

ORIGINAL RESEARCH

Metabolic markers in male infertility: a pilot study

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Abstract

Male infertility is an emerging health problem and there is an urgent need to search for therapeutic options. Altered metabolic states such as obesity and metabolic syndrome have been linked with male infertility. Therefore, searching for metabolic biomarkers is vital. Present work aimed to study metabolic alterations in infertile males. The seminal levels of L-Carnitine (LC), Kisspeptin (Kiss1) and Ghrelin (GHRL) were evaluated in 14 infertile and 10 fertile males. Baseline semen analysis was performed for both cases and controls. The levels of LC and Kiss1 were found to be lower in infertile patients as compared to fertile controls and the difference was found to be statistically significant. There was no significant difference observed in GHRL levels between patients and controls. The study presents a preliminary data where LC and Kiss1 levels could possibly be studied further for their association with male infertility. But to confirm their association with pathogenesis of male infertility, future studies need to be conducted in larger cohorts.

Keywords

L-carnitine (LC); Assisted reproductive technology; Male infertility; Intra cytoplasmic sperm injection (ICSI); Ghrelin (GHRL); Kisspeptin (Kiss1)

1. Introduction

Infertility is now considered a global health issue. It is defined as the inability to conceive even after one year of unprotected sexual intercourse [1]. Both male and female factors are almost equally contributing the global burden of infertility cases. Overall infertility affects approximately 10–15% of couples.

Male infertility is mostly related to seminal quality or sperm parameters [2]. Seminal parameters get altered in different pathological states such as viral infections, surgical conditions *etc.* [3, 4]. Infertility incidence varies in primary and secondary cause and, among developing and underdeveloped countries. Parameters such as age, obstetrical history, smoking and alcohol intake, body mass index (BMI), lifestyle and environmental factors have been considered the major risk factors to infertility. Altered metabolic states such as obesity and metabolic syndrome have been linked with male infertility. Studies have shown that both overweight and low BMI conditions impact reproductive function [5–7].

L-Carnitine (LC), Kisspeptin (Kiss1) and Ghrelin (GHRL) are the common metabolic hormones regulating reproductive functions [8–10]. Studies have shown that LC along with its acetylated form, acetyl L carnitine function as an antiapoptotic agent, antioxidant and fatty acid transporter in the process of beta oxidation. LC is considered an essential molecule for gamete health [11]. It has also been shown that seminal LC levels usually correlate with the semen quality [12].

Kisspeptin system consists of cleavage products of the Kiss1 precursor and the kisspeptin receptor (Kiss1R).

Initially, observed in cancer metastasis research, the discovery of *KISS1/KISS1R* gene mutations leading to hypogonadotropic hypogonadism revealed its effects on reproductive function. Presently, kisspeptin serves as the central regulator of the reproductive axis during puberty and adulthood, also exerting a broad functional influence on endocrine system. Kiss1 and Kiss1R are also expressed in the testes; however, understanding of the pathways of kisspeptin signaling during spermatogenesis are lacking [13, 14].

Ghrelin, a gut hormone was initially linked to feeding behavior and energy balance, but recent research has shown its significant involvement in a wide range of biological functions such as motivation, memory, vascular function, neuroprotection following brain injury, stress response and reproductive regulation [15].

Infertility and metabolic syndrome have been seen to share common risk factors. Reactive oxygen species (ROS) leading to oxidative stress, endothelial dysfunction, and altered semen parameters might contribute to metabolic abnormality and reproductive dysfunction in infertile males [5]. Therefore, the current research aimed to study association between metabolic markers and fertility status in males.

2. Material & methods

24 Men aged 25–45 years were recruited from infertility clinic (Obstetrics & Gynaecology, AIIMS, New Delhi, India) out of which 14 were infertile (cases) and 10 were fertile males (controls). Infertile males having at least single subnormal semen

parameter with no live birth, no genital infection, endocrinological disorders (hypothyroidism, hypogonadotropic hypogonadism, diabetes mellitus *etc.*), genital tract obstruction, varicocele or known chromosomal aneuploidy/Yq microdeletion were selected as cases. Sperm concentration <15 million/mL, vitality <54%, total motility <40%, morphology <4% were considered subnormal. The female partners of these males were normal based on clinical and biochemical assessment. Fertile normozoospermic males having at least one live birth with no genital tract abnormality, endocrinological disorder were selected as controls. Fresh semen sample was collected from both cases and controls. Routine semen analysis was done, and seminal plasma was separated to assess LC, Kiss1 and GHRL levels.

