

# Evaluation of trace metals in follicular fluid in ICSI-treated patients

Artur Wdowiak<sup>1</sup>, Edyta Wdowiak<sup>2</sup>, Iwona Bojar<sup>3</sup>

<sup>1</sup> Diagnostic Techniques Unit, Faculty of Health Sciences, Medical University, Lublin, Poland

<sup>2</sup> International Scientific Association for the Support and Development of Medical Technologies

<sup>3</sup> Department for Woman Health, Institute of Rural Health in Lublin, Poland

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## Abstract

This study investigated the influence of cadmium (Cd), copper (Cu), iron (Fe), lead (Pb), selenium (Se), and zinc (Zn) in follicular fluid on the effectiveness of *in vitro* fertilization (IVF) treatment and the dynamics of embryo development. The study involved 221 women aged 25–35 years in whom intracytoplasmic sperm injection (ICSI) was performed. Analysis of the effects of the average concentrations of Se, Zn, and Cu on the developmental dynamics of embryos showed that higher concentrations of these metals were accompanied by shorter lead times for each of the various stages of development, while the reverse was true in the case of cadmium and lead. No relationship was observed between the mean concentrations of Fe and the dynamics of the human embryo. In order to discover whether the fact of becoming pregnant is affected by the investigated variables, a logistic regression model was applied. The dependent variable was the fact of achieving pregnancy, and the independent variable tested was the level of trace metals. Ultimately, only one variable remained statistically significant in the model: the level of Se.

## Key words

trace metals, intracytoplasmic sperm injection (ICSI), embryo development dynamics, time-lapse monitoring, follicular fluid

## INTRODUCTION

Problems in having children are affecting an increasing number of couples of childbearing age. Disturbing trends towards decreased fertility can be seen in many European countries [1, 2]. The origin of this phenomenon, however, is no longer a mystery with no leads. One of the factors that may be responsible for this phenomenon is contamination of the environment with heavy metals [3, 4]. The pollution of air, water and soil with heavy metals and their compounds leads to their presence in foods. Given the use of different chemicals in industry, the presence of heavy metals in food is thus inevitable. Plants and plant products, such as bread and cereals, fish and seafood, are the most vulnerable to the effects of pollution [5].

*In-vitro* fertilization (IVF) is the treatment method of last resort for many couples with reproductive disorders. The negative impact of the environment in which the oocyte grows affects the quality of the embryo and the time at which subsequent developmental stages are reached, and can ultimately affect whether pregnancy is achieved or not [6, 7]. The oocyte can probably be damaged by a toxic microenvironment during the primordial stage of follicle development and at the time of oocyte maturation [8]. One suggested cause of egg-cell damage is the deleterious effects of excess amounts of heavy metals [9].

Two of the most widely-recognized reproductive toxins are lead (Pb) and cadmium (Cd), which humans are exposed to occupationally and environmentally, leading to deleterious effects on chromatin integrity [10, 11]. Both these metals are prevalent in the human environment and accumulate

in the human body over the whole lifetime, including the prenatal phase [9]. Heavy metals possess a powerful oxidative-stress-inducing potential in body cells through lipid membrane disintegration, and gametes are to a certain extent prone to oxidative stress. This may be caused by the weakening of cellular-based defensive mechanisms [12, 13]. Free-radical processes encompass numerous overlapping mechanisms that have not been researched in depth. Human exposure to Pb and Cd often coincides with considerable exposure to zinc (Zn). Low doses of metals such as zinc and copper (Cu) may safeguard male reproductive results, and may help counteract the effects of Cd, Pb, and other metals. Copper, zinc, and selenium (Se) are essential for good health and are components of many enzymes crucial for human reproduction. On the other hand, they may be harmful above certain levels [14]. Lead, which works in an antagonistic or competitive fashion with selenium, copper, and zinc, may additionally impede the function and degrade the antioxidative defenses of cells [15, 16, 17, 18].

