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ORIGINAL RESEARCH

Relationship Between Heavy Metal levels in Seminal Plasma and Sperm Quality in Iranian Men

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Abstract: **Introduction:** During the last decades, frequent reports on the poor semen quality in humans have raised many researchers' concerns to study the possible impact of lifestyle or environmental factors on semen quality. The debate is continuously growing on the adverse reproductive effects of exposure to heavy and trace metals found in the environment, even at their relatively low levels. **Materials and Methods:** This study was carried out from July 2018 to February 2019. A total of 40 men were divided into two groups (idiopathic oligo– and/or asthenozoospermia and normozoospermic men) to determine the correlation between arsenic (As), cadmium (Cd), copper (Cu), and manganese (Mn) levels in the seminal plasma with sperm quality parameters including concentration, total motility, progressive motility, viability, mitochondrial membrane potential (MMP), sperm plasma membrane integrity (SPMI), acrosome integrity (AI) and DNA fragmentation in Iranian men with idiopathic oligo– and/or asthenozoospermia and normozoospermic individuals. **Results:** A significant positive or suggestive correlation was found between as concentrations in the seminal plasma and sperm concentration, motility, progressive motility, viability, MMP, SPMI, and DNA fragmentation. Moreover, seminal plasma Cd concentrations were also correlated negatively with sperm viability. We also found a positive correlation between the seminal plasma Mn levels and sperm concentration, motility, progressive motility, morphology, viability, acrosome integrity, and DNA fragmentation. **Conclusion:** We showed that the levels of As, Cd, and Mn levels in the seminal plasma are associated with the sperm functional parameters. Considering the unpleasant effects of the studied metals on semen quality, it is suggested that long-term contact with these metals be avoided, especially by people at their reproductive age.

Keywords: Infertility, Metals, Trace Elements, Semen quality, Sperm

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1. Introduction

All humans are at daily risk of exposure to various environmental factors such as pesticides, exogenous estrogens, and heavy metals, that may negatively affect their fertility potential (1-4). In recent decades, heavy metal exposure has increased dramatically due to environmental pollution and lifestyle change. Some heavy metals such as cadmium, mercury, and lead are toxic to humans and may lead to nutritional deficiencies, hormonal imbalances, system dysfunctions, and infertility (1, 5). On the other side, some metals such as copper, manganese, selenium, and zinc are essential for health maintenance; however, excessive exposure to

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these beneficial metals may be hazardous (6).

Among all infertility cases, approximately 40–50% result from male factors such as suboptimal sperm parameters (7, 8). Moreover, semen could be an important criterion for assessing fertility and diagnosing male reproductive disorders (9). Seminal plasma is the liquid part of semen, secreted by epididymis and the accessory glands before and during ejaculation. Seminal plasma components like cytokines and carbohydrates play an important role in the regulation of maternal immunological tolerance, the factor necessary for successful fertilization, and embryo implantation (10).

Several studies have reported a decline in semen quality over the past decades. A meta-analysis by Carlsen et al. showed a trend of decreasing sperm count and volume over the last 50 years (11). Although the exact mechanism for such decline in sperm quality is not fully understood, environmental factors such as heavy metals are possible contributing agents that may be harmful to sperm production. Environmental and occupational exposure to several metals, including arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg), are known to alter semen quality (1, 12). Cadmium and arsenic are some of the most commonly found metals associated with several reproductive adverse effects like decreased semen quality and increased DNA damage (13, 14). These metals have widespread environmental distribution and accumulate in the human body over a lifetime. Arsenic exposure through occupational sources like welding, metallurgy, furnace, and groundwater contamination has been linked to abnormal semen quality and sperm DNA damage (15); cadmium exposure may impair semen quality, cause sperm DNA damage, decrease fertility rates, increase spontaneous abortion or male infertility (16, 17). Copper and manganese, which often function as cofactors for metal-activated enzymes, have been associated with reduced semen quality in animals and in humans (18-20). Several studies have been shown that levels of these metals increased in the environment, drinking water, air exposure, food, and soil in Tehran, Iran. For example, in the street dust samples collected from Southern and Eastern parts of Tehran, risk assessment code results showed a high risk for Pb, Cd, and Zn (21). Analytical results from agricultures soil in Teheran indicated that in the winter and summer seasons, Mn, Zn, Pb and Cu are maximum in concentration in some stations (22). Findings from a systematic review showed Cd contamination in Iranian food groups such as rice, cereal and legumes, canned tuna fish, vegetables, fruit juice, and egg (23).

Acknowledging the influence of geographical variations on sperm quality and heavy metal exposure, this study was carried out to determine the correlation among arsenic, cadmium, copper, and manganese levels in the seminal plasma with sperm quality parameters including concentration, total motility, progressive motility, viability, mitochondrial membrane potential (MMP), sperm plasma membrane integrity (SPMI), acrosome integrity (AI) and DNA fragmentation in Iranian men with idiopathic oligo– and/or asthenozoospermia and normozoospermic individuals.

2. Materials and Methods

2.1. Study design

This study is a hospital-based, case-control study conducted from July 2018 to February 2019 in Tehran, Iran. The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study (IR.SBMU.RETECH.REC.1396.256), and written informed consent was obtained from all participants.

2.2. Subject recruitment

Our study subjects were male partners of infertile couples undergoing in vitro fertilization treatment. Twenty patients with idiopathic male infertility (idiopathic oligo– and/or asthenozoospermia) and twenty normozoospermic individuals were recruited. They were subject to thorough general medical and genital examination. We chose the participants among the normal BMI men with no underlying disease. The exclusion criteria were men diagnosed with normal Body Mass Index (BMI) or specific diseases that may impair reproductive capacities such as a serious structural disorder of the pelvic organs, clinical Varicocele, genital infection, and testicular trauma, cryptorchidism, mumps orchitis, and testicular atrophy.

2.3. Sample collection

Fresh semen samples were obtained by masturbation in polypropylene containers after a 3-5 day sexual abstinence period. After liquefaction at 37°C, An aliquot of semen samples was used for semen analysis, and then the seminal plasma was obtained by centrifugation at 1500g for 10 minutes at room temperature. An aliquot of approximately 1 mL of seminal plasma was frozen and stored at -20 °C until analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (7900 Agilent Technologies, Santa Clara, CA). Before analysis, seminal plasma samples were thawed at room temperature and then mixed gently for homogenization.

2.4. Routine semen analysis

According to WHO guidelines, the volume and pH of the semen samples were measured after semen liquefaction (24). An aliquot of semen sample (2 μL) was transferred to a pre-warmed SpermTrack® chamber (Proiser®, Spain), and sperm concentration, total sperm motility, and sperm progressive motility was evaluated by using a Sperm Class Analyzer® software (SCA, version 6, Microptic Co., Spain).

2.5. Morphological evaluation

Sperm morphology was evaluated using the Papanicolaou staining method. An aliquot of semen sample (20 μL) was transferred to slides, smeared, and air-dried. Air-dried slides were fixed and stained with Papanicolaou staining reagent. Two hundred sperm from each slide were evaluated and classified as normal and abnormal according to the strict sperm morphology criteria and expressed as the percentage of normal and abnormal sperm morphology (25).

