Effects of Various Elements in Seminal Plasma on Semen Profiles

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Abstract: *This study aimed to investigate the effects of metals on semen profiles. The concentrations of 50 elements in seminal plasma collected from 128 infertile men were measured. Eleven (Na, Mg, P, K, Ca, Fe, Cu, Zn, Se, Rb, Sr) elements were positively detected in all samples. Another eight elements (V, Mn, Co, As, Mo, Cd, Sn, Ba) were detected in over 75% of the samples. In these 19 elements, significant correlations were observed only between copper concentration and sperm motility. The presence of cadmium and zinc in seminal plasma was associated with a low total sperm number (p=0.067) and low sperm motility (p=0.052), respectively. Higher concentrations of cadmium were observed in the Brinkmann index under 100 than in that over 100 (p=0.055). Recovery of sperm motility after EDTA treatment was observed with in vitro exposure to 300 μg/ml of zinc sulfate. Declines in sperm motility after exposure to 50 μg/ml of copper sulfate were irreversible, even with EDTA treatment. It was suggested that excess copper and zinc in seminal plasma was detrimental for male reproductive capacity by reducing sperm motility. It also appeared that cadmium may exert toxic effects on spermatogenesis, after long-term exposure, as occurs with cigarette smoking.*

Key words: *Copper, Zinc, Cadmium, Seminal plasma, Semen parameter*

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Introduction

Recently, it has been suggested that environmental factors may have harmful effects on reproductive organs [1]. Some chemical substances such as dioxin apparently act as endocrine disrupters, which have been confirmed to result in harmful effects on reproductive systems and embryonic development [2–5]. Damage to human fertility, specifically a decline in male reproductive capacity has been suggested in many other reports, and the influence of environment factors including chemical substances and other pollutants in air, water, and soil have been examined [6–10]. Among the substances named elsewhere, many metals are discharged as environmental pollutants in developed countries.

This study sought to investigate the influence of elements (metals) on human male reproductive capacity. First, the relationship between the concentration of elements in seminal plasmas and semen profiles was examined. Second, the correlation between the concentration of elements in seminal plasma and smoking habit, which was thought to represent a source of metal exposure, was investigated. Finally, the effects of chelater on sperm motility after exposure of sperm to metallic elements such as copper and zinc *in vitro* was investigated.

Materials and Methods

Collection of seminal plasma

Ejaculated semen was collected by masturbation from

128 subjects between the ages of 23 and 50 years (mean ± SD = 38.8 ± 5.3 years), who visited the hospital of Fukushima Medical University during January 1996 to December 1998. After liquefaction of the semen samples for 30 min at 37°C, general semen profiles were analyzed with a computer-assisted sperm analyzer (CASA, Cell Soft 3000, Cryo Resources Co., USA). Seminal plasmas were obtained by centrifugation (300 g, 5 min), and each 1.5 ml of supernatant was preserved in a sampling tube (1.5 ml, Eppendorf-IATRON, Tokyo, Japan), determined to be free of metals by preliminary assessment, at –30°C until measurement.

Semen evaluation

Sperm concentration and sperm motility were measured with CASA. The total sperm number (TSN: volume (ml) X sperm concentration (/ml)) in a single ejaculation was calculated. According to WHO criteria [11], the 128 samples were sorted into two groups, consisting of cases of normozoospermia (n=50) and impaired semen profile cases (n=78).

Measurement of element concentration in seminal plasma

Fifty elements from thawed samples were analyzed at the Department of Epidemiology and Environmental Health of Juntendo University. After thawing, 400 μl of the sample and the same amount of HNO_3 (TAMAPURE-AA-100, Tama Chemical Co., Japan) were mixed and allowed to react overnight in the vial (Taf-tainer vial, G.L.Science, Japan), which had been treated with nitric acid to remove any trace of metals. Metals in the samples were measured by the following methods:

1) Flame atomic absorption spectrometry

The concentrations of sodium (Na), potassium (K), calcium (Ca), zinc (Zn), and magnesium (Mg) were determined with a flame atomic absorption spectrometer (Z-6100, Hitachi Co., Tokyo, Japan), and an acetylene-air-based flame source. Samples were aspirated at 5.5 ml/minute. The detection limits were 50 ng/ml for Na, 100 ng/ml for K, Ca and Zn and 10 ng/ml for Mg.

