Original Article

Insight into the Serum Kisspeptin Levels in Infertile Males

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Abstract

Background: Regulation of reproduction is now considered to be carried out by the kisspeptin and its receptor, GPR54 or Kiss1r. Mutations of either *Kiss1* or *Kiss1r* in humans and mice result in profound hypogonadotropic hypogonadism. The present study was aimed to determine whether the levels of kisspeptin are associated with male infertility.

Methodology: The study involved 176 male subjects aged 18 – 50 years including 26 fertile and 150 infertile. Infertile subjects were further subdivided according to WHO guidelines of semen analysis into 22 asthenozoospermia, 08 asthenoteratozoospermia, 18 azoospermia, 58 normozoospermia, 06 oligozoospermia, 12 oligoasthenozoospermia and 26 oligoasthenoteratozoospermia. Thorough clinical examinations excluded those suffering from chronic health problems. Serum kisspeptin levels were measured by enzyme immunoassay (EIA) and follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were estimated by chemiluminescence assay (CLIA).

Results: The results of the present study have revealed that kisspeptin levels were significantly lower in all infertile males as compared to the fertile males. Significantly low LH and testosterone levels were observed in all infertile groups as compared to fertile group. FSH levels were significantly lower in normozoospermic and azoospermic as compared to fertile males, while no significant difference was observed between the other infertile and fertile group.

Conclusion: The study revealed that serum kisspeptin levels were observed significantly lower in the infertile as compared to fertile males, indicating that the kisspeptin might be associated with the fertility problems in males.

Keywords: FSH, kisspeptin, LH, male infertility, testosterone

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Introduction

Infertility is regarded as a social problem amongst all cultures and societies. It affects about 10% – 15% couples of reproductive age. Male infertility is directly or indirectly responsible for the 60% of cases involving the reproductive-age couples with fertility related issues.[1] The hormone based treatment for infertility work through the manipulation of the hypothalamic–pituitary–gonadal (HPG) axis at the level of gonadotropin releasing hormone (GnRH) or below. The discovery of *Kiss1* in 1996 and the successive identification of the kisspeptin receptor (previously known as G-protein-coupled receptor 54, GPR54) added a new dimension to our understanding of the physiology of the HPG axis, reproduction and fertility.[2,3]

The *Kiss1* gene encodes a 145 amino acid protein that is cleaved to produce a 54 amino acid peptide called kisspeptin, which possesses a distinct RF-amide motif (Arg-Phe-NH_2) in its C-terminal region. Shorter fragments (*e.g.* kisspeptin-14, kisspeptin-13, and kisspeptin-10) of kisspeptin-54 which are generated by further cleavage of the preprohormone, also bind to GPR54.[4–6]

Kisspeptin-54 was originally identified as a metastasis suppressor peptide hence named metastin.[4–6] Later on, dysfunctional or deletional mutations in the gene encoding for the G protein-coupled receptor, GPR54 were shown to be the cause of hypogonadotropic hypogonadism. Hypogonadotropic hypogonadism is a condition which is characterized by absent or delayed spontaneous pubertal maturation and of reproductive function due to the deficiency of pituitary secretions of gonadotropic hormones in humans and mice.[7,8] This finding unveiled the previously unsuspected reproductive dimension of this signaling system, as a key player in the regulation of the gonadotropic axis.[7,8] Further support for these essential reproductive functions came from the demonstration that mice engineered to lack functional Gpr54 or *Kiss1* genes were a complete phenocopy of the affected humans,[8,9] thus demonstrating the conserved roles of kisspeptins in the control of the HPG axis in mammals. Neurons that express kisspeptin are present in those areas of the hypothalamus, which are involved in the regulation of the gonadotropin secretion; the arcuate nucleus (Arc), the periventricular nucleus (PeN), and the anteroventral periventricular nucleus (AVPV) in mice.[10,11] In addition to their prominent expression at hypothalamic levels, evidences suggest that *Kiss1* and/or *Kiss1r* mRNAs or proteins are also present in several peripheral reproductive tissues including the ovary,[12,13] the testes[5] and the spermatozoa.[14] Intraperitoneal chronic administration of the kisspeptin has been shown to cause the dose dependent degeneration of the testicular tissue, seminal vesicles and the prostate gland.[15–17] In support of a role for kisspeptin-GPR54 signaling as a gatekeeper of puberty, prolonged activation of *Kiss1r* signaling due to a gain of function mutation has been described by Teles and his colleagues in an 8-year old girl who presented with

