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The expressions in oxytocin and sex steroid receptors in the reproductive tissues of normal and unilateral cryptorchid dogs

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ABSTRACT

In males, oxytocin is involved with various physiological functions, such as reproductive tract contractility and testicular steroidogenesis. Due to the relationship between sex steroid hormones, oxytocin receptor (OTR) expression and cryptorchidism pathogenesis, this study aimed to investigate the mRNA expression and the localization of OTR in relation to sex steroid receptors in the male reproductive tract of both normal and unilateral abdominal cryptorchid dogs using quantitative PCR and immunohistochemistry. Male dogs were divided into two groups of normal and cryptorchid dogs. Samples from each cryptorchid dog were separated into two subgroups: scrotal and abdominal subgroups. The results showed that a lower percentage of positive OTR immunostaining in the testis and epididymis was observed in the cryptorchid group compared to the normal group. Within the cryptorchid group, the mRNA expression and the localization of OTR in the testis and epididymis of the abdominal subgroup was less than that of the scrotal subgroup. Moreover, the localization of OTR and estrogen receptor beta (ERβ) in reproductive tissues was positively correlated only in the normal group and not in the cryptorchid group. In conclusion, this study proposed that OTR expression, as well as the correlation between the OTR and ERβ in reproductive tissues of male dogs, can be disturbed by cryptorchidism. Furthermore, the OTR, ERβ and their correlation may be involved with the pathogenesis of cryptorchidism. Therefore, the study of gene knockout models to confirm the effect of OTR and sex steroid receptors on canine cryptorchidism should be of interest for further investigation.

1. Introduction

Cryptorchidism is a common congenital defect of the reproductive system in male dogs that results from an autosomal sex-linked gene [1] and may be associated with endocrine disorders such as testosterone deficiency and may be gonadotropins deficiency [2]. Previous studies in humans showed that more than half of cryptorchidism cases responded to stimulation of the hypothalamic-pituitary-gonadal (HPG) axis; therefore, an androgen deficiency might be responsible for the cryptorchidism in these cases [3]. Moreover, the excess of estrogen in male mouse fetuses led to a suppression of insulin-like factor-3/relaxin-like factor, which is a major product of fetal and adult Leydig cells, and potentially cause a feedback inhibition of the HPG axis resulting in hypoandrogenaemia [4]. Consequently, many investigators have studied the expression of sex steroid receptors in cryptorchidism cases of various species, including pigs [5], humans [6] and rats [7]. Besides sex steroid hormones, several studies have described that binding of oxytocin to its receptor initiates the signal transduction which alters the physiologic state of the target cells [8]. Oxytocin is involved with many physiological functions of the male reproductive system, such as ejaculation and sperm transport by stimulating contraction of the seminiferous tubules [9], epididymis [10] and vas deferens [11]. Oxytocin is also implicated in testicular steroidogenesis [12]. Oxytocin has an effect on the activity of 5α-reductase which cause a reduction of testosterone but an increase in dihydrotestosterone (DHT) in plasma and testis [13]. In females, the sex steroid hormones, mainly estrogen, were suggested to have a major role in regulating the transcription of the *OTR* gene [14]. Interestingly, a study of the rabbit epididymis suggested that the expression of OTR in males can also be regulated by estrogen [15]. To date, OTR localization in the testis and epididymis has been found in humans, monkeys [16] and sheep [17]. Moreover, the study in sheep showed the OTR localization to be on epithelial cells and the

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muscular layer of the ductus deferens [17]. Due to the roles of androgen and estrogen in the expression of the OTR, as well as their possible roles in the pathogenesis of cryptorchidism, the investigation of the oxytocin, androgen and estrogen receptor localization may lead to a better understanding of how these hormones regulate the reproductive tract of male dogs and may also provide more information about canine cryptorchidism. Therefore, the present study aimed to compare the localization and mRNA expression of OTR in relation to sex steroid receptors (androgen and estrogen receptors) in the reproductive tissues (including the testis, epididymis and vas deferens) of normal male dogs to dogs with unilateral abdominal cryptorchidism. The knowledge from this study about the changes of OTR, estrogen receptors (ERs) and androgen receptor (AR) expression and their correlation in male reproductive tissues may lead to the pathophysiological changes mechanism in cryptorchid dogs.

2. Materials and methods

2.1. Animals

Normal male dogs (normal group, $n = 30$) and unilateral abdominal cryptorchid dogs (cryptorchid group, $n = 10$) of various breeds between 1 and 7 years old were used in this study. The unilateral abdominal cryptorchid dog was present by one testis was found within the scrotum while another testis was not palpable in the scrotum or inguinal area but in the abdominal region. From cryptorchid dogs, scrotal and abdominal testes were surgically collected for scrotal ($n = 10$) and abdominal ($n = 10$) subgroup, respectively. All tissues sampling was reviewed under the Chulalongkorn University Animal Care and Use Committee (CU-ACUC) approved protocol No. 1431006 with owners providing consent form. All dogs from each group were used for the investigation of OTR localization by immunohistochemistry while six randomly chosen dogs from each group were used for immunolocalization of sex steroid receptors (ERα, ERβ and AR). Moreover, five dogs from each group were used for the analysis of *OTR* mRNA expression by the RT-qPCR technique. None of the normal dogs had reproductive problems such as cryptorchidism or prostatic disease. For the cryptorchid group, none of the samples were neoplastic.

