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## Evaluation of effect of selected trace elements on dynamics of sperm dna fragmentation

ocena wpływu wybranych pierwiastków śladowych na dynamikę fragmentacji DNA w nasieniu.

### Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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### Summary

#### Introduction:

Lead and cadmium can lead to negative effects on sperm chromatin DNA integrity. Copper, zinc and selenium are essential components of many enzymes which are important for reproduction. The aim of this research was to evaluate the influence of lead, cadmium, zinc, copper and selenium on the dynamics of semen DNA fragmentation.

#### Material and methods:

The present study concerned 85 fertile and 131 infertile men aged 25-35. DNA fragmentation in the samples was determined after 3 h, 6 h and 12 h. The Pb, Cd, Cu, Zn, and Se measurements were performed by the electrothermal-atomic absorption spectrometry method.

#### Results:

We found that sperm DNA fragmentation was a dynamic process which was intensified with an increase in the level of lead in seminal plasma. The levels of lead and cadmium were higher in seminal plasma of infertile men, compared to fertile men. The levels of zinc, copper and selenium in seminal plasma were higher in men with proven fertility, compared to infertile men, and did not exert a significant effect on the dynamics of sperm DNA fragmentation. The level of cadmium had no significant effect on intensification of sperm DNA fragmentation in time.

#### Discussion:

Reports in the literature which concern the effect of trace elements on human reproduction are equivocal. The present study confirmed an unfavourable effect, especially that of lead, on the dynamics of sperm DNA fragmentation; however, these studies need to be expanded and continued in the future.

#### Key words:

lead • cadmium • zinc • copper • selenium • DNA fragmentation dynamics

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## INTRODUCTION

Recently, a number of reports have been published concerning a considerable decrease observed in male fertility [2,5]. The low sperm count in contemporary males, apart from deterioration of the quality of semen, results in the reduction of the chance to reproduce, which constitutes a great problem from the aspect of public health, and may be among the causes of the negative birth rates in many European countries [16].

The causes of the decrease in male infertility are not fully understood. This is due to the fact that it is very difficult to select individual hazardous factors. The main causes of the decrease in male infertility which are taken into consideration are: exposure to plant protection products, heavy metals, increasing frequency of overweight and obesity among men, sedentary life style causing overheating of the testicles, tobacco smoking habit, alcohol consumption, and narcotics, as well as the effect of the surrounding sources of magnetic waves [15,17,28,32].

Lead (Pb) and cadmium (Cd) are two of the well-known reproductive toxicants to which humans are exposed occupationally and environmentally and can lead to negative effects on the sperm chromatin DNA integrity [27,29]. Both are pervasive in the human environment and accumulate in the human body over a lifetime, including prenatal life [3,10]. Heavy metals have a strong capacity to induce oxidative stress in body cells by disintegration of the lipid membrane, and spermatozoa are quite sensitive to oxidative stress. This may be caused by the weakening of cellular-based defensive mechanisms [26,31]. The free radical processes comprise numerous overlapping procedures which have not been thoroughly investigated. Human exposure to Pb and Cd is often accompanied by considerable exposure to zinc (Zn). Low doses of metals such as zinc and copper (Cu) may have protective effects on male reproductive outcomes, and may assist in counteracting the effects of Cd, Pb, or other metals. Copper, zinc and selenium (Se) are essential for good health because they are components of many enzymes which are important for reproduction, but may be harmful above certain levels [29]. Selenium is essential for normal spermatogenesis in humans, and its critical role is mainly mediated by two selenoproteins, namely, phospholipid hydroperoxide glutathione peroxidase (PHGPx/GPx4) and selenoprotein P. PHGPx/GPx4 is the major selenoprotein expressed by germ cells in the testis, having multiple functions and representing the pivotal link between selenium, sperm quality and male fertility [20]. Lead, which works antagonistically or competitively with selenium, copper and zinc, may additionally disturb the function and lower the anti-oxidative defence of cells [7,26,31].

The damage done to the integrity of semen chromatin DNA, caused by oxidative stress among others, may directly in-

fluence fertility, as well as causing long-term epigenetic effects which induce men's infertility, cancers and premature menopause in future generations [6,9,21]. The process of semen DNA fragmentation is a phenomenon which intensifies and is individual for each person. Its causes are not fully investigated; exposure to heavy metals may be one of them. The aim of this research was to determine the influence of lead, cadmium, zinc, copper and selenium on the dynamics of semen DNA fragmentation.