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idiopathic central precocious puberty.[18] In addition, it has been shown that the serum kisspeptin levels were significantly higher in girls with the central precocious puberty and with premature thelarche than in age matched prepubertal control.[19–21] It has also been demonstrated that systemic administration of kisspeptin-54 can acutely increase circulating levels of LH, FSH, and testosterone release in human males.[22]

In the last, however, the serum kisspeptin levels in the girls with central precocious puberty and premature thelarche are available, but data on the kisspeptin levels in infertile and their age matched fertile males are lacking. The present study was aimed to determine whether the levels of kisspeptin are associated with male infertility.

Materials and Methods

The study included 176 male subjects aged 18 – 50 years. Among 176 male subjects 26 were fertile (proven fathers) and 150 were infertile (whose post marital interval was more than one year and had failed to procreate during the last one year of regular unprotected sexual intercourse). Infertile subjects were further subdivided into 22 asthenozoospermia, 08 asthenoteratozoospermia, 18 azoospermia, 58 normozoospermia, 06 oligozoospermia, 12 oligoasthenozoospermia and 26 oligoasthenoteratozoospermia. This division of infertile subjects into different groups was based strictly on the semen analysis according to the nomenclature of the WHO Laboratory manual for the Examination and Processing of Human Semen (2010). All the subjects were subjected to thorough clinical examination to exclude those suffering from the chronic health problems (tuberculosis, asthma, liver / renal disease, hypertension, severe obesity and diabetes). Among the 150 subjects with fertility problems; 32 were affected by varicocele, 16 from nonobstructive azoospermia, 10 hypergonadotropic hypo-gonadism, 53 had hypogonadotropic hypogonadism and 39 from idiopathic infertility.

The study was approved by the Institutional Review Board (IRB) and Advanced Studies and Research Board (ASRB), Khyber Medical University, Peshawar, Pakistan. Written informed consent was obtained from the subjects and the participation in the study was voluntary. Fertile and infertile subjects were recruited from the two private clinics in Dera Ismail Khan, Khyber Pakhtunkhwa (KPK), Pakistan. The semen samples were collected by masturbation after a sexual abstinence of at least three days and were analyzed for different parameters at the Bilal Clinical Laboratory according to the WHO guidelines. Non-fasting venous blood samples were collected from the subjects using disposable sterile syringes. Serum was separated through centrifugation at 1600×*g* and stored at -80°C for subsequent hormonal analysis.

Follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were quantitatively determined using chemiluminescence assay (CLIA) kits manufactured by Monobind Inc. USA according to manufacturer's instruction.

The kisspeptin was determined quantitatively by using enzyme immunoassay (EIA) that has been described previously [20,21,23,24] using KiSS–1 (68–121) Amide / Metastin (1–54) Amide / Kisspeptin 54 (Human) (Catalog # EK–048–59; Phoenix Pharmaceuticals, Inc., Burlingame, California, USA) according to the manufacturer's instructions and the absorbance was read at 450 nm. The kit showed 100% cross reactivity with kisspeptin 54.The minimum detectable concentration was 0.12 ηg/ml. The intra-assay variation and inter-assay variation was < 6% and < 9% respectively.

Statistical analysis

Results are presented as mean ± SEM. The obtained results were compiled and analyzed by Statistical Package for Social Sciences (SPSS, version 16, Inc, Chicago, Illinois, USA) using one way ANOVA followed by post hoc Tukey's test. The difference was considered statistically significant at $P < 0.05$.

Results

Mean age (years) and BMI (kg/m^2) of fertile and their age matched infertile subjects are listed in Table 1. Mean age (years) and BMI (kg/m^2) did not differ significantly in fertile and all infertile groups.

Sperm motility and sperm morphology of fertile and infertile subjects are shown in Table 2. Sperm motility was observed significantly lower in all infertile subgroups except oligozoospermic as compared to fertile ones. Normal sperm forms were observed significantly lower in all infertile groups as compared to fertile group. The results of our study revealed that head and midpiece malformations were observed significantly lower in all infertile subgroups except oligozoospermic as compared to fertile males. Tail defects showed no significant difference between the fertile and all infertile males.

