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Investigation the mRNA expression of KISS1 and localization of kisspeptin in the testes of Shiba goats and its relationship with the puberty and steriodogenic enzymes

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ABSTRACT

The objectives of the present study were to investigate the mRNA expression of KISS1 and localization of kisspeptin in the testes of Shiba goats. Additionally, its relationship with the puberty was investigated. Testes from prepubertal (1 month; $n=3$), and postpubertal Shiba goats (17 ± 1.5 months; $n=4$) were collected by surgical castration. Plasma testosterone (T) and estradiol (E2) were measured just before the castration using radioimmunoassay. Testicular expression of mRNAs encoding *KISS1*, *GPR54* and the following steroidogenic enzymes: cytochrome P450 side-chain cleavage (*P450scc;CYP11A1 gene*), 3beta-hydroxysteroid dehydrogenase (*3βHSD; HSD3B1 gene*), cytochrome P450, 17alpha-hydroxylase/17, 20 lyase (*P450c17; CYP17 gene*), and cytochrome P450 aromatase (*P450arom; CYP19 gene*) were quantified by real time PCR. The localization of kisspeptin in testes was determined by immunohistochemistry. Results revealed high plasma T and E2 in postpubertal goats compared to prepubertal goats. High significant increases ($P<0.05$) of mRNAs encoding *KISS1, GPR54, CYP11A1,* HSD3B1, CYP17, and *CYP19* genes in the testes of postpubertal goats compared to prepubertal goats. In addition, kisspeptin was immunolocalized in Leydig cells of the testis. The staining was mild in the interstitial cells of the prepubertal goats while strong staining was found in the postpubertal goats. In conclusion: Leydig cells are responsible for kisspeptin production in the goat testis and this expression is puberty dependent.

1. Introduction

Kisspeptin is a secreted protein encoded by *KISS1* gene which was originally identified as a metastasis suppressor gene (Lee and Welch, 1997). Kisspeptin, and its receptor (G protein-coupled receptor, known also *GPR54*), have an important role in the regulation of gonadotropin-releasing hormone (GnRH) secretion (Gottsch et al., 2006). Mutations of the genes that encode kisspeptin or its receptor have been identified in patients with delayed puberty or hypogonadotropic hypogonadism (Sonigo and Binart, 2012). Moreover, it was reported that *KISS1/GPR54* knockout mice demonstrate many reproductive abnormalities such as underdevelopment of gonads, decreasing levels of gonadotropins and steroid hormones, impaired gametogenesis, smaller testes and reduced the percentage of sperm (Funes et al., 2003; Seminara et al., 2003; Lapatto et al., 2007). In the goat, as a model of ruminants, few researches have been done to explore the central functional role of kisspeptin. It was demonstrated that intravenous injections of kisspeptin-10 stimulates the release of gonadotropins, not only in the female goats (Hashizume et al., 2010), but also, in the male goats (Saito et al., 2012).

Extrahypothalmic expression of KISSI mRNA has been reported in the testis of mice (Anjum et al., 2012; Mei et al., 2013) and human (Ohtaki et al., 2001). However, the functional role of KISSI mRNA expression in the testis remains a mystery. In goats, other than the central role of kisspeptin as GnRH secretagogue, no study investigated the testicular expression of KISS1 mRNA and kisspeptin in the male goat.

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During puberty various endogenous factors play an important role in the resurgence of gonadotropin-releasing hormone secretion from the hypothalamus, which triggers a cascade of hormone-dependent processes. Kisspeptin triggers puberty, because the reciprocal interaction between kisspeptin and its receptors has a role in the control of GnRH pulses and sex steroid negative feedback (Navarro et al., 2011).

Regarding to the relationship between hypothalamic kisspeptin and puberty, it was proposed that both male and female rats showed a marked increase in KISS1 and GPR54 mRNAs at the hypothalamic level coinciding with the onset of puberty (Navarro et al., 2004). Likewise, in both male and female rhesus monkeys, hypothalamic KISS1 mRNA levels detected by real-time PCR increased with puberty (Shahab et al., 2005). To the best of our knowledge, studies investigating the change of KISS1 expression at the testicular level during puberty are very rare especially in small ruminants.

There are many evidences explaining the relationship between hypothalamic KISS1 expression and steroids outputs. For example, Smith et al. (2007) stated that bilateral gonadectomy in sheep enhances KISS1 expression in the arcuate nucleus (ARC). However, there is no enough information about the local testicular expression of KISS1 and the steriodogenic capability of the testis especially in small ruminant. Therefore, the objectives of the current study were to investigate the expression of *KISS1/GPR54* mRNAs and localization of kisspeptin in the testes of goats by real time PCR and immunohistochemical techniques, respectively. In addition, we aimed to determine whether there are changes in mRNAs expressions of *KISS1/GPR54* and kisspeptin immunoreactivity in the goat testes from prepubertal to postpubertal stages, and identify whether the local KISS1 expression in the testis has a direct relationship with the steroidogenic capability in male goats.

2. Material and methods

All chemicals and reagents unless otherwise specified, were purchased from Sigma, St. Louis, MO, USA.

2.1. Animals management

Seven male Shiba goats (*Capra hircus*) were used in this experiment during the period between February and June 2014. Goats were kept under normal management program at a barn belonging to the Laboratory of Reproductive Physiology, Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, Japan. Clean water and mineralized salt licks were available ad libitum. Depending on the age, the animals were divided into two main groups: prepubertal or young group ($n = 3$), aging one month and weighing 3.30 ± 0.2 kg and postpubertal or adult group ($n = 4$), aging 17 ± 1.5 months and weighing 23 ± 0.9 kg.

