Zagazig Veterinary Journal, ©Faculty of Veterinary Medicine, Zagazig University, 44511, Egypt.
Volume 46, Number 2, p. 136-145, June 2018
DOI: 10.21608/zvjz.2018.14386

The Efficiency of Kisspeptin and GnRH as Stimulators of Gonadotrophins and Testosterone in Prepubertal Male Cattle

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Article History: Received: 23/4/2018 Received in revised form: 10/5/2018 Accepted: 15/5/2018

Abstract

Our study aimed to compare between the efficiency of kisspeptin-10 (Kp10) and gonadotropin-releasing hormone (GnRH) as a trigger stimulator for gonadotropins; luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone (T) in male calves. Four prepubertal male Japanese Black calves were used. The animals were given GnRH or Kp10 separately. Plasma LH and FSH concentrations were measured by radioimmunoassay (RIA) and T concentrations were assayed by enzyme-linked immunosorbent assay (ELISA). Gonadotropins were significantly increased in response to injections of GnRH and Kp10. However, the response of gonadotropins-release was significantly greater to GnRH than to Kp10 ($P<0.05$) throughout the 180-min period. Plasma T concentrations increased significantly following injection of Kp10 or GnRH ($P<0.05$) in comparison with the pre-injection levels (0.75 - 0.47 ng/m*l*) but no significant difference was observed in T-releasing in response to GnRH and Kp10 throughout the 180-min period. Histopathologically, the testicular tissue had seminiferous cords, essentially without lumina, rather than seminiferous tubules. The basal lamina was very thick and surrounded the immature ill-developed Sertoli as well as spermatogonia cells. Neither spermatid cells nor sperms were seen (no spermatogenesis) with abundant interstitial tissue. No differences in structure of testes in Kp10 or GnRH-injected claves and those of control. Conclusively, GnRH and kisspeptin stimulated equally the secretion of T in prepubertal bulls but did not affect process of spermatogenesis.

Keywords: Kisspeptin, GnRH, LH, FSH, Testosterone

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Introduction

Substantial efforts have been paid to unveil the key regulator in the hypothalamic-pituitary-gonadal (HPG) axis at initiation of puberty. Kisspeptin is encoded by KISS1 gene and acts through G-protein coupled receptor ligand (*Kiss1r*) [1-4]. Kisspeptin-10 is the shortest form of Kiss1 which elicits the gonadotrophin releasing hormone (GnRH) secretion as measured in hypophysial portal blood [5]. Consistent with its role within the brain, the *Kiss1r* is expressed by GnRH neurons [6]. Parenteral administration of kisspeptin to rodents, ruminants and primates stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the effect was suggested to be mediated at the level of GnRH [7-11]. Expression of kisspeptin in pituitary cells [12], and testicular tissue of mouse [13-15] and humans [1,4] indicates a direct effect of kisspeptin on reproduction. Parenteral administration of kisspeptin evokes the gonadotropin secretion in male calves [8]. Controversial reports have been existed on the expression of kisspeptin in the testicular tissue [14,15], but its function on testicular tissue is not studied yet. The LH and FSH were known to stimulate the testosterone (T) secretion and spermatogenesis. Our study aimed to compare between the efficiency of K10 and GnRH as trigger stimulators for the LH, FSH and T in prepubertal male calves.

Material and Methods

Peptides

Human kisspeptin-10 amide (Kp10; amino acid sequence: YNWNSFGLRF-NH2) was synthesized and purified in the laboratory of biochemistry, Iwate University, Japan by using an automated peptide synthesizer (Shimadzu PSSM-8; Shimadzu, Kyoto, Japan) and the reversed-phase HPLC using a C18 column (Jupiter 250mm×10mm, Phenomenex, CA, USA). Gonadotropin releasing hormone (GnRH) was purchased from Peptide Institute Inc., Osaka, Japan.

Animals

Four male Japanese black calves (age, 28-35 weeks old; body weight 139.2 ± 10.2 kg (mean ± SEM)) were used repeatedly for each group. The animals were housed in pens, provided with natural light through windows. They were fed on hay and concentrated rations and supplied with a free access to water. On the day of the experiment, the calves were not fed till after the experiment. All animal care and experimental protocols were approved by the Animal Care and Use Committee of Iwate University.

