

# Environmental exposure to zinc and copper influences sperm quality in fertile males

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## Abstract

**Introduction.** The presented study was designed to investigate the associations between environmental exposure to zinc and copper and levels of the parameters of oxidative stress and antioxidant defence system and selected cytokines in the seminal plasma of fertile males.

**Materials and methods.** The study population consisted of 65 fertile male volunteers from the southern region of Poland. Based on the medians of the levels of copper and zinc in seminal plasma, the study subjects were divided into 4 subgroups: groups with low and high environmental exposure to copper (Cu-L and Cu-H), groups with low and high environmental exposure to zinc (Zn-L and Zn-H).

**Results.** Semen volume, pH, count, motility and morphology of sperm cells in the Cu-L and Cu-H groups did not differ significantly. In the Cu-H group, TOS was increased significantly by 243% when compared to the Cu-L group. Similarly, median of IL-10 level in the Cu-H group was increased by 144% compared to the Cu-L group. Spearman correlation showed positive correlations between the levels of copper and TOS and IL-10. Negative correlations between copper and G-CSF and GM-CSF were also shown. In the Zn-L group, the percentage of progressively motile sperm cells after 1 hour was significantly higher by 17%, compared to the Zn-L group. In the Zn-H group, levels of G-CSF and MCP-1 were significantly higher by 70% and 145%, respectively, compared to the Zn-L group. The level of IL-10 was significantly lower in the Zn-H group by 60%, compared to the Zn-L group. Spearman correlation indicated that there is a positive correlation between the level of zinc and thiol groups, G-CSF and GM-CSF. Negative correlations between zinc and TOS and IL-10 were also shown.

**Conclusion.** Zinc enhances motility in fertile men. This beneficial effect of zinc may be due to zinc-induced reduction in the plasma oxidative stress intensity and modulations of the immune response. This study confirms the antagonistic relationship between zinc and copper.

## Key words

zinc, copper, semen, oxidative stress, cytokines, fertility

## INTRODUCTION

It is well known that certain trace elements are involved in male fertility. Elements, such as zinc, magnesium, copper, and calcium, are important for the maintenance of normal spermatogenesis, sperm maturation, DNA metabolism and repair and gene expression in germ cells [1]. In humans, the concentration of zinc is high in the prostate compared to the other tissues and body fluids. Zinc is a marker of prostatic function, regulates the functions of spermatozoa, acts as a co-factor for most enzymatic reactions, and helps in the preservation of sperm motility [2].

It has been reported that the majority of copper present in seminal plasma originates from the prostate. However, unlike zinc, copper is also released by other structures of the reproductive tract, e.g. epididymis and seminal vesicles [3]. Copper is an important trace element for numerous metalloenzymes and metalloproteins which are involved in energy or antioxidant metabolism. On the other hand, the ionic form of copper rapidly becomes toxic for a variety of

cells, including human spermatozoa. Copper can promote the formation of reactive oxygen species (ROS) and induce the oxidation and crosslinks between proteins leading to the inactivation of enzymes and structural proteins impairment [4].

The generation of ROS in the male reproductive tract has become a real concern because of their potential toxic effects at high levels on sperm quality and function. Spermatozoa require a minimal level of ROS for normal capacitation and acrosome reaction [4]. Because the concentration of ROS is critical for spermatogenesis and sperm maturation, it must be continuously regulated to retain only the small amount necessary to maintain normal cell function within the male reproductive tract and seminal fluid [5]. As a result, human seminal plasma contains high levels of non-enzymatic and enzymatic oxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) [6].

The function of testicular tissue is also controlled by the neuroendocrine-immune network integrated by cytokines [7]. Seminal fluid contains a complex array of cytokines, chemokines, and other bioactive molecules [8]. Under pathologic conditions, pro-inflammatory cytokines may induce oxidative stress [9]. As a result, anti-inflammatory

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and anti-oxidant properties of zinc [10] may be beneficial for sperm quality.