Kisspeptin levels (ng/ml) are presented in the Figure 1. In fertile subjects, kisspeptin levels were observed significantly higher ($P < 0.001$) [23.32 (11.08 – 36.55)], as compared with infertile normozoospermic [6.37 (1.01 – 11.49)], azoospermic [4.41 (2.69 – 6.82)], asthenozoospermic [5.34 (1.67 – 8.98)], asthenoteratozoospermic [4.41 (2.49 – 5.78)], oligozoospermic [3.43 (1.58 – 4.48)], oligoasthenozoospermic [4.72 (0.98 – 10.16)] and oligoas-

Table 1. Mean age (years) and BMI (kg/m^2) of fertile and infertile normozoospermic (NZ), azoospermic (AZO), asthenozoospermic (AZ), asthenoteratozoospermic (ATZ), oligozoospermic (OZ), oligoasthenozoospermic (OAZ) and oligoasthenoteratozoospermic (OATZ) males.

Parameter	Fertile (n=26)	Infertile (n = 150)						
		NZ (n=58)	AZO (n=18)	AZ (n=22)	ATZ (n=08)	OZ (n=06)	OAZ (n=12)	OATZ (n=26)
Age (years)	33.23±1.14	30.24±0.753	34.89±1.582	31.64±1.214	26.75±1.858	32.67±2.35	31±1.435	31.08±1.227
BMI (kg/m^2)	24.98±0.647	23.36±0.356	23.20±1.001	25.32±0.603	23.49±0.877	24.77±1.452	24.06±1.075	24.58±0.875

Values = Mean ± SEM

Table 2. Sperm motility and sperm morphology of fertile and infertile normozoospermic (NZ), azoospermic (AZO), asthenozoospermic (AZ), asthenoteratozoospermic (ATZ), oligozoospermic (OZ), oligoasthenozoospermic (OAZ) and oligoasthenoteratozoospermic (OATZ) males.

Parameter	Fertile (n = 26)	Infertile (n = 150)						
		NZ (n = 58)	AZO (n = 18)	AZ (n = 22)	ATZ (n = 08)	OZ (n = 06)	OAZ (n = 12)	OATZ (n = 26)
Total Sperm Motility	73.92±8.06	64.83±13.92*	0.00	28.27±10.96***	32.5±19.09***	70±8.94	38.33±12.67***	14.38±13.83***
Progressive Motility	64.77±6.72	50.86±12.07***	0.00	14.18±10.03***	17.5±11.02***	53.33±6.83^{NS}	15.83±7.02***	0.54±1.42***
Normal Form	77.46±9.84	60.97±15.42***	0.00	28.64±11.77***	2.75±0.46***	55±11.83***	19.17±7.64***	1.31±1.35***
Abnormal Head	11.08±5.58	18.38±6.97***	0.00	29.55±7.71***	38.75±7.91***	20±0.01^{NS}	36.67±11.55***	47.85±12.34***
Abnormal Midpiece	7.31±3.78	14.72±8.94*	0.00	30.91±6.84***	35±12.54***	16.67±9.31^{NS}	25.83±8.75***	33.23±13.44***
Abnormal Tail	4.15±3.73	6.45±3.73^{NS}	0.00	9.55±4.06^{NS}	11.25±5.83^{NS}	10±4.47^{NS}	21.67±15.28^{NS}	16.77±19.67^{NS}

Values = Mean ± SD; NS represents statistically non-significant as compared to fertile; *P< 0.05, **P< 0.01, ***P< 0.001 compared to fertile

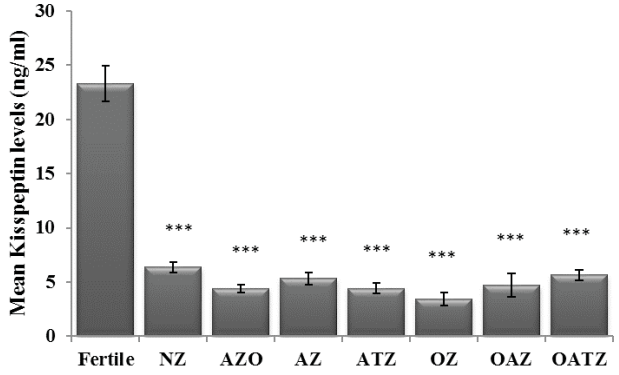

Figure 1. Mean kisspeptin levels (ng/ml) of fertile and infertile normozoospermic (NZ), azoospermic (AZO), asthenozoospermic (AZ), asthenoteratozoospermic (ATZ), oligozoospermic (OZ), oligoasthenozoospermic (OAZ) and oligoasthenoteratozoospermic (OATZ) male (Values are expressed as mean ± SEM. ***P < 0.001 compared to fertile).