2.2. Surgical castration and tissue sampling

Following anaesthesia by atropine sulphate (Atropine Sulfate Monohydrate 0.022 mg/kg; Tokyo Chemical Industry Co., Ltd. Japan) and xylazine (xylazine hydrochroride 0.05 mg/kg intramuscular; Tokyo Chemical Industry Co., Ltd. Japan), testes from all goats were collected by surgical castrations. After castration, testicular tissues were divided in two samples. One sample was snap frozen on dry ice or liquid nitrogen and stored at −8 0 °C for mRNAs isolation. The other sample was fixed in 4% paraformaldehyde in 0.05 M phosphate buffer saline (PBS, pH 7.4) for 24 h at 4 °C, dehydrated through series of alcohol concentrations (70%, 80%, 90%, 95%, 99%, and 100%), embedded in paraffin, and sectioned at 6 mm for kisspeptin immunohistochemistry. All procedures were carried out and approved by the committee of the animal ethics in accordance with the guidelines established by the Tokyo University of Agriculture and Technology, Japan for the use of animals.

2.3. Blood sampling

A venous blood sample was collected from the jugular vein into an evacuated heparinized tube (Terumo Venoject II, Tokyo, Japan) just before the castration. The blood samples were centrifuged at 3200 rpm for 15 min at 4 °C. Plasma was separated and stored at −20 °C until hormonal analysis.

2.4. Hormone analysis

Plasma concentrations of testosterone (T) and estradiol (E2) were measured in triplicates by a double antibody radioimmunoassay system using I^{125} labeled radioligands as described by Taya et al. (1985). The intra- and interassay coefficients of variation were 8.4 and 9.5% for T, and 6.1 and 7.8 % for E2, respectively.

2.5. Quantitative real time PCR

2.5.1. Total RNA isolation

Total RNA from each sample was extracted using TRIzol Reagent (Invitrogen Co., CA, USA) as described previously (Chomczynski and Sacchi, 1987). Briefly, and according to the protocol, approximately 0.1 g of testicular tissues was homogenized in 1 ml of TRIzol™ Reagent. The homogenate was incubated for 5 min at room temperature to allow the complete dissociation of nucleoprotein complexes. After the addition of 0.2 ml of chloroform, the mixture was vigorously shaken for 20 s at room temperature and centrifuged at $14{,}000 \times g$ for 15 min at 4 °C. The aqueous phase was then transferred carefully to a new tube and an equal volume of 100% isopropanol was added. RNA was precipitated by centrifugation at $14{,}000 \times g$ for 10 min at 4 °C. The RNA pellet was washed once with 1 ml of 80% ethanol, briefly dried under air, and dissolved in 50 μl of diethyl procarbonate-treated water. The concentration and purity of the isolated RNA were determined spectrophotometrically using a NanoDrop Lite (Thermo Fisher Sciencific Inc., Walthman, MA, USA).

2.5.2. cDNA synthesis

Complementary DNA (cDNA) was synthesized using PrimeScript™ Reverse Transcriptase (TaKaRa Bio, Shiga, Japan) according to the manufacturer's protocol (http://www.takara-bio.com). PrimeScript™ 1st strand cDNA synthesis kit contain all reagents necessary to synthesis 1st strand cDNA. Briefly, a mixture of 1 μl of random 6 mers (50 μM), 1 μl of dNTP mixture (10 mM each), template RNA, and RNase free distal water up to 10 μl was kept for 5 min at 65 °C, then cool rapidly on ice. After that, combining the previous reaction with 4 μl of 5× PrimeScript™ II buffer, 0.5 μl of RNase Inhibitor, 1 μl of PrimeScript™ II RTase, and RNase free distal water up to 20 μl, and two incubations at 30 °C, and 42 °C for 10 min, and 60 min, respectively. Inactivation the enzymes was then by incubation at 95 °C for 5 min, followed by cooling in ice.

2.5.3. Real time PCR

Testicular expression of mRNAs encoding *KISS1*, *GPR54* and the following steroidogenic enzymes: cytochrome P450 side-chain cleavage (*P450scc; CYP11A1* gene), 3beta-hydroxysteroid dehydrogenase (*3βHSD; HSD3B1* gene), cytochrome

Table 1
Primers sequences of target mRNAs for real-time quantitative PCR in goat testis.

mRNA	References	Primer sequence	Primer accession (sources)	Target species	Primer efficiency
KISS1	(An et al., 2013)	Forward: GTTTCCATGCTGTGTCGGTT Reverse: AGACAGAGAGAGGGAGGAGG	Gen Bank: JX047312.1	Goat	100%
GPR54	(Chu et al., 2012)	Forward: GGCCACGGACTTAATGTTCC Reverse: GAACACTGTCACGTACCAGC	Gen Bank: HM135393.1	Sheep	99%
CYP11A1	(Okuyama et al., 1996)	Forward: TGGAGGATGTCAAGGCCAAT Reverse: CACGGAGATAGGGTGGAGTC	Gen Bank: D50058.1	Goat	100%
HSD3B1	(Goosen et al., 2010)	Forward: CGGCATCCTGACCAATTACT Reverse: TTTGGTGTGGTGTGTCGTCT	Gen Bank: FJ007375.1	Goat	100%
CYP17	(Hough et al., 2013)	Forward: GGTGCTCTTGGCTGTCTTTC Reverse: GATGGGGCCATATTTTTCCT	NCBI reference NM-001009483.1	Sheep	98.80%
CYP19	(Chaves et al., 2012)	Forward: AAGCCAAGAGCAACAAGCAT Reverse: TGCATTTTTCCACGGTTACA	NCBI reference NM-001285747.1	Goat	100%
Beta actin		Forward: CTGCGGCATTCACGAAACTA Reverse: ATGCCAGGGTACATGGTGGT	Gen Bank: JX046106.1	Goat	100%