2.1 LC assay

Liquefied semen sample (without any dilution) was centrifuged at 2000 rpm for 10 minutes. The supernatant was transferred into microcentrifuge tube and stored at -20°C till further use. A master mix was made for samples and standard mixes. Carnitine standards were prepared with concentrations ranging from 0–10 nmole according as per manufacturer instructions (Sigma Aldrich MAK063, 3050 Spruce Street, Saint Louis, MO 63103, USA) for colorimetric detection. The well plate was incubated for 30 minutes at room temperature with gentle shaking. The absorbance was measured at 570 nm with a multiwell plate reader (TECAN, InfiniteM200 PRO, Hombrechtikon Zurich (canton), Switzerland). The absorbance measurement of the blank (0 nmole) was subtracted from all the other individual standard and sample measurements. The standard curve was plotted and then used to determine the LC concentration of each seminal plasma sample.

2.2 ELISA for the detection of seminal Kiss1 and GHRL levels

Seminal plasma levels of Kiss1 and GHRL (without any dilution) were measured using the commercially available ELISA kits, namely Human Kiss1 (Kisspeptin1) ELISA Kit (ITLK04058; Immunotag, 9800 Page Avenue, Saint Louis, MO 63132-1429, USA) and Human GHRL (Ghrelin) ELISA kit (ITLK01943; Immunotag, 9800 Page Avenue, Saint Louis, MO 63132-1429, USA) respectively. ELISA assays were performed according to the manufacturers' protocols. The detection ranges of Kiss1 and GHRL were 31.25–2000 pg/mL and 156.25–10,000 pg/mL respectively.

2.3 Statistical analysis

The levels of LC, Kiss1 and GHRL between cases and controls were compared using unpaired student *t*-test with Welch's correction using a version 8.0.1 program of GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). *p* value < 0.05 was considered statistically significant.

3. Results

3.1 Semen parameters

Semen analysis was performed for patients and controls according to the World Health Organisation (WHO) manual 2021. Sperm motility, vitality, morphology (Kruger's criteria) and concentration were assessed. Semen parameters were found normal in all 10 controls. In 6 out of 14 infertile cases, all semen parameters were found normal whereas remaining 8 cases showed subnormal values in one or more parameters (Table 1). The separated seminal plasma was further used for biochemical evaluation. Each experiment of all the assays were performed in duplicates and repeated twice.

TABLE 1. Semen parameters in controls and cases.

Controls, Age (yr)	Motility (%)	Vitality (%)	Sperm conc. ($10^6/\text{mL}$)	Morphology (%)	Cases, Age (yr)	Motility (%)	Vitality (%)	Sperm conc. ($10^6/\text{mL}$)	Morphology (%)
Control A (29)	55%	61%	37	34%	Case A (27)	65%	69%	72	13%
Control B (37)	73%	74%	133	29%	Case B (29)	65%	71%	4	2%
Control C (41)	60%	77%	122	23%	Case C (31)	36%	47%	214	37%
Control D (38)	70%	77%	123	31%	Case D (33)	60%	61%	110	23%
Control E (28)	66%	71%	118	27%	Case E (25)	23%	41%	6	7%
Control F (44)	68%	71%	57	33%	Case F (38)	57%	68%	124	14%
Control G (33)	73%	74%	135	32%	Case G (41)	48%	61%	15	8%
Control H (32)	51%	61%	204	35%	Case H (34)	58%	67%	6	5%
Control I (28)	77%	79%	128	27%	Case I (26)	70%	86%	148	17%
Control J (36)	81%	84%	119	35%	Case J (31)	64%	65%	12	4%
					Case K (42)	71%	78%	92	3%
					Case L (39)	57%	59%	98	6%
					Case M (37)	46%	50%	121	5%
					Case N (34)	58%	62%	114	11%
Mean	67%	73%	117.60	31%		56%	63%	81.14	11%
Standard Deviation	0.09	0.07	42.82	0.04		0.13	0.11	62.30	0.092

Sperm conc.: sperm concentration.

The participants had a normal BMI and none of them had any metabolic disorder.

