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RESEARCH ARTICLE

Associations between biochemical components of human semen with seminal conditions

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ABSTRACT

The aim of the study was to assess whether abnormal levels of seminal biochemical components could be associated with semen alterations and infertility. In this study, 92 human ejaculates from selected men were analyzed. Albumin, estradiol, ferritin, total proteins (TP), folic acid (FA), vitamin B12, alkaline phosphatase (ALP), creatine kinase (CK), gamma-glutamyl transpeptidase (GGT), lactate dehydrogenase were evaluated. Semen parameters and biochemical components of the 92 samples were correlated bySpearman's rho coefficient. Albumin showed a negative correlation with sperm progressive motility and vitality ($P < 0.05$), CK with sperm concentration and vitality ($P < 0.05$), ferritin with sperm morphology ($P < 0.05$). FA negatively correlated with sperm concentration ($P < 0.05$) and GGT with sperm motility ($P < 0.05$). The values of biochemical components were compared for each semen parameters (concentration, motility, morphology, vitality) in samples ≤5th percentile with those >5th percentile and in patients with/without leukocytospermia, presence/absence of germ cells, increased/normal viscosity by Mann Whitney U test. The albumin ($P < 0.001$) and TP ($P < 0.05$) levels and the GGT activity ($P < 0.001$) were significantly higher in patients with sperm motility ≤5th percentile. Patients with sperm vitality ≤5th percentile showed increased albumin concentration ($P < 0.01$) and the CK activity ($P < 0.001$). The presence of germ cells in semen was concomitant with high values of ferritin ($P < 0.01$); the ALP activity ($P < 0.01$) and FA level ($P < 0.001$) were decreased in hyperviscous semen. The FA and estradiol levels were significantly decreased in the smoker group compared to those measured in the non-smoker group. Subjects were grouped in infertile patients and men with unknown reproductive potential. Infertile patients albumin and ferritin were significantly increased ($P < 0.05$). This study suggests that some biochemical components may be associated with human seminal pathological conditions.

Abbreviations: ALP: alkaline phosphatase; LDH: lactate dehydrogenase; GGT: γ-glutamyl transferase; CK: creatine kinase; ACP: acid phosphatase; ALB: albumin; TP: total proteins; FERR: ferritin, E: estradiol; FOL: folic acid; B12: vitamin B12; FSH: follicle stimulating hormone; LH: luteinizing hormone; T: testosterone; BMI: body mass index; WHO: World Health Organization.

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Introduction

Seminal plasma is the fluid portion of semen, secreted by both the epididymis as well as the accessory glands before and during ejaculation. Seminal plasma is a complex fluid that contains different components, such as proteins, enzymes, macro-and microelements, lipids and nutrients, and it plays an important role in spermatozoa motility, viability, and the maintenance of fertilizing capacity; moreover, the proteins contained in seminal plasma may influence the fertilization process (Anel-López et al. 2017).

The effects of biochemical components in human seminal plasma are still debated (Feng et al. 2015; Zhang et al. 2015; Vickram et al. 2016). Several studies were conducted on the biochemical composition of seminal plasma in different domestic livestock species (Intasqui et al. 2016), in horses (Talluri et al. 2017) and in boars (Žaja et al. 2016). For example, the enzymes alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) have a significant role in the metabolic processes that provide energy for spermatozoa survival, motility, and fertility potential of the cells (Diaconescu et al. 2014). The activity of enzymes such as γ-glutamyl transferase (GGT), creatine kinase (CK) and ALP in seminal plasma are related to semen quality and spermatozoa membrane

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function, and also are involved in various metabolic processes during maturation.

GGT seems to exert a protective effect against oxidative stress in the epididymis preserving motility (Seligman et al. 2005), CK catalyzes the regeneration of ATP from the chemical shuttle between creatine and creatine phosphate, and it is important in sperm function (Banihani and Abu-Alhayjaa 2016). In boar ALP is secreted by epididymis and prevents a precocious capacitation (Bucci et al. 2014). Hence, the determination of the biochemical components and enzymes in seminal plasma may serve as a biological marker for semen quality since their levels/values indicate the sperm function, integrity, and damage (Pesch et al. 2006).