2) Colorimetric method

Phosphorus (P) concentration was determined by a colorimetric method; 600 μl of a sample solution, 200 μl of 2M sulfuric acid (Wako Pure Chemical, Tokyo, Japan) and 200 μl of a 7.8 mM ammonium molybdate solution (Wako) were mixed, after which 160 μl of a mixture containing 5% ascorbic acid (Wako) and 2 mM antimony potassium tartrate (Wako), and 840 μl distilled water were added. After 10 minutes, absorption at 880 nm was measured. The concentrations were obtained from a standard calibration curve. The detection limit was 5 ng/ml.

3) Microwave induced plasma-mass spectrometry (MIP-MS)

Another 44 elements shown in Table 1 were measured by MIP-MS according to the method described by Shinohara *et al.* [12]. Then 200 μl of H_2O_2 (Wako) was added to the vial and microwaved to induce a reaction (mls 1200 mega, Milestone s.r.l., Italy, 250 W for 2 min, 0 W for 1 min and 400 W for 5 min). The resulting solutions were mixed with pure water (specific resistance, 18.3 MΩ) to a volume of 2.0 ml and analyzed. The digestion blank was measured under the same conditions without seminal plasma. A Type P-7000 plasma-mass spectrometer (Hitachi Co., Tokyo, Japan), which requires pure N_2 gas (99.999%) as plasma gas and carrier gas was used to perform analyses.

Smoking habits

Thirty-nine subjects responded to questionnaires concerning smoking habit, the age at which the subject began smoking, duration of smoking habit in years, the number of cigarettes smoked daily, and the Brinkmann index (duration (years) × cigarette numbers per day). Subsequently, the relation between smoking and metal concentrations was examined.

Influence of in vitro exposure to metals on sperm motility

After obtaining consent from 3 subjects, who were then treated with IVF-ET, the surplus spermatozoa collected by swim-up procedure was used as experimental samples. Based on previous results, Cu and Zn were chosen for the following analysis. Stock solutions of Zn sulfate ($ZnSO_4 \cdot 7H_2O$) and Cu sulfate ($CuSO_4 \cdot 5H_2O$) were prepared at concentrations of 500 mg/ml and 50 mg/ml, respectively, and stored in metal-free plastic tubes at room temperature until use. According to our preliminary experiments, 300 μg/ml of Zn sulfate (68.3 μg/ml of Zn) and 50 μg/ml of Cu sulfate (12.8 μg/ml of Cu) were prepared by adding each stock solution to a culture medium (human tubal fluid (HTF), Irvine Scientific, Santa Ana, CA, USA) supplemented by 10% SSS (Serum Substitutes Supplements, Irvine Scientific, Santa Ana, CA, USA) as an incubation medium. Motile sperm was exposed to each metal in the plastic culture dish (FALCON, 35-3037 center-well

Table 1. Results of element concentrations (μg/ml) in seminal plasma

Element	Mean	SD	Maximum	Minimum	Rate of detection (%)
Li	0.010	0.011	0.055	ND	83/128 (65)
Na	3088.094	474.437	3980.000	1870.000	128/128 (100)
Mg	147.280	72.115	347.000	14.600	128/128 (100)
P	1053.094	211.133	1510.000	276.000	128/128 (100)
K	694.000	409.877	2519.000	283.000	128/128 (100)
Ca	365.878	136.316	781.600	49.800	128/128 (100)
V	0.005	0.003	0.011	ND	110/128 (86)
Mn	0.007	0.005	0.027	ND	122/128 (95)
Fe	0.300	0.293	2.086	0.048	128/128 (100)
Co	0.004	0.002	0.009	ND	116/128 (91)
Ni	0.005	0.017	0.140	ND	40/128 (31)
Cu	0.164	0.084	0.457	0.002	128/128 (100)
Zn	210.012	101.347	494.900	30.000	128/128 (100)
As	0.009	0.012	0.070	ND	105/128 (82)
Se	0.103	0.031	0.198	0.025	128/128 (100)
Rb	1.138	0.457	2.889	0.280	128/128 (100)
Sr	0.083	0.043	0.296	0.014	128/128 (100)
Zr	0.036	0.046	0.219	ND	70/128 (65)
Mo	0.003	0.003	0.020	ND	111/128 (87)
Cd	0.004	0.004	0.020	ND	97/128 (76)
In	0.001	0.002	0.010	ND	63/128 (49)
Sn	0.061	0.069	0.333	ND	107/128 (84)
Ba	0.033	0.071	0.566	ND	99/128 (77)