2.6. Acrosome integrity assessment

Acrosomal status was assessed by labeling fixed sperm with fluorescein is othiocyanate-conjugated Pisumsativum (FITC-PSA) (Sigma, USA). Briefly, an aliquot of sperm sample (30 μL) was transferred to slides, smeared, and air-dried. The air-dried sperms were permeabilized by cold methanol for 30 minutes at room temperature. Subsequently, they were dried and incubated with 50 μl of FITC-PSA in DPBS for 1 hour and washed with a stream of double-distilled water and dried at room temperature. Finally, in order to avoid the fading of fluorescence, the slides were mounted with glycerol. At least 200 sperms were evaluated and scored per slide by means of a fluorescence microscope (Olympus BX51, Tokyo, Japan) at 1000× magnification. Sperm with FITC-PSA fluorescence in the acrosome region was scored as acrosome intact, whereas sperm without FITC-PSA fluorescence or with only equatorial segment fluorescence was considered as acrosome-reacted.

2.7. Mitochondrial membrane potential detection

For detection of MMP, 5, 50, 6, 60, tetrachloro-1, 10, 3, 30 - tetraethylbenzimidazolcarbocyanine iodide (JC-1; SigmaAldrich Chemical Co., Germany), a lipophilic cationic dye, was used. JC-1 was stored in the dark at -20 C. According to the manufacturer's instruction, an aliquot of semen samples were centrifuged at 800 g for 5 min, and sperm were resuspended with phosphate-buffered saline (PBS) to adjust sperm density to 1 × 106/mL. For each sample, sperms were mixed and incubated with 5 μM of JC-1 (dissolved in DMSO; Sigma-Aldrich, USA) for 15 min at 37°C in the dark. Afterward, sperm cells were washed with PBS to remove the dye and resuspended in minimum volume to prepare the slide and were observed under the fluorescence microscope at an excitation of 488 nm and emission at 590 nm for red/green fluorescence. At high MMP, JC-1 forms J-aggregates inside the mitochondria and emits orange/red fluorescence while in low MMP state; it will remain in the monomer form and emit green fluorescence.

2.8. Sperm plasma membrane integrity evaluation

The hypo-osmotic swelling test (HOST) was used for analyzing the functional integrity of the sperm membrane. This test is based on the semi-permeability of the intact cell membrane, which allows the sperm to swell under hypo-osmotic conditions when an influx of water results in an expansion of cell volume. HOST was carried out, according to Ziarati et al. (26). In brief, 10 μL of sperm samples were added to 100 μL of hypo-osmotic solution (comprised of 0.73 g sodium citrate tri-hydrate and 1.35 g fructose dissolved in 100 ml of distilled water; 150 mOsm/L) and incubated at 37 °C for 30 min in a 5% CO2 incubator. Subsequently, a drop (15 μL) of incubated sperms was mixed with 5 μL of 2% eosin Y solution on a glass slide, covered with a coverslip, and valuated under a phase-contrast microscope (×40). A least 200 spermatozoa were evaluated per slide, and the percentages of spermatozoa with coiled tails were recorded as cells with the intact plasma membrane.

2.9. Sperm DNA integrity assessment

Sperm DNA integrity was examined by the sperm chromatin dispersion (SCD) technique using the sperm DNA fragmentation assay kit (SDFA; ACECR, Tehran, Iran) according to the manufacturer's instructions. The SCD assay is based on the principle that sperm with fragmented DNA fails to produce the characteristic halo of dispersed DNA loops observed in sperm with non-fragmented DNA following acid denaturation and removal of excess nuclear protein. Five SCD patterns have been defined: Sperm with large (the halo width is the same or larger than the smallest diameter of the core), medium (the halo size ranges from similar or larger than one-third of the minimum core diameter to a width of less than the smallest core diameter), small (the halo width is smaller than the minimum core diameter) and without halo and sperm cells without a halo and degraded (27). The Sperm DNA fragmentation index (DFI) is defined as the percentage of sperm with fragmented DNA (the sperm nuclei show either a small halo or no halo) in the sample.

2.10. Determination of metals in seminal plasma by ICP-MS

Based on a previously conducted method (10, 28). Briefly, seminal fluid aliquots (0.5 mL) were digested with 70% nitric acid (HNO3) (Fisher Scientific, USA), filtered, and diluted to 5 mL; digestion was performed in a closed vessel microwave system. Double distilled deionized water from the Milli-Q system was used for dilution before performing the analyses. All glassware was washed and immersed in concentrated HNO3 overnight and then rinsed with deionized water before use. Metals analysis was conducted via inductively cou-

pled plasma mass spectrometry (7900 Agilent Technologies, Santa Clara, CA). The operating parameters of ICP-MS were presented in Table S1.

2.11. Quality assurance

The concentration of As, Cd, Cu, and Mn in each sample was detected at three replications, and the average was calculated. The values of the limit of quantitation (LOQ), limit of detection (LOD), and recovery rate of heavy metals was presented in Table S2. To determining the recovery, blank samples at levels of 5, 25, 75, 150, 250, and 500 μg/mL of heavy metals (As, Cd, Cu, and Mn) were prepared in triplicates (29, 30).

2.12. Statistical analysis

The data were analyzed using the SPSS/PC Program version 25 (SPSS, Chicago, USA). All values are presented as means ± standard deviations (SD). $p \leq 0.05$ was considered significant. Student's t-test was used to analyze the difference in the groups. Correlation analysis between variables was performed by Pearson's test.

3. Results

3.1. Sociodemographic data of patients

This study included 20 men with idiopathic oligo– and/or asthenozoospermia and 20 age-matched healthy controls. Patients and controls were comparable regarding age (32±4.03 years for patients with idiopathic oligo– and/or asthenozoospermia versus 33.4±4.9 years for controls). None of the subjects had obvious occupational exposure to heavy metals. Semen parameters did not vary according to the job, age, or smoking habits. They all belonged to the same population.

3.2. Comparison of sperm parameters in oligo– and/or asthenozoospermia men and controls

Liquefaction time, pH, and semen volume were not different between these two groups (Table 1), while sperm concentration, normal sperm morphology, progressive motility, sperm viability, mitochondrial membrane potential (MMP), sperm plasma membrane integrity (SPMI), and sperm acrosome integrity showed a statistically significant decrease in oligo– and/or asthenozoospermia male semen samples when compared to the control group with p-value <0.001 (Table 1, Figure 1). Sperm DNA fragmentation percentage showed a statistically significant increase in oligo– and/or asthenozoospermia male semen samples compared to the control group with p-value <0.001 (Table 1, Figure 2). The frequency of each sperm morphological abnormality was presented in Table 2. Between two groups of men studied, the percentage of the double head, giant head, round head, pinhead, double tail, coiled tail, and without tail sperm were similar. The percentage of other categories of sperm abnormalities such as amorph sperms, short tail sperms, and sperms with excess residual cytoplasm (ERC) were significantly increased in oligoasthenozoospermic men compare to normozoospermic men (Table 2).

