oviduct was collected (see below) for expression analysis. Blood samples were also collected from these birds for serum steroid hormone estimation.

2.2. Blood sample collection and hormone estimation

Blood samples (1 ml) were taken from the wing vein for serum separation from all the birds. Serum samples were stored at −20 °C for further assay. Samples were collected on a weekly basis from 4 to 8 weeks period. Serum estradiol and progesterone concentrations were estimated by radio immunoassay (RIA) method using commercial RIA kits procured from Immunotech Pvt. Ltd., France and Omega Diagnostics Ltd., U.K.

2.3. Isolation of UVJ and identification of sperm storage tubules

The quail oviduct was isolated and removed from the reproductive tract immediately after sacrifice according to Brillard and Bakst (1990). The connective tissue surrounding the vagina and uterus was carefully removed by lifting and cutting the connective tissue away from underlying muscularis mucosa. The vagina and uterus were excised longitudinally, spread out on a dissecting board, and rinsed with ice cold PBS of pH 7.4. Beginning at the vaginal end, the vaginal mucosa was scraped with a scalpel, through the UVJ until the uterus was reached. The isolated mucosa was spread out in a Petri dish containing PBS and viewed under a stereomicroscope. The UVJ mucosa contained the SST embedded in longitudinal folds. The vaginal mucosa was removed and discarded, leaving only the UVJ mucosa containing the SST, which was used for RNA isolation.

2.4. Extraction of total RNA and cDNA synthesis

Total RNA was extracted from the homogenized UVJ mucosa containing SSTs using TRIzol denaturing solution (Invitrogen, USA) as per manufacturer's instructions. Approximately 15–25 mg of tissue sample was used for RNA isolation. The concentration and purity of RNA preparation was determined spectrophotometrically at A260 and A280. For all samples, the RNA 260/280 nm absorbance ratio was ≥2.0. Extracted total RNA samples were treated with RNase-free DNase (Biogene, CA, USA) to remove any genomic DNA contamination. Final RNA concentration was adjusted to (5.0 μg/μl). Each DNase treated, total RNA sample (1 mg) was reverse transcribed using the 'RevertAid First Strand cDNA Synthesis Kit' (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions, and resulted cDNA was stored at −20 °C until used.

2.5. Expression analysis of genes involved in UVJ development by qRT-PCR

The relative expression of specific gene mRNA's was studied by IQ5 Real-time PCR Detection System (Bio-Rad laboratories Inc. USA) in nuclease free optically clear PCR strips with flat caps (Axygen Scientific, Inc. USA) as per the guidelines of MIQE (Minimum Information for Publication of Quantitative Real-time PCR Experiments). The specific primer pairs for genes of interest except ER-α (published literature) and the reference gene (β-actin and GAPDH) were designed as suitable for SYBR green chemistry using Beacon designer software (Premier Biosoft International, USA). These designed primers were commercially synthesized (IDT, India) and used for qRT-PCR quantification (Table 1). A standard curve was generated for each gene using serially-diluted cDNA or purified PCR product as a template to calculate PCR reaction efficiency for each gene using REST 2009 Software. PCR reaction efficiencies were 0.9–1.0 for all seven genes.

SYBR Green-based reaction was performed in triplicate with a final volume of 25 μl. Each reaction mixture containing 1X SYBR Green PCR master mix (2x DyNAmo™ HS, Finnzymes, USA), 0.25 pM concentration of each gene-specific primer and 100 ng of cDNA template was run with cycling conditions of initial denaturation at 95 °C/5 min followed by 40 cycles of denaturation at 95 °C/20 s, annealing at 56–58 °C/20 s (as per the gene of interest, Table 1) and extension at 72 °C/20 s. No template control (NTC) was included for PCR reaction to check DNA contamination if any. For each sample, a dissociation curve (melt curve) was generated after completion of amplification. The threshold cycle (Ct) values of each sample and reference gene were normalized against β-actin and GAPDH Ct values. The relative mRNA expression level for each gene was calculated in pairwise comparison using the delta-delta Ct method in the REST 2009 Software.

2.6. Statistical analysis

The relative m-RNA expression of each target gene was normalized by using the two reference genes i.e. β-actin and GAPDH as internal controls. The fold expression for the gene of interest was calculated by delta-delta Ct method in the REST 2009 Software (Pfaffl et al., 2002). The mathematical model used in REST 2009 was based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group(s) and control group(s). Subsequently, the expression ratio results of the investigated transcripts are tested for significance by a Pair Wise Fixed Reallocation Randomisation Test and plotted using standard error (SEM) estimation via a complex Taylor's algorithm. Probability values of $P < .05$ were considered significant.

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