The presented study was designed to investigate the associations between environmental exposure to zinc and copper and levels of oxidative stress (OS) markers, such as malondialdehyde (MDA) and total oxidant status (TOS) in the seminal plasma of fertile males. Additionally, the following were measured: plasma levels of protein sulfhydryl groups (PSH), uric acid (UA) and activities of several antioxidant enzymes, such as SOD, CAT, GR, glutathione S-transferase (GST), and glucose 6-phosphate dehydrogenase (G6PD). The relationships between zinc and copper levels and levels of cytokines (IL-10, G-CSF, GM-CSF, MCP-1, MIP-1 $\beta$ ) in the seminal plasma were also determined.

## MATERIALS AND METHOD

**Study population.** The study population consisted of 65 healthy, non-smoking, fertile male volunteers from the southern region of Poland. Patients were drug tested and no drug consumption (including antioxidant medications) was reported at the time of the study. All participants had normal semen specimens according to the WHO standards [11]. Based on the medians of the levels of copper (CuS=21.7  $\mu$ g/dl) and zinc (ZnS=135.4 mg/l) in seminal plasma, the study subjects were divided into 4 subgroups:

- 1) with low environmental exposure to copper (Cu-L) – Cu concentration in seminal plasma between 10.2–21.7  $\mu$ g/dl (n=33);
- 2) with high environmental exposure to copper (Cu-H) – concentration in seminal plasma between 21.8–228  $\mu$ g/dl (n=32);
- 3) with low environmental exposure to zinc (Zn-L) – concentration in seminal plasma between 8.10–135.4 mg/l (n=33);
- 4) with high environmental exposure to copper (Zn-H) – concentration in seminal plasma between 135.5–256 mg/dl (n=32).

**Samples collection.** Semen was collected on the same day in the morning before the first meal. Semen samples (2–6 ml) were collected by masturbation after at least 3 days of sexual abstinence (number of days elapsed since last ejaculation was recorded for each volunteer), at home or at laboratory research facilities.

**Semen analysis.** All semen specimens were analysed according to the WHO standards [11], including assessment of seminal volume, sperm cells density, total sperm cells count, motility, supra-vital staining (for percentage of live spermatozoa). Additionally, sperm motility after 24 hours was assessed. Sperm morphology was examined after Papanicolaou staining. The semen samples (1.5 ml) were centrifuged at 6,000 g for 10 minutes in order to separate the spermatozoa from the seminal plasma. The seminal plasma was transferred to other tubes and stored at -75°C until required for the biochemical and lead analysis. Additionally, 10% lysate of spermatozoa in bi-distilled water was performed.

**Determination of zinc.** Concentration of zinc in seminal plasma (ZnS) was measured by atomic absorption spectrophotometry using Unicam 929 and 939OZ Atomic

Absorption Spectrometers with GF90 and GF90Z at a wavelength of 213 nm [12]. Before each assay, 1,500  $\mu$ l deionization water was added to 30  $\mu$ l of sample and mixed thoroughly before analysis. The calibration curve was prepared according to Merck standards. The spectrophotometer was calibrated after each series of assays and calibration curve. The certified Merck company standard was used as an internal control – Zn concentration of 0.1 mg/ml. Data are shown in mg/l.

**Determination of copper.** Concentration of copper in seminal plasma (CuS) was measured by atomic absorption using spectrophotometry Unicam 929 and 939OZ Atomic Absorption Spectrometers with GF90 and GF90Z at wavelength of 324.8 nm [12]. Before each assay, 500  $\mu$ l deionization water was added to 50  $\mu$ l of sample and mixed thoroughly before analysis. The calibration curve was prepared according to Merck standards. The spectrophotometer was calibrated after each series of assays and calibration curve. The certified Merck company standard was used as an internal control – Cu concentration of 10 mg/l. Data are shown in  $\mu$ g/dl.