Experimental design

Drug administration and sampling

The animals were given a single I.V. injection of Kp10 (5 µg/kg BW (3.85 nmol/kg BW.)) or GnRH (5 µg/kg BW (4.23 nmol/kg BW)). These peptides were injected into freely moving animals via an indwelling catheter previously inserted into one of the external jugular veins. The experiments were carried out at 2-3-days intervals. All calves received all the treatments. The order in which each calf received the treatments was determined at random. Blood samples (3 m*l* each) were drawn at - 60, 0 (just before injection), 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160 and 180 min after the injection. Individual plasma samples were obtained after centrifugation and stored at – 30°C until hormonal assay.

Testicular tissue collection for histopathology

Testicular tissues representative of the four prepubertal male calves were surgically collected by castration and prepared in the laboratory of Pathology for paraffin embedding. Samples were fixed in 10 % formalin and processed for paraffin embedding. Samples were sectioned with microtome at 4 µm thickness and stained with Haematoxylin and Eosin [16].

Hormone assay

Plasma concentrations of LH and FSH were measured by a double-antibody radioimmunoassay (RIA) [17,18]. The LH standard preparation and the hormone for iodination were USDA-bLH-B-6. The FSH standard preparation and the hormone for iodination were AFP5346D and AFP5318C, respectively. Assay sensitivities for LH and FSH were 0.21 and 0.12 ng/mL, respectively. All samples were assayed in a single run. The intra-assay coefficient of variation was 5.1 and 10.3 % for LH and FSH, respectively. The

plasma concentrations for T were measured by the double-antibody enzyme immune-assay (EIA) kit (BioCheck, Inc 323 Vintage Park Dr. Foster City, CA 94404, Catalog number: BC-1115) after extraction with diethyl ether. Ninety six-well ELISA plates already coated with 2nd antibodies received the following; sample (10 µL) + T-HRP (100 µL) + 1st anti-body solution (50 µL), then shacked for 1 min and incubated at 37°C for 90 min. The wells were washed five times with distilled water. TMP reagent (100 µL) was added to each well and shaked for 10 sec. Finally, the samples were incubated at room temperature for 20 min and assayed at 450 nm.

Statistical analysis

All data are presented as the mean ± SEM. The statistical significance of differences in plasma LH and FSH concentrations over time in each treatment group was analyzed by repeated measure ANOVA, and differences between specific groups were determined using the subsequent use of the Newman-Keuls test. The statistical significance of differences in plasma LH and FSH, and T concentrations at each sampling time between the Kp10-and GnRH-treated groups were determined using Student's t-test. All data were analyzed using the Graph-Pad Prism (GraphPad Software, San Diego, CA, USA). Results were considered significant at the P<0.05 level.

Results

The I.V. injection of Kp10 or GnRH significantly stimulated both the LH (Figure 1A) and FSH (Figure 1B) in prepubertal calves (P<0.05). Area under the LH-response curve (AUC) after GnRH injection was significantly greater than that after KP10 injection (210.7 ± 42.7 vs. 73.2 ± 13.3 /ng.min.mL, respectively) (P<0.05) throughout the 180-min period. Furthermore, the AUC of FSH after GnRH injection was significantly greater than that after Kp10 injection (384.9 ± 78.7 vs. 164.9 ± 34.3 /ng.min.mL, respectively) (P<0.05) throughout the 180-min period (Figure 1).

In comparison to pre-injection level, plasma T concentrations significantly increased after injection of Kp10 or GnRH (P<0.05). The T-releasing response to GnRH post-injection showed a non-significant difference from that of Kp10 throughout 180 min period. The maximum values of T were observed at 120-180 min after each injection of both peptides. The maximum values of T were recorded at 120 min after injection of Kp10 and GnRH; 4.70 ± 2.20 and 5.60 ± 2.10 ng/ml, respectively (Figure 2).