## MATERIAL AND METHODS

The present study was conducted in 2013 and 2014 at the Medical University of Lublin, Poland, and concerned 216 men aged 25-35. They were divided into two groups, 85 proven fertile men (group 1) and 131 infertile patients (group 2), who had been treated due to infertility for a period longer than 1 year (the couples previously had 4-6 intrauterine inseminations performed). From the study group were excluded men with the symptoms of systemic diseases, as well as patients with clinically diagnosed features of an inflammatory state of the reproductive organ, and smokers. In addition, patients with body weight disorders, i.e. BMI (body mass index) below 17 or over 30, were also excluded from the study group. Prior to enrolment, all patients (n=218) signed a written consent form allowing the use of their data for research purposes. The study was approved by the Ethics Committee.

The sperm was obtained by way of masturbation, and examined directly after liquidation according to the 2010 criteria by the World Health Organization [30]. Prior to the examination, the men maintained 4-day abstinence from sex and alcohol. In order to determine the percentage of fragmented DNA in sperm, the SCD test was used (sperm chromatin dispersion), according to instructions provided by the producers (Dyn-Halosperm kit, Halotech DNA SL, Madrid, Spain) [8]. Sperm cells suspended in agarose gel were treated with an acidic solution and then a lysing solution. It was noted that sperm cells with fragmented DNA had very little or no "halo" of the decompressed DNA, whereas the non-fragmented sperm DNA cells contained long loops forming a rich nucleic acid "halo". There were three hundred sperm cells counted in each sample. As a result, the sperm DNA fragmentation index (DFI) was obtained – the percentage of cells that had detectable sperm fragmentation. DFI in the samples was determined immediately after liquefaction of semen (DFI 0h), and after 3 h (DFI 3h), 6 h (DFI 6h) and 12 h (DFI 12h). Based on the acquired samples, the rate of change between individual measurements (in percent per hour) was established:  $DFI(3h/h) - DFI\%$  per hour during the first three hours;  $DFI(6h/h) - DFI\%$  per hour during the next three hours (6 h in total), and so on in individual intervals. Additionally, the DFI growth rate over

the duration of the study was determined from 0 h to 12 h – DFI (%/h).

Seminal plasma was obtained by centrifugation of the cellular elements. The Pb (mg/dL), Cd (mg/L), Cu (mg/dL), Zn (mg/L), and Se (mg/dL) measurements were performed by the electrothermal-atomic absorption spectrometry (AAS) method [13].

The results of the study obtained were subjected to statistical analysis. The values of measurable parameters analyzed were presented using the mean value, whereas non-measurable parameters are presented as numbers and percentages. The Mann-Whitney U test was applied to compare the levels of elements in the examined groups of patients. The relationships between the concentrations of elements and times of embryo development were tested using Pearson  $r$  correlations.  $P$  values  $p < 0.05$  were considered statistically significant. The database and statistical analyses were made using the software Statistica 9.1 (StatSoft, Poland).

## RESULTS

the study showed that the levels of lead and cadmium were higher in the plasma of men with reproductive disorders, compared to the control group, whereas an opposite relationship concerned the levels of selenium, zinc, and copper, and the differences between groups were statistically significant (Fig. 1).

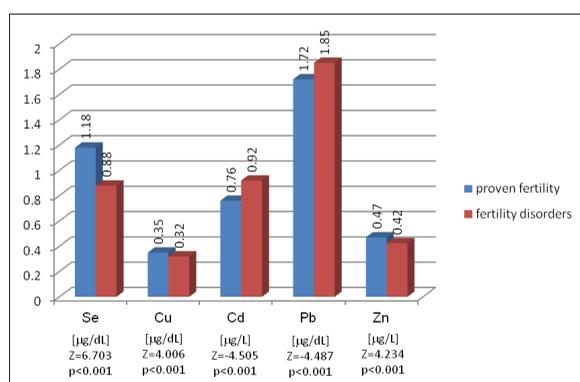


Fig. 1. Mean concentration of Se, Cu, Cd, Pb and Zn in seminal plasma

In both groups in the study, intensification of DNA fragmentation was observed during the incubation. In the group of men with proven fertility, at the beginning of observations, the DFI value was 10.32, on average; then, within 3 h this value increased at the rate of 10.48%/h to 41.75. Between 3 and 6 h of incubation, the rate of the process decreased to 3.03%/h, until the mean DFI reached the value of 49.94. At the last stage of measurements, the rate of fragmentation increase was 2.73, and the DFI value stabilized at the level of 62.33. During analogous measurements performed among men with decreased fertility, the initial DFI value was 25.20, on average, and increased at the rate of 6.22%/h within 3 h to the level of 43.86. After

3 h, the rate of the process was 3.03%/h, until DFI at the 6 h of measurement reached the level of 53.05. Between 6 and 12 h, the rate of fragmentation was 7.77%/h, and its last value measured was 76.37.

