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Kisspeptin and attributes of infertile males and females: A cross-sectional study in a subset of Pakistani population

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Running Title:

Infertility and Kisspeptin

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Abstract

Kisspeptin and attributes in infertile males and females: a cross sectional study in a subset of Pakistani Population.

Kisspeptin; a peptide hormone, plays a pivotal role in fertility and neuroendocrine regulation of hypothalamo-pituitary gonadal axis. Increased kisspeptin and reproductive hormones are responsible for fertility in male and females. This study aimed to explore the role of kisspeptin on hypothalamo-pituitary-gonadal axis by comparing the levels of kisspeptin in fertile and infertile subjects and identifying single-nucleotide polymorphisms(SNP) of *KISS1* gene in exon 2 and 3 of infertile male and female cohorts. A cross-sectional study was carried out on 80 males (44 infertile and 36 fertile) and 88 females (44 in each group). Significantly high levels of kisspeptin(KP), follicle stimulating hormone(FSH), luteinizing hormone and testosterone were observed in fertile male and female subjects except low FSH levels in comparison to infertile female subjects. One polymorphism in exon 2 [E1225K (G/A 3673)] and three in exon 3 [P1945A (C/G 5833); Insertion of T at 6075; G2026G (C/G 6078)] in infertile group were detected; with low KP and hormonal levels. Male subjects had abnormal sperm parameters and unsuccessful attempt of Intracytoplasmic sperm injection in females. Expression of SNP in exon 2 and 3 of *KISS1* could be responsible for alteration in release of reproductive hormones and gonadal functions, hence causing infertility.

Key words: Kisspeptin, Infertility, *KISS1*, polymorphism

1. Introduction:

Kisspeptin (KP) encoded by *KISS1* belongs to a family of peptide hormones which play a principal role in fertility and neuroendocrine regulation of hypothalamo-pituitary gonadal axis (Vaziri, Rafeie et al. 2017). KP secreting neurons are present in the different nuclei of hypothalamus; arcuate nucleus (ARC) also known as infundibular nucleus, the anteroventral peri-ventricular nucleus (AVPV), anterodorsal preoptic nucleus (APN), stria terminalis and Amygdale (Funes, Hedrick et al. 2003). The pulsatile secretion of Gonadotropin Releasing Hormone (GnRH) in central regulation of the Hypothalamo-pituitary gonadal (HPG) axis is played by hypothalamic *KISS1/KISS1R* (receptor of KISS 1) system (Skorupskaite, George et al. 2014). Consequently, the cross-talk between Kisspeptin and the receptor (*KISS1R*) stands crucial in regulating the commencement of puberty and release of hormones from the involved reproductive axis (Luan, Zhou et al. 2007)

KISS1 translates for a 145 amino acid long protein identified as kisspeptin-145 which produces a peptide containing 54 residues after cleavage, called Kisspeptin 54 or metastin that can further be sliced into much smaller amino acid sequences, recognized as kisspeptin-14, kisspeptin-13 and kisspeptin-10, however they represent a common structural motif (ArgPhe-NH2) in their C-terminal (de Tassigny, Fagg et al. 2007). Along the length of gene *KISS1*, more than approximately 294 single nucleotide polymorphism (SNPs) are already identified; among which the untranslated region (UTR) contributes to have 42 mutations, exon for 30 and the rest by intronic regions. [“A database of human single nucleotide polymorphisms” 2014, http://www.ncbi.nlm.nih.gov/SNP/].

KISS1R gene mutations result in loss of function of the *KISS1R*, leading to down-regulation of GnRH pulsatile secretion as well as infertility. On the other

hand, activating mutations cause prevention of desensitization of the *KISS/KISS1R* pathway and ultimately lead to precocious puberty. Two *KISS1* mutations, p.P74S and p.H90D, have been recognized as genetic causes of Central Precocious Puberty (Silveira, Noel et al. 2010). Furthermore, polymorphism of the *KISS1* gene with amino acid substitution (P110T) documented to have significant association with central precocious puberty (CPP) in Korean girls (Luan, Zhou et al. 2007) (Ko, Lee et al. 2010)

The role of KP in feedback regulation of GnRH secretion and hence release of gonadal hormones required for normal reproductive functions has been elucidated (Irwig, Fraley et al. 2004). Evidence advocates the loss of gene functionality or presence of SNPs in *KISS1* and *KISS1R* to be a risk factor for sexual immaturity and infertility axis in humans (Ko, Lee et al. 2010).

