Original Article

Detection of Lactoferrin and Iron in Seminal Plasma and Their Possible Relation to Semen Parameters and Infertility in Varicocele: A Cross-sectional Study

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Abstract

Background: Male infertility emerges as an important cause during the evaluation of infertile couples. Varicocele is a well-known cause of male infertility. The role of seminal lactoferrin, an iron-binding glycoprotein, in male fertility is unclear and needs further investigation. Recently, it has been linked to some sperm abnormalities and subfertility. **Aim:** This study aims to detect seminal lactoferrin levels in infertile men with varicocele and their relation to semen parameters and fertility status. We investigated a possible link between seminal lactoferrin and iron. **Settings and Design:** A cross-sectional study was conducted through the outpatient clinic. **Materials and Methods:** Seventy-five individuals were included in 3 groups (33 infertile men with varicocele, 25 infertile men without varicocele and 17 fertile participants without varicocele). Conventional semen analysis was conducted, and seminal plasma was obtained in all groups to detect lactoferrin and iron levels. **Statistical Analysis Used:** Statistical analysis was performed by SPSS version 24. **Results:** In infertile men with varicocele, seminal lactoferrin (155.92 ± 8.4 ng/ml, P = 0.296) and iron levels (260.71 ± 38.3 µg/dl, P = 0.409) were not significantly different from other groups. There was a positive correlation between seminal lactoferrin, iron levels and sperm concentrations and counts. Seminal iron and lactoferrin were significant independent predictors of sperm concentration. A negative correlation was reported between seminal lactoferrin levels and age. Lactoferrin in seminal plasma was not correlated with seminal iron. **Conclusion:** Infertile men with varicocele have seminal lactoferrin levels comparable to other infertile men and possibly fertile individuals. Iron concentrations are not linked to lactoferrin levels in seminal plasma.

Keywords: *Infertility, iron, lactoferrin, semen analysis, varicocele*

Introduction

Approximately, 15% of couples (48.5 million couples) are affected by infertility worldwide.[1,2] During infertility evaluation, a relevant male factor is present in more than one-third of couples.[3] Male factor infertility is thus important, with many aspects involved. The research is ongoing to determine different variables that affect sperm functions.[4]

Unilateral/bilateral varicocele is a known frequent risk factor for male infertility; it can cause direct or indirect damage to spermatogenesis and sperm functions.[5] The prevalence of varicocele in the general adult population is about 15%. For infertile males, it rises to 40% in the case of primary infertility and 80% in the secondary type.[6-8]

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The proposed pathogenetic mechanisms for reduced fertility in varicocele are decreased sperm counts, motility and normal morphology, increased oxidative stress levels, abnormal DNA fragmentation (up to 8 times more than the healthy individuals), raised fractions of necrotic and apoptotic sperms as well as reduced volume of corresponding testes.[9,10]

Semen analysis is the standard test for diagnosis of male infertility. Seminal plasma encloses important molecules from male reproductive glands that have crucial rules in sperm functions. It is therefore considered an invaluable resource for male fertility.[4]

Lactoferrin is an iron-binding globular glycoprotein with immunomodulatory actions, mainly at the mucosal level.[11] Lactoferrin has an essential role in iron absorption regulation and immune reaction modulation. In addition, it possesses antimicrobial, antioxidant, anti-inflammatory and anticancer properties. Lactoferrin is present in blood plasma and different exocrine fluids.[12,13] Three isoforms are identified for lactoferrin. Lactoferrin-α is the form that binds iron, whereas isoforms-β and -γ have ribonuclease activity and are not iron binding.[14] The essential biological actions of lactoferrin are exerted through receptor interaction with the target cells. Lactoferrin receptors are present in several cell types and tissues.[15]

In humans, seminal lactoferrin is secreted by seminal vesicles and the prostate. However, the exact physiological role of lactoferrin in the male reproductive system is not yet fully understood.[13] In general, it can guard against male genital tract infections and control sperm iron levels. Lactoferrin thus affects sperm quality and seems to be a potential good marker for it.[11,16] The effect of lactoferrin on semen parameters has been investigated in a few studies, mainly animal studies.[17] Previously, seminal lactoferrin was found associated with abnormal sperm concentration, morphology and motility in humans.[11,16]

