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# Follicular PB levels in women attending in vitro fertilization: role of endometriosis on the outcome

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## ABSTRACT

**Objectives**: the pathogenesis of adverse reproductive outcomes and Pb exposure has not yet been explored, despite the evidence linking Pb and impaired ovarian function. The aim of this study was to evaluate the relationship between serum and follicular concentrations of Pb and IVF treatment outcome.

**Methods**: the levels of intrafollicular Pb of women who attended the IVF program at the Fertility Center of University of Campania "Luigi Vanvitelli" from January 2015 to January 2017 were analyzed. Of these women 22 (47%) had male factor, 9 (19%) had tubal factor, 3 (6%) had ovarian factor, 6 (13%) had unexplained infertility and 7 (15%) had endometriosis problems.

**Results**: the levels of intrafollicular Pb showed a non-statistically significant trend toward higher values in patients with endometriosis (p=0.498). The median Pb level in follicles from pregnant patients showed trend towards lower values than that in the follicles from the non-pregnant patients but did not show significant variations according to IVF technique outcome (p=0.358). Similar results were obtained by correlating the levels of blood Pb according to the outcome of the IVF technique (p=0.360).

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## SOMMARIO

**Obiettivo**: la patogenesi degli esiti riproduttivi sfavorevoli e l'esposizione al piombo non è stata ancora ben esplorata nonostante vi siano evidenze che collegano il piombo e la funzione ovarica alterata. Lo scopo di questo studio è stato quello di valutare la relazione tra il siero e le concentrazioni follicolari di piombo nel trattamento di fecondazione in vitro (IVF).

**Metodi**: sono stati analizzati i livelli di piombo intrafollicolare delle donne che hanno partecipato al programma di fecondazione in vitro presso il Centro di Fertilità dell'Università della Campania "Luigi Vanvitelli" da gennaio 2015 a gennaio 2017. Di queste donne 22 (47%) avevano un problema di natura maschile, 9 (19%) avevano un problema tubarico, 3 (6%) avevano un problema ovarico, 6 (13%) avevano un'infertilità inspiegata e 7 (15%) avevano problemi di endometriosi.

**Risultati**: i livelli di piombo intrafollicolare hanno mostrato una tendenza verso valori più alti nei pazienti con endometriosi ma non statisticamente significativa (p = 0.498). Il livello medio di piombo nei follicoli delle pazienti in stato di gravidanza ha mostrato una tendenza verso valori più bassi rispetto a quelli nei follicoli delle pazienti non gravide, ma non ha mostrato variazioni significative in base all'esito della tecnica IVF (p = 0.358). Risultati simili sono stati ottenuti correlando i livelli di piombo nel sangue in base al risultato della tecnica di IVF (p = 0.360).

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**Conclusions**: our results suggest that there may be a significant relationship between IVF success and the degree of Pb contamination in the blood and follicular fluid and indicate that there is a need for more detailed analysis and larger study population to clarify the effects of Pb on women reproductive outcomes.

**Key words**: endometriosis, lead, fertilization in vitro, infertility.

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**Conclusioni**: i nostri risultati suggeriscono che potrebbe esserci una relazione significativa tra successo dell'IVF e il grado di contaminazione da piombo nel sangue e nel fluido follicolare e indicano la necessità di analisi più dettagliate e di una popolazione di studio più ampia per chiarire gli effetti del piombo sugli esiti riproduttivi nelle donne.

## INTRODUCTION

Pb is a heavy metal that has many different industrial, agricultural and domestic applications.

The general population is exposed to Pb via the ingestion of contaminated food and water, and inhalation of airborne Pb [1-3]. Moreover, Pb belongs to endocrine disrupting chemicals (EDCs) that are exogenous agents that interfere with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones, therefore may potentially impair homeostasis, reproduction, and developmental process [4].

It has been shown that Pb is a powerful disruptor of adrenal and ovarian steroidogenesis, inhibiting synthesis and activity of some hormones like as progesterone, 17-hydroxyprogesterone, 17,20-dihydroxyprogesterone, corticosterone, deoxycorticosterone, and 21-deoxycortisol in a dose-dependent manner [5-6].

It has been shown that, regarding its effects on 17β-estradiol, testosterone and cortisol, there are stimulatory effects after low-levels exposure while there are inhibitory effects after high-level exposure [7-8].