Twenty-three of 50 elements are shown in this table. Another 27 elements (Be, Cr, Ga, Ge, Y, Ag, Sb, Te, La, Ce, Pr, Nd, Sm, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hg, Tl, Pb, Bi, Th, U) are not shown, because they were under the detectable level.

organ culture dish, Becton Dickinson, NJ, USA) under these conditions: 5% CO_2, 5% O_2, and 90% N_2, 100% humidity. For the controls, 2 μl of distilled water was added to 1 ml of sperm in suspension. The pH of the medium was measured with a pH meter (TOA pH meter, HM-30S, TOA Electronics Ltd., Tokyo, Japan), and osmotic pressure was measured with an osmotic pressure meter (Osmotic Pressure Auto & STAT OM-6030, Kyoto First Science Co., Kyoto, Japan). Motile sperm was added to the incubation medium to attain concentrations of 20×10^5/ml, and the sperm was then cultured for 3, 5 and 24 hours. At 3, 5 and 24 hours after culture, sperm motility was analyzed by CASA.

After three hours of exposure, 12 mM of EDTA (ethylene diamine tetraacetic acid, disodium salt, Kanto Chemical Co. Ltd., Tokyo, Japan) was added to the same dose of sperm suspension (the final concentration of EDTA was 6 mM). Metal-free suspensions were treated in the same way as the control. Immediately after the addition of EDTA, the treated sperm was washed with fresh HTF supplemented by 10% SSS by centrifugation (300 g, for 5 min) and cultured for another 2 and 22 hours, followed by measurement of motion profiles with CASA.

Statistical evaluation

StatView J-4.11 (Macintosh) was used to perform statistical analyses. Statistical differences in elements between men with normal and impaired semen profiles were analyzed by ANOVA, with p values <0.05 considered significant. The correlation between semen profiles (sperm concentration, TSN, sperm motility) and element concentrations was studied by linear regression analysis. Paired *t*-test was used for the analysis of sperm motility after metal and chelate exposure, with p values <0.05 considered significant. The data were presented as the means $\pm$ SD, unless otherwise stated.

Results

Element concentrations in seminal plasma

Table 1 gives the basic measurement values for the concentrations of 23 elements in seminal plasma collected from 128 men. Zirconium (Zr) was measured in samples collected from 107 men. Concentration of another 27 elements including Be, Cr, Ga, Ge, Y, Ag, Sb, Te, La, Ce, Pr, Nd, Sm, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu, Hg, Tl, Pb, Bi, Th and U in the seminal plasma were

Table 2. Differences in element concentrations in normozoospermic men and men with impaired semen profiles

Element	Normozoospermic	Impaired	p-value
Na	3092.780 ± 460.192	3085.090 ± 486.275	0.929
Mg	139.388 ± 65.997	152.338 ± 75.759	0.324
P	1033.140 ± 197.287	1065.885 ± 219.842	0.394
K	1247.720 ± 422.019	1268.538 ± 404.452	0.780
Ca	357.116 ± 135.064	371.495 ± 137.687	0.563
V	0.005 ± 0.003	0.004 ± 0.003	0.197
Mn	0.008 ± 0.005	0.007 ± 0.005	0.423
Fe	0.318 ± 0.305	0.290 ± 0.286	0.595
Co	0.004 ± 0.002	0.004 ± 0.002	0.813
Cu	0.150 ± 0.065	0.173 ± 0.093	0.126
Zn	192.120 ± 92.006	221.481 ± 105.891	0.110
As	0.008 ± 0.011	0.010 ± 0.013	0.492
Se	0.104 ± 0.032	0.103 ± 0.031	0.816
Rb	1.100 ± 0.493	1.162 ± 0.434	0.458
Sr	0.082 ± 0.046	0.084 ± 0.041	0.878
Mo	0.002 ± 0.002	0.003 ± 0.004	0.195
Cd	0.004 ± 0.004	0.004 ± 0.005	0.730
Sn	0.060 ± 0.062	0.061 ± 0.074	0.910
Ba	0.028 ± 0.039	0.036 ± 0.085	0.524