Iron is an essential trace nutrient that plays a vital role in general health and fertility. In addition, it is one of the trace elements present in semen. Iron is highly toxic if accumulated in large quantities. Either its excess or deficiency can be hazardous to spermatogenesis with fertility impairment.[18] Alternatively, iron acts as a cofactor for the catalase enzyme that induces hydrogen peroxide decomposition. Hydrogen peroxide is one of the most toxic oxidising agents for human spermatozoa, thus its breakdown is mandatory for protection against its toxic effects.[19] Previous research that studied the impact of iron on sperm functions and male infertility has shown contradictory results. Some studies reported the association of seminal iron with abnormal sperm morphology and motility.[19,20] On the other hand, seminal iron was found to be significantly raised in normozoospermic men.[21]

Seminal lactoferrin has been studied sparsely in humans; previous reports have linked it to some sperm abnormalities and subfertility. Varicocele is a well-known cause of impaired male fertility. Thus, the current research aimed for the first time to assess seminal lactoferrin levels in varicocele and a possible relation between seminal lactoferrin and iron levels.

Materials and Methods

A cross-sectional, hospital-based study was conducted from June 2022 to July 2023. A sample of 75 male participants who attended the andrology outpatient clinic of the dermatology, venereology and andrology department was included. The current research was carried out after the principles of Good Clinical Practice and the Declaration of Helsinki. The study design was approved by the Institutional Ethics and Research Committee (N: IRB17101622). All study participants were requested to give written informed consent for inclusion. The participants were assigned into 3 groups: Group I involved 33 infertile men with varicocele, Group II involved 25 infertile men without varicocele as an infertile control group and Group III involved 17 fertile participants with a history of childbirth within the previous year to ensure fertility and without a history of varicocele as a control group.

The current study's inclusion criteria were as follows: age ranges from 20 to 50 years of age and clinical as well as scrotal Doppler ultrasonographic diagnosis of varicocele. Exclusion criteria were as follows: infertile men who had normal semen analysis with history/evidence of female factor infertility and concurrent or previous use of iron/lactoferrin in the last 3 months from all groups. Men were excluded from Group I if there was a history/evidence of genital tract infection, hormonal/chromosomal abnormalities, chronic debilitating diseases/systemic diseases affecting fertility (including diabetes), hormonal treatment or chemotherapy, severe oligozoospermia less than 5×10^6 per ml or azoospermia.

Each participant was subjected to a detailed clinical history and examination. Clinical history especially verified fertility, sexual and medical histories. Varicocele laterality and severity grades were detected by genital examination for Group I. Scrotal Doppler ultrasonography was performed for all participants; for diagnosis and grading severity of varicocele in Group I (I: mild, reflux <2 s, II: moderate, reflux >2 s and III: severe, spontaneous reflex increasing

with Valsalva),[22] and for exclusion of varicocele in Group II and III.

Conventional semen analysis was performed for all participants according to the WHO laboratory manual.[23] Fresh semen samples were collected by masturbation in a sterile plastic container after 2–7 days of sexual abstinence. Participants were instructed to collect all semen and report any semen loss. The specimens were analysed within 1 h of collection after complete liquefaction at 37°C. Evaluation of physical properties included semen volume, colour, odour, pH, liquefaction time and viscosity. Microscopic evaluation was done to report the following: sperm concentration and total sperm count by examining 20 µL of liquified semen using a conventional blood haemocytometer, progressive and total motility using the 3 grades system (progressive, non-progressive and immotile), morphology by Spermac stain to detect abnormal sperm forms and peroxidase test if round cell count >10/HPF.

After completion of semen analysis, the specimens were centrifuged at 3000 rpm for 20 min to obtain seminal plasma. The seminal plasma was transferred to sterile tubes and stored at −20°C. Seminal lactoferrin levels were measured by ELISA technique (Human Lactoferrin [Lf] enzyme-linked immunosorbent assay Kit, SinoGeneClon Biotech Co., Ltd, catalog no SG-10383). Furthermore, seminal iron levels were measured by the colorimetric CAB (Chromazurol B Single Reagent) method (SPECTRUM kit REF: 269 003).

Sample size calculation

Sample size was calculated using G*Power 3 software[24] (Heinrich Heine University Düsseldorf, Düsseldorf, North Rhine-Westphalia, Germany), with a power of 80% and type I error of 5% ($\alpha = 0.05$ and $\beta = 80\%$) on one-tailed test; the minimum required sample was 51; the individuals were divided into three groups: each includes 17 participants to detect an effect size of 0.5[16] in the mean seminal lactoferrin level (13.3 ± 1.6 vs. 13.4 ± 2.1 and 11.2 ± 1.4 mg/100 ml).