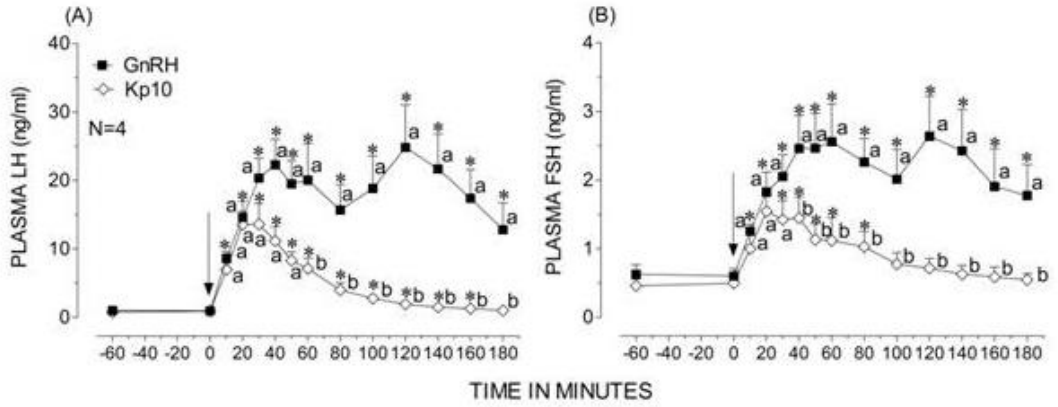

Figure 1: Plasma concentrations of LH and FSH in response to a single intravenous (I.V.) injection of GnRH (5 µg/kg body weight (BW): 4.23 nmol/kg BW) and Kp10 (5 µg/kg BW: 3.85 nmol/kg BW) in male calves are shown in Figure 1A and Figure 1B, respectively. Arrow indicates the injection time. Each value refers to the mean ± SEM for 4 male calves (age, 28-35 weeks old; BW, 139.2 ± 10.2 kg). P<0.05 compared with the pre-injection values. Asterisks on the bars denote significant difference from the pre-injection values (P<0.05). Letters (a, and b) on bars denotes the significant difference between the respective values of both groups (P<0.05).

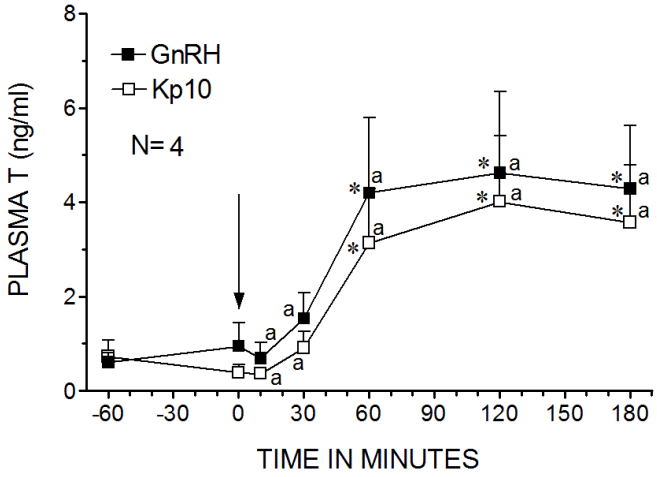

Figure 2: Plasma concentrations of T in response to a single I.V. injection of GnRH (5 µg/kg BW) or Kp10 (5 µg/kg BW) in 4 male calves. Other explanations are shown in Figure 1.