Many pathological conditions such as the presence of infection/inflammation or varicocele may cause oxidative stress that adversely alters the seminal fluid composition and sperm function by affecting membrane fluidity, permeability and impairing sperm functional competence (Collodel et al. 2015). It was reported that seminal plasma proteins could be considered as important biomarkers for male infertility (Macanovic et al. 2015). Feng et al. (2015) presented a pilot comparative study of biochemical markers in seminal plasma and serum of infertile men. Twenty four out of 26 evaluated biochemical markers were significantly different in seminal plasma and serum. The authors suggest that the differences might be associated with the selective secretion of testis, epididymis, and male accessory glands and the specific environment required for sperm metabolism and function maintenance. Among the conditions related to male infertility, include leukocytospermia a well-known indicator of infection or inflammation of urogenital tract (Fraczek et al. 2016) and the presence of germinal cells in ejaculates that may indicate an altered spermatogenesis (Rodríguez-Martínez et al. 2011). Furthermore, it was reported that seminal fluid hyperviscosity represents a peculiar aspect of prostate asymptomatic chronic inflammation (La Vignera et al. 2012). Another discussed risk factor for the alterations of seminal fluid is the smoking habit; although the real impact of tobacco on male fertility is still debated, a number of studies have claimed that cigarette smoking is correlated with alterations in sperm quality (Collodel et al. 2009).

In this study, we analyzed the semen parameters in 92 selected subjects. Tseminal plasma samples we assessed 10 biochemical components including albumin (ALB), total proteins (TP), GGT, ferritin (FERR), estradiol (E), folic acid (FOL), vitamin B12 (B12) and enzymes such ALP, LDH and CK to assess whether one or more of these components could be associated with semen abnormalities.

Results

Semen characteristics of the 97 selected men including pH, volume, sperm concentration, progressive motility, vitality, and morphology were evaluated (WHO 2010); moreover, the presence of germ cells, hyperviscosity and leukocytospermia were indicated for each sample. The seminal levels and the activity of enzymes were assessed. Among 97 patients (53 infertile, 44 unknown reproductive potential), 5 showed a seminal volume ≤1.5 ml and were excluded, finally 92 patients (aged 22–43 years; mean ± SD: 33.97 ± 4.73) were included in the study (50 infertile, 42 unknown reproductive potential).

In order to understand the possible correlations among the studied variables we used the Spearman's Rank Correlation Coefficient considering all the cases. Volume and pH did not correlate with any sperm parameters; however pH negatively correlated ($P < 0.05$) with ALB and CK. Where the correlations were significant, the biochemical variables negatively correlated with seminal parameters, except for LDH that showed a positive correlation with sperm concentration. In the evaluated men's population, ALP, TP, E and B12 did not correlate with seminal parameters (Table 1). ALB showed a negative correlation with sperm progressive motility (Figure 1) and vitality ($P < 0.05$); CK showed a negative correlation with sperm concentration and vitality (Figure 1, $P < 0.05$) and FERR with sperm morphology ($P < 0.05$). Negative correlations were also observed between FOL and sperm concentration ($P < 0.05$) as well as between GGT and sperm motility ($P < 0.05$).

The detected values of ALB, E, FERR, TP, FOL, B12 and enzymes ALP, CK, GGT and LDH were compared in patients with sperm concentration (32 men), motility (44 men), morphology (36 men), vitality (34 men) ≤5th percentile (respectively, <15 mil/ml; <32%; <4%; <58%, World Health Organization 2010) versus those obtained in patients with values of the same sperm characteristics >5th percentile (Table 2).

The values of seminal components did not significantly differ in patients with sperm concentration and morphology ≤5th percentile with respect to those observed in individuals where these parameters were >5th percentile. Sperm motility and vitality were influenced by the following biochemical components: the levels of ALB (0.66, $P < 0.001$) and TP (4.4, $P < 0.05$) and the GGT (6715, $P < 0.001$) activity were significantly higher in patients with sperm motility ≤5th percentile than in patients with motility >5th percentile (ALB, 0.49; TP, 3.64; GGT, 6567); in patients with sperm vitality ≤5th percentile, the CK activity (1216, $P < 0.001$) and the ALB level (0.6,

Table 1. Correlations (Spearman's rho coefficient) between semen biochemical components and sperm parameters.

Variable	Sperm concentration	Sperm morphology %	Sperm motility %	Sperm vitality %
ALB (g/dL)	0.009	0.023	**−0.536***	**−0.339***
ALP (U/L)	0.284	0.099	0.177	0.164
CK (U/L)	**−0.323***	−0.032	−0.199	**−0.939***
E (pmol/L)	−0.241	0.067	0.142	0.131
FERR (ng/mL)	0.027	**−0.316***	−0.251	−0.159
GGT (U/L)	0.076	−0.149	**−0.439***	−0.077
LDH (U/L)	**0.337***	0.023	−0.216	−0.202
TP (g/dL)	−0.094	−0.174	−0.081	−0.171
B12 (pmol/L)	−0.139	0.100	0.009	−0.040
FOL (ng/mL)	**−0.360***	0.005	−0.145	−0.225