Statistical analysis

Data analysis was performed using SPSS version 24 (IBM_SPSS, Statistical Package for Social Science, SPSS Inc., NY, USA). Descriptive statistics: means, standard errors and percentages were calculated. Shapiro–Wilk was used to test the normality of continuous variables. Test of significances: Chi-square test/Fisher's exact/Monte Carlo exact test was performed to compare the proportion differences in the baseline and clinical data among study groups. Furthermore, the McNemar test was used to compare the differences between right- and left-sided varicocele grades by Doppler ultrasonography in the varicocele group, independent sample *t*-test to test the mean differences in lactoferrin and iron concentrations between smoker and non-smoker groups and one-way analysis of variance for comparing seminal lactoferrin and iron levels and semen parameters between the three study groups. Spearman Ranked correlation test was calculated for the correlation between seminal lactoferrin and iron levels and different semen parameters. Multivariable linear regression analysis was used to detect independent predictors of sperm concentration. $P < 0.05$ was considered statistically significant.

Results

This cross-sectional study included 75 participants: 33 men with infertility and varicocele (Group I), 25 men having infertility without varicocele (Group II) and 17 fertile individuals without varicocele as controls (Group III). The mean age of infertile men without varicocele was 36.1 ± 5.6 years. This was significantly older than the mean age of infertile men with varicocele (31.7 ± 5.7, $P = 0.002$) and the fertile group (29.9 ± 4.3, $P < 0.001$). On the other hand, the distribution of groups according to residence, occupation or smoking status was comparable without statistical significance [Supplementary Table 1].

Regarding the differences in some clinical data between the study groups [Supplementary Table 2], it was noticed that the infertility duration for Group II (infertility without varicocele) was significantly longer (5.8 ± 0.8 years) than for Group I (infertility with varicocele, 3.55 ± 0.5 years) ($P = 0.012$). In addition, Group II had more previous assisted reproductive technology (ART) trials (7 males, 28%) than Group I (1 male, 3%) with a significant difference ($P = 0.002$). Insignificant differences were detected between infertile groups regarding infertility type, drug history or surgical history for infertility (other than ART). By scrotal Doppler ultrasound in the varicocele group [Supplementary Table 3], it was found that 22 males (66.7%) had bilateral varicocele and 11 males (33.3%) had unilateral left-sided varicocele. There was a significantly higher occurrence of moderate and severe varicocele on the left side (96.9%) compared to the right side (15.2%) ($P < 0.001$).

Regarding semen analysis parameters [Table 1], it was observed that Group III (fertile males) had significantly higher mean sperm concentration ($P = 0.002$) and total count ($P = 0.038$), percentage of progressive and total motility ($P = 0.008$ and 0.003, respectively), as well as the percentage of normal sperm forms ($P = 0.002$) than

Table 1: Semen parameters among different groups

Semen parameters	Group I (*n*=33)	Group II (*n*=25)	Group III (*n*=17)	*P**
Semen volume (mL), mean±SE	2.98±0.3	2.62±0.4	3.13±0.4	0.601
Sperm concentration (million/mL), mean±SE	30.55±3.1	26.74±4.5	48.06±3.7	0.002
*P***	I versus II=0.456	II versus III=0.001	I versus III=0.003	
Total sperm count (million/ejaculate), mean±SE	97.52±16.5	77.33±19.3	142.51±27.9	0.038
*P***	I versus II=0.411	II versus III=0.007	I versus III=0.037	
Progressive motility%, mean±SE	34.33±5.1	33.46±4.3	46.76±8.6	0.008
*P***	I versus II=0.819	II versus III=0.006	I versus III=0.005	
Total motility%, mean±SE	55.79±16.5	49.85±13.5	69.12±4.1	0.003
*P***	I versus II=0.221	II versus III=0.001	I versus III=0.011	
Normal form%, mean±SE	3.22±0.4	3.24±0.6	5.78±0.5	0.002
*P***	I versus II=0.970	II versus III=0.003	I versus III=0.001	

*ANOVA test was used to compare the difference in mean between groups, ***Post-hoc* test with Tukey's correction was used for pairwise comparisons. SE=Standard error

Group I and Group II. On the other hand, insignificant differences were detected in these parameters between infertile males with varicocele and those without varicocele. The predominant sperm abnormal forms were not different between Group I and Group II in a significant way.