A number of studies have verified role of Kisspeptin on reproductive axis, unexplained infertility and as a therapeutic agent to trigger oocyte maturation and ovulation (Abbara, Jayasena et al. 2014), (Mumtaz, Khalid et al. 2017). Literature has proved that mutations which inhibit the action of *KISS1* and *KISS1R* in idiopathic hypo-gonadotropic-hypogonadism (IHH) subjects resulted in delayed puberty and subfertility (Semple, Achermann et al. 2005) .

Although mutations inhibiting *KISS1* gene and its receptor activity can instigate infertility yet, the information about *KISS1* gene mutations and its polymorphisms are scarce. A research done on Q36R (rs35431622) *KISS1* gene in infertile female subjects in northern Iran documented that it has no association with female infertility and suggested that variation of results might be possible due to genetic variations on account of different geographic situations (Vaziri,

Rafeie et al. 2017). In addition to that, low levels of KP have also been identified in infertile male subjects of our region (Haris Ramzan, Ramzan et al. 2015).

We thus aimed to explore role of KP on HPG axis by comparing KP levels in fertile and infertile male and female subjects and identify the sequence variations, including mutations and single-nucleotide polymorphisms (SNPs) of *KISS1* gene in exon 2 and 3 of infertile males and female cohorts.

Subjects and Methods:

This cross-sectional study was conducted in the Department of Biological & Biomedical Sciences, Aga Khan University, Karachi, from April 2016 till March 2018 after acquiring ethical approval (3331-BBS-ERC-14). The estimated sample size by observing 94% power, with prevalence of 21.9% infertility and 22%+/-6 confidence limit, was 79. To avoid drop out of subjects we recruited 88 infertile (male and female) subjects and matched 80 fertile subjects, who fulfilled our inclusion criteria and consented to be part of our study.

Phenotypic Characterization:

Inclusion Criteria for Male Subjects;

All males between the ages of 25 to 55 years during the study phase who concurred to take part in the study were selected. A comprehensive history related to the diagnosis of infertility was obtained for excluding secondarily infertile males. A semen analysis report was obtained on request (6 to 9 months old). In case of failure to do so, male subjects were demanded a fresh semen samples by masturbation following a 3 - 5 days of asceticism. Samples were then processed and stored in sterilized containers, later were analyzed as per World Health Organization guidelines.

Fertile Group:

The "fertility status" of the recruited subjects was established according to the semen parameters observing the World Health Organization criteria "2010" which states "had total sperm number (TC) >39 million per ejaculate, total sperm motility (Progressive and Non-progressive) measured within 60 minutes of collection of more than 40%, and normal morphology of ≥ 4%." (Cooper, Noonan et al.)

Infertile group:

Men with history of primary infertility; sperm number (TC) less than 39 million/ejaculate, decreased sperm motility of all sperms (less than 40%) and having normal sperm morphology of less than 4% (Cooper, Noonan et al. 2010) were included in the study.

Exclusion Criteria for Male Subjects:

Subjects having diabetes, hypertension, arthritis, malignancy, epilepsy, tuberculosis, endocrinal disorders, liver/renal disease, cryptorchidism, testicular trauma, orchitis, testicular hypotrophy along with those who had general health issues were excluded. Additionally, those who suffered from secondary infertility were discounted. Moreover, subjects who were receiving any hormonal or steroids therapy were also excluded.

Inclusion Criteria for Female Subjects:

Fertile females:

All healthy females between the ages of 18 - 35 years, with a child less than 2 years of age from all ethnic groups were recruited as controls.

Infertile females:

All females who fulfill the criteria of primary infertility (never conceived in last more than one years) between the ages of 18 - 35 years, from all ethnic backgrounds, were enrolled in the study as cases.

Exclusion criteria for Female subjects:

The females who were diagnosed with secondary infertility, being treated with oral contraceptive pills, having thyroid disorders, preexisting diabetes and hypertension were excluded from the study.