The mean DFI values observed in individual time intervals were higher in the group of men with reproductive disorders, and when comparing DFI values in individual measurement times in both groups of men, a statistically significant difference was found between groups according to the initial DFI value ( $Z = -12.006$ ,  $p < 0.001$ ;  $p$  – statistical significance,  $Z$  – Mann-Whitney test result), measured after 3 h ( $Z = -2.980$ ;  $p = 0.028$ ), after 6 h ( $Z = -3.134$ ,  $p = 0.000$ ) and after 12 h ( $Z = -7.342$ ,  $p = 0.000$ ) (Fig. 2).

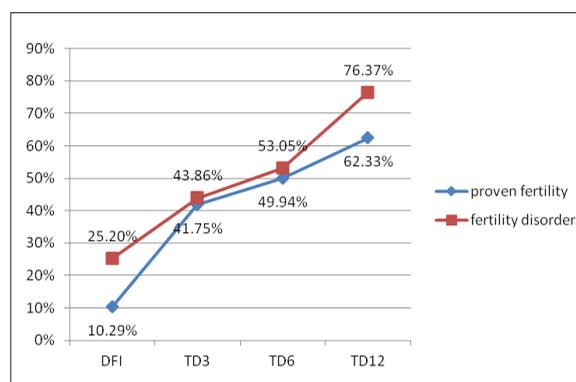


Fig. 2. Comparison of sperm DNA fragmentation index (DFI) at different time points after sperm donation in men with proven fertility (blue shade) and men treated for infertility (red shade)

When analyzing the effect of individual elements on the dynamics of sperm DNA fragmentation, no statistically significant correlations were found between the levels of Se, Zn, Cu and Cd, and DFI measured at various times in the group of fertile men. In the case of men with reproductive disorders, weak negative correlations were observed between Zn and Cu, and initial DFI, as well as between Se and Cu, and fragmentation examined after 12 h. Also, weak positive correlations were noted between Se and fragmentation measured after 6 h and Cu, and initial DFI (Table 1). The analysis performed showed that in both groups of men, higher concentrations of lead were accompanied by higher DFI values measured in the last minutes of observation (high correlations) (Fig. 3), while no such strong relationships were found with DFI measured earlier.

## DISCUSSION

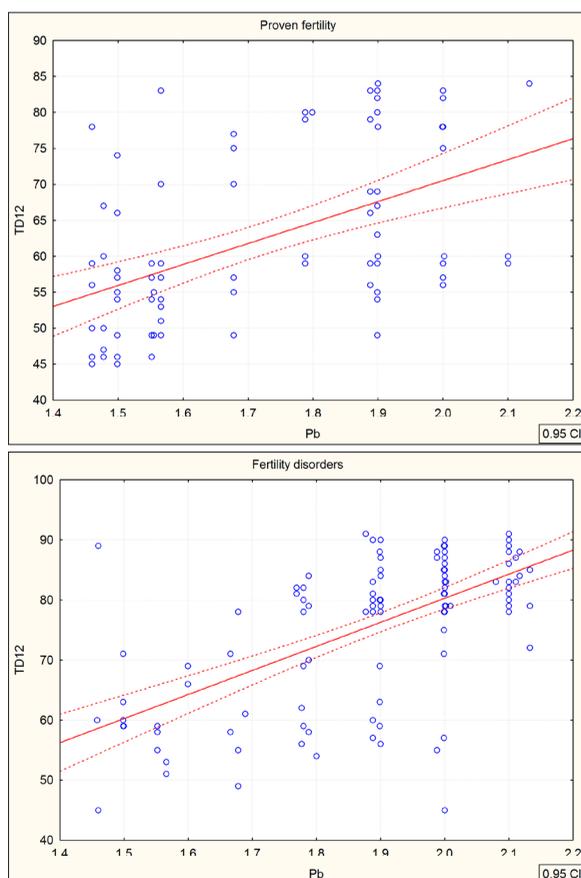
the present study indicates that the intensification of DNA fragmentation in human semen is a dynamic process, and its final level depends, to a great degree, on the concentration of lead in seminal plasma.