Iron is of vital importance in the synthesis of nucleic acids and proteins, electron transport, and cellular respiration, proliferation, and differentiation [19]. The expression of three mammalian genes is directly controlled by iron [19], and two of these influence reproduction. Fe is associated with a large number of redox reactions catalyzed by cytochromes, affecting energy production, drug and hormonal metabolism, and propagation and activation of defence systems via nicotinamide adenine dinucleotide phosphate (NADP) oxidase [19]. The link between iron and the Krebs cycle is further consolidated by means of mitochondrial aconitase enzyme [20]. Under conditions of ROS overproduction, or in a state of iron deficiency, cellular respiration is hindered by the nitrosylation of heme through the mitochondrial enzymes aconitase and glyceraldehyde-3-phosphate dehydrogenase [21], which depletes adenosine triphosphate (ATP).

Address for correspondence: Artur Wdowiak, Diagnostic Techniques Unit, Faculty of Health Sciences, Medical University, Staszica 4/6, 20-081 Lublin, Poland  
E-mail: wdowiakartur@gmail.com

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## OBJECTIVE

The aim of this study was to assess the influence of Cd, Cu, Fe, Pb, Se, and Zn in the follicular fluid on the effectiveness of ICSI and the dynamics of embryo development.

## MATERIALS AND METHOD

**Studied population.** The present study was conducted in 2014 and 2015 at the Ovum Reproduction and Andrology Private Clinic (NZOZ) in Lublin, Poland. Women (n=221) aged 25–35 undergoing new IVF treatment with basal antral follicle count  $\geq 8$ , basal follicle-stimulating hormone (FSH)  $< 10$  IU/mL, and  $\geq 8$  normally fertilized oocytes were included in the study.

Patients with severe endometriosis, premature ovarian failure, or severe asthenoteratozoospermia were excluded from the study. In addition, females with body weight disorders, as indicated by BMIs below 17 or over 30, were also excluded from the study group. Prior to enrollment, all patients (n = 221) signed a written consent form allowing the use of their data for research purposes.

**ICSI procedure.** All patients underwent ICSI treatment with fresh oocytes and freshly ejaculated spermatozoa ( $\geq 1$  million/mL). Ovarian stimulation was carried out by administering luteal gonadotrophin-releasing hormone analogue (GnRHa) (Diphereline; Ipsen Pharma, Melbourne, Australia) followed by recombinant follicle stimulating hormone (FSH) (Gonal-F, Merck-Serono, Darmstadt, Germany, and Puregon, Organon, Netherlands) from cycle day 3 in a short protocol. Recombinant human chorionic gonadotrophin (r-hCG) (Ovitrelle; Merc-Serono, Darmstadt, Germany) was administered 24–28 h after the final FSH administration. Vaginal ultrasound-guided aspiration of oocyte–cumulus complexes was performed 36 h later.

If the follicular fluid had no egg cell or had been contaminated with blood, the sample was excluded from the study. Contamination with blood occurred in 12% of samples and a lack of egg cells was found in 1% of patients.

Oocyte denudation and ICSI were performed 3 hours after retrieval, and the *in vitro* culture was carried out in 25  $\mu$ L of cleavage medium (Cook, Sydney IVF, Sydney, Australia) under mineral oil until day 2 (2–5 cells stage) in automated incubators with 5% CO<sub>2</sub> at 37°C, fitted with time-lapse image acquisition equipment (Primo Vision EVO Microscope, Cryo-Innovation, Hungary). Fifty hours after ICSI, the *in vitro* culture media was changed to blastocyst medium (COOK, Sydney IVF, Australia). The risk of multiple pregnancies is reduced by the appropriate selection of a single blastocyst for embryo transfer. The growth of all the embryos from each patient (n=221) was continuously monitored, with images being obtained at 10 min intervals [22]. This was achieved by using a compact time-lapse microscope system placed inside a regular incubator, combined with a microwell embryo culture dish. The embryos were not moved during the time-lapse observation. Between image acquisitions, the system was turned off completely to avoid exposure to electromagnetic radiation. The time-lapse recording was used to choose a single blastocyst for embryo transfer, which resulted in a singleton pregnancy.