The difference in sperm motility, vitality and morphology between cases and controls was found to be statistically significant ($p = 0.0193$, $p = 0.0223$ and $p < 0.0001$ respectively) (Fig. 1). The difference in sperm concentration in cases and controls was not statistically significant.

3.2 L-Carnitine levels

LC assay showed increased levels of seminal plasma LC in controls as compared to infertile patients and the difference was found to be statistically significant ($p < 0.0001$) (Table 2, Fig. 2). On comparing seminal LC concentration between infertile patients with subnormal and normal semen parameters the difference was not statistically significant ($p = 0.65$) (Table 3, Fig. 3).

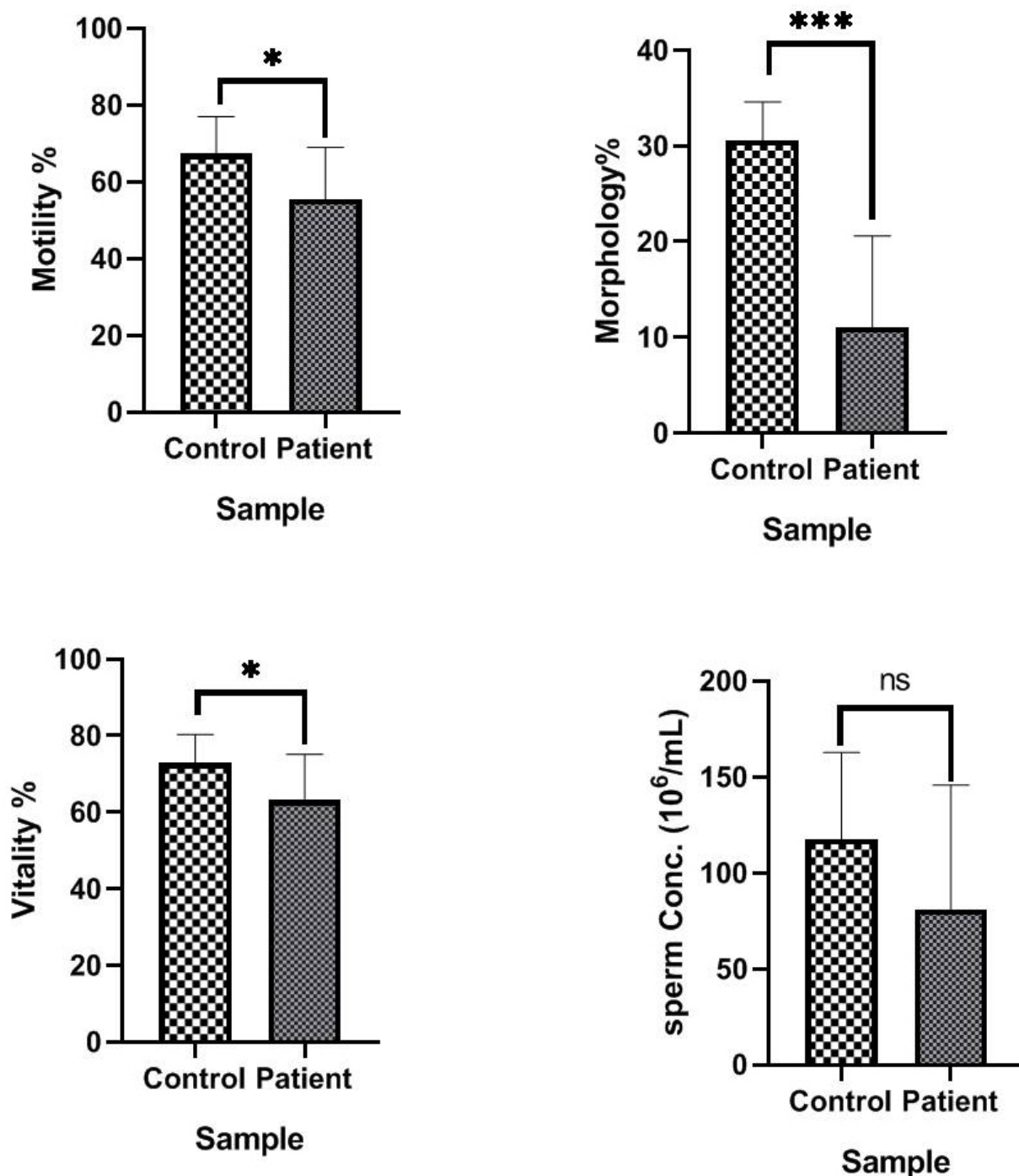


FIGURE 1. Comparison of semen parameters of controls and patients/cases. (Motility with p value = 0.0193*, Vitality with p value = 0.0223*, Sperm concentration with p value = 0.1180 (ns: not significant) and morphology with p value < 0.0001***).