The histological changes of the testicular tissues of the male calves treated with Kp10 and GnRH were shown in Figure 3. The testicular tissues of the calves treated with a single I.V. injection of Kp10 and GnRH had shown a non-spermatogenic response and those lumens of seminiferous tubules did not contain sperm cells. All calves had immature testes. The testicular tissue had seminiferous cords, essentially without lumina, rather than seminiferous tubules (Figure 3a-d). The basal lamina was very thick and surrounded the immature Sertoli cells and the only present spermatogenic cell i.e. spermatogonia (Figure 3a-d). Spermatogonia were the only germ cells present and they were big round cells with a pale cytoplasm and one conspicuous nucleolus. Nuclei of immature Sertoli cells had an oval shape with a regular border. Spare degenerated primary and secondary spermatocytes rarely seen in addition to spermatogonia in calf No. B 9-33 (little bit bigger in weight). Abundant interstitial tissue interspersed between the seminiferous cords consisted mostly of interstitial Leydig cells and fibroblasts (Figure 3a-d). Neither spermatid cells nor sperms were seen. No spermatogenesis was observed and spermatogenic cells proliferation with few interstitial tissue among seminiferous tubules are clear (Figure 3e-f). There is no histological difference between kisspeptin/GnRH - non-inoculated calf (B. 9-30, Figure 3a) and inoculated calves (B. 03, Figure 3b; B. 04, Figure 3c; B. 9-26, Figure 3d; B. 9-27 and B. 9-33, Figure 3e-f).

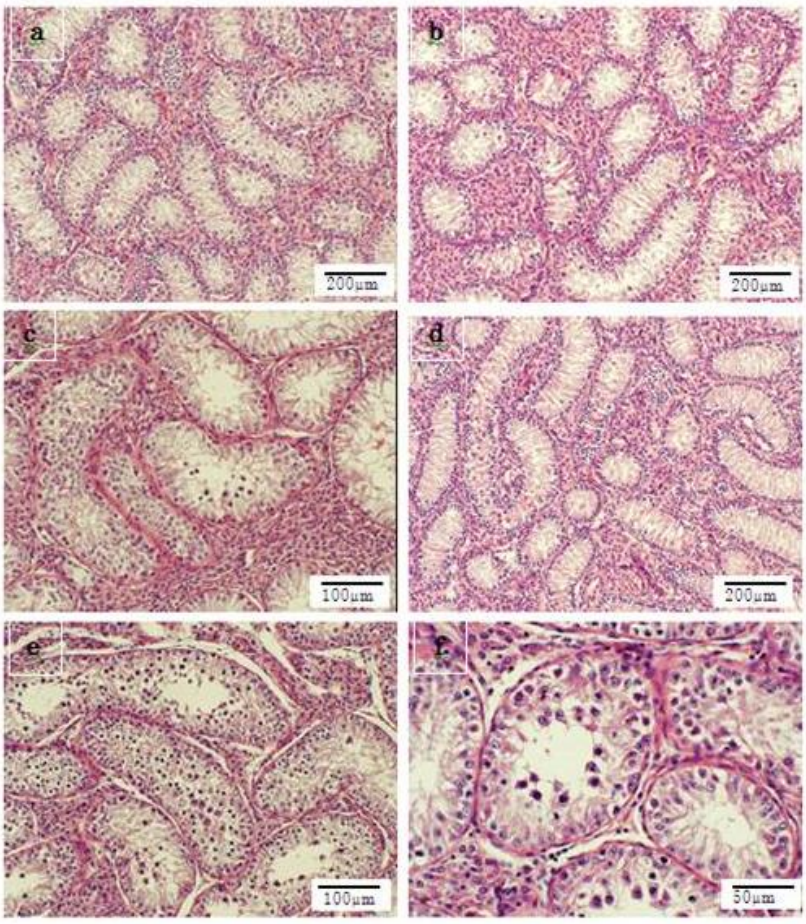

Figure 3: Testes of pre-pubertal calves inoculated with kisspeptin and GnRH revealed that the testicular tissue had seminiferous cords, essentially without lumina. The basal lamina was very thick and surrounded the immature ill-developed single row of Sertoli and spermatogonia cells. Neither spermatid cells nor sperms were seen (no spermatogenesis) with abundant interstitial tissue (a-d). Spermatogenic cells proliferation with few interstitial tissue among seminiferous tubules are clear (e-f). There is no histological difference between non-inoculated control calf (Figure 3a, H&E, bar = 200µm) and kisspeptin/GnRH-inoculated calves (B. 03, Figure 3b; B. 04, Figure 3c; B. 9-26, Figure 3d; B. 9-27 and B. 9-33, Figure 3e-f), H&E.

