Pakistan J. Zool., vol. 44(1), pp. 7-14, 2012.

Peripheral Administration of the Human Kisspeptin-10 and 26RF-Amide Inhibits Plasma Testosterone Levels in the Adult Male Broiler Breeder Birds (*Gallus domesticus*)

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Abstract.- Kisspeptin (KP), a family (belonging to the RFamide superfamily of peptides) of related-peptide hormones encoded by the *KISS1* gene, is recently identified as the major player in the neuroendocrine regulation of the mammalian reproduction. Despite the well established role of KP in mammals not much is known about their action in non-mammalian species especially in the avian reproductive physiology. Therefore, the present study was designed to examine the effect of peripheral administration of human kisspeptin-10 (KP10) on the plasma testosterone (T) concentration in the broiler breeder birds. Three different doses (11.5, 23, 38-nmol; intravenous injection) of KP10 were tested in the intact adult male broiler breeder birds under normal fed conditions. In addition, hCG (30IU) and vehicle (0.5 ml) were administered for control purposes. A single dose (38-nmol) of human 26RFamide (26RFa; another member of RFamide superfamily) was also tested. KP10 administration dose-dependently inhibited ($P<0.05$-0.005) plasma T levels. Likewise, 26RFa administration also significantly decreased ($P<0.05$) plasma T concentration. Vehicle administration did not alter plasma T levels. hCG administration stimulated ($P<0.01$) plasma T levels. Thus, the present study demonstrated that human KP10 and 26RFa administration suppressed plasma T levels in the adult male broiler breeder birds. Our findings of the present study, therefore, assign a novel role to KP10 and 26RFa, as inhibitor of plasma T secretion in the broiler breeder birds. However, the mechanisms (receptor and intracellular signaling) by which KP10 and 26RFa suppressed the plasma T level in the broiler breeder birds are currently unknown.

Keywords: Hypothalamus-pituitary-gonadal axis, kisspeptin-10, 26RFa, plasma testosterone, chicken.

INTRODUCTION

Kisspeptin (KP) is a family (belonging to the RFamide superfamily of peptides) of related-peptide hormones encoded by the *KISS1* gene. The *KISS1* gene transcribes into a 145-amino acid precursor peptide, which by post-translational modification is proteolytically processed into shorter amidated C-terminal 54, 14, 13 and 10 amino acid products known as KP54, KP14, KP13, KP10 (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). The consensus C-terminal decapeptide, common to all KPs, is the minimum amino acids sequence mandatory both for binding and activation of G protein-coupled receptor, GPR54 (recently named as KISS1R in human and kiss1r in other animals) (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001; Gottsch *et al.*, 2009).

KPs and their receptors have been implicated as major regulators of the neuroendocrine GnRH secretion in the mammals (Roa and Tena-Sempere, 2007; Seminara, 2007; Popa *et al.*, 2008; Seminara and Crowley, 2008). Mutation and targeted deletion of *KISS1* as well as *KISS1R* result in the hypogonadotropic hypogonadism in human and rodents (de Roux *et al.*, 2003; Seminara *et al.*, 2003). *Kiss1r* is expressed in the hypothalamic GnRH neurons and several other areas involved in the regulation of hypothalamus-pituitary-gonadal

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0030-9923/2012/0001-0007 $ 8.00/0

(HPG) axis (Messager *et al.*, 2005; Shahab *et al.*, 2005; Clarkson and Herbison, 2006). Central as well as peripheral injections of KP potently stimulate the HPG axis in GnRH-dependent manner (Thompson *et al.*, 2004; Shahab *et al.*, 2005; Patterson *et al.*, 2006; Wahab *et al.*, 2008), while acyline, a GnRH receptor antagonist, blocks the KP-induced release of the key reproductive hormones (Gottsch *et al.*, 2004; Shahab *et al.*, 2005). Therefore, available evidence suggests a central role for KP in the regulation of HPG axis in mammals.

26RFamide (26RFa) peptide, another member of the RFamide superfamily of peptides, originally isolated in frog, has also been cloned in various mammals (Chartrel *et al.*, 2003). This peptide is the ligand of a previously orphan G-protein-coupled receptor, GPR103 (Fukusumi *et al.*, 2003; Jiang *et al.*, 2003). The genes encoding 26RFa and GPR103 are expressed in various brain areas including hypothalamus (Chartrel *et al.*, 2003; Jiang *et al.*, 2003). Recently, 26RFa and its N-terminal elongated form, 43RFa, have been implicated in the central regulation of the HPG axis in mammals (Navarro *et al.*, 2006).