In the literature there are many reports concerning the dynamics of human sperm DNA fragmentation. In one of them, Gosálvez J. et al. [12] investigated fresh and thawed

**Table 1.** Correlations between the levels of elements and DNA fragmentation measured at individual times

Elements	Group=proven fertility				Group=fertility disorders				
	DFI	TD3	TD6	TD12	DFI	TD3	TD6	TD12	
Se	r	-0.073	0.194	0.023	0.005	0.012	-0.083	0.187	-0.220
	p	0.509	0.076	0.833	0.967	0.891	0.344	0.032	0.011
Zn	r	-0.074	0.039	-0.036	-0.062	-0.214	-0.053	0.042	-0.168
	p	0.502	0.725	0.746	0.576	0.014	0.549	0.634	0.054
Cu	r	-0.106	-0.177	0.014	0.036	0.185	-0.005	0.027	-0.216
	p	0.333	0.105	0.897	0.744	0.033	0.956	0.760	0.013
Cd	r	0.099	0.047	0.060	0.044	-0.196	0.070	0.022	0.087
	p	0.368	0.668	0.584	0.689	0.025	0.428	0.799	0.323

Cd – cadmium; Cu – copper; DFI – DNA fragmentation index; p – statistical significance; r – correlation rate; Se – selenium; Zn – zinc; TD3, TD6, TD12 – DNA fragmentation in 3rd, 6th and 12th hours; Red-marked – statistical significances



**Fig. 3.** Correlations between Pb concentration activity, and the rate of DNA fragmentation 12 h after sperm donation in the group of men with proven fertility (3.a) and men treated for infertility (3.b). CI – confidence interval

sperm from five donors of proven fertility. In their study, they measured the rate of DFI increase within the first 6 h of incubation, and obtained the value of 1.6% per hour for fresh sperm, and 4.3% per hour for thawed sperm. In

the present study, in fertile men, the mean rate of fragmentation increase was 6.86%/h within the first 6 h of measurement. In other studies, Gosálvez J. et al. found an increased rate of DNA fragmentation in a group of 10 infertile men, compared to sperm donors of proven fertility [11]. The mean rate of DFI increase among men treated due to infertility was 10.13% per hour within 4 h. In the current study, the mean rate of DFI increase in the group of men treated due to infertility in the first 3 h was 6.22%/h. The discrepancies between the results obtained in the present study and those by Gosálvez[11]. J. et al. may result from the fact that the examined groups considerably differed with respect to numbers. Also, the clinical past history is not known, nor the possible burden of other diseases which may affect the fertility of the patients examined by Gosálvez J. et al., and as is known, based on studies conducted by García-Peiró A. et al., the rate of DFI increase is significantly affected by, among other things, varices of the sperm cord [10,11,12]. García-Peiró A. et al. [10] in their studies confirmed that basal DFI levels for clinical and subclinical groups were similar, but the increasing rate of sperm DNA damage was higher in patients with clinical varicocele. According to the researchers, this finding could be related to the higher levels of reactive oxygen species (ROS) associated with the clinical varicocele condition. Similar considerations concerning the effect of ROS on the dynamics of fragmentation were presented by Santiso R. et al., who investigated the effects of agents that cause genetic damage, in fresh semen samples from different donors exposed *in vitro* to increasing acute doses of ionizing radiation, elevated temperature (41°C and 45°C), acidic pH (pH 4) and nitric oxide (NO), and donor sodium nitroprusside (SNP) [25]. Sperm DNA fragmentation was analyzed after an incubation period of chronic (24 h), or acute (1 h) exposure to each treatment followed by incubation at 37°C over a period of 24 h. All agents, except for ionizing radiation, accelerated DNA fragmentation kinetics following chronic exposure over a 24 h period. Transient exposure to NO and heat, but not acidic pH, increased the basal (T0) level of DFI. Despite

the removal of the three toxicants, the remaining sperm following acute exposure showed a decrease in their expected DNA longevity. The researchers explained the effect of hyperthermia and the nitric oxide donor sodium nitroprusside on an increased rate of DNA fragmentation by the concept of oxidative stress. The effect of hyperthermia on fertility was also confirmed by studies carried out on an animal model by Rahman M.B. et al. [22]. The objectives of their study were to investigate the dynamics of DNA methylation reprogramming in the paternal pronucleus, and subsequent fertilisation potential of heat-stressed bull spermatozoa having altered chromatin condensation. Heat-stressed spermatozoa showed a greatly reduced ( $p < 0.01$ ) fertilisation rate compared with non-heat-stressed or normal control spermatozoa (53.7% vs. 70.2% or 81.5%, respectively).