**Kinetic parameters of embryo development.** The following staging system was used for describing embryo growth:

- t0 – time of ICSI procedure; tF – time of the first frame in which both pronuclei could be observed;
- tC – time of the frame with the last observation of both pronuclei;
- t1 – the time when one cell stage was observed;
- t2, t3, t4, t5, t6, t7, t8, t9 – the time of the stages for the corresponding number of cells—e.g., t2 for 2 cells, and so on (stages were annotated at the first frame in which the cells were seen to be separated by membranes);
- tM – time of the first frame in which the embryos were compacting into the morula stage;
- tB – time of the frame in which a crescent-shaped area began to emerge from the morula;
- tEB – time of the first frame showing the expanded blastocyst with increased volume and expansion of the blastocele cavity.

Based on the analysis of time-lapse records, and in accordance with the guidelines of the American Society for Reproductive Medicine and the European Society of Human Reproduction and Embryology [23], a single blastocyst was selected to be transferred into the uterus.

Clinical pregnancy was confirmed with a TVUSG examination for the presence of gestational sac and live embryo in the uterine cavity.

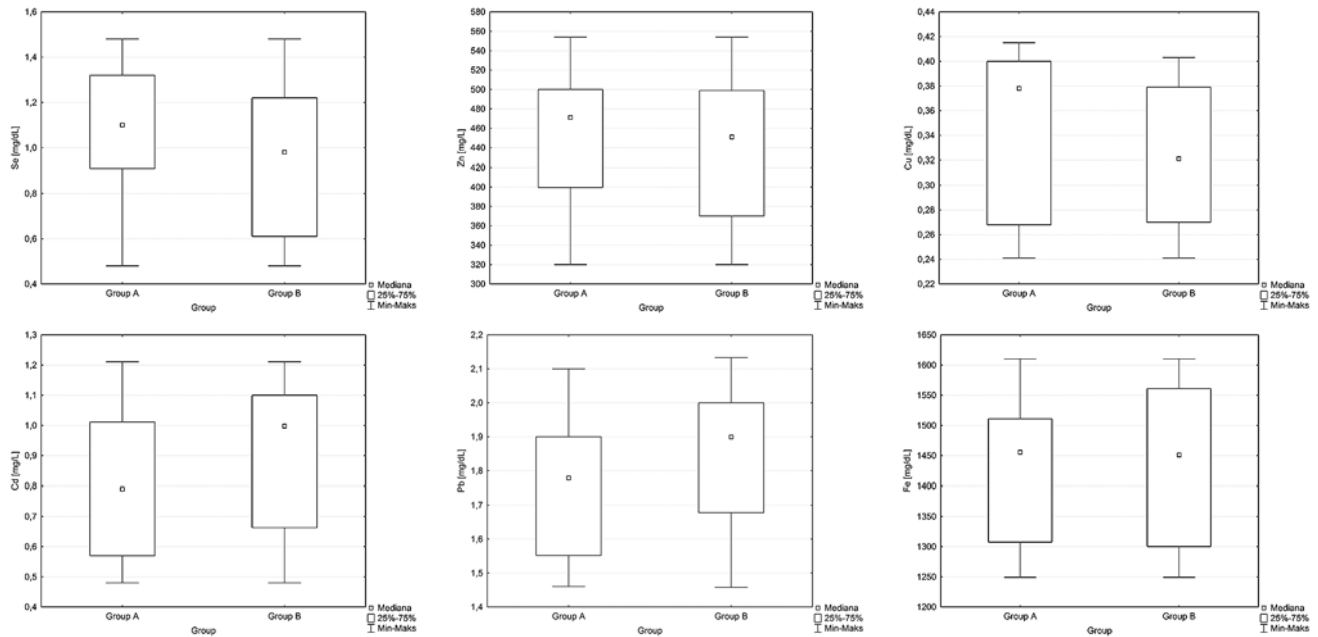
**Determination of Pb, Cd, Cu, Zn, Fe, and Se in follicular fluid.** Measurements of Pb ( $\mu$ g/dL), Cd ( $\mu$ g/L), Cu ( $\mu$ g/dL), Zn ( $\mu$ g/L), Fe ( $\mu$ g/dL), and Se ( $\mu$ g/dL) in the follicular fluid were performed by the electrothermal-atomic absorption spectrometry (AAS) method [13].