Sperm concentration: number of sperm/ml $\times 10^6$; **sperm morphology %**: percentage of sperm with normal morphology; **sperm motility** %: percentage of sperm with rapid + slow progressive motility; **sperm vitality** %: percentage of unstained sperm after Eosin Y treatment; **ALB**: albumin; **ALP**: alkaline phosphatase; **CK**: creatine kinase; **E**: Estradiol; **FERR**: ferritin; **GGT**: gamma-glutamyl transpeptidase; **LDH**: lactate dehydrogenase; **TP**: total protein; **B12**: vitamin B12; **FOL**: folic acid. Significant correlations are in bold; *P < 0.05, **P < 0.01, ***P < 0.001.

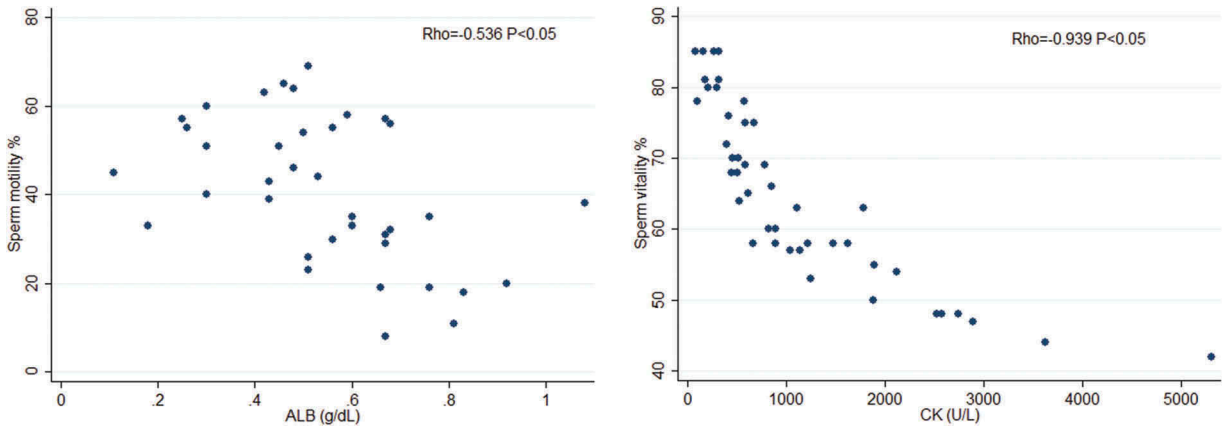

Figure 1. Scatter plots displaying the relationship between sperm motility and ALB and between sperm vitality and CK.

P < 0.01) increased respect to those measured in individuals with sperm vitality >5th percentile (CK, 318; ALB, 0.5).

The levels of the semen components in patients with leukocytospermia (32 patients), presence of germ cells (36 patients), hyperviscosity (36 patients) were compared with those obtained in patients without leukocytospermia (60 patients), absence of germ cells (56 patients) and normal viscosity (56 patients), respectively (Table 3). No significant differences were observed in patients with or without leukocytospermia. The FERR levels showed significantly higher values in individuals with germ cells (316.4, P < 0.01) in the ejaculates compared to those measured in semen lacking germ cells (203.4). ALP activity (139, P < 0.01) and the FOL level (8.53, P < 0.001) were significantly lower in hyperviscosous semen than those observed in semen with normal viscosity (ALP, 238; FOL, 12.91; Table 3).

A comparison of the seminal components was performed in a group of mild smokers (32 men) with respect to non smokers (30 men, Table 4). Sperm concentration (42.5; P < 0.05), morphology (6; P < 0.001), motility (36; P < 0.05), FOL (12.6; P < 0.001) and E (13.6; P < 0.001) levels were significantly decreased in the smoker group (non smoker: sperm concentration, 79.25; morphology, 22; motility, 43.5; FOL, 6.94; E, 13.6).

The patients were divided in two groups: infertile patients (N = 50) and subjects with unknown reproductive potential (N = 42). Differences in terms of patient's characteristics, seminal and biochemical parameters were evaluated and the results were reported in Table 5. Infertile patients showed a significant reduction in sperm concentration, morphology, motility (P < 0.001) and an increased presence of leukocytospermia (P = 0.043) and germ cells (P = 0.05). Among biochemical components, ALB (P = 0.022) and FERR (P = 0.025) were higher in the group of infertile patients than those measured in semen of individuals of unknown reproductive potential. In these two groups, no significant differences were detected for characteristics as age and smoking habit (Table 5).