**Determination of superoxide dismutase (SOD) activity.** The method of Oyanagui [13] was used to measure the activity of SOD in seminal plasma. In this method, xanthine oxidase produces superoxide anions which react with hydroxylamine forming nitric ions. These ions react with naphthalene diamine and sulfanilic acid generating a coloured product. The concentration of this product is proportional to the amount of produced superoxide anions and negatively proportional to the activity of SOD. Absorbance was measured using an automated Perkin Elmer analyzer at a wavelength of 550 nm. The enzymatic activity of SOD was expressed in nitric units. The isoenzymes of SOD, such as Mn-SOD and CuZn-SOD, were also indicated, using KCN as the inhibitor of the CuZn-SOD activity. The activity of SOD is equal to 1 nitric unit (NU) when it inhibits nitric ion production by 50%. Activities of SOD in seminal plasma were expressed in NU/ml.

**Determination of protein sulfhydryl groups (PSH).** PSH concentration was determined as described by Koster [14] using DTNB, which undergoes reduction by compounds containing sulfhydryl groups, yielding the yellow anion derivative, 5-thio-2-nitrobenzoate, which absorbs at a wavelength of 412 nm using an automated Perkin Elmer analyzer. The results are shown in  $\mu$ mol/l.

**Determination of catalase (CAT) activity.** Catalase activity in seminal plasma was measured by the method of Johansson and Borg [15] using an automated Perkin Elmer analyzer. The method is based on the reaction of the enzyme with methanol in the presence of optimal concentrations of hydrogen peroxide. The formaldehyde produced is measured spectrophotometrically at 550 nm as a dye purpald. The activity of Cat-Px is expressed as U/l.

**Determination of glutathione reductase (GR) activity.** The activity of GR in seminal plasma was measured according to Richterich [16] using an automated Perkin Elmer analyzer. Activity was expressed as  $\mu$ moles of NADPH utilized per minute per l in seminal plasma (U/l).

**Determination of glutathione S-transferase (GST) activity.**

The activity of GST in seminal plasma was measured according to the kinetic method of Habig and Jakoby [17] using an automated Perkin Elmer analyzer. The activity of GST was expressed as  $\mu$ moles of thioether produced per minute per l in seminal plasma (mU/l).

**Determination of G6PD.** The activity of G6PD in seminal plasma was measured according to Richterich [16] using an automated Perkin Elmer analyzer. G6PD activity was expressed as  $\mu$ moles of NADPH produced per minute per g protein in seminal plasma (U/g protein).

**Determination of malondialdehyde (MDA).** The product of lipid peroxidation – MDA was measured fluorometrically as a 2-thiobarbituric acid-reactive substance (TBARS) in seminal plasma, according to Ohkawa [18], with modifications. Samples were mixed with 8.1% sodium dodecyl sulfate, 20% acetic acid and 0.8% 2-thiobarbituric acid. After vortexing, samples were incubated for 1 hour at 95°C, and butanol-pyridine 15:1 (v/v) added. The mixture was shaken for 10 minutes and then centrifuged. The butanol-pyridine layer was measured fluorometrically at 552 nm and 515 nm excitation (Perkin Elmer, USA). TBARS values are expressed as malondialdehyde (MDA) equivalents. Tetraethoxypropane was used as the standard. Concentrations are given in  $\mu$ mol/l plasma.

**Determination of Total oxidation status (TOS).** Total oxidant status was measured in seminal plasma according to Erel [19]. The assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium. The change in colour of the ferric ion by xylenol orange is measured as a change in absorbance at 560 nm. This process is applied in an automated Perkin Elmer analyzer and calibrated with hydrogen peroxide. Data are shown in  $\mu$ mol/l.

**Determination of uric acid.** This parameter was determined in seminal plasma using a biochemical photometric assay analyzer. Uric acid in allantoin is oxidized to form hydrogen peroxide which reacts in the presence of peroxidase, N-ethyl-N-(2-hydroxy-3-sulphopropyl)-3-metyloaniline and 4-aminofenazone to a red form. The colour intensity is directly proportional to the uric acid concentration and is measured photometrically.