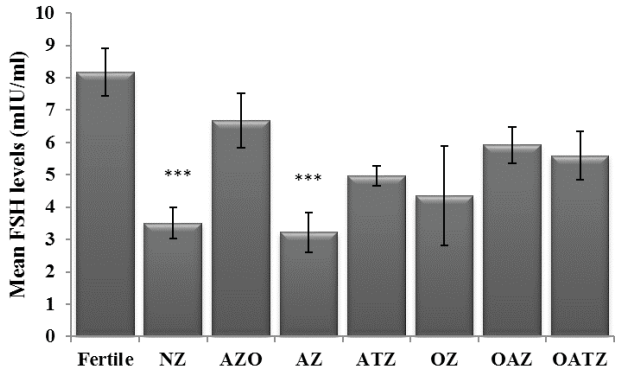

Figure 2. Mean follicular stimulating hormone (FSH) levels (mIU/ml) of fertile and infertile normozoospermic (NZ), azoospermic (AZO), asthenozoospermic (AZ), asthenoteratozoospermic (ATZ), oligozoospermic (OZ), oligoasthenozoospermic (OAZ) and oligoasthenoteratozoospermic (OATZ) male (Values are expressed as mean ± SEM. ***P < 0.001 compared to fertile).

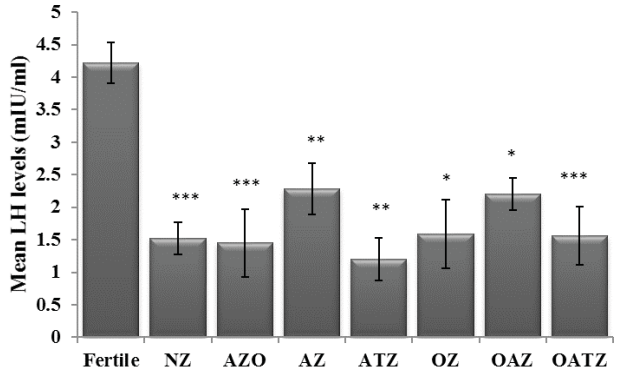

Figure 3. Mean luteinizing hormone (LH) levels (mIU/ml) of fertile and infertile normozoospermic (NZ), azoospermic (AZO), asthenozoospermic (AZ), asthenoteratozoospermic (ATZ), oligozoospermic (OZ), oligoasthenozoospermic (OAZ) and oligoasthenoteratozoospermic (OATZ) male (Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared to fertile).

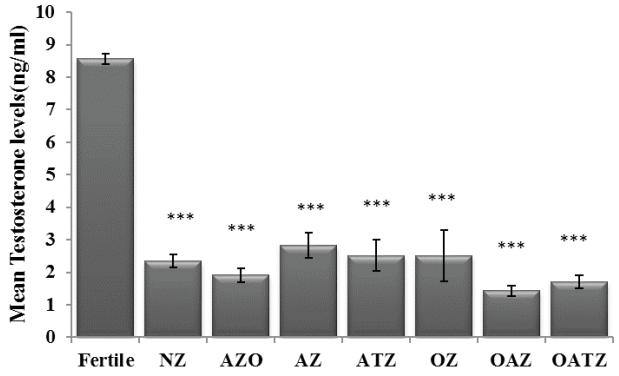

Figure 4. Mean testosterone levels (ng/ml) of fertile and infertile normozoospermic (NZ), azoospermic (AZO), asthenozoospermic (AZ), asthenoteratozoospermic (ATZ), oligozoospermic (OZ), oligoasthenozoospermic (OAZ) and oligoasthenoteratozoospermic (OATZ) male (Values are expressed as mean ± SEM. ***P < 0.001 compared to fertile).

thenoteratozoospermic [5.67 (1.41 – 8.77)] males.