Seminal lactoferrin and iron levels were measured in the three groups [Table 2]. In the varicocele group, the mean seminal levels of lactoferrin (155.92 ± 8.4 ng/ml) and iron (260.71 ± 38.3 µg/dl) did not show significant differences from other groups (P = 0.296 and 0.409, respectively). When the total study sample was divided according to smoking status [Table 2], mean seminal lactoferrin (156.24 ± 8.7 versus 163.92 ± 8.3 ng/ml) and iron levels (215.77 ± 21.9 vs. 251.34 ± 38.5 µg/dl) also did not show significant differences between smoker and non-smoker groups (P = 0.523 and P = 0.401, respectively).

The correlation between seminal levels of lactoferrin, iron, age and semen parameters in the total sample was performed [Table 3]. A significant negative correlation was detected between seminal lactoferrin levels only and participant ages (r = −0.306, P < 0.001). Furthermore, a significant positive correlation was found between lactoferrin levels and both sperm concentrations (r = 0.280, P = 0.008) and total sperm counts (r = 0.310, P = 0.003). There was a significant positive correlation between iron levels and both sperm concentrations (r = 0.374, P < 0.001) and total sperm counts (r = 0.264, P = 0.011). Notably, an insignificant correlation was observed between seminal lactoferrin and iron levels in the whole study sample (r = −0.081, P = 0.264) [Figure 1]. When the same correlations were performed in the varicocele group [Table 3], similar findings were observed as in the total sample. However, a barely significant positive correlation was detected between seminal lactoferrin levels and sperm concentrations (r = 0.265, P = 0.068) [Figure 2].

By multivariable linear regression analysis of the total sample [Table 4], after adjusting for age and smoking status, seminal iron and lactoferrin levels were independently associated with sperm concentration, i.e., a 1-µg/dl increase in seminal iron was correlated with a 34% (16%–56%, P = 0.017) increase in sperm concentration. Likewise, a 1-ng/ml increase in seminal lactoferrin was correlated with a 78% (19%–94%, P = 0.047) increase in sperm concentration.

Discussion

Lactoferrin was observed to be abundantly present in human semen plasma as well as on the surface of sperms.[25] After secretion, it adheres to the head of sperms and other molecules,[26] Wang *et al*.[25] detected the presence of lactoferrin receptor in human sperms, localised to the sperm head and mid-piece, and binds to lactoferrin in a saturable manner. The mean seminal lactoferrin levels in our participants showed insignificant differences between the 3 study groups. We observed a significant positive correlation between lactoferrin levels and both sperm concentration and total sperm count in the total sample. Furthermore, a positive correlation was found for lactoferrin with total sperm count in the varicocele group. Likewise, seminal lactoferrin levels were detected as a significant independent predictor of sperm concentrations in the total sample.

In the literature, few studies are available on human seminal lactoferrin with no previous research studying seminal lactoferrin in varicocele. Inconsistent with our results, a recent study of 39 semen samples of infertile males has demonstrated a significant negative correlation between seminal lactoferrin expression assessed by cytofluorimetry and sperm concentration and morphology.[11] In addition, in the study of Buckett *et al*.[16] on 368 semen samples, seminal lactoferrin

concentrations by single radial immunodiffusion assay were found significantly raised in groups with oligozoospermia and oligo-asthenozoospermia above other groups. The inconsistency of our results with the two previous studies could be due to different measurement techniques of lactoferrin and different sampling sizes. The precise definition and inclusion criteria of research groups are considered strengths of the current study. Furthermore, the detection of our findings by both correlation and linear regression analysis endorses the current results.

On the other hand, some previous animal research showed consistent results with our study. Two studies conducted on dogs and horses reported a positive correlation between seminal plasma lactoferrin levels and both sperm concentration and total sperm count. In addition, there was an insignificant correlation between seminal plasma lactoferrin concentration and sperm motility.[13,27] Other earlier animal studies showed variable effects of lactoferrin on male fertility. For example, the addition of lactoferrin to frozen

Table 2: Seminal lactoferrin and iron levels in different groups and according to fertility and smoking status