3.3. Comparison between seminal plasma arsenic, cadmium, copper, and manganese levels (in μg/L) in oligo– and/or asthenozoospermia men and controls

Table 3 shows the mean concentration of As, Cd, Cu, and Mn in seminal plasma in oligo– and/or asthenozoospermia and controls subjects. As and Mn levels were significantly higher in oligo– and/or asthenozoospermia patients (p-value < 0.01 and p-value < 0.05, respectively) compared to control. There was, however, no significant difference in the seminal plasma Cd and Cu concentrations between groups (Table 3).

3.4. Correlations between semen seminal plasma arsenic, cadmium, copper, and manganese levels (in μg/L) and sperm parameters

Initially, correlations between the sperm parameters and the metal concentrations were studied after combining the data from two groups. Pearson's correlation coefficients for these data are given in Table S3. As shown in Table S3 and Figure 3, there was significant positive correlation between seminal plasma as concentration and sperm DNA fragmentation (r = 0.52, p-value <0.05). There were however significant negative correlation between seminal plasma As concentration and sperm count (r = -0.32, p-value <0.05), motility (r = -0.50, p-value <0.01), progressive motility (r = -0.40, p-value <0.05), viability (r = -0.45, p-value <0.01), MMP (r = -0.37, p-value <0.05), AI (r = -0.40, p-value <0.01) and SPMI (r = -0.36, p-value <0.05). Additionally, a significant negative correlation was noted between seminal plasma Mn concentration and sperm count (r = -0.43, p-value <0.01), motility (r = -0.40, p-value <0.01), progressive motility (r = -0.41, p-value <0.01), normal sperm morphology (r = -0.47, p-value <0.01), viability (r = -0.32, p-value <0.05)and AI (r = -0.31, p-value <0.05). A significant positive correlation was also showed between sperm DNA fragmentation and seminal plasma Mn concentration (r = 0.54, p-value <0.01) (Table S3). Seminal plasma Cd concentration were negatively correlated with sperm viability (r = -0.33, p-value <0.01). In accordance to our finding, Wang et al. reported a significantly inverse dose-dependent relationships between Mn, As and Cd levels and progressive motility and between As and Cd levels and total motility (31). There was no correlation between the seminal plasma concentrations of Cu with any parameter of the semen. There were significant positive correlations between seminal plasma Mn level and sperm DNA fragmentation in-

dex in control group (r = 0.55, p-value <0.05) (Table S4, Figure 3). Significant positive correlations were also noted between seminal plasma Mn level and SPMI among oligo– and/or asthenozoospermia subjects (r = 0. 57, p-value <0.01) (Table S4, Figure 4). As shown in Table 4, in our samples, high concentrations (ppb) of Cu were determined in the seminal plasma of subjects. If we observe Table 3, Cu concentrations in our population were in agreement with Chinese subjects (18, 19). The levels of Cu in seminal plasma collected in Turkey (32) and Nigeria (33) were higher than reported levels in our study. The levels of Cd reported in this study were comparable to those reported in Turkey (32) and lower than Cd levels in seminal plasma measured in people of Lebanon (10), Pakistan (34), and Egypt (35). Seminal plasma Mn concentrations in our population were in agreement with those reported in Pakistan (34), but higher Mn levels were found in the seminal plasma of Chines cases (18). For seminal plasma levels of As, our results were in agreement with those reported in Lebanon (10) but lower than those reported in China (18).

4. Discussion

In the current study, we investigated the association between arsenic, cadmium, copper, and manganese levels in seminal plasma and sperm parameters like mitochondrial membrane potential (MMP), plasma membrane integrity (SPMI), and acrosome integrity. Taken together, statistically significant inverse correlations were found between the sperm quality and the levels of As, Mn, and Cd. Besides, the concentrations of As and Mn in the seminal plasma of the abnormal group were significantly higher than those of the normal group. Since the 1990s, reports about the declining trend of semen quality in humans have focused researches on the possible impacts of environmental or lifestyle factors like environmental and occupational exposure to trace elements, environmental pollution, and pesticides on semen quality and male reproductive health (11, 36). There is a growing concern for adverse reproductive effects associated with exposure to heavy and trace metals encountered in the environment, even at relatively low levels. Although heavy metals have variable distributions in body fluids (37), the common practice of measuring these metals in the blood may not necessarily be truly reflective of the actual exposure of the male reproductive tract. Some metals have a preferential predisposition to male reproductive organs (38, 39). A study showed that measurements of heavy metals in the seminal plasma are more predictable for semen quality than conventional blood measurements (10). Therefore, we conducted this study to explore correlation between these metals and semen quality. This was achieved using ICP–MS and compared with semen qualities such as sperm concentration, motility, progressive motility, normal morphology, viability, mitochondrial membrane potential (MMP), plasma membrane integrity (SPMI), acrosome integrity and DNA fragmentation. We found a significant or suggestive correlation among arsenic concentration in seminal plasma and sperm concentration, motility, progressive motility, viability, mitochondrial membrane potential (MMP), plasma membrane integrity (SPMI), and DNA fragmentation. Some studies showed arsenic adversely affects the reproductive performance and semen quality that is characterized by abnormal sperm morphology, decreased sperm count, and decreased sperm motility and viability that is consistent with our findings (40-42). In a study conducted by Huang et al., integrated proteomics and metabolomics analysis revealed that arsenic exposure mainly impaired spermatogenesis and fertilization via aberrant modulation of male reproduction-related proteins and metabolites, which may be mediated by the ERK/AKT/NF-KB-dependent signaling pathway (43). In our study, seminal plasma Cd concentration was also negatively correlated with sperm viability. Our results go in line with Sukhn et al., which found that Cd in seminal fluid to be significantly associated with low sperm viability. Cd by increasing peroxidation of membrane lipids results in sperm death (10). In support of our findings, Wang et al. (2017) reported that seminal plasma As and Cd were associated with reduced semen quality in a linear dose-dependent manner (31). They found significantly inverse dose-dependent relationships between Mn, As, and Cd levels and progressive motility and between Us and Cd levels and total motility (31). Cd can act as an endocrine-disrupting chemical that leading to the inhibition of steroidogenesis and spermatogenesis. This metal has potent estrogen- and androgen-like activities and binds to androgen and estrogen (44, 45). Several studies suggested that Cd exposure may contribute to impaired semen quality, decreased fertility rates, increased frequencies of spontaneous abortion, and male infertility (44, 46). We found a correlation between seminal plasma Mn levels and sperm concentration, motility, progressive motility, normal morphology, viability, acrosome integrity, and DNA fragmentation. Several studies have demonstrated the deleterious effects of Mn on reproductive potential in men without occupational Mn exposure (36, 47). In an in vitro study using semen from healthy male volunteers, researchers showed that 500 ppm Mn2+ significantly inhibited sperm motility with no accompanying change in seminal malondialdehyde levels (48). So, long-term Mn exposure should be avoided in life, especially for people at reproductive age.

5. Conclusion

Exposure to environmental pollutants such as As, Mn, and Cd, which may occur occupationally or indirectly, affects se-

men quality and male infertility. There is a need, therefore, to understand conditions that may predispose some people to accumulate high amounts of toxicants from exposures no higher than what most of us encounter. It may be better to develop new strategies to prevent the continuous contamination of the environment by these toxicants. Besides, every society should have its own epidemiological data to establish the necessary precautions. Future works with larger sample sizes, human epidemiologic studies, and mechanistic studies, are needed to confirm these findings.