TABLE 2. LC concentration in infertile patients and controls.

Patient ID (control)	Seminal plasma LC levels (mM) in controls	Patient ID (Infertile patients)	Seminal plasma LC levels (mM) in cases	
Control A	0.24	Case A	0.22	
Control B	0.28	Case B	0.04	
Control C	0.32	Case C	0.13	
Control D	0.37	Case D	0.02	
Control E	0.32	Case E	0.24	
Control F	0.25	Case F	0.04	
Control G	0.23	Case G	0.04	
Control H	0.31	Case H	0.01	
Control I	0.25	Case I	0.01	
Control J	0.30	Case J	0.23	
		Case K	0.02	
		Case L	0.01	
		Case M	0.22	
		Case N	0.24	
(Mean \pm SEM)	0.28 \pm 0.13	(Mean \pm SEM)	0.08 \pm 0.02	<i>p</i> value < 0.0001

SEM: Standard Error of the Mean.

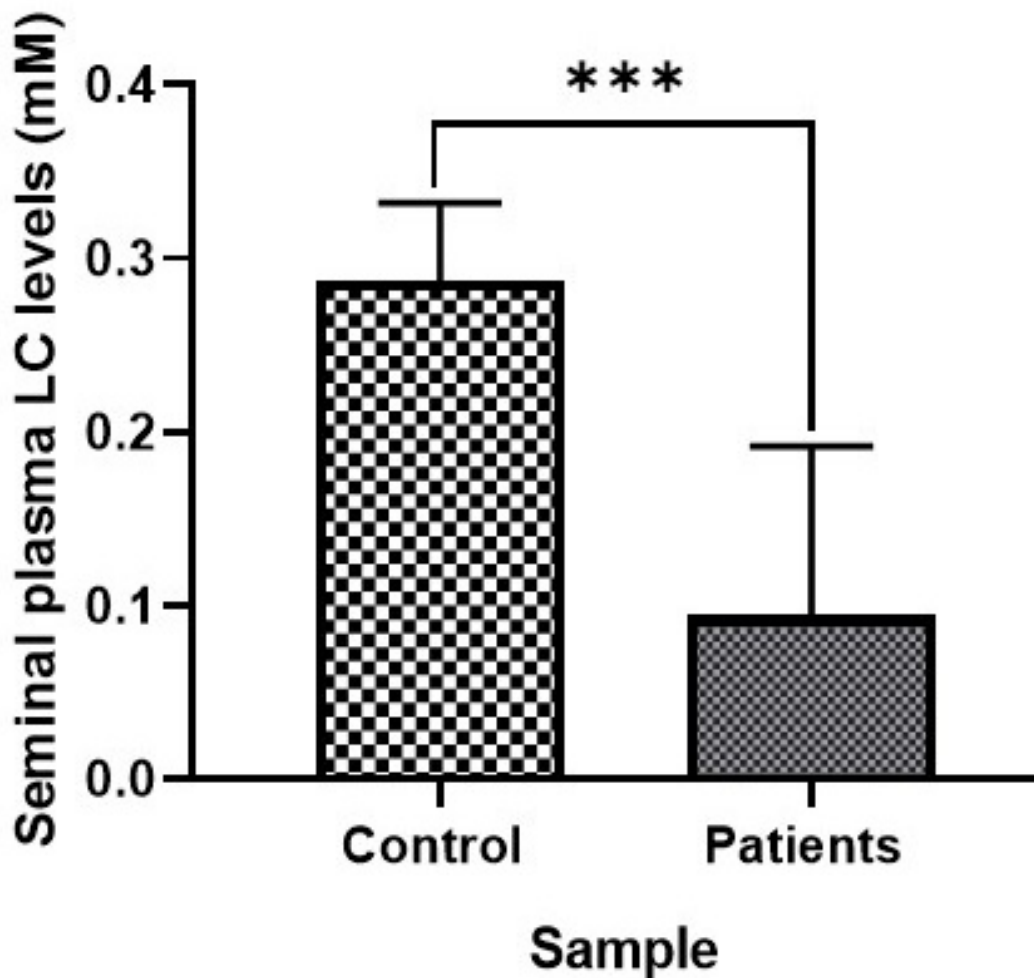


FIGURE 2. L carnitine (LC) concentration in seminal plasma of control & patients. The levels of LC between patients and controls were compared using unpaired *t*-test. *p* value < 0.05 was taken as significant (***) *p* < 0.0001).