Discussion

The aim of this study was to investigate if the level/activity of some biochemical components in human ejaculates could be suggested as having an association with semen

Table 2. Medians and interquartile ranges (IQR: 75°–25° centile) of semen biochemical values in patients with sperm concentration, morphology motility and vitality ≤5°perc vs those obtained in patients with sperm concentration, morphology, motility and vitality >5°perc respectively.

	Sperm concentration		Sperm morphology%		Sperm motility%		Sperm vitality%	
Variable	≤5°perc (32/92)	>5°perc (60/92)	≤5°perc (36/92)	>5°perc 56/92)	≤5°perc (44/92)	>5°perc (48/92)	≤5°perc (34/92)	>5°perc (58/92)
ALB (g/dL)	0.51 (0.24)	0.56 (0.24)	0.66 (0.25)	0.49 (0.18)	**0.66 (0.25)*** **	**0.49 (0.18)**	**0.6 (0.2)** **	**0.5 (0.3)**
ALP (U/L)	116 (59)	175.5 (290)	156 (126)	171.5 (301)	156 (126)	171.5 (301)	154 (301)	167 (200)
CK (U/L)	1178 (1386)	642.5 (715)	753.5 (1210)	731.5 (1031)	753.5 (1210)	731.5 (1031)	**1216 (1296)*** **	**318 (365)**
E (pmol/L)	17 (4.51)	14.2 (9)	15 (4.71)	14.61 (9)	15 (4.71)	14.61 (9)	15 (6.11)	14.61 (9.98)
FERR (ng/mL)	248.3 (196.4)	221 (156.3)	264.2 (167.4)	210.3 (147.1)	264.2 (167.4)	210.3 (147.1)	265.6 (178.8)	205.8 (150.7)
GGT (U/L)	6205 (1652)	6973 (2883)	6715 (3146)	6567 (2961)	**6715 (3146)*** **	**6567 (2961)**	6720 (2630)	6437 (3016)
LDH (U/L)	1496 (697)	1563 (684)	1536 (592)	1558 (684)	1536 (592)	1558 (684)	1633 (638)	1473 (611)
TP (g/dL)	4 (1.32)	3.73 (1.76)	4.4 (1.25)	3.64 (1.19)	**4.4 (1.25)***	**3.64 (1.19)**	4.06 (1.44)	3.73 (1.47)
B12 (pmol/L)	748 (313.6)	565.1 (374.8)	661.4 (359.7)	550.1 (413)	661.4 (359.7)	550.1 (413)	618 (311.5)	526 (698)
FOL (ng/mL)	12.75 (3.31)	9.24 (8.26)	12.49 (5.08)	10 (8.26)	12.49 (5.08)	10 (8.26)	12.37 (6.68)	9.24 (8.2)

Sperm concentration: number of sperm/ml x10^6; **sperm morphology %**: percentage of sperm with normal morphology; **sperm motility** %: percentage of sperm with rapid + slow progressive motility; **sperm vitality** %: percentage of unstained sperm after Eosin Y treatment; **ALB**: albumin; **ALP**: alkaline phosphatase; **CK**: creatine kinase; **E**: Estradiol; **FERR**: ferritin; **GGT**: gamma-glutamyl transpeptidase; **LDH**: lactate dehydrogenase; **TP**: total protein; **B12**: vitamin B12; **FOL**: folic acid. Significant comparisons are in bold; *P < 0.05, **P < 0.01, ***P < 0.001.

Table 3. Medians and interquartile ranges (IQR: 75°–25° centile) of semen biochemical values in patients with presence/absence of leukocytospermia, germ cells, hyperviscosity.

	Leukocytospermia		Germ cells		Hyperviscosity	
Variable	>1mil/ml (32/92)	<1mil/ml (60/92)	Presence (36/92)	Absence (56/92)	Presence (36/92)	Absence (56/92)
ALB (g/dL)	0.52 (0.25)	0.6 (0.2)	0.60 (0.23)	0.51 (0.24)	0.48 (0.37)	0.56 (0.21)
ALP (U/L)	167 (246)	158.5 (325.5)	271 (299)	156 (259)	**139 (160)** **	**238 (376)**
CK (U/L)	628.5 (934)	785 (1165)	731.5 (708)	628.5 (1390.5)	588 (594)	784 (1855)
E (pmol/L)	14.81 (7.89)	14 (7.36)	14 (4.71)	16.8 (10.1)	17.14 (9.58)	13.7 (7.61)
FERR (ng/mL)	221 (198.8)	245.7 (114.1)	**316.4 (137.3)** **	**203.4 (104.7)**	188.4 (211.5)	260.4 (129.1)
GGT (U/L)	6647 (3285)	6574 (2030)	6841 (3347)	6567 (2640)	6034 (3182)	6974 (2630)
LDH (U/L)	1478 (800)	1535 (482.5)	1609 (421)	1412 (810)	1453 (756)	1578 (782)
TP (g/dL)	3.64 (1.18)	4.4 (1.28)	4.24 (1.35)	3.61 (1.18)	3.72 (1.76)	3.99 (1.27)
B12 (pmol/L)	569 (329.6)	690.7 (340.5)	649.7 (385.7)	588.6 (354.3)	532 (370.6)	659 (349.2)
FOL (ng/mL)	11.9 (8.51)	11 (4.76)	11.9 (5.04)	10 (8.76)	**8.53 (6.97)*** **	**12.91 (9.12)**