6. Appendix

6.1. Acknowledgements

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6.2. Author contribution

All the authors have the same contribution.

6.3. Funding/Support

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6.4. Conflict of interest

No conflict of interest.

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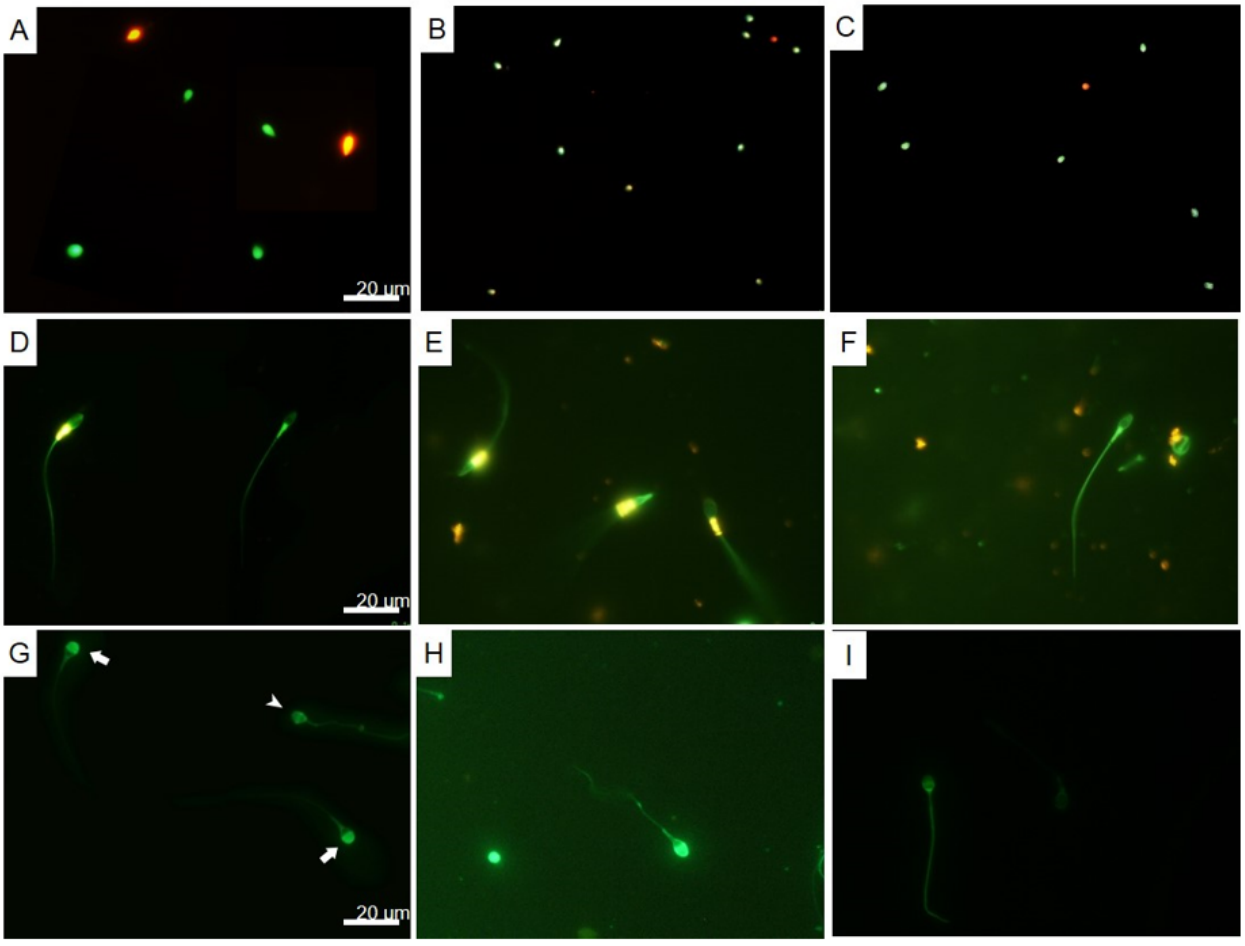

Figure 1: Fluorescent microscopic analysis of sperm characteristics. (A) Live and Dead assay, sperm displaying green fluorescent was considered as viable, while sperm displaying red fluorescent was considered as non-viable. (B) Representative images showing the Live and Dead staining for normal and (C) oligo– and/or asthenozoospermia individuals. (D) Mitochondrial membrane potential assessed by JC 1 stain, orange and green florescent indicated high and low mitochondrial membrane potential respectively. (E) Representative images of JC 1 staining for normal and (F) oligo– and/or asthenozoospermia men. (G) Sperm Acrosome integrity analysis by FITC-PSA stain, arrow indicated intact acrosome and arrowhead indicated reacted acrosome. (H) Representative images of FITC-PSA staining for normal and (I) oligo– and/or asthenozoospermia men. Scale bar 20 µm.

Table 1: Basic statistics of various semen parameters in the investigated groups.

Semen parameters	Normozoospermia (n=20)	Oligozoospermia (n=20)	P–value
Age (year)	33.4±4.9	32±4.03	ns
Semen volume (ml)	4.70±1.32	4.51±1.87	ns
Liquefaction time (minutes)	22±5.5	18±6.5	ns
pH	7.4±0.5	7.5±0.6	ns
Sperm Concentration (mil/ml)	74.45±36.19	5.05±2.50	<0.001
Normal sperm morphology (%)	6.30±0.92	3.05±0.60	<0.001
Progressive sperm motility (%)	52.05±12.76	23.73±11.30	<0.001
Sperm viability (%)	75.15±8.39	47.85±10.95	<0.001
Sperm DNA fragmentation (%)	21.20±6.64	38.75±8.9	<0.001
Mitochondrial membrane potential (MMP) (%)	68.20±8.95	44.25±11.59	<0.001
Sperm plasma membrane integrity (SPMI) (%)	64.50±10.30	47.75 ±8.45	<0.001
Sperm acrosome integrity	66.55±9.53	47.40±9.73	<0.001