TABLE 3. Comparison of LC levels between patients with normal and subnormal parameters.

Infertile men with subnormal parameters (n = 8) (Mean ± SEM)	Infertile men with normal parameters (n = 6) (Mean ± SEM)	<i>p</i> value
(0.11 ± 0.03)	(0.09 ± 0.04)	0.65

SEM: Standard Error of the Mean.

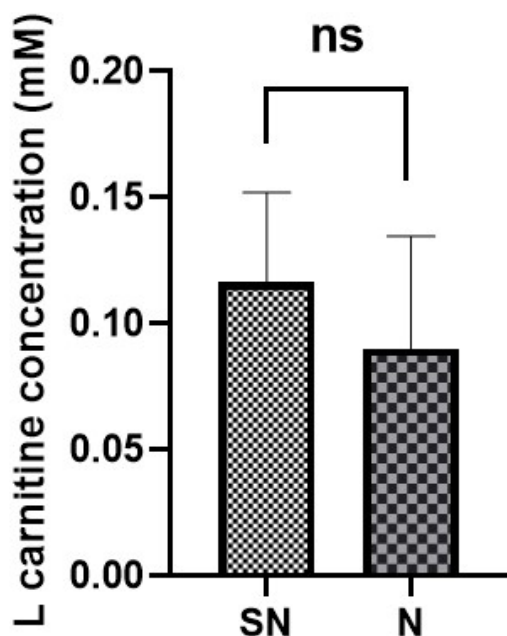


FIGURE 3. Comparison in LC concentration between infertile patients with subnormal. (SN) & normal (N) semen parameters ($p = 0.65$). ns: not significant.

3.3 Kisspeptin levels

Kisspeptin (Kiss1) ELISA assay showed increased levels of seminal plasma Kiss1 in controls as compared to infertile cases and the difference found to be statistically significant ($p < 0.0001$) (Table 4). There was no statistically significant difference of Kiss1 concentration among infertile patients with subnormal and normal parameters ($p = 0.81$) (Table 5, Figs. 4,5).

3.4 Ghrelin levels

Ghrelin (GHRL) ELISA assay showed no significant difference in seminal plasma GHRL in controls as compared to infertile patients ($p = 0.71$) (Table 6). Among infertile patients with subnormal and normal parameters there was no statistically significant difference in GHRL levels ($p = 0.55$). The results obtained are shown in the Table 7 and (Figs. 6,7).

4. Discussion

There has been constant need for biomarkers predicting causes of male infertility. Recent research has shown multiple biomarkers predicting infertility status in males [16, 17]. Results of present study showed that 60% infertile males

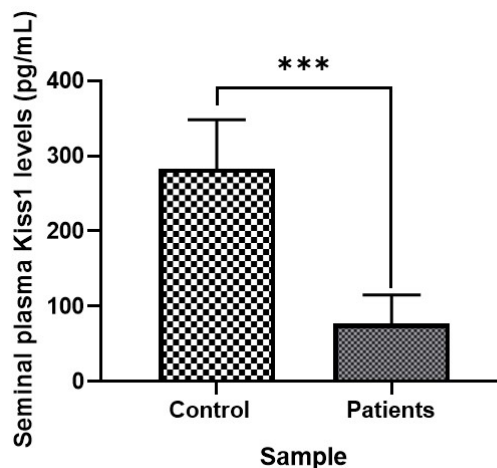


FIGURE 4. Kiss1 concentration in seminal plasma of control & patients. The levels of Kiss1 between Infertile men with normal and subnormal semen parameters were compared using unpaired *t*-test. p value < 0.05 was taken as significant (***) ($p < 0.0001$).