Nineteen elements detected in all samples were compared.

under the detectable level. The following elements were detected in all samples: Na, Mg, P, K, Ca, Fe, Cu, Zn, Se, Rb, Sr. These elements plus the other eight elements including V, Mn, Co, As, Mo, Cd, Sn, and Ba were detected in over 75% of the samples. A total of 19 elements were selected to undergo the following statistical analysis.

Comparison of element concentrations in men with normozoospermic and those with impaired semen profiles

Table 2 shows the differences between element concentrations in normozoospermic subjects (n=50) and subjects with impaired semen profiles (n=78). The average ages (in years) of the normozoospermia and impaired profile subjects were 38.4 ± 5.2 and 39.1 ± 5.4, respectively (p=0.573). There was no statistical differences between the two groups with regard to concentrations of the 19 elements specified above.

Correlation between element concentrations and semen profiles

No significant correlation was observed between the sperm concentration and concentrations of 19 elements (Table 3). The TSN and Cd concentrations appeared to be correlated negatively, but no clear-cut statistical proof was obtained (r^2=2.9, p=0.067) (Table 4). On the other hand, it was clearly demonstrated that higher concentrations of Cu had adverse effects on sperm motility (r^2=7.4, p=0.003) (Table 5). Moreover, although no statistical correlation was observed, it appeared that the concentrations of Zn and Cd had some suppressive

Table 3. Linear analysis of regression between element concentrations (μg/ml) in seminal plasma and sperm concentration ($\times 10^6$/ml)

Elements	b	r^2 (%)	F	P
Na	0.005	0.1	0.093	0.761
Mg	0.042	0.2	0.190	0.664
P	0.021	0.3	0.401	0.528
K	0.020	1.1	1.392	0.240
Ca	–0.008	0.0	0.027	0.870
V	75.522	0.0	0.888	0.348
Mn	1449.015	0.9	1.107	0.295
Fe	26.181	1.0	1.214	0.273
Co	1402.049	0.1	0.147	0.702
Cu	–87.577	0.9	1.110	0.294
Zn	–0.022	0.1	0.104	0.748
As	–525.377	0.7	0.859	0.356
Se	342.534	1.9	2.398	0.124
Rb	19.056	1.2	1.552	0.215
Sr	–23.695	0.0	0.021	0.885
Mo	–2035.778	0.6	0.791	0.375
Cd	–2339.126	1.7	2.207	0.140
Sn	–69.402	0.4	0.474	0.493
Ba	19.851	0.0	0.041	0.841

No significant correlation was observed between sperm concentration and concentrations of 19 elements.

Table 4. Linear analysis of regression between element concentrations (μg/ml) in seminal plasma and total sperm number ($\times 10^6$/ejaculate)

Elements	b	r^2 (%)	F	P
Na	0.049	0.4	0.414	0.521
Mg	–0.101	2.1	0.050	0.823
P	0.013	0.8	0.007	0.933
K	0.029	0.1	0.132	0.717
Ca	–0.037	0.0	0.024	0.878
V	262.166	0.0	1.189	0.278
Mn	4323.612	0.4	0.438	0.503
Fe	100.210	8.6	0.848	0.359
Co	3270.902	0.0	0.037	0.848
Cu	–329.847	0.6	0.705	0.403
Zn	–0.071	0.0	0.049	0.825
As	–3097.140	1.3	1.463	0.229
Se	1349.840	1.5	1.702	0.195
Rb	11.165	0.0	0.024	0.877
Sr	–292.591	0.1	0.153	0.697
Mo	–11116.520	1.0	1.159	0.284
Cd	–13663.562	2.9	3.429	0.067*
Sn	–67.642	0.0	0.018	0.892
Ba	63.453	0.0	0.019	0.889

*The TSN and Cd concentrations appeared to be correlated negatively.