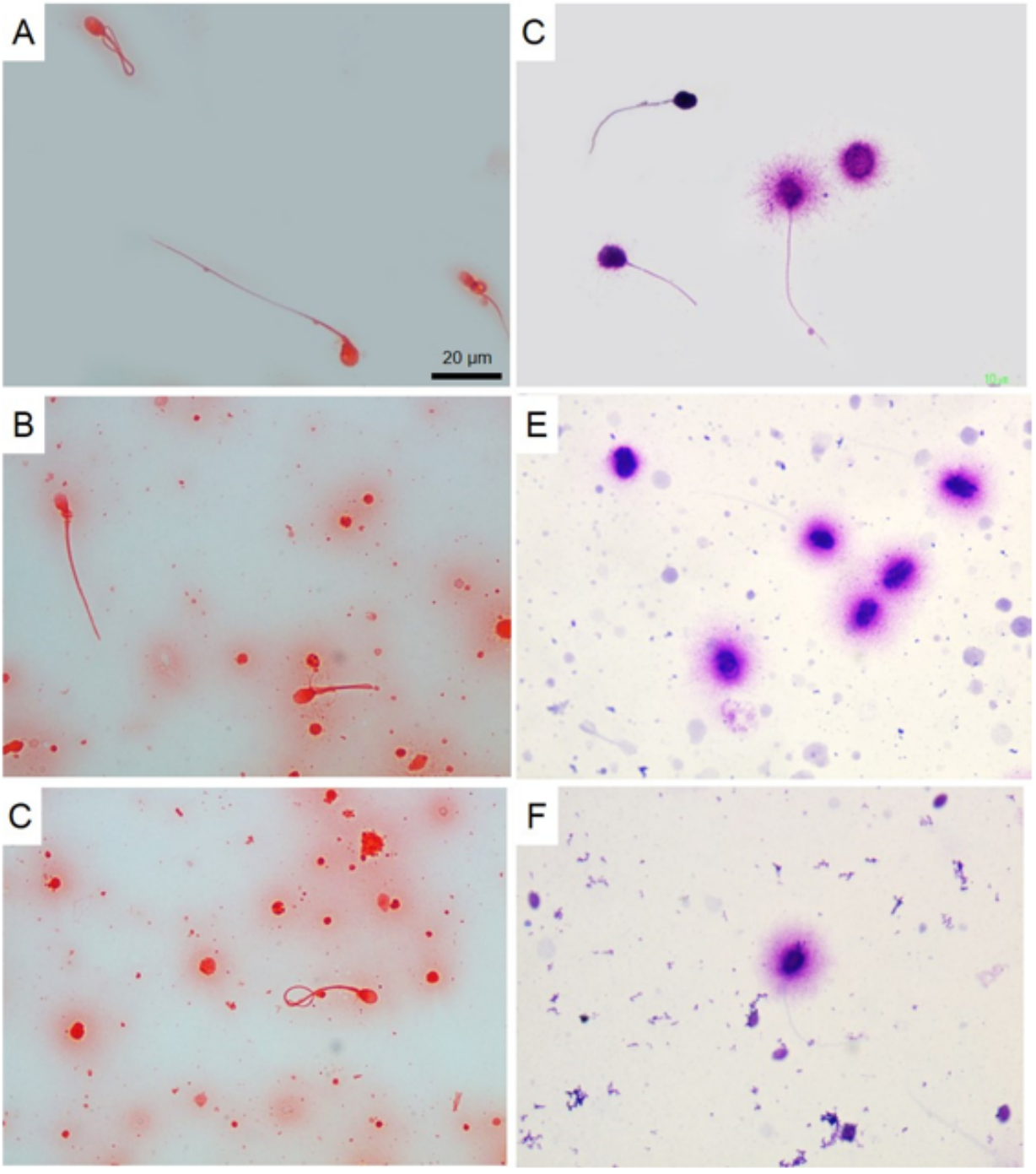

Figure 2: light microscopic analysis of sperm characteristics. (A) Assessment of membrane integrity by hypo-osmotic swelling test, arrow indicated intact membrane and arrowhead indicated damage membrane. (B) Representative images showing hypo-osmotic swelling test for normal and (C) oligo– and/or asthenozoospermia individuals. (D) Sperm DNA fragmentation analyzed by sperm chromatin dispersion test (SCDT) indicated sperm with Large halo (I), Medium halo (II), Small halo (III) and No halo (IV). (E) Representative images of SCDT for normal and (F) oligo– and/or asthenozoospermia men. Scale bar 20 µm.

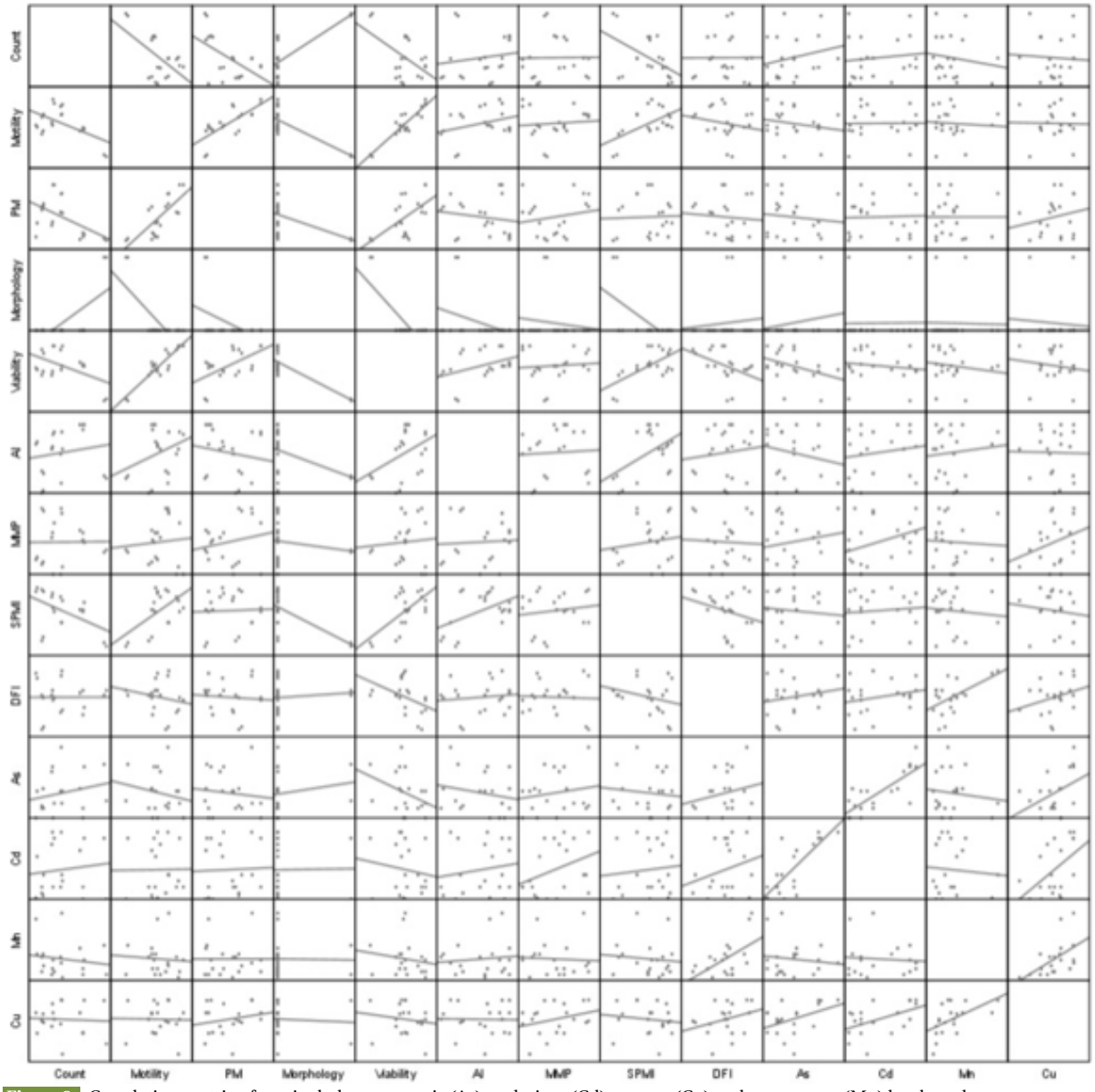

Figure 3: Correlation matrix of seminal plasma arsenic (As), cadmium (Cd), copper (Cu) and manganese (Mn) levels and sperm parameters in control individuals. PM; progressive motility, AI; sperm acrosome integrity, MMP; sperm mitochondrial membrane potential, SPMI; sperm plasma membrane integrity, DFI; DNA fragmentation index.