Leukocytospermia: presence of leukocytes (>1 × 10^6 leukocytes/ml) in the ejaculate; **germ cells**: presence of germ cells in ejaculate; **hyperviscosity**: presence of hyperviscosity in ejaculate **ALB**: albumin; **ALP**: alkaline phosphatase; **CK**: creatine kinase; **E**: Estradiol; **FERR**: ferritin; **GGT**: gamma-glutamyl transpeptidase; **LDH**: lactate dehydrogenase; **TP**: total protein; **B12**: vitamin B12; **FOL**: folic acid. Significant comparisons are in bold; *P < 0.05, **P < 0.01, ***P < 0.001.

abnormalities and presence of conditions known to alter semen quality itself, such as leukocytospermia, hyperviscosity, presence of germ cells, and smoking habit. In addition these, variables were compared between smokers and non smokers, infertile patients and individuals of unknown reproductive potential.

In this study, ALB, CK, FERR, GGT, and FOL negatively correlate with human seminal characteristics indicating a possible involvement in, or marker of semen quality. To understand the role of these seminal components in different seminal conditions, we compared their level/activity grouping ejaculates according to normal and altered semen parameters.

CK activity was significantly increased in semen of patients with sperm vitality ≤5th percentile. In clinical analysis, the serum evaluation of CK indicates the presence of skeletal muscle damage, in semen the patterns of CK-immunocytochemistry was used to demonstrate cytoplasmic retention in diminished-maturity spermatozoa (Huszar et al. 2004). Our data indicate that CK may be associated with low-sperm vitality in semen since the sperm plasma membrane is broken, and the enzyme is released into the seminal plasma.

Table 4. Comparison of biochemical values and semen parameters in smokers and non smokers. Medians and interquartile ranges (IQR: 75°–25° centile) are reported.

	Smokers (32)	Non smokers (30)
pH	7.8 (0.3)	7.8 (0)
Sperm concentration	**42.5 (108.5)***	79.25 (120)
Sperm morphology %	**6 (7)*****	22 (8)
Sperm motility %	**36 (30)***	43.5 (24)
Sperm vitality %	65.5 (20)	64.5 (21)
ALB (g/dL)	0.50 (0.25)	0.56 (0.21)
ALP (U/L)	162.5 (297)	161 (317)
CK (U/L)	628.5 (647)	731.5 (1321)
E (pmol/L)	**13.6 (8.09)*****	18.07 (9)
FERR (ng/mL)	175.4 (120.3)	263 (152.5)
GGT (U/L)	6493 (2883)	6715 (2734)
LDH (U/L)	1631 (812)	1464 (593)
TP (g/dL)	3.66 (1.42)	4.02 (1.64)
B12 (pmol/L)	649.7 (375.2)	690.8 (360.8)
FOL (ng/mL)	**6.94 (3.86) *****	12.6 (5.9)

Smokers (≥15 cigarettes day); **non smokers** (never smoked); **pH**: pH of the sample; **sperm concentration**: number of sperm/ml $\times 10^6$; **sperm morphology %**: percentage of sperm with normal morphology; **sperm motility** %: percentage of sperm with rapid + slow progressive motility; **sperm vitality** %: percentage of unstained sperm after Eosin Y treatment; **ALB**: albumin; **ALP**: alkaline phosphatase; **CK**: creatine kinase; **E**: Estradiol; **FERR**: ferritin; **GGT**: gamma-glutamyl transpeptidase; **LDH**: lactate dehydrogenase; **TP**: total protein; **B12**: vitamin B12; **FOL**: folic acid. Significant comparisons are in bold; *P < 0.05, **P < 0.01, ***P < 0.001.

Table 5. Comparisons between patient's characteristics, biochemical markers, and semen parameters of infertile patients and unknown reproductive potential men. Medians and interquartile ranges (IQR: 75°–25° centile) are reported. The number of individuals is reported in characteristics as smokers, hyperviscosity, leukocytospermia, presence of germ cells.