Moreover, Pb exposure results in inhibition of cytochrome P-450 aromatase activity and estrogen receptors in the granulosa cells of the ovarian follicles and may interfere with pubertal development in girls [9-12]. Indeed, data about the interference of Pb on reproductive system are scarce: in pregnant women, exposure to high doses of Pb may increase the risk for spontaneous abortion Pb to abortion, while in males to disturbances in sperm production and decrease in fertility [13-14]. Despite the evidence linking Pb and impaired ovarian function, the pathogenesis of adverse reproductive outcomes and Pb exposure has not yet been explored.

Moreover, in vitro fertilization (IVF) may be a good in vivo model with which we can evaluate directly the effects exerted by Pb on the reproductive outcome. The aim of this study was to evaluate the relationship between serum and follicular concentrations of Pb and IVF treatment outcome.

## MATERIALS AND METHODS

This was a 3-year retrospective cohort study of women who attended the IVF program at the Fertility Center of University of Campania "Luigi Vanvitelli" from January 2015 to January 2017. Only normogonadotrophic patients aged < 38 years with regular menstrual cycles, body mass index (BMI) > 20 and < 28.0 kg/m2, normal karyotype and hysteroscopic evidence of a normal uterine cavity within the last 6 months were included. Exclusion criteria were: acute illness, diabetes mellitus, hypopituitarism, hyperprolactinemia, luteal insufficiency, dysthyroidism, chronic inflammatory diseases, autoimmune disorders, genetic diseases, polycystic ovary syndrome (PCOs), endometriosis, those with less than three months of follow-up and any potential risk of exposure to heavy metals according to a specific questionnaire investigating the potential environmental and occupational exposure to Pb [6].

All the IVF/ICSI (intracytoplasmic sperm injection) cycles were performed according to routine clinical practice. Clinical records of women who were referred for IVF program were collected prospectively in a dedicated database. All charts that were recorded in the database were reviewed carefully by two authors. Data were anonymized before analysis. Institutional Review Board approval was obtained by local ethic committee. A comprehensive written consent form was signed by all participants before enrolment.

Women were classified in 5 groups according to the indications of IVF: male factor, tubal factor, endometriosis, diminished ovarian reserve (DOR), and unexplained infertility. We considered DOR as an abnormal ovarian reserve test (i.e., antral follicular count (AFC) <5–7 follicles or anti-Müllerian hormone (AMH) <0.5–1.1 ng/ml) [15].

Patients underwent a standard down-regulation with GnRH analogue hormone at a dose of 0.1mg/day (Triptoreline, Decapeptyl, Ipsen, Milan, Italy) until estradiol levels ≥40 ng/mL and no follicle >7 mm; patients over 35 years old received a sequential stimulation protocol starting with uFSH (Fostimon, IBSA, Switzerland) for 6 days according to a step-down approach (225IU for 4 days and 150 IU for the last two days) and then shifting to rFSH at the standard dosage of 150 IU; patients under 35 years old received a standard protocol with rFSH (Gonal- F; Serono, Rome, Italy), at a daily dose of 225 IU for 4 days and 150IU for the last two days.

By the seventh day therapy was personalized according to the hormonal and ultrasonographic assessment. Ovulation induction was monitored by vaginal ultrasound and hormonal assessment every second-third day. When at least three follicles had reached a diameter of 18 mm, a single sub cutaneous (SC) bolus of 10.000 IU of hCG (Gonasi HP 10000; IBSA, Rome, Italy) was administered. Transvaginal follicular aspiration was performed 34-36 hours after hCG administration.

Oocytes retrieved were cultured in Petri dishes in IVF HTF Buffer (Cook Medical) at 37° C in a humidified 5% carbon dioxide/95% air environment. The oocytes were denuded enzymatically using 80 IU/ml hyaluronydase (Sage) and mechanically. The semen was processed with 90% - 45% discontinuous Gradient (SAGE In-Vitro Fertilization) centrifugation at 1700 g for 12 minutes. After IVF, the resulting embryos were cultured in IVF Continuous Medium (Cook Medical) at 37° C under 5% carbon dioxide in air environment until the day of embryo transfer.

The luteal phase was supported with the administration of 33 mg/day of natural progesterone starting from pick-up day and then 50 mg/day from embryo transfer day. Serum levels of hCG were measured 14 days after ET and, if positive, were obtained every 3-6 days until an intrauterine gestational sac was demonstrated by US examination.

After oocytes retrieval, follicular fluid (FF) was pooled for each patient and centrifuged to separate cells. Supernatant was immediately aspirated and stored at -80° C until analysis of Pb. Venous blood sample (5 mL) was drawn from each participant into Vacutainer tubes containing 10.5 mg of tripotassium-ethylene diaminetetraacetic acid (K3-EDTA) as an anticoagulant and stored at 4°C for analyses of Pb. Statistical analysis was performed with Statistical Package for Social Sciences (SPSS) software (version 19.0; IBM Inc, Armonk, NY).