	Lactoferrin (ng/mL), mean±SE	*P*	Iron (µg/dL), mean±SE	*P*
Group				
Group I (*n*=33)	155.92±8.4	0.296*	260.71±38.3	0.409*
Group II (*n*=25)	152.68±10.9		196.46±24.8	
Group III (*n*=17)	176.97±13.5		225.99±40.2	
Fertility status				
Infertile (*n*=58)	154.53±6.7	0.121**	233.01±24.5	0.882**
Fertile (*n*=17)	176.97±13.5		225.99±40.2	
Smoking				
Non-smoker (*n*=33)	163.92±8.3	0.523**	251.34±38.5	0.401**
Smoker (*n*=42)	156.24±8.7		215.77±21.9	

*ANOVA test was used to compare the difference in mean between groups, **Independent sample *t*-test was used to compare the difference in mean between groups. SE=Standard error

Table 3: Univariate correlation between seminal lactoferrin and iron levels with some clinical data and semen parameters in total sample and varicocele group (Group I)

	Lactoferrin (ng/mL), *r* (*P*)*		Iron (µg/dL), *r* (*P*)*	
	Total sample (*n*=75)	Group I (*n*=33)	Total sample (*n*=75)	Group I (*n*=33)
Iron level (µg/dL)	−0.081 (0.264)	−0.095 (0.299)	1	1
Age (years)	−0.306 (<0.001)	−0.314 (0.038)	−0.091 (0.220)	−0.146 (0.208)
Disease duration (years)	-	−0.108 (0.274)	-	−0.191 (0.144)
Semen volume (mL)	0.068 (0.282)	−0.054 (0.283)	0.171 (0.071)	−0.009 (0.497)
Sperm concentration (million/mL)	0.280 (0.008)	0.265 (0.068)	0.374 (<0.001)	0.358 (0.020)
Total sperm count (million/ejaculate)	0.310 (0.003)	0.300 (0.045)	0.264 (0.011)	0.364 (0.019)
Progressive motility%	−0.029 (0.407)	0.116 (0.260)	−0.080 (0.255)	−0.126 (0.243)
Total motility%	−0.079 (0.258)	0.213 (0.118)	−0.092 (0.229)	−0.144 (0.213)
Normal form%	0.049 (0.344)	0 069 (0.352)	0.040 (0.372)	0.48 (0.396)

*Spearman-ranked correlation was used to test the univariate correlations

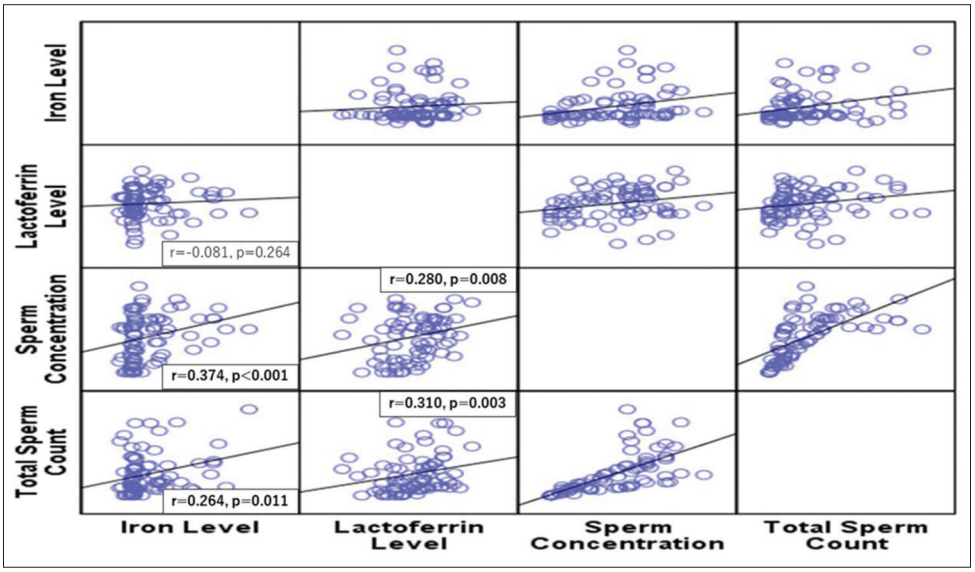

Figure 1: Correlation matrix of lactoferrin, iron and semen parameters in the total sample

Table 4: Independent predictors of sperm concentration in total sample, multivariable linear regression model

	Estimate	SE	*t*-statistic	*P*
Intercept (sperm concentration)	70.98 (45.4–96.5)*	12.8	5.53	<0.001
Age/year	−0.92 (−1.7–−0.2)	0.4	−2.41	0.019
Smoking status (smoker)	−6.34 (−11.2–−1.5)	2.4	−2.62	0.011
Iron (µg/dL)	0.34 (0.16–0.56)	0.11	2.45	0.017
Lactoferrin (ng/mL)	0.78 (0.19–0.94)	0.47	2.02	0.047