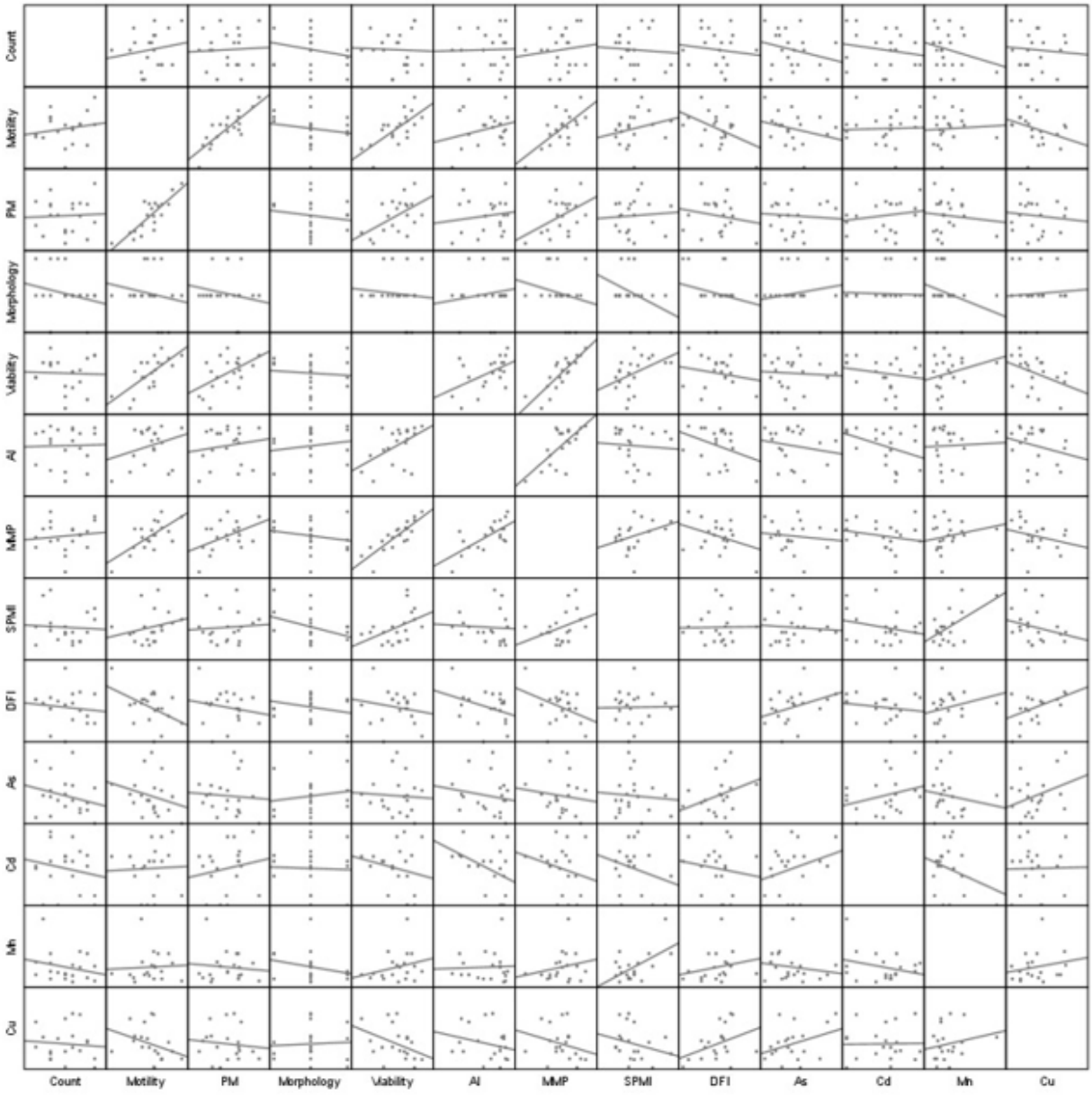

Figure 4: Correlation matrix of seminal plasma arsenic (As), cadmium (Cd), copper (Cu) and manganese (Mn) levels and sperm parameters in oligo– and/or asthenozoospermia individuals. PM; progressive motility, AI; sperm acrosome integrity, MMP; sperm mitochondrial membrane potential, SPMI; sperm plasma membrane integrity, DFI; DNA fragmentation index.

Table 2: Sperm morphology in normozoospermic and oligasthenozoospermic individuals.

Sperm morphology parameters	Normozoosperm (n=20)	Oligoasthenozoosperm (n=20)
Amorph	82.20 ± 5.78 *	74.85 ± 5.77
Double Head	0.20 ± 0.42	0.25 ± 0.55
Giant Head	1.10 ± 0.73	1.40 ± 1.09
Round Head	1.70 ± 1.15	1.50 ± 1.27
Pin Head	3.60 ± 2.27	5.05 ± 5.67
Double Tail	0.10 ± 0.31	0.40 ± 0.68
Coiled Tail	1.70 ± 1.49	2.40 ± 1.81
Short Tail	5.70 ± 3.40 *	10.95 ± 5.88
Without Tail	0.20 ± 0.42	0.40 ± 0.59
Excess residual cytoplasm (ERC)	3.70 ± 2.45 *	6.10 ± 2.86

Table 3: Concentrations (ug/L) of metals in human seminal plasma of investigated groups.

Metal	Normozoospermia (n=20)	Oligozoospermia (n=20)	P–value
As	10.25±8.30	22.70±15.29	<0.01
Cd	0.48±0.41	0.75±0.46	ns
Cu	144.80±51.46	118.65±44.52	ns
Mn	7.35±5.92	19.15±13.89	<0.05

Table 4: Comparison of mean concentrations of toxic metals in semen samples of different countries with our results.

First author	Year	Samples		Age (year)		Semen Cd conc/(μg/l)		Semen Cu conc/(μg/l)		Semen As conc/(μg/l)		Semen Mn conc/(μg/l)	
		normal-quality	low-quality	normal-quality	low-quality	Normal-quality	low-quality	normal-quality	low-quality	normal-quality	low-quality	normal-quality	low-quality
This study	2020	20	20	32±4.03	33.4±4.9	0.48±0.41	0.75±0.46	144.80±51.46	118.65±44.52	10.25±8.30	22.70±15.29	7.35±5.92	19.15±13.89
Sukhn C(10)	2018	61	55	37.6±0.8	37.4±0.8	3.67 (11.3±2.7)	6.22 (55.6±29.8)	-	-	17.87 (31.15±4.7)	17.80 (43.2±7.54)	-	-
Zafar A (34)	2015	25	25	-	-	1.71±0.75	8.15±9.97	77.74±31.61	232±353	-	-	23.88±19.9	5.93 ± 7.73
Taha EA (35)	2013	30	30	34.6±6.47	32.5±6.1	2.77±0.51	3.77±0.29	-	-	-	-	-	-
Kahraman S (32)	2012	10	42	-	-	0.65±0.30	1.2±0.37	765 (510-928)	560 (560-692)	-	-	-	-
Li (18)	2012	28	21	33.8±3.1	34.5±2.6	-	-	195.±45.2	222.7±0.9	68.62±41.66	53.16±16.39	103.9±25	127.5±33.6
Akinloye O (33)	2011	40	20	36.6±1	35.2±1	-	-	392.7±38.7	362.2±24.7	-	-	-	-

Table 5: Operating parameters of ICP-MS (7900 Agilent Technologies, Santa Clara, CA) the determine concentration of As, Cd, Cu and Mn in seminal plasma.