These reports emphasize the importance of oxidative stress in the intensification of sperm DNA fragmentation in time, and, as is known, heavy metals contribute to the generation of this phenomenon, which has been confirmed in the studies by Taha E.A. et al. [27]. These researchers examined 30 infertile male patients with idiopathic oligo- and/or asthenozoospermia, and 30 healthy fertile men, who formed the control group. Lead and cadmium levels in seminal plasma, semen parameters, sperm DNA fragmentation percentage and semen ROS assay were measured in all subjects. Significant positive correlations were noted between seminal lead and cadmium levels on the one hand and sperm DNA fragmentation percentage and semen ROS level in infertile men and controls on the other hand. In the present study, no direct effect of the level of lead on initial DFI was confirmed, but an effect was confirmed on DFI after 12 h of incubation, which is indirectly in accordance with the results presented by Taha E.A. et al. [27].

Mínguez-Alarcón L. et al did not notice any significant differences between the concentrations of lead, cadmium and copper in body fluids (blood, blood plasma and seminal plasma) [18]. We can thus predict that concentrations observed in blood serum will be equivalent to those in the seminal plasma.

The present study showed that the levels of lead and cadmium in seminal plasma are higher in the groups of men with fertility disorders, which has also been confirmed by other researchers. Pant N. et al. conducted a study of healthy fertile and infertile men, aged 20-43, for semen analysis. In their study, the lead and cadmium values were significantly higher in infertile subjects, which is consistent with the results of this study. In addition, a negative relationship was found between seminal lead and cadmium concentration and sperm concentration, sperm motility and percentage of abnormal spermatozoa [19]. Similar to the study by Kiziler A.R. et al., concentrations of Cd and Pb in the infertile group of smokers were significantly higher than those in the fertile male and non-smoker infertile male groups, which also confirms the effect of the smoking habit on the

distribution of heavy metals [14]. Studies by Saaranen M. et al. indicated that the serum selenium level was significantly higher in infertile than in fertile men, but the seminal fluid did not show such a difference, which is inconsistent with the present results [23]. The same studies showed that the seminal fluid lead concentration was significantly higher in infertile than in fertile men, which is confirmed by the results of the current study. Akinloye O. et al. in their study investigated Zn and Cu concentrations in sera and seminal plasma of 60 infertile men (40 oligozoospermic and 20 azoospermic), and 40 men with evidence of fertility (normozoospermic; controls) [1]. The Cu/Zn ratio in seminal plasma was significantly higher in controls compared with other groups, which is in accordance with the observations of this study. In studies by Aydemir B. et al. [4], who examined semen and blood obtained from 60 subfertile men and from 40 fertile volunteers, Cu levels in serum and seminal plasma in the subfertile male group were significantly higher than those in the fertile male group, which is contradictory to the results of the present study and studies by Akinloye O. et al. [1].

Reports in the literature which concern the effect of trace elements on human reproduction are equivocal. This may result from the fact that in the case of elements which exert a favourable effect on male fertility and play an important role as components of many enzymes, such as zinc, copper, or selenium, the phenomenon of their deficiency will cause reproductive disorders, whereas an excess may hypothetically be harmful. Therefore, it seems justifiable to seek optimum concentrations of these elements, and possibilities of their detection in sperm, for patients undergoing infertility treatment not to overdose the dietary supplements used in order to improve sperm parameters. Literature data pertaining to the effect of heavy metals are more coherent, and confirm a definitely hazardous effect of these elements on reproductive processes. The present study confirmed an unfavourable effect, especially that of lead, on the dynamics of sperm DNA fragmentation; however, these studies need to be expanded and continued in the future.

## CONCLUSIONS

1. Sperm DNA fragmentation is a dynamic process which is intensified with an increase in the level of lead in seminal plasma.
2. Levels of lead and cadmium are higher in seminal plasma of men with fertility disorders, compared to those with proven fertility.
3. Levels of zinc, copper and selenium in seminal plasma are higher in men with proven fertility, compared to patients treated due to infertility, and do not exert a significant effect on the dynamics of sperm DNA fragmentation.
4. The level of cadmium has no significant effect on intensification of sperm DNA fragmentation in time.

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