	Infertile (N = 50)	Unknown reproductive potential (N = 42)
Patient's characteristics		
Age (years)	37 (4)	35 (7)
Smokers	19	13
Seminal parameters		
pH	7.8 (0.2)	7.8 (0.2)
Sperm concentration	**16 (52.9)*****	93.7 (175.3)
Sperm morphology %	**6 (2.5)*****	18 (9)
Sperm motility %	**31 (17.5)*****	51 (18)
Sperm vitality %	60 (17.5)	68 (20)
Hyperviscosity	16	20
Leukocytospermia	**22***	10
Germ cells	**24***	12
Biochemical markers		
ALB (g/dL)	**0.6 (0.19)***	0.49 (0.16)*
ALP (U/L)	155 (305.75)	238 (286)
CK (U/L)	853 (1210)	669 (805)
E (pmol/L)	14.77 (6.68)	13.57 (7.89)
FERR (ng/mL)	**260.4 (167.4)***	188.4 (147.1)
GGT (U/L)	6710 (2907)	6575 (2379)
LDH (U/L)	1493 (581)	1482 (691)
TP (g/dL)	4.04 (1.26)	3.61 (1.55)
B12 (pmol/L)	560.2 (293.7)	525.05 (373.9)
FOL (ng/mL)	**9.62 (7.49)***	12.53 (5.22)

Age: patient's age; **smokers**: number of smoker patient (>15 cigarettes day), **pH**: seminal pH; s**perm concentration**: number of sperm/ml x10^6; **sperm morphology %**: percentage of sperm with normal morphology; **sperm motility** %: percentage of sperm with rapid + slow progressive motility; **sperm vitality** %: percentage of unstained sperm after Eosin Y treatment; **ALB**: albumin; **Hyperviscosity**: number of patients with increase of semen viscosity; **Leukocytospermia**: number of patients with a presence of seminal leukocytes >10^6/ml; **Germ cells**: number of patients with a presence of seminal germ cells; **ALP**: alkaline phosphatase; **CK**: creatine kinase; **E**: Estradiol; **FERR**: ferritin; **GGT**: gamma-glutamyl transpeptidase; **LDH**: lactate dehydrogenase; **TP**: total protein; **B12**: vitamin B12; **FOL**: folic acid. Significant differences are showed in bold. *P < 0.05, **P < 0.01, ***P < 0.001.

In addition, FERR levels were significantly increased in semen samples that also contained germ cells in comparison to samples lacking them. A study of Santambrogio et al. (2007) showed that FERR was highly expressed in human and mouse testis tissue. Behrouzi et al. (2013) found FERR only in the supernatant of a control group of men with normal semen parameters, but it was not reported in spermatozoa or in relation to sperm DNA integrity. Germ cells should not be present in normal ejaculates, in pathological conditions, on the contrary, the seminiferous epithelium may release immature germ cells in the ejaculate (Baccetti et al. 2002). Thus, the simultaneous presence of significant levels of FERR and germ cells in ejaculates suggests that FERR can be considered associated with pathological conditions related to germ cells release as

confirmed in the group of infertile men considered in this study.

ALB and TP levels as well as GGT activity were related to different seminal conditions mainly concerning motility and viability. ALB arises from prostate, testis, and epididymis; an association between ALB, determined in seminal plasma, and sperm morphology has been reported (Elzanaty et al. 2007). Zorn et al. (2003) observed an increase of ALB concentration, complement component C3, caeruloplasmin, immunoglobulins IgG and IgA, and cytokines interleukins-8 and -6 in cases of urogenital tract inflammation; increased levels of ALB have been observed in a group of Reactive Oxygen Species (ROS) positive patients (Sharma et al. 2013). Among our selected subjects, ALB levels were increased in patients with both reduced sperm motility and vitality, parameters generally decreased in case of oxidative damage due to infections or caused by inflammation (Fraczek et al. 2016). The presence of oxidative stress may alter the ALB effect in the sperm membrane influencing mechanisms as capacitation and/or acrosomal reaction (Signorelli et al. 2012). In addition, in the group of infertile patients the number of subjects with leukocytospermia, inseparably connected with ROS production (Fraczek and Kurpisz 2007), was increased as well as ALB levels.