*Confidence interval. SE=Standard error, *t*-statistic=*t*-test value

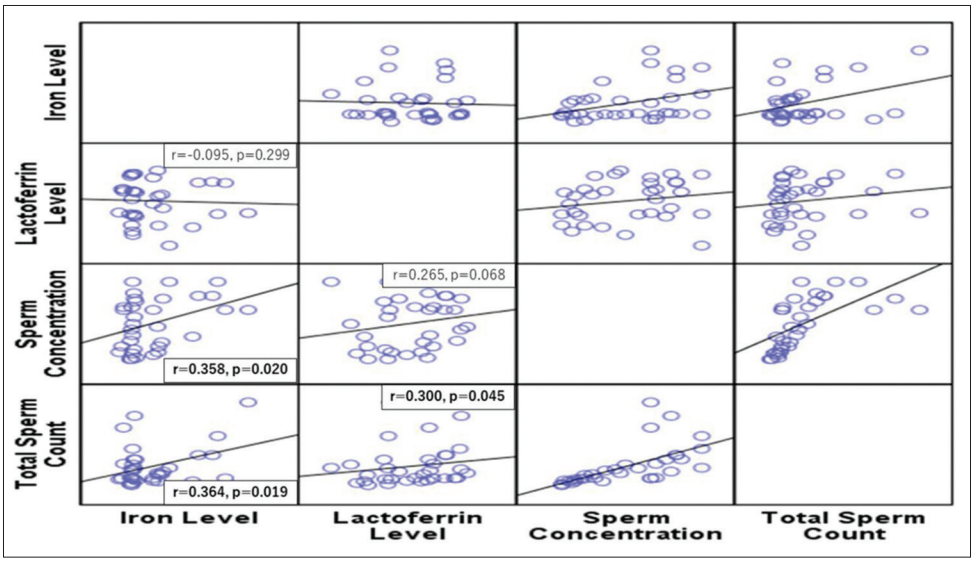

Figure 2: Correlation matrix of lactoferrin, iron and semen parameters in the varicocele group

equine semen was observed to protect the spermatozoa from membrane dysfunction by hypoosmotic swelling test through the anti-oxidative properties of lactoferrin.[17] Recently, Li *et al*.[28] revealed lactoferrin protective effects on dietary deoxynivalenol-induced male reproductive malfunctions. Oral administration of lactoferrin restored the spermatogenesis disorder in mice fed a deoxynivalenol inclusion diet and eliminated the resultant oxidative stress.

As lactoferrin is considered an iron-binding glycoprotein,[29] seminal levels of iron were evaluated in our study. Insignificant differences in mean seminal iron levels were detected between the 3 study groups. Aydemir *et al*.[30] found that seminal plasma iron concentrations significantly raised in 60 subfertile males than in 40 fertile males, suggesting iron as a mediator of oxidative damage. In contrast, the study of Sun *et al*.[31] on 20 healthy men and 26 men with bilateral varicocele reported decreased seminal iron levels in men with bilateral varicocele in a significant way. They suggested that decreased iron levels may be due to the activation of the ferroptosis pathway which is an iron-dependent, non-apoptotic cell death. Shukla *et al*.[32] also showed close results with significantly decreased seminal iron in all semen groups from 75 infertile men in comparison with 75 healthy controls.

Regarding semen parameters, the current study documented a significant positive correlation between seminal iron levels and both sperm concentrations and total sperm counts in the total sample and the varicocele group. In addition, seminal iron was found as significant independent predictor of sperm concentration in the total sample. Consistently, Jia *et al*.[33] reported a positive significant correlation between iron levels and sperm concentration and total count in 841 male volunteers. In addition, Liu *et al*.[34] observed the same correlation in 1136 subjects regardless of their fertility status. However, varicocele was an exclusion criterion in that study. Inconsistent with our results, earlier studies detected the associations of increased seminal iron levels with asthenozoospermia[35] and teratozoospermia.[20]

The correlation between seminal lactoferrin and iron levels in this study was insignificant in the total sample and varicocele group. Lactoferrin properties on sperms and in seminal plasma were shown to be different from lactoferrin in its purified form. In semen, lactoferrin seems to bind unidentified molecules that may affect its biological actions.[26]

The current study's pros are the assessment of seminal lactoferrin in a specific disease-causing infertility rather than random sampling in previous studies, the clear

definition of research groups and the inclusion of two control groups.