Data were shown as means ± standard deviation or as median. A p value <0.05 was considered statistically significant The Mann–Whitney U test was used to assess inter-group differences in relation to non-parametric continuous variables: median Pb level in follicles from pregnant patients and in the follicles from the non-pregnant patients. The Kruskal-Wallis H test was used to compare data among more than two groups: to assess the relationship among the five groups of women with different indications of the IVF technique and the levels of intrafollicular Pb and also correlating the outcome of the IVF technique to the levels of blood Pb.

The study was performed according to the strengthening the reporting of the observational studies in epidemiology (STROBE) guidelines [16].

## RESULTS

Data for 47 women who underwent IVF were analyzed. Three patients were excluded: 1 was lost to follow up and 2 were no data recorded. Of these women 22 (47%) had male factor, 9 (19%) had tubal factor, 3 (6%) had ovarian factor, 6 (13%) had unexplained infertility and 7 (15%) had endometriosis problems as shown in **Table 1**.

The reference value for the Pb concentrations of subjects not professionally exposed according to the list of the Italian Reference Values Society (SIVR) is 11-30 µg/l in blood [17,18]. A reference value for intrafollicular concentration is not available.

The distribution of Pb levels within follicles and plasma ranged from 0 to 47,4 µg/l. In this study, we thought it is more appropriate to include zero values in the statistical analyses to avoid overestimation of exposure risk, especially as there is no defined source of exposure.

Comparing the data according to the indication of the IVF technique, the levels of intrafollicular Pb showed only a non-statistically significant trend toward higher values in patients with endometriosis (Kruskal-Wallis test for independent samples: p=0.498). Similar results were serum Pb levels.

Table 1.
*Main characteristics, follicular fluid concentrations and IVF outcome of the study population according to the indication for IVF.*

| | Indications for IVF | | | | |
|---|---|---|---|---|---|
| | **Male factor** (no=22) | **Tubal factor** (no=9) | **Diminished ovarian reserve** (no=3) | **Endometriosis** (no=7) | **Idiopathic factor** (no=6) |
| **Age** (years) | 33.8 ± 3.7 | 35.4 ± 2.3 | 35.1 ± 2.5 | 34.0 ± 2.9 | 35.0 ± 3.4 |
| **BMI** (kg/m2) | 25.7 ± 2.1 | 26.3 ± 3.4 | 28.2 ± 4.1 | 24.5 ± 3.5 | 27.5 ± 1.9 |
| **Follicular fluid concentration of Pb** (μg/l) | | | | | |
| Mean | 1.50 | .88 | 1.10 | 2.21 | .37 |
| Median | .61 | .79 | .93 | .77 | 0.00 |
| SD | 3.54 | .95 | .34 | 3.87 | .60 |
| **Beta-hCG positive** | 5/22 | 3/9 | 0/3 | 2/7 | 4/6 |

*Table 1 BMI Body Mass Index, SD standard deviation*

Table 2.
*Follicular and blood Pb levels according to the beta-hCG in the different study groups.*

| | Male factor | | Tubal factor | | DOR | | Endometriosis | | Idiopathic factor | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Beta-hCG** | pos | neg | pos | neg | pos | neg | pos | neg | pos | neg |
| **Follicular Pb** (μg/l) | | | | | | | | | | |
| Mean | 1.3 | 1.9 | 0.6 | 0.7 | NA | 0.8 | <lod | 3.3 | 0.7 | NA |
| SD | 0.9 | 2. | 0.7 | 0.5 | | 0.1 | | 4.5 | 0.2 | |
| **Blood Pb (μg/l)** | | | | | | | | | | |
| Mean | 22.3 | 21.5 | 17.7 | 22.0 | NA | 27.9 | <lod | 21.5 | 14.3 | <lod |
| SD | 16.0 | 13.6 | 16.0 | 19.0 | | 17.5 | | 19.0 | | |

*Table 2 DOR diminished ovarian reserve, LOD limit of detection, POS positive, NEG negative, SD standard deviation*

Table 3.
*Follicular and blood Pb levels according to the beta-hCG in the total population.*

| | **Blood Pb level** (μg/l) | | | **Follicular Pb level** (μg/l) | | |
|---|---|---|---|---|---|---|
| | Mean | Median | SD | Mean | Median | SD |
| **Negative beta-hCG** | 15.8 | 12.0 | 14.8 | 3.0 | 0.7 | 1.7 |
| **Positive beta-hCG** | 15.8 | 16.8 | 16.6 | 2.0 | 2.0 | 2.0 |

*Table 3 SD standard deviation*

Even if the median Pb level in follicles from pregnant patients showed trend towards lower values than that from the non-pregnant patients, the analysis with Mann-Whitney U test for independent samples did not show significant variations according to IVF technique outcome (p=0.358) as shown in **Table 2**. Similar results were obtained by correlating the levels of blood Pb according to the outcome of the IVF technique when Kruskal-Wallis test for independent samples (p=0.360) as shown in **Table 3**.