Despite the well established role of KP and partly that of 26RFa in mammals not much is known about their actions in non-mammalian species. Although recently, a functional KP–Kiss1r system has been unveiled in fish (Kanda *et al.*, 2008; van Aerle *et al.*, 2008), but currently, to the best of our knowledge, no data are available for the role of KP and 26RFa in the avian reproductive physiology. Therefore, the present study was conducted to examine the effect of the peripheral injections of the human KP10 and human 26RFa on the plasma testosterone (T) concentration in the adult male broiler breeder birds.

MATERIALS AND METHODS

Birds

Sexually mature male broiler breeder birds (Ross, a commercial hybrid; *Gallus domesticus*), at 25 weeks of age, weighing 5-6.5 kg were purchased from a poultry farm in Islamabad, Pakistan. The birds were kept in the poultry facility of our Departmental animal house and were left for 2 weeks to acclimatize to their surroundings. The experimental birds were maintained under standard condition of management and feeding (North, 1984). Water was available *ad libitum* to all birds. The experimental protocol was approved by the Departmental Committee for Care and Use of Animals.

Chemical agents

Heparin (Rotex media, Trittau, Germany) and human chorionic gonadotropin (hCG; Pregnyl®, N.V Organon Oss Holland) were purchased locally. Human KP-10 (amino acids 45-54) was purchased from Calbiochem (La Jolla, CA, USA). Human 26RFa (TSGPLGNLAEELNGYSRKKGGFSFRF-NH2) was synthesized in the lab of one of the authors (Dr. Hubert Vaudry, European Institute for Peptide Research, Laboratory of Cellular and Molecular Neuroendocrinology, INSERM, University of Rouen, France; Chartrel *et al.*, 2003). Working solutions of KP10/ hCG were made in normal saline (0.9% NaCl).

Experimental design

The experimental design of this study consisted of blood samplings before and after KP10, 26RFa, vehicle (normal saline), and hCG intravenous (*i.v.*) treatments. A total of 7 samples (0.5 ml) were collected from each bird during sampling. Two samples were obtained before the treatment at 15 min intervals (-15 and 0 min) while five samples were taken after the treatment at 15, 30, 60, 90, and 120 min.

The treatments were given in the following order:

KP10, vehicle and hCG treatment

Three different doses (11.5-, 23-, 38-nmol/ bird; injected in dilution of 0.5 ml saline) of KP10 were administered into groups of five birds. Another group of five birds received vehicle treatment as control. hCG (30 IU) was administered as a positive control to another group of five birds. The KP10, vehicle and hCG treatments were given on same day.

26RFa and vehicle treatment

A single dose of 26RFa (38-nmol) was administered to a group of four birds. Another group

of five birds received the vehicle treatment as control. 26RFa treatment was given in November 2009.

The doses of KP10 and 26RFa were selected from the neuroendocrine effective doses of these peptides in monkeys. Recently we had shown that 38-nmol dose of KP10 stimulated plasma T and adiponectin in monkey (Wahab *et al.*, 2008, 2010). Likewise, 38-nmol dose of 26RFa stimulated plasma prolactin and growth hormone concentrations in adult male monkey (Qaiser *et al.*, 2009). The monkeys used in these experiments were of 6-9 kg in body weight.

Blood collection

Using sterile heparinized syringe fitted with 26-gauge needle, blood was collected from wing vein in all birds. Blood collection was always carried out between 1100 h to 1500 h and with a minimum of handling stress. The blood samples drawn were immediately transferred into pre-sterilized tubes and spun down at 3000 rpm for 10 min at 4 °C to separate the plasma. The plasma was stored at -20°C until assayed.

Radioimmunoassay of T

Plasma T concentrations were determined by using a solid phase competitive RIA. The T kits were purchased from Immunotech, Marselle Cedex 9, France. The RIAs were performed as per the manufacturers' instructions. The sensitivity of the T assay was 0.025 ng/ml and intra- and inter-assay coefficients of variation were both < 10%.

Statistical analysis

Statistical comparisons of the mean pre- and post-treatment T levels were made by nonparametric student's t tests. Data at different doses of KP10, and vehicle were compared for significance by the analysis of variance (ANOVA) followed by the Dunnett test. A *P-value* ≤0.05 was considered statistically significant in all the cases.