Table 5. Linear analysis of regression between element concentrations (μg/ml) in seminal plasma and sperm motility (%)

Elements	b	r^2 (%)	F	P
Na	0.009	1.9	2.297	0.132
Mg	–0.039	1.1	1.273	0.262
P	0.005	0.2	0.208	0.649
K	–0.002	0.1	0.089	0.766
Ca	–0.014	7.4	0.653	0.421
V	1816.456	0.0	3.926	0.050
Mn	182.843	0.1	0.145	0.704
Fe	–3.711	0.2	0.206	0.651
Co	664.009	0.2	0.268	0.606
Cu	–86.328	7.4	9.417	0.003*
Zn	–0.046	3.2	3.850	0.052**
As	–316.891	2.3	2.734	0.101
Se	–60.673	0.5	0.612	0.436
Rb	0.491	0.1	0.008	0.927
Sr	–18.525	0.1	0.108	0.743
Mo	–1415.677	2.8	3.323	0.071
Cd	–1064.516	3.1	3.766	0.055
Sn	70.940	2.6	3.154	0.079
Ba	–6.692	0.0	0.040	0.842

*Statistical correlation was observed. **Tendency was observed.

effects on sperm motility (r^2=3.2, p=0.052 and r^2=3.1, p=0.055, respectively) (Table 5).

Relationship between smoking habit and metal concentrations

We examined the relationship between Brinkmann indexes and the concentrations of Cu, Zn, and Cd in seminal plasmas, which appeared to have some effect on semen profiles. For smoking habit, we found no significant differences with Cu, Zn and Cd concentrations. Relatively higher concentrations of Cd were observed for Brinkmann index values over 100 (0.004 ± 0.001 μg/ml, n=25) compared to values under 100 (0.002 ± 0.001 μg/ml, n=14) (Fig. 1), but this was not statistically significant (p=0.055).

Changes in sperm motility after exposure to metallic elements in vitro

Cu and Zn, which appeared to have suppressive effects on sperm motility were chosen for analysis. Figure 2 shows the effects of these two metals on sperm motility. For concentrations of 300 μg/ml (68.3 μg/ml of Zn) of Zn sulfate, significant declines in sperm motility were observed from 3 hours after initial exposure compared to the control. The pH of the medium containing the 300 μg/ml of Zn sulfate was 7.3–7.0, and osmotic pressure was 275–310 mOsm/kg.H_2O.

In the case of 50 μg/ml (12.8 μg/ml of Cu) of Cu sulfate, significant declines in sperm motility were observed from 3 hours after initial exposure compared to the control. The pH of the medium containing 50 μg/ml of Cu sulfate was 7.2–7.4, and osmotic pressure was 275–310 mOsm/kg.H_2O.

As shown in Fig. 2, some differences were noticed in changes in sperm motility after EDTA treatment during *in vitro* exposures to Zn and Cu. EDTA *per sei* did not have adverse effects on sperm motility. Recovery of sperm motility was noted in cases of 300 μg/ml exposure to Zn, but declines in sperm motility after 50 μg/ml exposure to Cu were irreversible, even after treatment with EDTA.

Discussion

It has been suggested in recent years that environmental factors such as an endocrine disrupters may adversely affect the reproductive organs [1]. In addition to the substances discussed elsewhere, many metals are discharged as environmental pollutants from the combustion of fossil fuel, such as diesel fuel. While certain trace amounts of metals are essential for physiological homeostasis, it is well known that