Clinical Data Collection: The clinical data including age, height, weight, blood pressure, menstrual/obstetric and gynecological history with general physical examination was recorded in all study subjects. The height in centimeters (converted to meters) and weight in kilograms of all the recruited subjects were noted to calculate the body mass index (BMI), and categorized consulting cutoffs for Asians, where 18 - 22.9 kg/m² was normal weight, overweight 23 - 24.9 kg/m² was overweight and BMI ≥ 25 kg/m² was considered obese (WHO 2004). Gender, age and height of every participant was entered manually into the BIA machine by a digital keyboard, and it immediately revealed the percentage fat mass (% FM) of the individual (Lazzer, Boirie et al. 2003)

Biochemical Measurement

Serum samples were used to detect the hormones levels using commercially available Enzyme Linked Immuno Sorbent Assay (ELISA) kits, following the manufacturer's protocol. Follicle stimulating hormone (FSH) by Human FSH Enzyme Immunoassay (Kit Cat. No DKO010; DiaMetra), LH by Human LH Enzyme Immunoassay (Kit Cat. No DKO010; Dia Metra). Immunoassay for

FSH, the inter assay coefficient of variation, <8% and intra assay coefficient of variation, <9.7% and similarly LH immunoassay, the inter assay coefficient of variation was <7.91%; intra assay coefficient of variation was <9.21%. Serum KP was measured by ELISA kit (Cat. No: 95611, Glory BioScience, USA). The analytical sensitivity was 10.16 ng/L and intra and inter assay coefficients of variation was less than 10% and 12%, respectively. For assessing total testosterone levels commercially available Human Total Testosterone (TT) immune-enzymatic kit for serum analysis was utilized (Cat. No DKO002 by Diametra).

Genotype Characterization:

Blood Sampling and genotyping:

Ten ml of venous blood was collected form all study subjects. DNA was isolated from the leukocytes in the peripheral blood of the study subjects using a DNA isolation kit (Genomic DNA Purification Kit Cat. No A1125 by Promega, USA). The isolated DNA was quantified by measuring the ultraviolet (UV) absorbance and determining the absorbance ratio (A280/A260) for 2 µ L samples, employing a Nanodrop-ND1000 (Thermo Fisher Scientific, Waltham, MA). Extracted DNA was considered pure at an absorbance ratio of ~1.8. Furthermore, gel electrophoresis was run to visualize the PCR products on 5µL of sample in 2% agarose gel against a 100bp ladder on approximately 15% of all samples for confirmation. Gel was observed in Gel Doc Imaging system (Biorad, United Kingdom) (Figure 1a & b).

For exon 2: Polymerase chain reaction (PCR) was executed using the 2X PCR Hotstart Master Mix (Cat# G906, ABM (Applied Biological Materials Inc, Canada) according to the instructions mentioned on the provided manual. The cycle conditions during PCR were: 1 cycle for 5 min at 95 °C for initial denaturation

followed by 40 cycles at 95 °C for 20 seconds, 65°C for 15 seconds s, 72 °C for 15 seconds, followed by a final extension of 1 min at 72 °C.

For exon 3: PCR was executed employing the Go Taq (R) Hotstart Green Master mix (Cat #M5122, Promega Corporation, USA) according to the instructions mentioned on the provided manual. The cycle conditions during PCR were: 1 cycle for 5 min at 95 °C for initial denaturation followed by 35 cycles at 95 °C for 30 s, 58°C for 45 s, 72 °C for 45 s followed by a final extension of 10 min at 72 °C.

Purification of the PCR products was done using PCR Clean Up for DNA Sequencing (Cat. No BT5100, Bio Basic Inc, Canada) following the manufacturer protocol. Genotypic analysis was performed to detect mutations responsible for infertility by PCR amplification of the fertile and infertile male (n=80) and female subjects (n=88) within the region of exon 2 (214bp) and exon 3 (606bp) of KISS1 gene. The obtained sequences were directly compared to previously published *KISS1* gene sequence using the MEGABLAST search tool in the National Center for Biotechnology Information (NCBI) database. Sequence files were imported into Chromas Lite, and then assembled using Molecular Evolutionary Genetic Analysis version 6.0.