**Statistical analysis.** The results were processed with Statistica 9.1 software system (StatSoft, Poland). The values of the measurable parameters were shown by the mean and standard deviation, median, minimum, and maximum values, as well as lower and upper quartile. The analysis of difference between groups A and B was performed using the Mann–Whitney test. To check the relationship between the concentrations of trace metals and embryo kinetic data, the Pearson correlation coefficient was used. In order to verify the relationship between the achievement of pregnancy and the concentration of metals, a logistic regression model was used. The level of significance of  $p < 0.05$  was taken to indicate a statistically significant difference or dependency.

**Ethical statement.** The study was approved by the Ethics Committee of the Institute of Rural Health in Lublin, Poland. All women were provided with oral and written information on the study and signed a written consent form allowing the use of their data for research purposes.

## RESULTS

**Characteristics of the studied population and the effectiveness of IVF.** The mean age of the women included in the study was  $30.80 \pm 2.95$  years and ranged from 25–35 years. In turn, the BMI of the studied women was  $22.18 \pm 3.28$  kg/m<sup>2</sup> and ranged from 17–30 kg/m<sup>2</sup>. Patients were divided into 2 groups: those where pregnancy was confirmed by a heart



**Figure 1.** The comparison of the concentrations of trace metals between group A and group B

rate (group A;  $n = 68$ ), and those where a living pregnancy was not obtained (group B;  $n = 153$ ).

**Trace metals in follicular fluid** (Fig. 1). In the comparison of the concentrations of trace metals between the group, statistically significant differences were not determined for the average concentrations of Zn ( $Z=1.557$ ,  $p=0.115$ ), Cu ( $Z=1.761$ ,  $p=0.078$ ), Cd ( $Z=-1.559$ ,  $p=0.119$ ), and Fe ( $Z=0.858$ ,  $p=0.391$ ). In the group where pregnancy was obtained, a higher mean level of selenium was observed in the follicle fluid ( $1.09 \mu\text{g/dL}$ ) than in the other group ( $0.94 \mu\text{g/dL}$ ), with these differences being statistically significant ( $Z=3.203$ ;  $p=0.001$ ). Furthermore, the significant difference ( $Z=-2.501$ ;  $p=0.012$ ) involved lead, whose mean concentration in the

group that obtained pregnancy was lower ( $1.74 \mu\text{g/dL}$ ) than in the group where treatment was unsuccessful ( $1.83 \mu\text{g/dL}$ ).

**Kinetic parameters of embryo development** (Tab. 1). The embryo development times  $t_C$ ,  $t_1$ ,  $t_2$ ,  $t_3$ ,  $t_4$ ,  $t_5$ , and  $t_B$  were significantly shorter in the cases where pregnancy was achieved than where it was not ( $t_C$ ,  $p<0.001$ ;  $t_1$ ;  $p=0.002$ ;  $t_2$ ;  $p=0.003$ ;  $t_3$ ;  $p=0.024$ ;  $t_4$ ;  $p<0.001$ ;  $t_5$ ;  $p=0.002$ ; Mann-Whitney  $U$ -test). The remaining times ( $t_F$ ,  $t_6$ – $t_M$ ) showed no significant differences.

**Correlations between trace metals and stages of embryo development** (Tab. 2). Analysis of the effects of the average concentrations of Se, Zn and Cu on the developmental