Serum GGT might also be considered an inflammatory marker (Shin et al. 2015). GGT is a cell surface glycoprotein involved in antioxidant defense by maintaining GSH and cysteine homeostasis (Ehala-Aleksejev and Punab 2016). In reproductive tract, it is expressed in the Sertoli and Leydig cells, epididymis, seminal vesicles, and vas deferens. In stallion semen, increased GGT activity indicates a function for cell protection against free radicals (Pesch et al. 2006); GGT could play the same function in human semen since we found that its activity was increased in semen of patients with reduced sperm motility.

The high level of TP observed in semen of patients with reduced sperm motility and vitality may be explained by the presence of dead or altered sperm that can release cellular constituents, including proteins in the fluid. Moreover, an over-expression of some seminal proteins was observed in men with altered sperm function and in presence of seminal oxidative stress (Camargo et al. 2018). Recently, Sharma et al. (2016) reported that in buffalo semen TP values was negatively correlated with motility, as observed in humans, and suggested that TP play an important role in sperm membrane stability and then in viability and motility.

Semen hyperviscosity is one of the factors involved in sperm function deficiency and it is reported to impair seminal plasma total antioxidant capacity (Layali et al. 2015). In this paper, the presence of seminal hyperviscosity was associated with decreased ALP activity and FOL level. ALP is an enzyme that catalyzes the detachment of phosphate groups from several substrates. It is present in male genital tract fluids and its activity could have a role in sperm metabolism. Bucci et al. (2014) demonstrated that ALP activity decreases during capacitation and that the addition of ALP to spermatozoa compromises the capacitating process itself. Therefore ALP seems to be involved in holding sperm quiescent and in controlling fertilizing ability that could be affected by hyperviscosity since ALP levels are reduced in presence of this condition.

Folic acid is a vitamin B (B9) that plays a vital role in nucleic acid synthesis and amino acid metabolism. It has free radical scavenging abilities which suggested its use as a potential antioxidant for the treatment of male subfertility (Majzoub and Agarwal 2017). The reduction of this seminal component may indicate a lower semen quality in ejaculates with hyperviscosity.

Finally, the smoking habit is associated with a significant reduction in sperm concentration, morphology, and motility, and these observations agreed with the data generally reported in the literature (Mostafa et al. 2017). Ghaffari and Rostami (2003) described a reduction of sperm CK activity in semen from smokers; in this study rather than the CK activity we observed a decrease of FOL and E levels in semen of smokers men. The discrepancy concerning the effect of cigarette smoke on seminal biochemical components could be due to the criteria used to select the population of men included in the studies. Other authors found that the seminal plasma of smokers contains a lower folate concentration compared with non smokers (Wallock et al. 2001).

It is well known that E is a pleiotropic hormone normally produced in the mammalian testis from testosterone by the enzyme cytocrome P450 which is expressed in Sertoli cells, Leydig cells and germ cells (Lardone et al. 2010). E is related to spermatogenesis because male germ cells possess estrogen receptors (ERα and ERβ) and male mice lacking ERα are infertile. In an *in vitro* culture system Kim and Park (2018) observed that E stimulates spermatogenesis by inducing expression of the spermatogenic genes SYCP1, encoding a protein associated with meiosis, and PRM1, encoding protamine, both required for a correct spermatogenesis. Recently Hagiuda et al. (2015) reported that in infertile couples male partners with low serum E levels had low motile sperm counts and altered sperm morphology. A direct toxic testicular damage in smokers may damage testicular endocrine and spermatogenetic functions reducing sperm quality (Harlev et al. 2015).

In conclusion, the reported data represent preliminary observations on the relationship between abnormal levels of biochemical components and seminal pathological conditions. High levels of ALB may be associated with reduced sperm motility, viability, and pH; in addition, they are increased also in the considered group of infertile patients and this is probably due at an inflammatory status as showed by the contemporary presence of leukocytospermia and germ cells. GGT activity and TP levels were increased in semen from patients with low motility and CK activity in subjects with reduced sperm vitality and pH. These data as well as the significant decrease of ALP and FOL in hyperviscous semen and in smokers suggest that some biochemical components may be involved in an imbalance of seminal oxidative status. Moreover, the increased amount of FERR, observed in presence of germ cells in ejaculates, seem to be related at an altered spermatogenesis and infertility.

We are aware that the study was carried out in a small size sample group and further studies, with an increased number of patients selected and grouped according to their reproductive pathologies, are required. Enrolling a large number of patients will enable us to use a multiple/logistic regression analysis to test whether the biochemical factors could be used as a model to predict seminal pathological findings.

Materials and methods

Patients

The study group consists in 97 selected Italian men (aged 22–45 years, mean age 34.13 ± SD 4.82) attending our laboratory for reproductive problems and semen analysis in the period between January 2015 and September 2017. They were men that did not seek pregnancy and attended our center to control their semen parameters (unknown reproductive potential, 44) and patients, who did not get pregnancy after two years of unprotected sexual intercourse (infertile, 53), the female factor was excluded.