Discussion

Reproduction is a network of sequential responsive actions of feedback mechanisms between the hypothalamus, pituitary gland, and gonads which coordinate the processes of gonadotropin secretion and in parallel; the production of gametes [19]. Puberty is a complicated scenario of biologic processes involving the sexual development and growth. From the neurobiological point of view, GnRH hypothalamic network serves as the common pathway through which the secretion of gonadotropins from the anterior pituitary (AP) gland is enhanced [20,21]. The precise mechanisms whereby GnRH regulates the gametogenesis and the reproductive endocrine function at different stages of reproduction are still partially unknown [22]. The clear action of GnRH is its effect on AP cells to synthesize and release the LH and FSH.

Kisspeptin has recently been implicated in the testicular function by its expression in the mouse [14] and primate's testes [1]. Recently, it was found to play a significant role in the sperm motility [23]. Our experiment studied the effects of kisspeptin on the gonadotropins and T secretion from one side and their effects on induction of spermatogenesis from another side as a sign of puberty. The role of gonadotropins and T on spermatogenesis had

been manipulated in several studies [24]. Neither kisspeptin nor GnRH injections in separate single doses made changes in the spermatogenic cells for production of spermatozoa. They could be three factors in a network including others such as; IGF-I, IGF-2, TGF-α, TGF β, basic FGF, interleukins [25]. Continuous administration of kisspeptins to monkeys and rodents desensitized the Kiss1r [26,27,28], and also had led to significant degeneration of the testes, germ and Sertoli cells in the adult rats [28]. Kisspeptin and its receptor expression has been detected in testicular tissue [1,3,4], but the kisspeptin function on testicular tissue is still unclear. It had been stated that Kp10 administered peripherally (50 µg, I.V. bolus) caused a robust increase in plasma T levels in adult male rhesus monkeys [29]. However, Kp10 did not affect the T release from the testicular tissue of monkeys and showed no effect on spermatogenesis *in vitro* [30].

Previous studies have shown that Kisspeptin is able to stimulate the release of T in different animal species such as rodents [11,31,32], monkeys [26] and male goats [33]. Our study is consistent with those results confirming the stimulatory action of kisspeptin on T secretion from the male calves. The effect of Kp10 on the release of T was completely blocked by the GnRH antagonist, acyline [31]. Therefore, the effect of kisspeptin on the T secretion appeared to be mediated via GnRH/LH. However, our results showed that GnRH and kisspeptin stimulate the secretion of T equally at the prepubertal age in cattle, but not equally in case of the gonadotropins. Although, kisspeptin was stated to stimulate the LH and FSH indirectly after stimulating the endogenous GnRH, the equalized response of both peptides on the T secretion confirms a direct action of kisspeptin on the Leydig cells and that kisspeptin could play an autocrine and/ or paracrine role in the testicular function. In future studies, we need more experiments using the GnRH antagonists to investigate if the blocking effect will also include kisspeptin or not.

Activation of GnRH neurons is the key event responsible for the initiation of puberty [34,35]. Kisspeptins are involved in regulation of gonadotropin secretions [36-38]. Those peptides are identified in several species and act via the G-protein coupled receptor GPR54 [38,39]. Kisspeptin is reported to play a pivotal role in induction of puberty in humans [36,38] and rodents [38] through awakening of the HPG axis. It seems plausible that kisspeptin-GPR54 signaling within the GnRH neuronal network is critical for the pubertal activation of GnRH neurons [27,40]. Although, kisspeptin was found to be expressed in the germ cell, spermatocytes and spermatids [41], the gonadotropins- and T-releasing responses to a single I.V. injection of Kp10 and GnRH could not stimulate the process of spermatogenesis in the prepubertal male cattle. The GnRH secretory pattern was stated to exhibit a diphasic ontogenetic pattern of activity including a perinatal releasing peak that consequently followed by quiescence until the onset of puberty [42,43].