**Table 1.** Embryo development kinetics for patients in the examined groups

Variable	Embryo kinetic data from all patients ( $n=165$ )				Embryo kinetic data from group in which pregnancy was achieved ( $n=57$ )				Embryo kinetic data from group in which the embryo echo was not achieved ( $n=108$ )			
	Mean $\pm$ SD	Median	Range	Lower-upper quartile	Mean $\pm$ SD	Median	Range	Lower-upper quartile	Mean $\pm$ SD	Median	Range	Lower-upper quartile
$t_F$	$9.6 \pm 3.2$	9.1	4.8–15.2	6.4–12.1	$9.7 \pm 3.2$	9.6	4.8–15.2	7.0–12.6	$9.6 \pm 3.1$	9.1	4.8–15.1	6.2–11.9
$t_C$	$24.5 \pm 3.6$	24.9	17.0–30.0	22.2–28.0	$23.8 \pm 3.6$	23.4*	17.0–29.9	21.1–26.3	$24.9 \pm 3.5$	25.1*	17.0–30.0	23.1–28.2
$t_1$	$24.8 \pm 3.6$	26.0	18.5–30.9	21.2–27.9	$24.3 \pm 3.5$	25.9	18.7–30.1	21.1–26.9	$25.0 \pm 3.6$	26.0	18.5–30.9	22.0–28.1
$t_2$	$26.9 \pm 3.7$	26.1	21.4–34.5	24.0–29.0	$26.3 \pm 3.4$	26.1	21.4–34.2	23.2–28.1	$27.2 \pm 3.8$	26.1	21.7–34.5	24.1–30.0
$t_3$	$37.5 \pm 4.9$	38.2	27.9–47.5	34.4–40.2	$37.0 \pm 5.0$	38.1	28.1–47.2	35.1–39.1	$37.7 \pm 4.8$	38.4	27.9–47.5	34.4–41.1
$t_4$	$39.2 \pm 5.6$	37.3	33.1–56.8	35.9–40.3	$37.8 \pm 4.9$	36.2**	33.2–55.1	34.8–39.0	$39.9 \pm 5.8$	37.9**	33.1–56.8	36.2–41.8
$t_5$	$53 \pm 7.2$	53.4	37.0–66.8	49.4–57.5	$52.9 \pm 8.0$	53.2	37.5–66.5	49.3–58.1	$53.0 \pm 6.8$	53.7	37.0–66.8	49.6–57.4
$t_6$	$55.3 \pm 7.7$	53.5	46.0–73.5	49.1–59.3	$55.4 \pm 7.9$	53.0	46.0–73.0	49.4–59.6	$55.2 \pm 7.7$	53.7	46.0–73.5	49.0–58.4
$t_7$	$57.9 \pm 8.9$	55.8	46.0–82.1	51.8–62.3	$56.9 \pm 7.6$	56.3	46.1–80.0	52.4–60.8	$58.5 \pm 9.6$	55.8	46.0–82.1	51.6–62.9
$t_8$	$62 \pm 12.5$	59.1	46.0–98.0	54.3–65.5	$61.9 \pm 12.5$	57.9	46.0–95.4	54.5–65.1	$62.1 \pm 12.6$	60.1	46.0–98.0	53.8–66.5
$t_9$	$76.1 \pm 11.4$	75.6	56.7–99.0	67.5–83.5	$76.3 \pm 11.1$	75.6	57.3–99.0	67.5–83.9	$76.0 \pm 11.6$	75.6	56.7–99.0	67.5–83.0
$t_M$	$84.9 \pm 11.4$	86.5	58.3–103.0	78.6–93.4	$84.4 \pm 11.2$	86.0	59.4–103.0	78.3–93.4	$85.2 \pm 11.5$	87.0	58.3–103.0	78.6–93.4
$t_B$	$105.7 \pm 3.2$	104.5	103.0–117.4	103.8–106.1	$105.0 \pm 2.7$	104.1*	103.0–117.4	103.6–105.2	$106.0 \pm 3.4$	104.7*	103.0–117.2	103.9–106.9

All data expressed in hours and fractions on an hour.  $p$  – statistical significance;  $SD$  – standard deviation;  $t_F$  – time of the first frame in which both pronuclei could be observed;  $t_C$  – the frame with the last observation of both pronuclei;  $t_1$ – $t_9$  – time for the corresponding number of 1–9 cells, respectively;  $t_M$  – first frame in which embryos were compacting into the morula stage;  $t_B$  – frame in which a crescent-shaped area began to emerge from the morula. Superscripts within rows denote statistical significance between the group in which pregnancy was achieved and the group in which pregnancy was not determined: \*  $p < 0.05$ , \*\*  $p < 0.01$  (Mann-Whitney  $U$  test).