**Determination of cytokines.** IL-10, G-CSF, GM-CSF, MCP-1, MIP-1b were detected in seminal plasma using a Bio-Plex 200 System (Bio-Rad Laboratories Inc., USA), which is based on 3 core elements. The first core element is a technology that uses fluorescently-dyed magnetic microspheres (beads), each with a distinct colour code to permit discrimination of individual tests within a multiplex suspension, and allows the simultaneous detection of diverse analyte molecules in a single well of a 96-well microplate. Moreover, the magnetic beads allow for magnetic separation during the washing steps. The second core element is a dedicated flow cytometer with 2 lasers (a 532 nm Nd-Yag laser used to excite phycoerythrin in the assay and a 635 nm solid state laser used to excite the dyes inside the beads to determine their 'color' or 'region') and associated optics to measure the different molecules bound to the surface of the beads. The third core element is a

higher-speed digital signal processor that efficiently manages the fluorescent output.

The principle of these bead-based assays is similar to capture sandwich immunoassays. The samples and standards were incubated with the coupled beads (antibodies directed against the desired cytokines were covalently coupled to internally-dyed beads) in the wells of 96-well plates and washed. Next, the biotinylated detection antibodies specific for different cytokine epitopes were added. After incubation and washing, streptavidin (phycoerythrin solution) was added to bind biotinylated detection antibodies on the beads. Next, the suspensions of washed beads were analyzed using the Bio-Plex System. Software presented data as both median fluorescence intensity as well as concentration (pg/ml).

**Statistical analysis.** A database was created in the MS Excel 2007. Statistical analysis was performed using Statistica 10.0 PL software. Statistical methods included mean and standard deviation (SD) for normal distribution and median and interquartile range (IQR) for subnormal distribution. Shapiro-Wilk's test was used to verify normality and Levene's test to verify homogeneity of variances. Statistical comparisons between groups were made by a t-test, t-test with a separate variance or Mann-Whitney U test (non-parametric test). Spearman's coefficient *R* for non-parametric correlation was calculated. A value of  $p < 0.05$  was considered significant.

**RESULTS**

Mean age in the Cu-L and Cu-H groups did not differ significantly. Similarly, no differences were observed in the semen volume and the pH, count, motility and morphology of sperm cells (Table 1).

In the Cu-H group, the Mn-SOD activity and TOS value were increased significantly by 125% and 243%, respectively, when compared to the Cu-L group. Similarly, the median of IL-10 level in the Cu-H group was increased by 144%, compared to the Cu-L group (Table 3). Other parameters did not differ significantly between the examined groups. Spearman correlation showed positive correlations between the levels of copper and TOS ( $R=0.39$ ;  $p=0.015$ ), IL-10 ( $R=0.53$ ;  $p=0.007$ ). Negative correlations between copper and G-CSF ( $R=-0.49$ ,  $p=0.013$ ) and GM-CSF ( $R=-0.57$ ;  $p=0.003$ ) were also shown.

Mean age in the Zn-L and Zn-H groups did not differ significantly. In Zn-L group, the percentage of progressively motile sperm cells after 1 hour was significantly higher by 17% compared to the Zn-L group. Analogically, there was a tendency toward higher values of sperm cells count in 1 ml by 35% (Table 2).

In the Zn-H group, the G6PD activity and levels of UA, G-CSF, and MCP-1 were significantly higher by 95%, 24%, 70%, and 145%, respectively, compared to the Zn-L group. Additionally, there was a tendency toward higher CuZn-SOD activity and levels of PSH and GM-CSF in the Zn-H group by 21%, 22%, and 23%, respectively, compared to the Zn-L group. At the same time, there was a tendency toward lower TOS value and Mn-SOD activity by 9% and 52%, respectively. The level of IL-10 was significantly lower in the Zn-H group by 60% compared to the Zn-L group (Table 3). Other parameters did not differ significantly between the examined groups. Spearman correlation indicated that there is a positive correlation