2.2. Collection of samples

Bilateral reproductive tissue samples including the testis, epididymis, and vas deferens were obtained after castration. Reproductive tissues from each dog were divided into two parts: (i) samples were snap frozen in liquid nitrogen and stored in cryogenic vials at −80 °C for RT-qPCR and (ii) samples were fixed in 4% (w/v) paraformaldehyde in a phosphate buffered saline (PBS) solution for 48 h and then embedded in a paraffin block which was cut into 4 μm thick sections for placement on gelatin-coated slides.

2.3. Isolation of RNA and reverse transcription

Frozen tissue samples was ground to a fine powder using a sterile mortar and pestle in liquid nitrogen and were then homogenized in a mixture of lysis buffer. Immediately, after RNA extraction, total RNA was isolated from the samples using a column-based method (RNeasy® Mini Kit, QIAGEN Ltd, West Sussex, UK). To determine RNA concentration and integrity, each RNA sample was evaluated using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Delaware, USA) and a 1% (w/v) agarose gel electrophoresis technique with Red safe staining (Red safe™, iNtRON biotechnology, Gyeonggi-do, Korea). Isolated RNA was treated with DNase to eliminate genomic DNA using RQ1 (RNA-Qualified) RNase-Free DNase (Promega®, Medison, USA). Subsequently, the DNase-treated RNA was generated into complementary DNA (cDNA) using random hexamer primers and avian myeloblastosis virus reverse transcriptase (Reverse transcription system, Promega®).

2.4. RT-qPCR technique

Primer pairs for the *OTR* gene were designed using sequences published in GenBank by Primer 3 (Version 0.4.0), which are available online. To normalize the quantification, set of qPCR assay were needed to perform for the expression of reference genes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 5S ribosomal RNA (RP5S) genes on all experimental samples. Primer pairs for the GAPDH and RP5S genes were designed according to previous studies [18,19]. Primers that were used in this study are shown in Table 1. Conventional PCR was performed, and the PCR product was used for the preparation of standards and analyzing the optimal melting and annealing temperature for each gene. A PCR purification kit (QIAquick® PCR purification kit, QIAGEN Ltd) was used to generate pure PCR products for use as standards and for sequencing (1st Base DNA Sequencing services, Selangor, Malaysia). The target and reference gene expression levels were detected by quantitative PCR (qPCR) using an automated fluorometer ABI PRISM®7300 Sequence Detection System (Applied Biosystem®, Life Technologies, Darmstadt, Germany) and amplified using KAPA SYBR® FAST qPCR Master mix (2×) universal (KAPA BIOSYSTEMS, MA, US). All assays for each gene were run in duplicate in the same reaction using 10 ng of cDNA sample together with the no template control and eight known concentrations of standards of a 10-fold serial dilution of the PCR product of each gene and the results used to generate a standard curve. Concentrations of the PCR product were calculated by comparing the threshold cycle (C_T) values of the unknown samples to the standard curve. The reference genes were evaluated for stability using geNorm VBA applet. From this algorithm, a gene expression normalization factor was calculated for each sample. The mRNA expression values of the measured genes were normalized by multiplying the sample value for each gene with the calculated normalization factor. The expression was estimated as picograms cDNA per micrograms of total RNA (pg/μg RNA). To produce an accurate and reproducible quantification of all qPCR assays, coefficient of determination (R^2) of each assay was more than 0.98 and the percentage of PCR efficiency (%E) of each gene was in range 90–110%.

2.5. Immunohistochemical staining of OTR, androgen receptor (AR), estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ)

Tissue sections were deparaffinized in xylene, followed by descending concentrations of ethanol. An antigen retrieval technique [20] was performed by heating the sample in a microwave oven with 0.01 M (w/v) citric acid buffer, pH 6.0, at 750 W (10 min for OTR, 30 min for AR, and 20 min for ERα and ERβ detection). Afterwards, slides were rinsed in PBS and incubated in 3% (v/v) hydrogen peroxide in methanol to block endogenous peroxidase action. The sections were then incubated with normal serum (Vector Laboratories, CA, USA) to reduce nonspecific reactions. The primary antibodies used were the mouse monoclonal antibody to ERα (Dako, Glostrup, Denmark; clone 1D5, dilution 1:50), rabbit polyclonal antibody to ERβ (Santa Cruz Biotech, TX, USA; clone H-150, dilution 1:50), rabbit polyclonal antibody to AR (Santa Cruz Biotech; clone N-20, dilution 1:100) and goat polyclonal antibody to OTR (Santa Cruz Biotech; clone N-19, dilution 1:200). Thereafter, all sections were incubated with biotinylated secondary antibody (Vector Laboratories). Antibody bindings were visualized by using a