## DISCUSSION

Our results show that Pb may concentrate in ovarian tissue and may interfere with IVF outcome: particularly low concentrations of follicular Pb are inversely associated with pregnancy, pointing to the necessity for more detailed studies. It's well known that chronic exposure to heavy metals may Pb to their accumulation in various organs and body tissues. The follicle basement membrane is very permeable to low- and high-molecular substances that can diffuse into follicular fluid in a matter of minutes [19-20] but there are very few published studies on the amount of Pb in follicular fluid [21-23].

Though the follicular fluid Pb levels in our participants were low, the contamination of the fluid by these chemicals shows that the reproductive organs and the fetuses have been potentially exposed to these substances, and that the germ cells and early embryos may be affected. The ovarian follicle is a fragile micro-environment where interactions between hormones, growth factors, the oocyte and its surrounding somatic cells are essential to generate a fully competent oocyte. In vitro experiments suggest that EDCs can disturb this finely tuned balance [24].

Concentration of Pb within follicular fluid has not been well characterized, and the effects of Pb exposure on female gametes are unknown. Taupeau et al. examined the ovaries of mouse exposed chronically to Pb and found that low Pb concentration in the ovary caused dysfunction of folliculogenesis, with fewer primordial follicles and an increase in atretic antral follicles [25].

An experimental study by Avazeri et al. revealed that Pb salt at low concentrations (p10pM) can affect in vitro the control of meiosis in mouse oocytes [26]. Authors hypothesized that the observed deleterious effects on oogenesis in vivo have been induced via the same calcium-dependent protein kinase C (PKC) pathway disturbance [27]. Regarding the specific function of Pb as an endocrine disruptor, it has been seen that it could be involved in the reduction of oocyte quality as in vitro studies have shown that Pb acts by blocking Estrogen receptor (ER) beta on granulosa cells thus influencing oocyte maturation [28]. It would also be able to reduce the mRNA and proteins of the enzymes involved in the biosynthesis of estrogens in these same cells.Other studies show that the accumulation of this metal at the ovarian level would be linked to increased atresia of the antral follicles [25,29]. An interesting aspect of the action of Pb is its ability to stimulate, at nanomolar concentrations, the activity of PKC replacing the Calcium.

Moreover, having the ability to bind to the sulfhydryl groups of proteins or substituting essential metal ions, is able to interfere with different enzyme and receptor systems such as CYP 450 and ERβ and almost all organs and systems are potential targets [25,30]. The effects induced by it can therefore be extremely varied. A particular aspect to consider is the difference in follicular Pb levels between the groups.

Considering the Pb as an environmental pollutant, we would expect higher values in the idiopathic infertility group or in the DOR group. On the contrary, the higher values were found in the group of patients affected by endometriosis. In fact, we observed that Pb level in patients with endometriosis and without an ongoing pregnancy was higher than other groups, while patients with endometriosis with an ongoing pregnancy Pb level was below the lower limit of detection. Indeed, there is some evidence that the endometriotic nodule could have the ability to store Pb, but its correlation with IVF outcome is not clear at all [31,32].

The most important limitation of our study is the retrospective and non-randomized design, the small samples and the wide variability of values; on the other hand, our patients were free from potential bias such as dietary, environmental, professional and voluntary exposure to heavy metals, therefore they may be useful for interpreting the relationship between Pb and IVF. Our results suggest that there may be a significant relationship between IVF success and the degree of Pb contamination in the blood and follicular fluid and indicate that there is a need for more detailed analysis and larger study population to clarify the effects of Pb on women reproductive outcomes. Such finding is alarming and priority for further studies are, urgently, needed [33,34].

The future objectives should therefore be aimed at developing experimental models for the study of the effects of heavy metals to characterize their mechanisms of action, establish cut-offs of toxicity for follicular fluids, evaluate the levels of exposure both in the general population and in specifically exposed groups, to study the effects at low doses since some heavy metals could cause specific endocrine effects at lower doses than those normally considered as non-toxic, deepen interactions between lifestyles and environmental contaminants, identify metabolic conditions and genetic polymorphisms as potential susceptibility factors in the population to the effects of specific heavy metals.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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