**Table 1.** Age, semen analysis, parameters related to oxidative stress intensity and antioxidant reserves in seminal plasma and copper concentration in seminal plasma

	Cu-L group (n=33)		Cu-H group (n=32)		p value	relative change
	Mean	SD	Mean	SD		
Copper concentration in seminal plasma (CuS) mg/dl	16.2	3.1	55.0	58.2	<b>&lt;0.001</b>	240%
Age (years)	33.0	4.83	32.1	5.69	0.476	-3%
Sperm volume (ml)	3.72	2.25	3.80	1.66	0.878	2%
pH value	7.56	0.07	7.58	0.07	0.223	0,3%
Sperm cells count in 1ml (mln/ml)	70.5	51.6	72.2	51.9	0.895	2%
Total sperm cells count (mln)	252	205	242	154	0.824	-4%
Motile sperm cells after 1 hour (%)	57.8	9.34	57.8	9.28	0.991	0%
Progressively motile sperm cells after 1 hour (%)	26.7	12.1	25.3	7.64	0.568	-5%
Motile spermatozoa after 24 hours (%)	18.9	18.8	21.3	14.4	0.562	13%
Progressively motile spermatozoa after 24 hours (%)	6.33	9.94	6.00	5.61	0.870	-5%
Normal morphology (%)	49.5	6.64	52.4	8.61	0.142	6%
SOD activity (NU/ml)	181	64.6	191	46.7	0.512	5%
Mn-SOD activity (NU/ml)	26.2	19.3	58.8	74.7	<b>0.029</b>	125%
CuZn SOD activity (NU/ml)	147	43.9	128	64.8	0.227	-13%
PSH concentration (μmol/l)	211	66.3	223	96	0.625	6%
CAT activity (U/l)	642	683	576	254	0.240	-10%
GR activity (U/l)	61.0	61.2	67.0	62.5	0.675	10%
GST activity (mU/l)	4.76	3.85	3.39	1.97	0.094	-29%
G6PD activity (U/l)	70.1	33.6	76.2	64.2	0.760	9%
MDA concentration (μmol/l)	2.72	1.37	2.40	0.73	0.254	-12%
TOS (μmol/l)	4.99	3.81	17.1	18.8	<b>0.011</b>	243%
Uric acid (mmol/l)	3.95	0.83	4.59	1.53	0.155	16%

between the level of zinc and PSH ( $R=0.30$ ;  $p=0.039$ ), G6PD ( $R=0.56$ ;  $p=0.001$ ), UA ( $R=0.32$ ;  $p=0.038$ ), G-CSF ( $R=0.50$ ;  $p=0.011$ ), GM-CSF ( $R=0.57$ ;  $p=0.003$ ). Negative correlations between zinc and TOS ( $R=-0.34$ ;  $p=0.035$ ) and IL-10 ( $R=-0.57$ ;  $p=0.003$ ) were also shown.

## DISCUSSION

Zinc plays an important role in the physiology of sperm cells. Although many papers have been published on the multiple functions of zinc in semen, its influence on the structure, motility and survival of spermatozoa is still controversial [20]. Some studies indicate that a high zinc concentration is associated with normal morphology and increased sperm cell motility and density. Consistently higher zinc levels were found in fertile men, compared to those who were infertile, and zinc supplementation was reported to be an effective method for treatment of the infertile males with chronic prostatitis. On the other hand, some papers indicated that high zinc concentration are associated with poor sperm quality, whereas other studies showed no significant association between plasma zinc level and sperm quality [21–23].