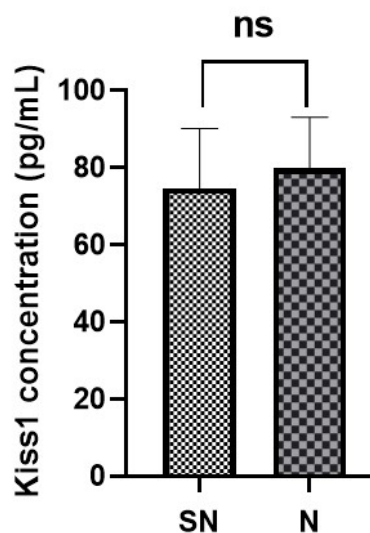


FIGURE 5. Comparison of Kiss1 concentration between infertile patients with subnormal. (SN) and normal (N) semen parameters ($p = 0.81$). ns: not significant.

had normal semen parameters. Remaining 40% cases had one or more subnormal parameters. On comparing the LC levels, cases showed lower concentrations of LC as compared to controls and the difference was statistically significant. Similar study was also carried out in oligozoospermic and

TABLE 4. Kisspeptin levels in cases and controls.

Patient ID (control)	Seminal plasma Kiss1 levels (pg/mL) in controls	Patient ID (Infertile/cases men)	Seminal plasma Kiss1 levels (pg/mL) in cases	
Control A	346	Case A	87	
Control B	398	Case B	149	
Control C	335	Case C	109	
Control D	248	Case D	62	
Control E	198	Case E	88	
Control F	206	Case F	117	
Control G	254	Case G	25	
Control H	256	Case H	55	
Control I	267	Case I	45	
Control J	325	Case J	28	
		Case K	43	
		Case L	50	
		Case M	100	
		Case N	118	
(Mean ± SEM)	283.30 ± 20.50	(Mean ± SEM)	76.94 ± 10.21	<i>p</i> value < 0.0001
Intra CV (n = 10)	0.015 (1.5%)	Intra CV (n = 14)	0.074 (7.4%)	
Average % CV		Average % CV		

CV: Coefficient of variation; SEM: Standard Error of the Mean.

TABLE 5. Comparison of Kiss1 levels between infertile patients with normal and subnormal parameters.

Infertile men with subnormal parameters (n = 8)	Infertile men with normal parameters (n = 6)	<i>p</i> value
(Mean ± SEM)	(Mean ± SEM)	
(74.63 ± 14.55)	(79.83 ± 12.14)	0.81

SEM: Standard Error of the Mean.

TABLE 6. Comparison of GHRL levels between infertile patients and controls.

Patient ID (control)	Seminal plasma GHRL levels (pg/mL) in controls	Patient ID (Infertile/cases men)	Seminal plasma GHRL levels (pg/mL) in cases	
Control A	2145.67	Case A	2244.09	
Control B	2037.4	Case B	2214.56	
Control C	2283.46	Case C	1820.86	
Control D	1820.86	Case D	1919.29	
Control E	1712.6	Case E	2135.82	
Control F	2076.77	Case F	2047.24	
Control G	2194.88	Case G	2125.98	
Control H	2155.51	Case H	1919.29	
Control I	2244.09	Case I	1909.45	
Control J	1870.08	Case J	2155.51	
		Case K	2214.56	
		Case L	2037.40	
		Case M	2204.72	
		Case N	2155.51	
(Mean ± SEM)	2054 ± 60.82	(Mean ± SEM)	2079 ± 36.77	<i>p</i> value = 0.71
Intra CV (n = 10)	0.025 (2.5%)	Intra CV (n = 14)	0.0189 (1.9%)	
Average % CV		Average % CV		

CV: Coefficient of variation; SEM: Standard Error of the Mean.

TABLE 7. Comparison of GHRL levels between cases with normal and subnormal parameters.

Infertile men with subnormal parameters (n = 8) (Mean ± SEM)	Infertile men with normal parameters (n = 6) (Mean ± SEM)	p value
(2099 ± 48.86)	(2052 ± 48.81)	0.55

SEM: Standard Error of the Mean.

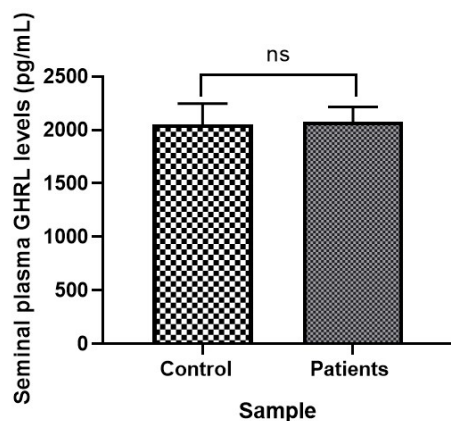


FIGURE 6. GHRL concentration in seminal plasma of control & infertile patients ($p = 0.71$). ns: not significant.