All coding exons (exons 2 and 3) of KISS1 gene were PCR amplified with specific primers as follows:

	Forward primer (5´ to 3´)	**Reverse primer (3´ to 5´)**
exon 2	CAGATCCTGTGCCTGACCT A	CCCACTCCTTTCCCCAGAG

exon 3	ATGGGATGACAGGAGGTGTTG	ACCATCCATTGAGGATGGAAG

Statistical Analysis: Statistical analyses were performed using IBM Statistical Package for the Social Sciences (IBM SPSS version 21; IBM Corp Inc, Armonk, NY). Continuous variables (such as age, LH, FSH etc.) were represented as Mean ± standard deviation and the evaluation of the categorical variables (BMI) was expressed in terms of frequencies and percentages. To compare continuous and categorical variables, Independent sample t-test and Pearson's chi square test was applied. Correlations were adjusted for age and BMI for hormonal associations (logistic regression). SNP data was calculated by chi-squared statistics, Odds ratio with 95% confidence interval was calculated for genotype and allele frequency analysis.

Results:

The results of the study showed that infertile males were obese and were at a lower age bracket as compared to fertile group ($p<0.001$). Furthermore, the infertile females also demonstrated a higher BMI as compared to the fertile females ($p<0.001$). The sperm count and motility was decreased along with increased abnormal morphology in the infertile males as compared to the fertile males ($p<0.01$) (Table 1). A similar trend in terms of hormonal profile was observed in the infertile female subjects; however, no difference for age was recorded (Table 1). Comparison of hormones in Table 2 show significantly high levels of KP, FSH, LH and Testosterone in fertile male, however KP, LH and Estradiol levels were significantly higher in fertile female subjects with low FSH levels. When tested for the correlations of Kisspeptin levels on the male hormones of hypothalamo-hypophyseal-gonotrophic axis; FSH showed

moderate positive relationship with KP levels (r=0.67; p=<0.001), while Testosterone (r=0.38; p=<0.01) showed weak correlation with fertility in all subjects. Similarly, KP showed weak positive correlation with Estradiol (r=0.466; p=0.001) while no relationship was observed with LH and FSH. All correlations were lost when adjusted for age and BMI (p>0.05).

Table 3 shows the genotype distribution of the polymorphic EXON 2 and 3 sequences. This study was able to identify one polymorphism in exon2 [E1225K (G/A 3673)] and 3 unique polymorphisms in exon3 [P1945A (C/G 5833); Insertion of T at 6075; G2026G (C/G 6078)] in the study population. Interestingly, these polymorphisms were observed in higher frequency in infertile group (both genders) versus the fertile; yet the presence or absence of polymorphic site in both exons of interest failed to reveal any significant difference in this study population (p>0.05).

This study documented one novel result in the study cohort. In a sample of n=80 males and n= 88 females; 03 subjects in each sex group tested positive for all 03 of the polymorphisms in exon-3 region. When their data was linked with the polymorphic status; it was observed that 04 out of 06 individuals (male female combined) were smokers with low KP and its related hormonal levels at various points during the study. Furthermore, their sperm parameters fell in the abnormal category (males) or attempt at Intracytoplasmic Sperm injection (ICSI) was unsuccessful (females) (Table 4 and 5). This result shows a probable effect of environmental changes on genetic alterations in the KP gene and its secondary effect on the reproductive axis.

Discussion:

The relationship of KP with the interruption of hypothalamic-pituitary-gonadal axis can be demonstrated by high concordance of the phenotypes between comparable genetic variants present in GnRH receptor, FSH and its receptor, LH and its receptor in mice and humans (Rehman, Jamil et al. 2015). In male subjects, we have observed low levels of KP with concomitant decrease in gonadotropin and sex steroid hormone levels, which is comparable to studies in male infertile subjects(Haris Ramzan, Ramzan et al. 2015). This can probably be an explanation of KP role in preservation of spermatogenesis and hence fertility. Low FSH levels was observed in normozoospermic and azospermic infertile male subjects by Ramzan et al which was not significantly different in fertile and infertile males (Haris Ramzan, Ramzan et al. 2015). The significant low FSH levels in infertile male subjects of our study may be explained by lack of stratification of subjects into infertile categories on the basis of sperm parameters. In the female infertile subjects a high FSH explains the negative feedback interplay on HPG axis due to decrease in Estradiol secretion. Literature also supports a raised FSH in infertile females (Prasad, Parmar et al. 2015).

Kisspeptin is now safely and successfully used in both healthy and infertile human subjects after trials in United Kingdom, and it is possible that in the future the Kisspeptin signaling may be used as a target in the treatment of reproductive disorders (Hameed, Jayasena et al. 2011).