**Table 2.** Age, semen analysis, parameters related to oxidative stress intensity and antioxidant reserves in seminal plasma and zinc concentration in seminal plasma

	Zn-L group (n=33)		Zn-H group (n=32)		p value	relative change
	Mean	SD	Mean	SD		
Zinc concentration in seminal plasma (ZnS) mg/l	85	42.5	174	23.9	0.000	105%
Age (years)	32.5	4.59	32.5	5.93	0.991	0%
Sperm volume (ml)	4.17	2.49	3.34	1.10	0.251	-20%
pH value	7.57	0.08	7.57	0.06	0.815	-0,1%
Sperm cells count in 1ml (mln/ml)	60.8	38.7	82.3	60.4	<b>0.092</b>	35%
Total sperm cells count (mln)	247	198	248	165	0.974	1%
Motile sperm cells after 1 hour (%)	57.3	9.23	58.4	9.36	0.644	2%
Progressively motile sperm cells after 1 hour (%)	24.0	9.3	28.1	10.63	<b>0.027</b>	17%
Motile spermatozoa after 24 hours (%)	17.3	16.1	23.0	17.2	0.176	33%
Progressively motile spermatozoa after 24 hours (%)	4.88	6.02	7.50	9.62	0.191	54%
Normal morphology (%)	50.7	7.07	51.2	8.49	0.776	1%
SOD activity (NU/ml)	188	67.0	183	43.6	0.767	-2%
Mn-SOD activity (NU/ml)	53.0	68.4	25.4	18.1	<b>0.061</b>	-52%
CuZn SOD activity (NU/ml)	126	60.7	153	42.2	<b>0.083</b>	21%
PSH concentration (μmol/l)	192	67.7	235	87	<b>0.072</b>	22%
CAT activity (U/l)	577	499	639	558	0.669	11%
GR activity (U/l)	67.9	58.5	60.3	65.0	0.631	-11%
GST activity (mU/l)	3.74	2.79	4.37	3.38	0.440	17%
G6PD activity (U/l)	50.2	40.5	97.9	57.9	<b>0.009</b>	95%
MDA concentration (μmol/l)	2.50	1.26	2.62	0.93	0.683	5%
TOS (μmol/l)	12.09	14.21	11.0	16.4	<b>0.089</b>	-9%
Uric acid (mmol/l)	3.71	0.95	4.60	1.30	<b>0.008</b>	24%

**Table 3.** Concentrations of cytokines in seminal plasma

	Cu-L group		Cu-H group		p value
	Median	IQR	median	IQR	
IL-10 (pg/ml)	0.57	0.15	1.39	2.66	<b>0.012</b>
G-CSF(pg/ml)	39.9	52.8	16.1	28.6	0.090
GM-CSF(pg/ml)	226	43.5	191	53.6	0.141
MCP-1 (MCAF) (pg/ml)	1493	1850	737	1194	0.114
MIP-1b(pg/ml)	56.3	31.0	44.6	41.6	0.305
	Zn-L group		Zn-H group		p value
	median	IQR	median	IQR	
IL-10 (pg/ml)	1.39	2.66	0.55	0.15	<b>0.010</b>
G-CSF(pg/ml)	10.4	36.7	34.8	17.7	<b>0.025</b>
GM-CSF(pg/ml)	187	53.6	230	40.3	<b>0.068</b>
MCP-1 (MCAF) (pg/ml)	909	981	2225	4048	<b>0.025</b>
MIP-1b(pg/ml)	46.4	45.0	55.3	30.7	0.598

In the presented study, a higher plasma zinc concentration was associated with an elevated sperm count (tendency toward significance) and enhanced motility. Colagar et al. [21] consistently showed significantly higher seminal zinc levels in

fertile subjects than in infertile groups. Besides, seminal zinc in fertile and infertile participants correlated positively with sperm count and normal morphology of sperm. In a study by Fuse et al. [24] there was a positive correlation of zinc level with sperm concentration and motility. Sørensen et al. [23] examined chelation of zinc in semen samples, and postulated that intracellular mitochondrial zinc ions play a crucial role in sperm cell motility. This is in agreement with a study by Kumar et al. [25] who stated that zinc influences sperm motility by improving sperm oxygen uptake and controlling energy utilization through the ATP system. Zinc may also increase the activities of zinc-dependent enzymes, such as sorbitol dehydrogenase and lactate dehydrogenase, which play a significant role in the maintenance of sperm motility [25].