The limitations of this research include the inability to match the age among all groups and unequal number of study groups. Furthermore, seminal lactoferrin levels were not correlated to serum iron and associated conditions such as anaemia. The impact of seminal lactoferrin on the occurrence of pregnancy and pregnancy outcomes was not evaluated. This study was powered to detect a large effect size. Thus, smaller effects could have been missed and that is a limitation of the study.

We recommend further studies to be performed on the role of seminal lactoferrin in varicocele and male fertility considering the present research limitations, studying the relation between serum and seminal levels of lactoferrin and the impact of lactoferrin supplementation on seminal lactoferrin concentrations as well as semen parameters.

Conclusion

Seminal lactoferrin and iron levels in infertile men with varicocele were not different from infertile individuals without varicocele and possibly fertile individuals. No relation could be detected between lactoferrin and iron levels in seminal plasma. Our findings concluded a positive association of seminal lactoferrin and iron levels with sperm concentrations and counts. Furthermore, iron and lactoferrin were observed as significant independent predictors of sperm concentration in seminal plasma. Increasing age was associated with a reduction in seminal lactoferrin concentrations.

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Authors contribution

All the authors contributed sufficiently to the study concept, design, literature search, data acquisition and interpretation, statistical analysis and manuscript preparation.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement

The research data are available from the corresponding author on a reasonable request.

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Supplementary Table 1: Baseline characteristics in the studied groups

	Group I (*n*=33)	Group II (*n*=25)	Group III (*n*=17)	*P*
Age (years)				
Mean±SD (range)	31.64±5.7 (20–44)	36.12±5.6 (22–46)	29.88±4.3 (21–40)	0.001*
*P***	I versus II=0.002	II versus III <0.001	I versus III=0.280	
Residence, *n* (%)				
Urban	12 (36.4)	10 (40)	11 (64.7)	0.142***
Rural	21 (63.6)	15 (60)	6 (35.3)	
Occupation, *n* (%)				
Non-worker	1 (3)	1 (4)	0	0.341***
Unskilled	28 (84.8)	19 (76)	17 (100)	
Skilled	4 (12.1)	5 (20)	0	
Special habits, *n* (%)				
Non-smoker	12 (36.4)	13 (52)	8 (47.1)	0.373***
Smoker	21 (63.6)	12 (48)	9 (52.9)	

*ANOVA test was used to compare the difference in mean between groups, ***Post-hoc* test with Tukey's correction was used for pairwise comparisons, ***Monte Carlo exact test was used to compare the proportion difference between groups. SD=Standard deviation

Supplementary Table 2: Clinical data in the studied groups

	Group I (*n*=33), *n* (%)	Group II (*n*=25), *n* (%)	Group III (*n*=17), *n* (%)	*P*
Infertility				
1ry	16 (48.5)	15 (60)	-	0.273*
2ry	17 (51.5)	10 (40)	-	
Infertility duration (years)			-	
Mean±SD (range)	3.55±0.5 (1–12)	5.80±0.8 (1.5–17)	-	0.012**
Drug history				
No	21 (63.6)	15 (60)	15 (88.2)	0.244***
Antioxidant	11 (33.3)	9 (36)	1 (5.9)	
Other	1 (3.1)	1 (4)	1 (5.9)	
Surgical history for infertility (other than ART)				
No	32 (97)	24 (96)	-	0.924*
Yes	1 (3)	1 (4)	-	
Previous ART				
No	32 (97)	18 (72)	-	0.002***
Yes	1 (3)	7 (28)	-	

*Chi-square test was used to compare the proportion difference between groups, **Independent sample *t*-test was used to compare the difference in mean between groups, ***Fisher's exact test was used to compare the proportion difference between groups. ART=Assisted reproductive technology, SD=Standard deviation

Supplementary Table 3: Scrotal Doppler ultrasound findings of varicocele in group I

n=33	Right side, *n* (%)	Left side, *n* (%)	*P*
No varicocele	11 (33.3)	0	<0.001*
Mild varicocele	17 (51.5)	1 (3.1)	
Moderate varicocele	5 (15.2)	17 (51.5)	
Severe varicocele	0	15 (45.4)	

*McNemar test was used to compare the proportion difference between groups