Parameter	Unit	Value
Plasma argon flow	L/min	12
Measurement replicate	-	3
Nebulizer argon flow	L/min	0.7
Auxiliary argon flow	L/min	0.3
Sample flow rate	mL/min	1
Sample uptake time	s	240
Plasma, auxiliary and nebulizer gas	-	Argon
Type of detector Solid state	-	CCD

Table 6: LOQ, LOD and recovery rate of heavy metals analyzed by ICP-MS analysis at 6 spiking levels (n = 3).

Metals	LOQ (μg/L)	LOD (μg/L)	Average recovery rate (%)	RSD (n = 6)
As	2.45	1.00	95.00	2
Cu	4.55	1.00	96.00	5
Cd	0.27	0.05	93.00	4
Mn	3.48	1.00	96.00	6

Table 7: Correlation of seminal plasma arsenic (As), cadmium (Cd), copper (Cu), and manganese (Mn) levels with sperm parameters.(Continuous)

		Count	Motility	PM	Morphology	Viability	AI	MMP	SPMI	DFI	As	Cd	Mn	Cu
Count	Pearson Correlation	1	.571**	.476**	.859**	.561**	.624**	.625**	.379*	-.611**	-.326*	-.205	-.437**	.193
	Sig. (2-tailed)		.000	.002	.000	.000	.000	.000	.016	.000	.040	.204	.005	.232
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
Motility	Pearson Correlation	.571**	1	.919**	.559**	.936**	.718**	.797**	.734**	-.761**	-.501**	-.241	-.411**	.142
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.001	.134	.008	.383	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
progressive motility	Pearson Correlation	.476**	.919**	1	.523**	.830**	.544**	.724**	.542**	-.634**	-.402*	-.168	-.414**	.246
	Sig. (2-tailed)	.002	.000	.001	.000	.000	.000	.000	.000	.010	.299	.008	.126	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
Morphology	Pearson Correlation	.859**		.559**	.523**	1	.544**	.531**	.576**	.341*	-.638**	-.311	-.251	-.476**
.212														
	Sig. (2-tailed)	.000	.000	.001	.000	.000	.000	.031	.000	.051	.118	.002	.189	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
Viability	Pearson Correlation	.561**	.936**	.830**	.544**	1	.766**	.834**	.773**	-.717**	-.454**	-.339*	-.326*	.069
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.003	.032	.040	.671	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
sperm acrosome integrity	Pearson Correlation	.624**	.718**	.544**	.531**	.766**	1	.735**	.602**	-.605**	-.441**	-.307	-.317*	.107
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.004	.054	.046	.510	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
sperm mitochondrial membrane potential	Pearson Correlation	.625**	.797**	.724**	.576**	.834**	.735**	1	.632**	-.680**	-.375*	-.220	-.297	.207
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.017	.172	.063	.199	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40

Table 7: Correlation of seminal plasma arsenic (As), cadmium (Cd), copper (Cu), and manganese (Mn) levels with sperm parameters. (Continuous)

		Count	Motility	PM	Morphology	Viability	AI	MMP	SPMI	DFI	As	Cd	Mn	Cu
sperm plasma membrane integrity	Pearson Correlation	.379*	.734**	.542**	.341*	.773**	.602**	.632**	1	-.563**	-.365*	-.252	-.137	.051
	Sig. (2-tailed)	.016	.000	.000	.031	.000	.000	.000	.000	.021	.117	.400	.755	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
DNA fragmentation index	Pearson Correlation	-.611**	-.761**	-.634**	-.638**	-.717**	-.605**	-.680**	-.563**	1	.525**	.231	.541**	.012
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000	.000	.000	.001	.152	.000	.943	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
As	Pearson Correlation	-.326*	-.501**	-.402*	-.311	-.454**	-.441**	-.375*	-.365*	.525**	1	.513**	.105	.178
	Sig. (2-tailed)	.040	.001	.010	.051	.003	.004	.017	.021	.001	.001	.521	.273	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
Cd	Pearson Correlation	-.205	-.241	-.168	-.251	-.339*	-.307	-.220	-.252	.231	.513**	1	-.035	.158
	Sig. (2-tailed)	.204	.134	.299	.118	.032	.054	.172	.117	.152	.001	.831	.329	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
Mn	Pearson Correlation	-.437**	-.411**	-.414**	-.476**	-.326*	-.317*	-.297	-.137	.541**	.105	-.035	1	.105
	Sig. (2-tailed)	.005	.008	.008	.002	.040	.046	.063	.400	.000	.521	.831	.518	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40
Cu	Pearson Correlation	.193	.142	.246	.212	.069	.107	.207	.051	.012	.178	.158	.105	1
	Sig. (2-tailed)	.232	.383	.126	.189	.671	.510	.199	.755	.943	.273	.329	.518	
	N	40	40	40	40	40	40	40	40	40	40	40	40	40

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 8: Correlation of seminal plasma arsenic (As), cadmium (Cd), copper (Cu), and manganese (Mn) levels with sperm parameters in oligo– and/or asthenozoospermia men and control individuals.(Continuous)

Groups			Count	Motility	PM	Morphology	Viability	AI	MMP	SPMI	DFI	As	Cd	Mn	Cu
Normozoo-spermia	Count	Pearson Correlation	1	-.568**	-.536*	.695**	-.510*	.156	.009	-.498*	.004	.225	.115	-.150	-.053
		Sig. (2-tailed)		.009	.015	.001	.021	.510	.970	.025	.988	.341	.629	.529	.824
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Motility	Pearson Correlation	-.568**	1	.745**	-.731**	.917**	.318	.087	.569**	-.202	-.189	.010	-.069	-.020
		Sig. (2-tailed)	.009		.000	.000	.000	.172	.714	.009	.392	.425	.965	.773	.932
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	progressive motility	Pearson Correlation	-.536*	.745**	1	-.412	.578**	-.160	.189	.035	-.078	-.114	.034	.000	.198
		Sig. (2-tailed)	.015	.000		.071	.008	.499	.425	.883	.744	.632	.887	.999	.404
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Morphology	Pearson Correlation	.695**	-.731**	-.412	1	-.759**	-.360	-.141	-.614**	.093	.175	.013	-.020	-.065
		Sig. (2-tailed)	.001	.000	.071		.000	.119	.552	.004	.698	.461	.956	.933	.785
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Viability	Pearson Correlation	-.510*	.917**	.578**	-.759**	1	.388	.081	.631**	-.425	-.363	-.140	-.156	-.146
		Sig. (2-tailed)	.021	.000	.008	.000		.091	.733	.003	.062	.115	.556	.513	.539
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	sperm acro-some integrity	Pearson Correlation	.156	.318	-.160	-.360	.388	1	.055	.489*	.113	-.206	.159	.104	-.018
		Sig. (2-tailed)	.510	.172	.499	.119	.091		.817	.028	.636	.384	.502	.661	.941
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	sperm mito-chon-drial mem-brane poten-tial	Pearson Correlation	.009	.087	.189	-.141	.081	.055	1	.143	-.045	.173	.355	-.055	.298
		Sig. (2-tailed)	.970	.714	.425	.552	.733	.817		.547	.851	.465	.124	.818	.201
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	sperm plasma mem-brane integrity	Pearson Correlation	-.498*	.569**	.035	-.614**	.631**	.489*	.143	1	-.271	-.105	.097	-.099	-.125
		Sig. (2-tailed)	.025	.009	.883	.004	.003	.028	.547		.247	.658	.685	.679	.599
		N	20	20	20	20	20	20	20	20	20	20	20	20	20