With respect to copper, there is still no full understanding of its role in male reproductive capacity [22]. In a study by Wong et al. [2] there was a positive correlation between blood copper concentrations and sperm motility. Significant correlations between semen copper level and sperm concentration, progressive motility and normal morphology were observed by Jockenhövel et al. [26]. However, Aydemir et al. [4] reported higher levels of plasma copper in a subfertile male group, compared with a fertile male group. In the presented study, no association we found was found between copper level and sperm volume, count, motility and normal morphology. This discrepancy between the results of the above-mentioned studies may be due to the redox activity of copper.

On one hand, copper plays a role as a trace element essential for the activity of numerous metalloenzymes and metalloproteins involved in energy or antioxidant metabolism, such as SOD, cytochrome oxidase, lysine oxidase, dopamine- $\beta$ -hydroxylase, and ceruloplasmin [4]. On the other hand, copper is known to be a catalyst for Fenton and Haber-Weiss reactions which generate hydroxyl free radicals from hydrogen peroxide and superoxide ion radicals [22]. Copper not only promotes ROS formation, but also can bind directly to the free thiol groups of cysteine. In consequence, copper can lead to oxidation and crosslinks between proteins, thus inactivating enzymes or impairing structural proteins [4]. In consequence, the ionic form of copper has been identified as a highly toxic element. Because spermatozoa contain high concentrations of polyunsaturated fatty acids and generate reactive oxygen species, mainly superoxide anion and hydrogen peroxide, they are particularly susceptible to peroxidative damage [2], including lipid peroxidation. Damage to lipid membranes induced by reactive oxygen species has been proposed as one of the major causes of human male infertility [22]. Consistently, Sakhaee et al. [27] showed reduced sperm concentration, motility and viability in rats poisoned with copper. The presented study shows elevated levels of TOS value in the Cu-H group compared to the Cu-L group. A positive correlation between copper levels and TOS values was also shown. These results indicate that some toxic effects of copper may also occur in fertile males.

In the light of this, the mechanisms for maintaining the balance between essential and toxic levels of copper are very important for good sperm quality. First of all, the copper ions in semen are mainly bound to proteins. As a result, copper present in seminal plasma from fertile males is catalytically inactive [28]. Besides, antioxidant protection at sites of gamete production, maturation and storage and embryo implantation has been developed [27]. Human seminal plasma contains high levels of non-enzymatic antioxidants, such as ascorbate,

thiol groups, and less substantial amounts of glutathione (GSH). In addition, the scavenging potential in gonads and seminal fluid is maintained by enzymatic oxidants, such as SOD, CAT, GPx, and GR. SOD is the first line of defence against superoxide radical. Superoxide radical is converted by SOD into hydrogen peroxide which, in turn, is utilized by CAT and GPx [6]. Both copper and zinc are co-factors for SOD (CuZn-SOD). It has been established that zinc only stabilizes SOD, while copper functions in the dismutation reaction by undergoing alternate oxidation and reduction. Therefore, it has been proposed that changes in CuZn-SOD activity are not influenced by zinc, but by copper [20]. The presented results are contradictory to this hypothesis, and showed no association between copper level and CuZn-SOD activity. At the same time, a tendency was shown towards elevated activity of CuZn-SOD in the Zn-H group, compared to the Zn-L group. Consistently, Marzec-Wróblewska et al. [29] observed a positive correlation between SOD activity and zinc levels.

There are more proposed mechanisms by which zinc could act as an antioxidant. First of all, zinc may be a direct scavenger of superoxide radical [30]. Secondly, zinc binds the sulfhydryl groups of proteins, protecting them from oxidation. Thirdly, zinc competes with copper for binding to cell membranes and some proteins, making it more available for binding to metallothionein. As a result, the Cu/Zn ratio has been used previously as an indicator of oxidative stress [31]. Antagonistically, copper inactivates zinc-containing alkaline phosphatase and carbonic anhydrase which are essential for sperm motility [32]. In the presented study, zinc and copper have the opposite effect on the activity of Mn-SOD. However, this interaction is difficult to interpret.