RESULTS

T secretion following administration of KP10

The pattern of T secretion before and after vehicle and 11.5-, 23-, 38-nmol doses of KP10 is shown in Figure 1A. The inhibitory effect of KP10 on the plasma T concentration was apparent after

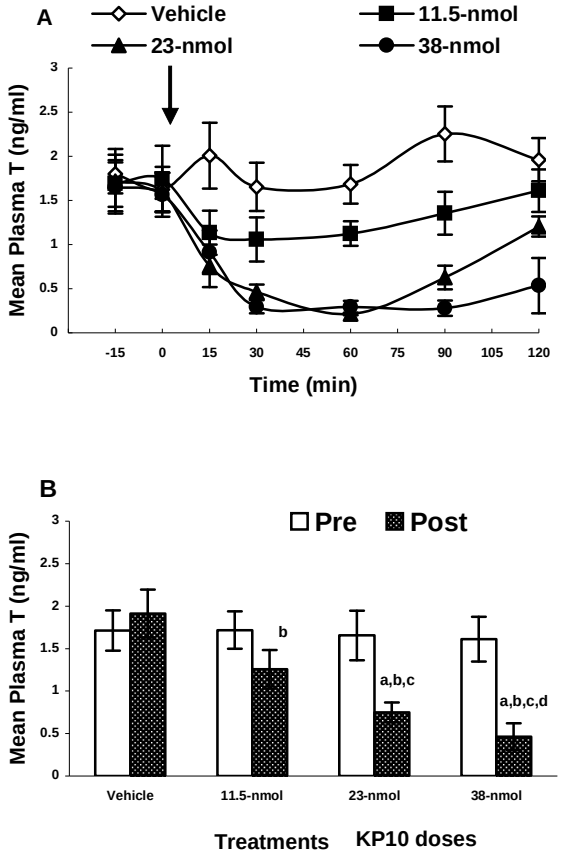

Fig. 1. (**A**) Changes in mean (±SEM) plasma T concentrations following bolus *iv* administration of vehicle and different doses of KP10 (arrow) in the adult male broiler breeder birds (n=5). (**B**) Comparison of the mean (±SEM) plasma T concentrations in the pre- (-15, 0 min) and post-KP10 treatment period (15-120 min) in the adult male broiler breeder birds (n=5). $^{a}P < 0.05$ versus pre-treatment, $^{b}P < 0.05$-0.005 versus post-vehicle, $^{c}P < 0.05$ versus post-11.5-nmol KP10, $^{d}P < 0.05$ versus post-23-nmol KP10.

15-30 min of KP10 administration and continued till 60-75 min in 23-nmol KP10 treated group while to 120 min in 38-nmol KP10 treated group. Vehicle injection did not significantly alter T levels in birds. Comparison of mean pre- and post-treatment T levels revealed that 23- and 38-nmol doses of KP10 significantly decreased (P<0.01-0.005) plasma T secretion in the broiler breeder birds (Fig. 1B).

Mean pre- and post-T levels were comparable between the vehicle and 11.5-nmol KP10 injection. Comparison of post-vehicle and post-KP10 levels showed that all doses of KP10 significantly reduced (P<0.05-0.005) T secretion. The pattern of T secretion before and after a single dose of hCG is shown in Figure 2. The hCG administration significantly stimulated (P<0.01) T release in the treated birds.

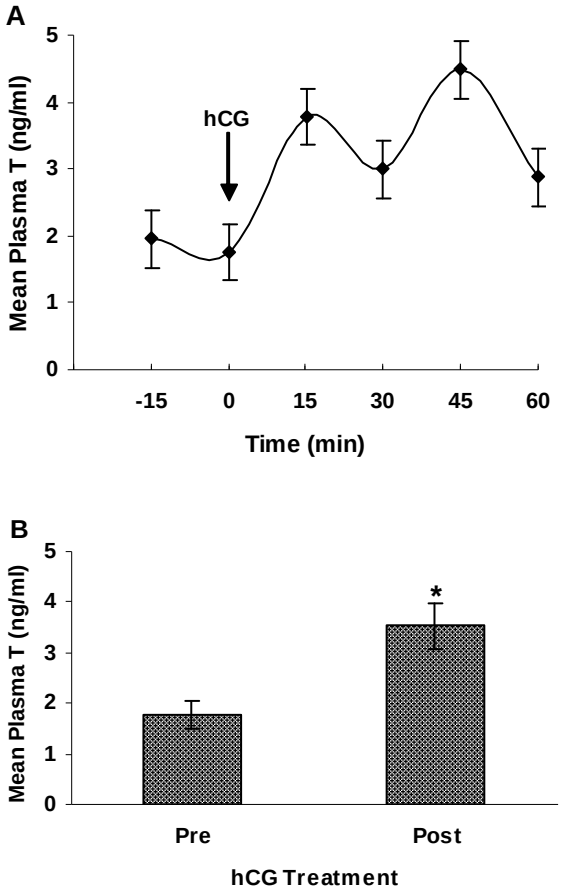

Fig. 2. (**A**) Changes in mean (±SEM) plasma T concentrations after bolus *iv* administration of hCG (arrow) in the adult male broiler breeder birds (n=5). (**B**) Comparison of the mean (±SEM) plasma T concentrations in the pre- (-15, 0 min) and post-hCG treatment period (15-60 min) in the adult male broiler breeder birds (n=5). hCG administration (30IU) significantly increased (*P<0.01) mean T.