The role of Kisspeptin injections to stimulate the secretion of LH and FSH in numerous mammalian species including rats, mice, sheep, cows and monkeys is supportive of our statement (Navarro, Castellano et al. 2005). Furthermore, a study done by Dhillo et al 2015, documents that KP infusion significantly increased plasma LH, FSH, and testosterone levels (Clarke, Dhillo et al. 2015)

289 is supportive of our study in which infertile study subjects had low KP, LH,
290 FSH and testosterone in male subjects. The correlation of KP with estradiol in
291 both genders explains that KP requires estradiol secretion to stimulate GnRH
292 secretion as suggested by the study in which ovariectomy abolished the KP-
293 induced GnRH release in pubertal monkeys, and estradiol replacement resulted
294 in partial recovery of KP- induced GnRH release (Guerriero, Keen et al. 2012).
295 Kisspeptin represents and should be investigated in the treatment of fertility
296 disorders characterized by low gonadotropins or anovulation. (Clarke, Dhillo et
297 al. 2015)

298 Higher KP in non-obese males and non-obese young females were observed in
299 our study, which might be due to the fact that KP effects negatively on body
300 weight and calorie consumption (Walewski, Ge et al. 2014, Stengel 2011 #214,
301 (Lin, 2015 #216). BMI and body fat % age were noted to be high in infertile
302 groups while KP was low which is perhaps due to certain changes in the sex
303 hormones which regulates obesity by increasing the serum triglycerides levels
304 (Shamai, Lurix et al. 2011, Kołodziejski, Pruszyńska-Oszmałek et al. 2018). It
305 is a well-established fact that there are various mechanisms how obesity causes
306 infertility (Talmor and Dunphy 2015).There is an ample evidence that KP
307 signaling tends to decrease the metabolic rate and initiates glucose intolerance
308 and increased body fat (Holmes 2014).

309 In this study, no clear-cut difference was observed in the genetic mutations
310 amongst fertile and infertile males and female subjects. Three unique *KISS1*
311 mutations were identified all together in unrelated subject in each gender
312 category. The absence of this variant in the fertile female group suggests that
313 this is a rare mutation which has a major qualitative effect on the KISS gene.

However only 1 fertile male tested positive for this mutation; when we checked the paternal age and status of fertility in the last 5 years, it was identified that this gentleman was 33-year-old and had a baby 4 years ago. Perhaps this substitution mutation is related to the age group and environmental or stress related changes. There are examples in literature where same SNPs resulted in diverse expressions. As an example in Chinese population a SNP of amino acid substitution (P110T) in KISS1 in females with CPP was found to be statistically interconnected to infertility whereas in Korean girls the same mutation had a protective effect on infertility (Luan, Zhou et al. 2007) (Ko, Lee et al. 2010). Yet, no data is available from the current literature to support this claim. Therefore, more work is required to assess the functionally or causality of this mutation with infertility.

In terms of mutations in exon 3, we found no link of the SNP's with infertility. These mutations i.e. insertion of Thiamine at position 6075 and substitution of Proline to Alanine at position 1945 may be explained as due to faulty gene regulation process related with age, since all these individuals were in the age range of 34 to 48 year. Interestingly, in the fertile group with these mutations; one female had delivered a baby within last 7 months. This finding suggests that silent mutations at these positions, does not affect the functional role of Kisspeptin protein. The study is limited in terms of being a uni-centric study with a small sample size in which the impact of polymorphism in infertile females on the basis of cause of infertility has not been taken into consideration. Furthermore, the association of gene variation of KISS1 could have been further validated in terms of impact on different altered sperm parameters.

However, this is the first study in this region that has attempted to explore a cause effect relationship of *Kiss1* gene variation with hormones of reproduction and impact on fertility status in both male and female infertile subjects.

Conclusion: Role of KP in regulation of normal reproductive functions can be explained on the basis of its effect on secretion of gonadotropins and sex steroids. Polymorphism in exon2 [E1225K (G/A 3673)] and 3 unique polymorphisms in exon3 [P1945A (C/G 5833); Insertion of T at 6075; G2026G (C/G 6078)] can be further explored as plausible cause of decreased KP production in infertile male and female subjects. Further detailed studies are warranted for understanding of the mechanistic role of genetic variations of KP in infertility.

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