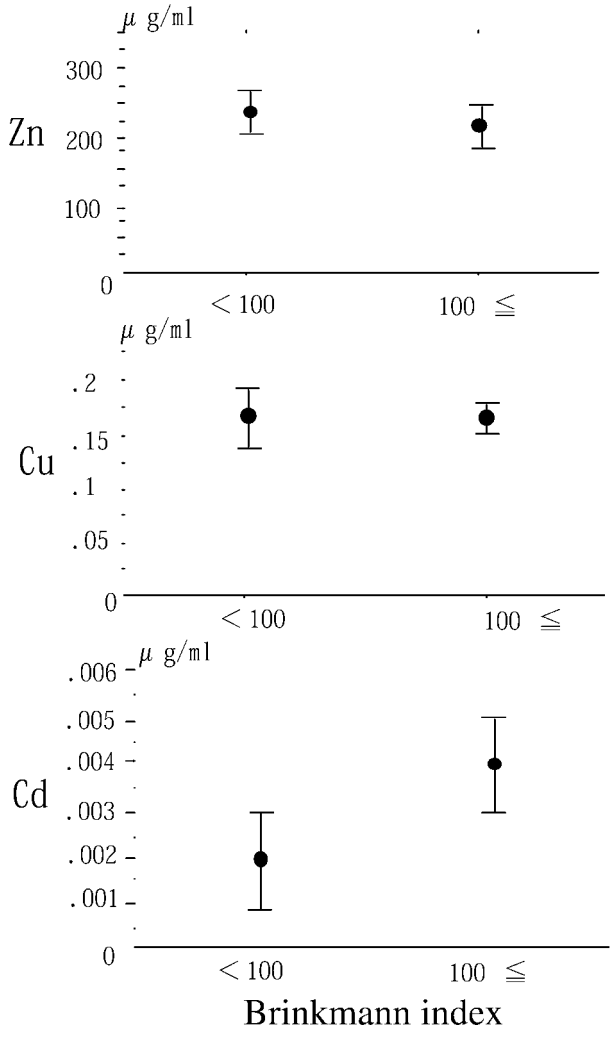

Fig. 1. Relationships between concentrations of zinc, copper and cadmium in seminal plasma, and the Brinkmann index. No significant differences between each metal and the Brinkmann index. There was a tendency for higher concentrations of Cd to be observed with a higher Brinkmann index (p=0.055, mean ± se).

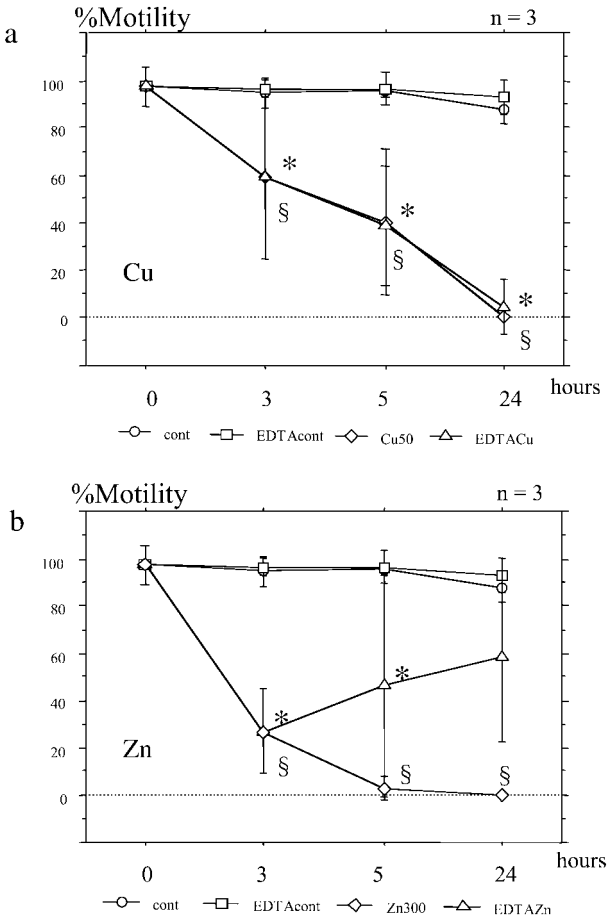

Fig. 2. Effects of zinc and copper exposure *in vitro* on sperm motility, and the effect of EDTA treatment on recovery from impaired sperm motility after exposure to these elements. a: Cu exposure *in vitro*, b: Zn exposure *in vitro*, cont: control, EDTAcont: 6 mM EDTA treated sperm, Cu50: sperm incubated in 50 μg/ml copper sulfate containing medium without 6 mM EDTA exposure, Zn300: sperm incubated in 300 μg/ml zinc sulfate containing medium without 6 mM EDTA exposure, EDTACu: Sperm incubated in 50 μg/ml copper sulfate for 3 hours treated with 6 mM EDTA, EDTAZn: Sperm incubated in 300 μg/ml zinc sulfate for 3 hours treated with 6 mM EDTA. *: P<0.01, §: P<0.01 (mean ± se).

excessive or insufficient concentrations of these elements will induce toxicity and deficiency symptoms, respectively. In addition, many elements have harmful effects on spermatogenesis in the seminiferous tubes of testis.