T secretion following administration of 26RFa

The pattern of T secretion before and after a single dose of 26RFa is shown in the Figure 3A. As compared to pre- or vehicle-treatment levels, 26RFa significantly inhibited (P<0.05) the plasma T concentration in the adult male broiler breeder bird (Fig. 3B). The inhibitory effect became prominent after 60 min of 26RFa administration.

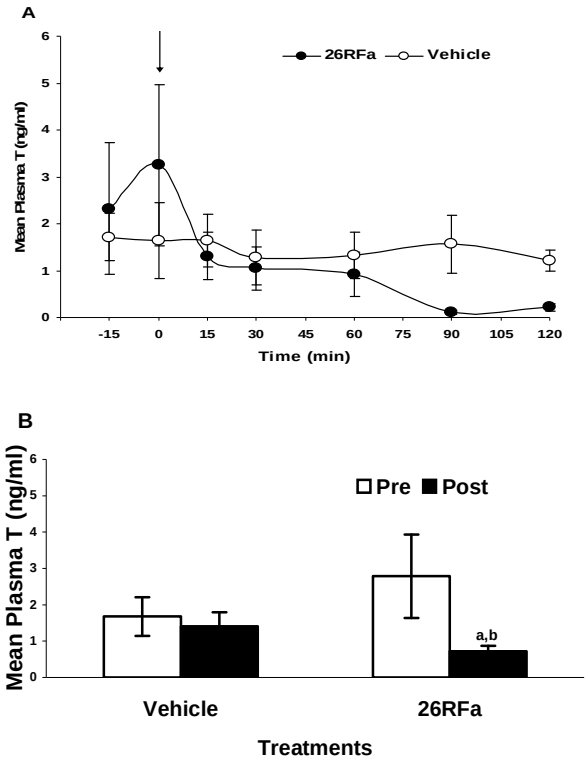

Fig. 3. (**A**) Changes in mean (±SEM) plasma T concentrations following bolus *iv* administration of 26RFa (arrow) in the adult male broiler breeder birds (n=5). (**B**) Comparison of the mean (±SEM) plasma T concentrations in the pre- (-15, 0 min) and post-26RFa treatment period (15-120 min) in the adult male broiler breeder birds (n=5). 26RFa administration (38-nmol) significantly decreased (^aP < 0.05) mean T. Post-26RFa mean T was also less (^bP < 0.05) than the post-vehicle T.

DISCUSSION

In recent years, several members of the RFamide superfamily of peptides have been implicated in the regulation of reproductive axis (Kriegsfeld, 2006). Recently, two new members of the RFamide superfamily of peptides, KP and

26RFa have been identified (Chartrel *et al.,* 2003, 2006; Kotani *et al.,* 2001). Available data show that KP and 26RFa stimulate the reproductive axis in the mammals (Gottsch *et al.,* 2004; Thompson *et al.,* 2004; Dhillo *et al.,* 2005; Shahab *et al.,* 2005; Navarro *et al.,* 2006; Wahab *et al.,* 2008). The role of KPs, and to some extent of the 26RFa, has been extensively studied in the mammals and some non-mammalian species (Chartrel *et al.,* 2006; Navarro *et al.,* 2006; Roa and Tena-Sempere, 2007; Seminara, 2007; Greives *et al.,* 2008; Popa *et al.,* 2008). Until now, to the best of our knowledge, no data are available for the role of KP and 26RFa in the avian reproductive physiology. In the present study, therefore, we have studied the effect of peripheral administration of human KP10 and human 26RFa on the plasma T levels in the adult male broiler breeder birds. Our results, unexpectedly, showed that peripheral administration of the human KP10 inhibits dose-dependently plasma T levels. Similarly, human 26RFa also decreased the plasma T levels. Vehicle injection did not alter plasma T levels, suggesting that the observed inhibitory effect was specific for KP10 and 26RFa. In contrast, hCG administration significantly stimulated plasma T levels. The stimulatory T response to hCG suggests that the testis in these birds were normally responsive to gonadotropins