The presented results confirm the above-described protective effects of zinc, and show a negative correlation between zinc level and TOS value, and a positive correlation between zinc level and the level of protein thiol groups. Additively, higher zinc levels are associated with a higher UA level and elevated activity of G6PD. These associations may be also beneficial for semen quality because UA acts as a non-enzymatic antioxidant, while G6PD provides reducing equivalents essential for GSH recovery [33]. Thus, the antioxidant properties of zinc may be one more explanation for the elevated motility of sperm due to zinc action [25].

It has been shown that pro-inflammatory cytokines induce lipid peroxidation and may contribute to the poor sperm quality [9]. In the light of this, the anti-inflammatory properties of zinc seem to be beneficial. Zinc via A20, a zinc finger-transactivating factor, inhibits IL-1 $\beta$ - and TNF- $\alpha$ -induced NF- $\kappa$ B activation. As a result, the generation of inflammatory cytokines and adhesion molecules is reduced. Zinc is also believed to regulate the Th1-response, while the Th2 cytokines are not affected by zinc, except for IL-10 production, which was reported to be increased in zinc-deficient elderly subjects. An increased level of IL-10 down-regulates the Th1-response. In consequence, in a zinc deficiency there is a shift from the Th1- to Th2-response [10]. In concordance with these findings, in the current study, a decreased level of IL-10 in the Zn-H group was found, compared to the Zn-L group. A strong negative correlation was also found between zinc level and IL-10 level. Simultaneously, significantly elevated levels of chemokines, such as G-CSF and MCP-1, were obtained in the Zn-H group, compared to the Zn-L group. G-CSF and GM-CSF levels correlated positively with zinc level. All of these chemokines may have an important role in the successful

fertilization. MCP-1 controls Th2 polarization to maintain the maternal immune tolerance toward the allogeneic foetus. CSFs have been also found to modulate the immune milieu of the female reproductive tract, enabling fertilization. Consistently, it has been reported that seminal fluid from normal fertile donors is characterized by higher levels of MCP-1, G-CSF, and GM-CSF, compared with seminal fluid from infertile men [34]. Besides, it has been shown that that bull spermatozoa express functional GM-CSF receptors that signal for increased glucose and vitamin C uptake. In an *in vitro* study, GM-CSF was able to enhance several parameters of sperm motility in the presence of glucose or fructose [35]. The associations between levels of zinc and GM-CSF, found in the presented study, and between GM-CSF and sperm motility, provide one more explanation for the increased sperm motility due to elevated zinc level.

This study indicates the beneficial effect of zinc action in seminal plasma on the IL-10 level and the levels of investigated chemokines, except for MIP-1 $\beta$ . Interestingly, copper seems to act antagonistically. An elevated level of IL-10 was observed in the Cu-H group, compared to the Cu-L group. Besides, a positive correlation was found between copper level and IL-10 level and negative correlations between copper level and levels of G-CSF and GM-CSF. This antagonism between zinc and copper is difficult to interpret because little is known about zinc- and copper-dependent immunological functions. Therefore, further investigation in this field is needed.

## CONCLUSIONS

Zinc enhances sperm motility in fertile men. This beneficial effect of zinc may be due to zinc-induced reduction in the oxidative stress intensity and modulations of the immune response.

In fertile males, copper levels do not influence sperm volume, count, motility and morphology. However, the threshold of copper toxicity seems to be relatively low because even in fertile males copper is associated with elevated level of oxidative stress, measured as TOS value. Besides, copper is supposed to have a negative effect on the levels of chemokines enabling fertilization.

The presented study confirms an antagonistic relationship between zinc and copper. However, the influence of this antagonism on sperm quality and function needs further investigation.

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