**Table 2.** Correlations between levels of trace metals and embryo kinetic data

	group in which pregnancy was achieved (n=68)						group in which the embryo echo was not achieved (n=153)					
	Se	Zn	Cu	Cd	Pb	Fe	Se	Zn	Cu	Cd	Pb	Fe
tF	r=-0.138 p=0.261	r=-0.027 p=0.823	r=-0.068 p=0.582	r=-0.026 p=0.828	r=-0.025 p=0.838	r=0.065 p=0.593	r=-0.274 p=0.001	r=-0.131 p=0.105	r=-0.279 p=0.000	r=0.1155 p=0.155	r=0.059 p=0.466	r=0.028 p=0.726
tC	r=-0.363 p=0.002	r=-0.091 p=0.460	r=-0.161 p=0.188	r=0.369 p=0.002	r=0.324 p=0.007	r=0.046 p=0.705	r=-0.449 p=0.000	r=-0.246 p=0.002	r=-0.364 p=0.000	r=0.376 p=0.000	r=0.269 p=0.001	r=0.076 p=0.345
t1	r=-0.335 p=0.005	r=-0.016 p=0.893	r=-0.318 p=0.008	r=0.422 p=0.000	r=0.255 p=0.035	r=0.077 p=0.532	r=-0.403 p=0.000	r=-0.198 p=0.014	r=-0.346 p=0.000	r=0.310 p=0.000	r=0.294 p=0.000	r=0.011 p=0.887
t2	r=-0.385 p=0.001	r=-0.330 p=0.006	r=-0.269 p=0.026	r=0.456 p=0.000	r=0.360 p=0.003	r=0.068 p=0.580	r=-0.416 p=0.000	r=-0.244 p=0.002	r=-0.315 p=0.000	r=0.356 p=0.000	r=0.322 p=0.000	r=0.028 p=0.729
t3	r=0.237 p=0.051	r=-0.053 p=0.664	r=-0.197 p=0.107	r=0.233 p=0.055	r=0.424 p=0.000	r=0.016 p=0.893	r=-0.389 p=0.000	r=-0.126 p=0.121	r=-0.285 p=0.000	r=0.288 p=0.000	r=0.231 p=0.004	r=0.090 p=0.266
t4	r=-0.423 p=0.000	r=-0.143 p=0.243	r=-0.226 p=0.063	r=0.313 p=0.009	r=0.2879 p=0.017	r=0.091 p=0.460	r=-0.439 p=0.000	r=-0.272 p=0.001	r=-0.211 p=0.009	r=0.278 p=0.001	r=0.174 p=0.031	r=0.049 p=0.543
t5	r=-0.136 p=0.267	r=-0.098 p=0.424	r=-0.417 p=0.000	r=0.096 p=0.434	r=0.084 p=0.493	r=0.002 p=0.981	r=-0.037 p=0.649	r=-0.042 p=0.599	r=-0.136 p=0.092	r=0.067 p=0.404	r=0.099 p=0.221	r=0.086 p=0.287
t6	r=0.015 p=0.904	r=-0.159 p=0.193	r=-0.336 p=0.005	r=0.136 p=0.266	r=0.152 p=0.214	r=0.051 p=0.678	r=-0.187 p=0.021	r=-0.119 p=0.141	r=-0.158 p=0.051	r=0.136 p=0.093	r=0.021 p=0.789	r=0.030 p=0.705
t7	r=0.030 p=0.804	r=-0.021 p=0.859	r=-0.171 p=0.162	r=-0.021 p=0.860	r=0.003 p=0.978	r=0.082 p=0.503	r=-0.024 p=0.768	r=-0.009 p=0.903	r=-0.037 p=0.646	r=0.019 p=0.810	r=0.087 p=0.283	r=0.147 p=0.069
t8	r=-0.174 p=0.155	r=0.128 p=0.298	r=0.132 p=0.280	r=-0.057 p=0.639	r=-0.008 p=0.949	r=-0.108 p=0.378	r=-0.048 p=0.554	r=-0.014 p=0.855	r=-0.066 p=0.415	r=0.111 p=0.169	r=0.1400 p=0.084	r=-0.063 p=0.437
t9	r=-0.048 p=0.696	r=-0.188 p=0.125	r=-0.007 p=0.949	r=-0.167 p=0.171	r=-0.137 p=0.264	r=0.116 p=0.343	r=-0.108 p=0.183	r=-0.015 p=0.849	r=-0.004 p=0.958	r=0.049 p=0.546	r=0.0881 p=0.279	r=0.041 p=0.615
tM	r=-0.309 p=0.010	r=0.004 p=0.971	r=0.005 p=0.966	r=-0.048 p=0.693	r=0.314 p=0.009	r=0.056 p=0.650	r=-0.004 p=0.952	r=-0.048 p=0.555	r=-0.007 p=0.927	r=0.006 p=0.936	r=0.0181 p=0.824	r=0.049 p=0.546
tB	r=-0.155 p=0.206	r=-0.426 p=0.000	r=-0.316 p=0.008	r=0.377 p=0.002	r=0.414 p=0.000	r=0.020 p=0.870	r=-0.244 p=0.002	r=-0.276 p=0.001	r=-0.101 p=0.214	r=0.249 p=0.002	r=0.170 p=0.035	r=0.062 p=0.445