Serum follicle stimulating hormone (FSH) levels (mIU/ml) are shown Figure 2. FSH levels were found significantly lowered (P < 0.001) in infertile normozoospermic (NZ) [3.51 (0.09 – 13.20)] and azoospermic [3.22 (0.09 – 20.9)] as compared to fertile males [8.17 (3.40 – 13.90)], while no significant difference (P > 0.05) was observed between the infertile asthenozoospermic [6.68 (2.20 – 13.70)], asthenoteratozoospermic [4.97 (3.90 – 6.0)], oligozoospermic [4.35 (0.40 – 8.80)], oligoasthenozoospermic [5.92 (2.60 – 17.4)] and oligoasthenoteratozoospermic [5.59 (1.20 – 21.6)],

compared to fertile males [8.17 (3.40 – 13.90)].

Luteinizing hormone (LH) levels (mIU/ml) are outlined in Figure 3. Significantly low serum luteinizing hormone (LH) levels were noticed in asthenozoospermic [2.28 (0.02 – 5.20)] and asthenoteratozoospermic [1.20 (0.30 – 2.30)] ($P < 0.01$), oligoasthenozoospermic [2.20 (0.60 – 3.10)] and oligozoospermic [1.76 (0.09 – 4.60)] ($P < 0.05$), normozoospermic [1.52 (0.02 – 7.20)], azoospermic [1.45 (0.04 – 10.50)] and oligoasthenoteratozoospermic [1.56 (0.04 – 13.50)] ($P < 0.001$), compared to fertile males [4.22 (2.10 – 7.10)].

Serum testosterone levels (ng/ml) are summarized in Figure 4. Testosterone levels were observed significantly lower ($P < 0.001$) in infertile normozoospermic [2.34 (0.70 – 7.80)], azoospermic [1.91(0.70 – 3.20), asthenozoospermic [2.82 (0.30 – 6.90)], asthenoteratozoospermic [2.52 (0.90 – 4.50)], oligozoospermic [2.50 (0.70 – 4.90)], oligoasthenozoospermic [1.43 (0.90 – 2.40)] and oligoasthenoteratozoospermic males [1.70 (0.04 – 3.0)], as compared to fertile males [8.57 (7.30 – 9.80)].

Discussion

Kisspeptins, a family of neuropeptides encoded by the *Kiss1* gene, were identified in 2001 as natural ligands of the previously orphan G protein-coupled receptor, GPR54. They are mainly expressed in discrete neuronal populations of the hypothalamus and have recently been emerged as an essential regulator of GnRH (gonadotropin-releasing hormone) neurons and, hence, are potent stimulators of gonadotropin secretion. Initially the known biologic function of the kisspeptin was to suppress the tumor metastasis.[25] Later on it was reported that mutations in the *Kiss1* or *Kiss1r* gene were shown to be the cause of hypogonadotropic hypogonadismin both males[7,8] and females.[26] Infact kisspeptins are now regarded as the key players in the different aspects of the maturation and functioning of the reproductive axis, which include the sexual differentiation of the brain, puberty timing, regulation of secretion of gonadotropins and gonadal hormones, as well as control of fertility.[25]

Several studies were carried out to determine the role of kisspeptin in the regulation of the reproductive axis. However, information regarding the kisspeptin concentration in infertile males was lacking. This is the first study to disclose the deficiency of the serum kisspeptin levels in infertile males; therefore comparison cannot be made with other ones.

We hypothesized that the serum kisspeptin concentrations might be decreased in infertile males as compared to the fertile males. To this end, we designed the study and determined the serum levels of kisspeptin in infertile males; whose post marital interval was more than one year, and their age matched fertile counterparts.

The results of the present study demonstrated that levels of kisspeptin, LH and testosterone were observed significantly lower in all infertile groups as compared to the fertile group (Figure 1, 3 and 4 respectively). On the other hand serum FSH levels were noted significantly lower in normozoospermic and azoospermic as compared to fertile males and no significant difference was observed between the other infertile groups and fertile group as shown in Figure 2.