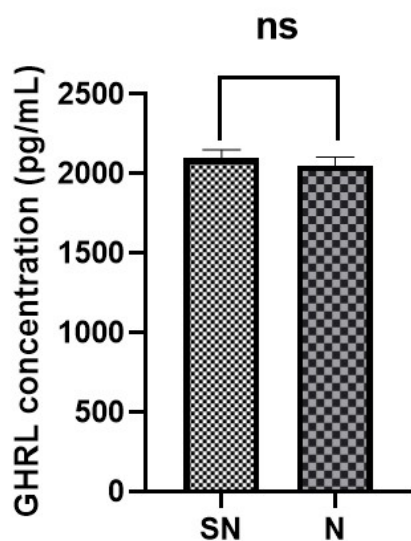


FIGURE 7. Comparison of GHRL concentration between infertile patients with subnormal. (SN) and normal (N) semen parameters. The levels of GHRL between Infertile men with normal and subnormal semen parameters were compared using unpaired t -test. p value < 0.05 was taken as significant ($p = 0.55$). ns: not significant.

azoospermic humans. The levels of LC in both the types of infertile samples was significantly lower than the control samples [18]. Present study showed no significant difference in LC levels of infertile group with normal and subnormal semen parameters. This result was in contrast to a study that showed LC levels were positively correlated with

semen parameters [19]. This difference can be attributed to overall smaller number of cases ($n = 8$ and $n = 6$ in each group). L-carnitine plays a crucial role in sperm function and maturation within the male reproductive tract, particularly in the epididymis. It acts as a carrier molecule, facilitating the transport of long-chain fatty acids into the mitochondria, where they undergo beta-oxidation to generate energy. This energy production is vital for the motility and viability of spermatozoa during their journey through the female reproductive tract. Furthermore, L-carnitine has been shown to exert antioxidant properties, protecting sperm from oxidative stress-induced damage, which is essential for maintaining sperm quality and DNA integrity [8].

The results of Kiss1 ELISA assay showed lower concentrations of Kiss1 in patients as compared to controls and the difference was statistically significant. Few other studies showed that Kiss1 levels were positively correlated with semen parameters [20]. Kisspeptin, a neuropeptide encoded by the KISS1 gene, has emerged as a key regulator of reproductive function, including sperm maturation and function. It acts on the hypothalamus to stimulate the release of gonadotropin-releasing hormone (GnRH), which in turn governs the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland. Studies have suggested that kisspeptin plays a direct role in sperm maturation and function through its effects on the testes and epididymis [21].

Our study showed no statistical difference in GHRL levels between patients and controls. Panidis *et al.* [22], 2008 also showed that there is no difference in seminal plasma ghrelin levels between men with normospermia and dyspermia.

Other studies have shown that GHRL improves integrity of sperm membrane, improved sperm motility and concentration. It was suggested that this ameliorative effect of ghrelin might be due to its antioxidant effects on sperm plasma membrane [23]. It was also shown that GHRL receptors (GHSRs-1a) were expressed in the spermatids of rats. This suggests that GHRL plays a role in intracellular signaling in spermatozoa that is required for motility [24]. In our study cases showed higher concentrations of GHRL as compared to controls though the difference was not statistically significant. This can be attributed to a smaller cohort used in the study.

5. Conclusions

Present study provides a preliminary data showing association of various metabolic markers with male infertility. Biomarkers that can suggest diagnostic as well as therapeutic options are vital to explore. As the numbers of cases were less in the present study therefore correlation among markers and reproductive status was not done. However, results indicated the possible association of metabolic markers with infertile cases.

This possibly indicates that metabolic system may emerge as a valuable indicator and focus for addressing male fertility challenges in the coming years.

AVAILABILITY OF DATA AND MATERIALS

The data underlying the findings of this article will be shared on reasonable request to the corresponding author.

AUTHOR CONTRIBUTIONS

MS and KS—designed the research study. KS—performed the research; wrote the manuscript. AH—provided help and advice on experiments. MS—analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol of the study was approved by the Institute PG Ethics Committee (IECPG-30/27.02.2020) and written consent was taken from all cases and controls.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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