The process of spermatogenesis in the seminiferous tubules have been found to be under the regulation of Sertoli cells, germ and Leydig cells, through sets of physical and biological interactions [41]. Expression of both Kiss1/ Kiss1r, in the seminiferous tubules of the adult rhesus monkey and human testis, refers to a possible direct action of the peptide in a regulatory network of the spermatogenesis and a modulatory communication among the germ cell, Sertoli cells and kisspeptin signaling in a paracrine and/or autocrine manner [40].

Controversial reports had studied the Kiss1 localization in those Leydig cells. Kiss1r was detected in the mouse germ cells but not detected in the Leydig cells [13], those results were contradicted by another report that documented the expression of kisspeptin in mouse Leydig cells [15]. Furthermore, immunolocalization of kisspeptin along with GnRH, GnRH receptor, and gonadotropin-inhibiting hormone were detected in the mouse testis Leydig cells [13]. Our results confirm the expression of kisspeptin and localization of Kiss1r in the Leydig cells which enhanced the direct secretion of T in response to Kp10 injection and that amount of T secretion in response to Kp10 showed no significant difference than that in response to GnRH in the prepubertal male cattle.

Conclusion

GnRH and kisspeptin stimulated equally the secretion of testosterone hormone through stimulatory effect on GnH in prepubertal bulls but did not affect process of spermatogenesis. However, histopathological results revealed no progress in spermatogenesis process indicating lacking GnRH and kisspeptin receptors in testicular tissue.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

We are thanking prof. T. Hashizume and Dr. Hirata, faculty of agriculture, Iwate university, Japan, for their continuous scientific work and cooperation. The authors express their deep appreciation to prof. Tetsuro Yamashita who synthesized Kisspeptin-10.

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الملخص العربي

كفاءة الكيسبيبتن والهرمون المحرر لموجهة الغدد التناسلية في تحفيز افراز موجهة الغدد التناسلية وهرمون التستوستيرون في عجول الماشية في عمر ما قبل البلوغ

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تهدف هذه الدراسة للمقارنة بين التأثير التحفيزي لكل من الكيسبيبتن 10 والهرمون المحرر لموجهة الغدد التناسلية(GnRH) في افراز موجهة الغدد التناسلية (GnH) , الهرمون اللوتيني (LH), هرمون التحوصل (FSH) وهرمون التستوستيرون في ذكور العجول الغير بالغة. تم حقن أربعة عجول يابانية صغيرة بالكيسبيبتن 10 والهرمون المحرر لموجهة الغدد التناسلية كلا على حدة وقياس مستوي الهرمون اللوتيني وهرمون التحوصل باستخدام المقايسة المناعية الإشعاعية وهرمون التستوستيرون باستخدام الإليزا. لوحظ ارتفاع مستوي هرمون موجهة الغدد التناسلية معنويا بعد حقن كلا من الكيسبيبتن 10 والهرمون المحرر لموجهة الغدد التناسلية الا أن تأثير الأخير كان أوضح وأقوي خلال فترة 180 دقيقة. ومن الجانب الأخر فإن مستوي هرمون التستوستيرون ارتفع معنويا بعد حقن كيسبيبتن 10 (0.75 نانو جرام/مل) مقارنة بفترة ما قبل الحقن (0.47 نانو جرام/ مل) ولكن ليس هناك فروق بين مستوي هرمون التستوستيرون خلال فترة 180 دقيقة من حقن كلا من الكيسبيبتن 10 والهرمون المحرر لموجهة الغدد التناسلية. على المستوي النسيجي، لا يوجد اختلاف بين نسيج الخصية في العجول الغير بالغة المحقونة والغير محقونة حيث أن نسيج الخصية يحتوي علي حبال منوية بدلا من الأنابيب المنوية بالإضافة إلي وجود خلايا بينية ومنوية غير ناضجة ولا وجود لأي خلايا نطفية أو حيوانات منوية. نستخلص من ذلك أن الكيسبيبتن 10 والهرمون المحرر لموجهة الغدد التناسلية لهما تأثير تحفيزي متساوي على مستوي هرمون التستوستيرون في عمر ما قبل البلوغ وبدون أي تأثير على تكوين الحيوانات المنوية.