Se – selenium; Zn – zinc; Cu – copper; Cd – cadmium; Pb – lead; Fe – ferrum; tF – time of the first frame in which both pronuclei could be observed; tC – the frame with the last observation of both pronuclei; t1–9 – time for the corresponding number of 1-9 cells, respectively; tM – first frame in which embryos were compacting into the morula stage; tB – frame in which a crescent-shaped area began to emerge from the morula; (r – Pearson's correlation coefficient; p < 0.05)

dynamics of embryos showed that, in both groups, higher concentrations of these metals were accompanied by shorter lead times for the various stages of development, while the reverse was true in the case of cadmium and lead. No relationship was observed between the mean concentration of Fe and the dynamics of the human embryos.

**Group A.** In group A, negative correlations were found between the mean level of Se and the embryonic developmental times tC–t2, t4, and tM. For the remaining times, no significant correlations were noted. In the case of Zn, similar correlations involved t2 and tB, while no statistically significant differences were determined for the rest of the times. The average level of Cu was negatively correlated with t1–t2, t5–t6, and tB, and in the remaining times, the relationships were not statistically significant. Positive correlations were observed between the level of Cd and the times tC–t2, t4, and tB, as well as with tC–t4 and tM–tB. No significant relationships were seen for the other times.

**Group B.** Negative correlations occurred for the Se levels with times tF–t4, t6, and tB, and for Cu with times tF–t4. With regards to other times, the relationships were not statistically significant. Both Pb and Cd positively correlated with tC–t4 and tB, while no statistical significance was seen in the other cases.

**Table 3.** Parameters of logistic regression model

$Chi^2 = 15,875, df = 6, p = 0,014$					
$R^2_{Pseudo} = 0,058, R^2_{Nagelkerke} = 0,098, R^2_{Cox-Snell} = 0,069$					
Metal	Wald	p	OR	-95% CI	+95% CI
intercept	0,411	0,522	0,14	0,00	54,38
<b>Selenium</b>	<b>4,113</b>	<b>0,043</b>	<b>3,02</b>	<b>1,04</b>	<b>8,76</b>
Zinc	0,416	0,519	1,00	1,00	1,01
Copper	0,094	0,759	2,44	0,01	716,50
Cadmium	0,019	0,890	0,91	0,23	3,60
Lead	2,764	0,096	0,26	0,05	1,27
Ferrum	0,632	0,426	1,00	1,00	1,00

CI – confidence interval; OR – odds ratio; p – statistical significance; r – correlation rate; df – degrees of freedom;  $R^2$  – goodness of fit

**Prognostic value of trace metals levels on the outcomes of IVF (Tab. 3).** In order to determine whether the fact of obtaining pregnancy is affected by the variables, a logistic regression model was employed. The dependent variable was the fact of achieving pregnancy, and the independent variable was the level of the trace metals. Ultimately, only one variable – the level of Se – remained statistically significant in the model.

## DISCUSSION

The presented study shows that the probability of pregnancy occurring as a result of the ICSI procedure is greater if higher concentrations of selenium are present in the follicle fluid. In the case of copper, zinc, iron, lead, and cadmium, No relationship was found between the concentration and the effect of ICSI therapy.