Determination of kisspeptin levels in humans has revealed it to be significantly higher in girls with central precocious puberty and those with premature thelarche than in their age matched controls.[19–21] Recently, it has also been shown that the plasma kisspeptin, LH and FSH levels of the fertile (control) males are significantly increased than males with isolated hypogonadotropic hypogonadism.[27] Furthur, these studies has revealed that there is significant elevation of serum kisspeptin; LH, FSH and testosterone in response to intravenous infusion of kisspeptin – 54 in human males.[22] The results of these studies and other similar literatures provide ample evidence that the kisspeptin has a central role in the control of reproduction and is critical for the normal development and maintenance of the reproductive axis. Our results are in agreement with this observation that the kisspeptin levels were significantly lower in all the infertile groups as compared to the fertile males. It has been reported that the *Kiss1* mRNAs are excessively expressed in hypothalamus in addition to testis. *Kiss1* mRNAs are also reported to be expressed in several non-reproductive tissues like stomach, small intestine, thymus, spleen, lung and kidneys. However, the level of mRNA expression in non-hypothalamic tissues are lower than that of the hypothalamus[4,5] and contributions of the peripheral tissues to the serum kisspeptin levels is insignificant. Therefore, it is assumed that the serum kisspeptin might be coming from hypothalamus.

Kisspeptin is a powerful stimulator of LH and FSH release, both after intracerebral and systemic administration of the peptide.[28–31] The sensitivity of LH release to the stimulatory effect of kisspeptin is manifold high[10,31] as compared to the sensitivity of FSH release in response to kisspeptin.[30] Further to this, kisspeptin also potentially increase the serum testosterone levels in normal males.[22] A number of studies revealed that severe testicular degeneration and desensitization of the HPG axis is caused by the continuous intraperitoneal kisspeptin administration to prepubertal rats, resulting in decreased testosterone concentration by the testis. This decrease in testosterone concentration is likely to be due to down regulation of LH secretion with kisspeptin treatment.[15] These finding is in agreement with our findings which showed that serum LH and testosterone levels were significantly lower in all infertile groups than fertile group while the FSH is lower significantly in normozoospermic and asthenozoospermic males as compared to fertile and did not significantly differ between the other infertile groups and fertile males. It has been previously reported that the serum levels of both LH and FSH are higher,[32–34] lower,[35] or unmodified [36] in infertile and azoospermic males as compared to normal males. On the other hand, no such change was observed in the serum level of FSH in oligozoospermic males relative to that of fertile ones.[37,38] It is for certain that spermatogenesis is assessed by sperm counts, motilities, and morphologies. Spermatogenesis is reinitiated and maintained at normal levels in men by introducing human chorionic gonadotropin (hCG) to stimulate Leydig cell function. This also restores the intratesticular testosterone concentration with undetectable FSH levels in blood after short-term suppression of exogenous testosterone, while using the FSH alone could partly restore the sperm output.[39,40] In addition, it has been shown that high levels of the intratesticular testosterone levels, secreted by the Leydig cells are necessary for spermatogenesis. Inside the Sertoli cells, testosterone selectively binds to the androgen receptor and leads to the activation and maintenance of spermatogenesis and inhibition of germ cell apoptosis while the action of FSH minimally serves to promote spermatogenic output by increasing the number of Sertoli cells.[41,42]

Kisspeptin might be a key contributory factor in the control of testosterone, FSH and LH levels in males. This study provides a link between the kisspeptin levels and male reproductive axis

depending on the fertility status of the subjects. The study showed that serum kisspeptin levels were significantly lower in the infertile as compared to fertile males therefore; infertility in these subjects might be due to the deficient release of kisspeptin.

As the concentration of kisspeptin is significantly lowered in infertile males than the fertile controls, it might be used as a diagnostic tool for infertility and treatment of infertility disorders. Further studies on *Kiss1* gene polymorphisms leading to an increased risk of suppression of kisspeptin are also needed.

Author Contributions

MHR perceived the idea; designed the study, conducted the work and drafted the manuscript. MS supervised the study. MR co-supervised the study. FW helped in study design. FR was instrumental in statistical analysis. MJ edited the manuscript. Semen analysis was carried out by MAK. All the authors have read and approved the manuscript.

Competing Interests

The authors have no conflict of interest of intellectual or financial nature with any individual or institution.

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