Table 8: Correlation of seminal plasma arsenic (As), cadmium (Cd), copper (Cu), and manganese (Mn) levels with sperm parameters in oligo– and/or asthenozoospermia men and control individuals.

Groups			Count	Motility	PM	Morphology	Viability	AI	MMP	SPMI	DFI	As	Cd	Mn	Cu
	DNA fragmentation index	Pearson Correlation	.004	-.202	-.078	.093	-.425	.113	-.045	-.271	1	.211	.244	.552*	.296
		Sig. (2-tailed)	.988	.392	.744	.698	.062	.636	.851	.247		.373	.300	.012	.204
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	As	Pearson Correlation	.225	-.189	-.114	.175	-.363	-.206	.173	-.105	.211	1	.788**	-.125	.417
		Sig. (2-tailed)	.341	.425	.632	.461	.115	.384	.465	.658	.373		.000	.600	.067
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Cd	Pearson Correlation	.115	.010	.034	.013	-.140	.159	.355	.097	.244	.788**	1	-.075	.500*
		Sig. (2-tailed)	.629	.965	.887	.956	.556	.502	.124	.685	.300	.000		.754	.025
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Mn	Pearson Correlation	-.150	-.069	.000	-.020	-.156	.104	-.055	-.099	.552*	-.125	-.075	1	.541*
		Sig. (2-tailed)	.529	.773	.999	.933	.513	.661	.818	.679	.012	.600	.754		.014
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Cu	Pearson Correlation	-.053	-.020	.198	-.065	-.146	-.018	.298	-.125	.296	.417	.500*	.541*	1
		Sig. (2-tailed)	.824	.932	.404	.785	.539	.941	.201	.599	.204	.067	.025	.014	
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
Oligo– and/or asthenozoospermia	Count	Pearson Correlation	1	.170	.049	-.210	-.036	.027	.121	-.064	-.119	-.254	-.180	-.239	-.084
		Sig. (2-tailed)		.473	.837	.373	.880	.909	.611	.789	.618	.280	.448	.309	.724
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Motility	Pearson Correlation	.170	1	.839**	-.172	.709**	.285	.695**	.244	-.465*	-.271	.039	.055	-.337
		Sig. (2-tailed)	.473		.000	.469	.000	.223	.001	.300	.039	.248	.872	.819	.146
		N	20	20	20	20	20	20	20	20	20	20	20	20	20

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 8: Correlation of seminal plasma arsenic (As), cadmium (Cd), copper (Cu), and manganese (Mn) levels with sperm parameters in oligo– and/or asthenozoospermia men and control individuals.

Groups			Count	Motility	PM	Morphology	Viability	AI	MMP	SPMI	DFI	As	Cd	Mn	Cu
	Progressive motility	Pearson Correlation	.049	.839**	1	-.167	.534*	.151	.462*	.073	-.181	-.075	.170	-.103	-.111
		Sig. (2-tailed)	.837	.000		.483	.015	.524	.040	.761	.445	.754	.474	.666	.642
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Morphology	Pearson Correlation	-.210	-.172	-.167	1	-.086	.148	-.197	-.368	-.201	.150	-.032	-.264	.061
		Sig. (2-tailed)	.373	.469	.483		.718	.532	.405	.111	.396	.529	.892	.261	.797
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Viability	Pearson Correlation	-.036	.709**	.534*	-.086	1	.504*	.844**	.446*	-.177	-.063	-.193	.268	-.399
		Sig. (2-tailed)	.880	.000	.015	.718		.023	.000	.048	.456	.793	.414	.253	.082
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	sperm acrosome integrity	Pearson Correlation	.027	.285	.151	.148	.504*	1	.701**	-.068	-.339	-.177	-.402	.043	-.248
		Sig. (2-tailed)	.909	.223	.524	.532	.023		.001	.775	.144	.454	.079	.856	.293
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	sperm mitochondrial membrane potential	Pearson Correlation	.121	.695**	.462*	-.197	.844**	.701**	1	.356	-.364	-.129	-.221	.215	-.260
		Sig. (2-tailed)	.611	.001	.040	.405	.000	.001		.124	.114	.588	.349	.363	.269
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	sperm plasma membrane integrity	Pearson Correlation	-.064	.244	.073	-.368	.446*	-.068	.356	1	.016	-.080	-.254	.571**	-.264
		Sig. (2-tailed)	.789	.300	.761	.111	.048	.775	.124		.947	.739	.279	.008	.261
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	DNA fragmentation index	Pearson Correlation	-.119	-.465*	-.181	-.201	-.177	-.339	-.364	.016	1	.347	-.145	.227	.385
		Sig. (2-tailed)	.618	.039	.445	.396	.456	.144	.114	.947		.134	.543	.336	.094
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	As	Pearson Correlation	-.254	-.271	-.075	.150	-.063	-.177	-.129	-.080	.347	1	.295	-.167	.351
		Sig. (2-tailed)	.280	.248	.754	.529	.793	.454	.588	.739	.134		.207	.480	.129
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Cd	Pearson Correlation	-.180	.039	.170	-.032	-.193	-.402	-.221	-.254	-.145	.295	1	-.291	.017
		Sig. (2-tailed)	.448	.872	.474	.892	.414	.079	.349	.279	.543	.207		.213	.943
		N	20	20	20	20	20	20	20	20	20	20	20	20	20

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 8: Correlation of seminal plasma arsenic (As), cadmium (Cd), copper (Cu), and manganese (Mn) levels with sperm parameters in oligo– and/or asthenozoospermia men and control individuals.

Groups			Count	Motility	PM	Morphology	Viability	AI	MMP	SPMI	DFI	As	Cd	Mn	Cu
	Mn	Pearson Correlation	-.239	.055	-.103	-.264	.268	.043	.215	.571**	.227	-.167	-.291	1	.205
		Sig. (2-tailed)	.309	.819	.666	.261	.253	.856	.363	.008	.336	.480	.213		.387
		N	20	20	20	20	20	20	20	20	20	20	20	20	20
	Cu	Pearson Correlation	-.084	-.337	-.111	.061	-.399	-.248	-.260	-.264	.385	.351	.017	.205	1
		Sig. (2-tailed)	.724	.146	.642	.797	.082	.293	.269	.261	.094	.129	.943	.387	
		N	20	20	20	20	20	20	20	20	20	20	20	20	20

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).