Selenium is a cofactor for GPx and plays a central role in removing lipid hydroperoxides and H<sub>2</sub>O<sub>2</sub>. Ceko et al. concluded that levels of Se and selenoproteins are elevated in large healthy follicles and may play a critical role as an antioxidant during late follicular development, which seems to justify the presented results regarding the importance of this element in achieving pregnancy [24]. A statistically significant positive correlation for selenium in follicular fluid with the GPx antioxidant enzyme, zinc with SOD, and copper with SOD was observed in the study by Singh et al. [25]. According to Paszkowski et al., diminished selenium-dependent GPx activity in follicular fluid is associated with unfertilized oocytes. Additionally, selenium activity in granulosa cells affects the fertilization potential of oocytes in women undergoing IVF, which seems to be in accord with the results of the current study [26]. Many other studies have also shown that oxidative stress in the follicular fluid is directly associated with poor IVF pregnancy outcome [27, 28, 29].

As in the current study, Bloom et al. found no statistically significant associations between follicular fluid levels of Pb or Cd and pregnancy. Neither was any association suggested for Cd and Pb concentrations and biochemical pregnancy during multivariable regression in the study by Al-Saleh et al. [30].

The presented research shows that heavy metals, such as cadmium and lead, impede the dynamics of human embryo development during ICSI, especially prior to stage 4. Although the available literature does not contain much research on this topic, indirectly similar results to those presented involve experiments on animals by Jorssen et al. In their study, an *in vitro* bovine embryo culture system was used to expose individual morulae to cadmium [31]. They investigated the sensitivity of the bovine morulae to various concentrations and exposure times. Morulae were exposed to different cadmium concentrations for 18 or 70 hours, and developmental competence, embryo quality, and the expression of cadmium exposure-related genes were evaluated. Cadmium exposure hampered embryonic developmental competence and quality. Compared with the 18-hour exposure, the 70-hour exposure induced a 20-fold higher toxic response with regard to developmental competence and a more 'cadmium-typical' transcript expression. The observed impediment to embryo development at higher concentrations of cadmium may be associated with the decreased reproductive potential demonstrated by Jorssen et al. In the research carried out on human embryos by Butts et al., the authors detected significant associations between toxic metal levels (Hg, Cd) and cortisol, and also in evaluating the impact of these metals on associations between cortisol and IVF outcomes [32]. Their data also raise the possibility of toxic metals modifying the associations between cortisol and IVF outcomes among women. The toxic effect of cadmium may explain its properties as a metalloestrogen, that is, an inorganic metal ion that binds to and activates estrogen receptors. Research by Pfeiffer et al.

confirms that these receptors play a significant role in early embryonic development [33].

In the research of Singh et al., the follicular fluid levels of cadmium, lead, and iron were positively correlated with reactive oxygen species levels [25]. Furthermore, uncomplexed iron, together with superoxide – which reduces Fe (III) and hydrogen peroxide and which is decomposed by the Fenton reaction – results in a lethal mixture containing hydroxyl radicals that can directly damage DNA, lipids, and proteins [34]. Iron can be delivered to granulosa cells by soluble transferrin, a transporter that captures iron released in interstitial spaces. Sanchez et al. determined that iron intoxication did not seem to affect embryo quality, since no correlation was observed between the levels of ferritin in individual follicles and embryo quality [35]. This might reflect the fact that, even though iron overload affects oocyte development, the mature oocytes retrieved were capable of generating good quality embryos. This seems to agree with the observations in the presented study concerning the concentration of iron, which did not have any effect on either the dynamics of embryo development or on achieving pregnancy.

Even though the influence observed here of Pb, Cd, and Zn on the dynamics of the embryo's early development does not translate directly into achieving pregnancy in ICSI, it should stimulate further research in this area. The pace at which the embryo achieves the subsequent developmental stages depends partly on the damage to the genetic material, which needs to be fixed at this time [36]. Based on the research of Hanna et al., it is known that Pb and Cd have an impact on DNA methylation in blood [37]. Hence, it can be assumed that a similar phenomenon may also affect the embryo. It is a well-known fact that decreased embryo development may be caused by genetic deficiencies and, as a consequence, may induce detrimental epigenetic effects in later generations [36, 38]. It also raises the need for further research into this problem by larger research groups.

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