The inclusion/exclusion criteria for this study consisted in non azoospermic men with a normal 46, XY karyotype evaluated by conventional cytogenetic analysis, a normal hormonal profile (follicle stimulating hormone FSH, luteinizing hormone LH, and testosterone T), no history of drugs and alcohol, radiotherapy, chemotherapy, diabetes, chronic illness nor medication, the absence of systematic sperm defects, the absence of cryptorchidism. Men with varicocele and/or positive semen bacteriology were excluded from the study as well as patients with a body mass index (BMI) >25 kg/m^2. The evaluation of the patients included information about their medical history and life style factors, such as the use of cigarettes; the smokers were included in the study. Smokers were individuals that smoked >15 cigarettes a day and non smokers were subjects that never smoked.

Among 97 selected men, 36 were smokers (>15 cigarettes a day) and 31 were non smokers. Selected men were not under pharmacological treatment for the past 3 months. In this study, we considered only ejaculates with a volume ≥1.5 ml to prevent potential influence of sampling and ensure all biochemical components to be detected. The individuals provided a written informed consent before the inclusion in this study that was approved by the Ethics Committee of Azienda Ospedaliera Universitaria Senese, CEAOUS.

Semen analysis

Participants were instructed to abstain from intercourse and masturbation for 3–5 days before the sperm collection. Semen samples were collected by masturbation in a sterile container and samples were examined after liquefaction for 30 min at 37°C. Volume, pH, concentration, morphology, and motility were assessed as recommended by World Health Organization (2010). Volume was read in a modified graduated glass measuring cylinder, pH with pH paper in the range 6.0–10.0, peroxidase stain was used for leukocytes identification; a value of $>1 \times 10^6$ leukocytes/ml was considered out of range; the seminal fluid hyperviscosity was diagnosed when the semen formed a thread longer than 2 cm (WHO 2010). The presence of germ cells, which are not present in normal samples, was also recorded.

Biochemical assays

Each semen samples was centrifuged at 12,000*g* for 5 min. The SP was collected by micropipette and stored in 2-ml cryotubes at −80°C until use. The stored SP was thawed at room temperature and again centrifuged at 12,000*g* to obtain a sample free of cells as reported by Feng et al. (2015).

SP samples, (1 ml at room temperature), were tested using a COBAS 8000 modular analyzer (Roche Diagnostics, Mannheim, GmbH, Germany) by means of two analytical modules: C702, the high throughput clinical chemistry module and E602, the immunoassay module. We measured the following parameters in SP: ALB (albumin g/dl), ALP (alkaline phosphatase U/L), CK (creatine kinase U/L), E2 (estradiol pg/µl), FERR (ferritin ng/ml), GGT (gamma-glutamyl transpeptidase ng/ml), LDH (lactate dehydrogenase; U/L), TP (total protein g/dl), B12 (vitamin B12 pg/ml), FOL (folic acid ng/ml).

For the analytes measured in module C702 (ALB, ALP, CK, GGT, LDH, TP), COBAS 8000 calibration was done with the human lyophilized serum Calibrator C.f.a.s. (Roche Diagnostics, Mannheim, GmbH, Germany). Human lyophilized serum PreciControl ClinChem Multi levels 1 and 2 (Roche Diagnostics, Mannheim, GmbH, Germany) were respectively used as normal and pathologic controls.

For the analytes measured in module E602 (FERR, E2, B12, FOL) we used specific calibrator for each analyte (Roche Diagnostics, Mannheim, GmbH, Germany). PreciControl Varia levels 1 and 2 (Roche Diagnostics, Mannheim, GmbH, Germany) and PreciControl Universal levels 1 and 2 (Roche Diagnostics, Mannheim, GmbH, Germany) were used as normal and pathologic controls respectively.

Statistical analysis

The Kolmogorov–Smirnov test was performed to verify the normality of distributions. Medians and interquartile ranges (IQR: 75°–25° centile) or absolute frequencies and percentages (%) were used to represent the data. The correlations between 10 seminal biochemical components and semen parameters were evaluated by Spearman's rho coefficient. The comparisons between groups -infertile /unknown reproductive potential, semen parameters ≤ or >5th percentile, with/without leukocytospermia, presence/absence of hyperviscosity and presence/absence of germ cells- were calculated by Mann–Whitney *U* test or Chi square test depending on variable distribution. A P value <0.05 was considered statistically significant; data analyses and graphic representations were performed by SPSS v. 20 (SPSS Inc., Chicago, USA).

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