The evidence of suppression of plasma T levels by KP10 in the male breeder birds in unique for two reasons. Firstly, as opposed to its action in other vertebrates, KP is causing inhibition rather than stimulation of reproductive hormones in birds. Secondly, KP10 is causing an effect in birds where no *Kiss1* and *GPR54* genes have been reported (Greives *et al.,* 2008; Felip *et al.,* 2008; Lee *et al.,* 2009). Sequence analysis revealed that human KP10 shares identity with GnIH, RFRP1 and RFRP2 especially the last three amino acids. The last amino acids Leu-Arg-Phe-NH2 (LRF-amide) sequence at the C-terminus is same in all these peptides. The C-terminal LRF-amide signature is most important for binding and activation of the GnIH receptor (Yin *et al.,* 2005). The neuropeptides such as GnIH, RFRP1, RFRP2 and chicken pentapeptide which possess this sequence can bind and activate GnIH receptor. Other neuropeptides such as Met-enkephalin-RF, galanin and neuropeptide Y, which lack the C-terminal LRF-amide motif, is unable to bind and activate GnIH receptor (Yin *et al.,* 2005). Since GnIH and its related peptides cause inhibition of gonadotropin (Tsutsui *et al.* 2000; Ikemoto and Park, 2005; Ubuka *et al.,* 2006, 2008), so, it is conceivable that their receptors could mediate, at least in part, the inhibitory effects of KP10 on T release in birds.

The mechanisms of the inhibitory action of 26RFa on plasma T secretion are not clear but it shares Arg-Phe-NH2 sequence at the C-terminus with other RFamide peptides (Chartrel *et al.,* 2003), having inhibitory effect on the HPG axis in the birds, therefore, it is possible that 26RFa may act through the receptors of these RFamide peptides. Alternatively, 26RFa may act through a homologous avian GPR103, the putative receptor of 26RFa, to inhibit T secretion in birds.

The site of action of inhibitory effect of KP10 and 26RFa on the T release in chickens is unknown. Available studies have shown the expression of GnIH receptor mRNA in the pituitary as well as in several brain regions in birds **(**Ikemoto and Park, 2005; Yin *et al.,* 2005; Maddineni *et al.,* 2008). The expression of GnIH receptor mRNA in the pituitary suggest that similar to GnIH, KP10 may act directly on the pituitary via GnIH receptor to inhibit LH release which inturn would lead to inhibition of plasma T concentration. This notion is supported by previous studies in the birds showing that GnIH administration causes reduction in plasma T concentration through modulation of LH synthesis and release (Ubuka *et al.,* 2006). Therefore, inhibition of plasma T concentration after KP10 and 26RFa administration is more likely to be a result of the decrease in LH secretion. Alternatively, as expression of GnIH receptor mRNA has been observed in the hypothalamus (Yin *et al.,* 2005), KP10 and 26RFa could also act at the level of the hypothalamus via GnIH receptor to inhibit GnRH secretion. More recently, GnIH receptor expression has also been demonstrated in the chicken testis (Bentley *et al.,* 2008), so the direct testicular action of KP10 and 26RFa could not be excluded.

The experimental design employed in this study precluded an assessment of a functional KP and 26RFa signaling in birds. But the inhibition of

the circulating T concentration after KP10 and 26RFa injection suggests a novel inhibitory action of these RFa peptides on plasma T levels in chickens. Further studies are needed to identify both an endogenous peptide like KP and 26RFa, and the receptors by which KP10 and 26RFa cause inhibition of circulating T levels in birds. Similarly, studies on the effect of other member of the mammalian KP family on the reproductive axis of birds are also necessary for better understanding the evolution of KP signaling in vertebrates. Recently, it has been demonstrated that the bull frog GPR54 responds strongly to the Xenopus KP (Kiss-12Y) form than human KP10. In contrast, hGPR54 prefers mammalian forms of KP10 (Moon *et al.*, 2009).

In summary, we have described the effects of three doses of human KP10 on the HPG axis in the adult male broiler breeder birds. Our data demonstrated for the first time that human KP10 administration inhibits dose-dependently circulating T concentration in the adult male chickens. Human 26RFa also decreases plasma T levels. The stimulation of T release by hCG and lack of the effect by vehicle administration suggest that the inhibitory effect was specific to KP10 and 26RFa. Further studies are necessary to identify the significance and functional basis of the inhibitory action of RFa peptides on the avian reproductive system.

ACKNOWLEDGEMENTS

The work presented here was funded by the Higher Education Commission, Islamabad, Pakistan. Fazal Wahab is an indigenous PhD fellow of HEC.

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(Received 7 September 2010, revised 13 June 2011)