For example, for lead (Pb), which passes through the blood-testis barrier, it has been reported that concentrations exceeding 35 μg/dl in the blood suppress spermatogenesis. Moreover, such concentrations reduce the steroid synthesis of Leydig cells [13]. Subsequent studies have shown that men exposed to lead at the workplace exhibited impaired semen qualities, such as teratozoospermia and oligozoospermia [14, 15]. In contrast, our study showed no relationship between concentrations of lead in seminal plasma and semen parameters.

Based on animal experiments on rodents, the per os administration of 1.1–1.2 mg/kg of Cd has been reported to induce damaging effects on the endothelial cells of blood vessels and necrotic change within the testes [16]. In contrast to other elements, reports

indicate that Cd is taken into the human body through the respiratory system rather than the digestive tract [17], so that cigarette smoking appears high on the list of suspects as activities leading to Cd intake, due to the Cd content per cigarette (1–2 μg). The effects of smoking on reproductive functions have been studied in detail. Reports indicate that smoking correlates with reduced female fertility and a higher incidence of spontaneous abortions [18, 19] delayed cleavage of embryos derived from the IVF-ET procedure [20]; and reduced fertility among men born from women who had smoked [21]. Although no statistically significant correlation was observed in this study between Cd concentrations in seminal plasma and TSN, Brinkmann index values, which precisely indicate the smoking habits of individuals, exhibited some correlation with Cd concentrations ($p=0.055$). Nevertheless, further investigation of Cd concentrations in blood is required, and follow-up studies may reveal that the TSN of smoking individuals declines with accumulated Cd.

Some levels of metals are essential for normal spermatogenesis and sperm functions. In particular, Zn secreted from prostate gland is found at concentrations 200 to 300 times that in blood, and contributes to the stabilization of the sperm plasma membrane and sperm nuclear structure [22, 23]. Thereafter, Zn is free from spermatozoa during capacitation in the female genital tract [24]. According to other reports, the relationship between the concentrations of Zn in seminal plasma and semen parameters appears to be controversial [25, 26]. This study suggested that excess Zn in seminal plasma should be highly detrimental to sperm mortility, moreover this effect could be alleviated by EDTA treatment. The effects of EDTA, which cannot cross the sperm plasma membrane, showed that impaired sperm motility was caused by excessive Zn levels outside the spermatozoa, and that Zn did not lead to irreversible damage to the spermatozoa in the brief time frame after ejaculation.

The role of Cu in male reproductive capacity appears to be largely unknown, although high concentrations of Cu in seminal plasma correlated with reduced sperm motility both in our study and in previous studies [27]. Excess levels of monovalent and divalent Cu ions in solution should result in lipid peroxidation in sperm plasma membrane, an effect that may render sperm immotile. Moreover, a chelater such as EDTA has no effect on recovery from sperm immobilization after exposure to Cu, in contrast to Zn.

It is known that exposure to excessive levels of Cu, Zn and Cd stimulates the synthesis in the human liver of a metal linkage protein called metallothionein. Metallothionein composed of 62 amino acids reduces the toxicity of elements such as Cu, Zn, Cd and Hg, and its presence has been confirmed in seminal plasma [28]. Therefore, it should be considered that metallothionein combined with Zn in seminal plasma might be produced after exposure to another metallic element. Such evidence calls for special care when investigating the action of metals on male reproductive capacity.

This study sought to verify that three metals had a suppressive effect on sperm function. The study suggests that excess amounts of Cu and Zn in seminal plasma reduce male reproductive capacity by reducing sperm motility. The study also suggests that Cd exerts toxic effects on spermatogenesis after long-term exposure due to activities such as cigarette smoking.

In conclusion, it was highly probable that metals reduced the reproductive capacity of men, exposure to which is associated with environmental pollution and changing lifestyles. Further studies are required to clarify the harmful effects of other materials on the reproductive system.

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