Effect of Heavy Metals Levels In Follicular Fluid on ICSI Outcome

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ABSTRACT

Heavy metals and trace elements adversely affect animal health and the reproductive system and its functions through direct or indirect effects on numerous organs and systems. This study was aimed to evaluate the effect of heavy metals levels in follicular fluid on a patient undergoing Intracytoplasmic Sperm Injection (ICSI) outcomes namely Cadmium (Cd), Lead (Pb), and Arsenic (As). In comparison between the normal concentration level of Cd and the high concentration level of Cd the present study showed a significant difference in embryos quality ($P=0.01611$), while no statistically significant difference in pregnancy rate was observed. Although Pb showed a significant difference in embryos quality; grade I embryos in normal level concentration than high-level group ($P=0.00021$), and grade III embryos were significant in a high-level concentration of Pb than low-level group ($P=0.0043$). The present study concluded that a high level of heavy metal concentration in the follicular fluid has a harmful effect on the grading of embryos and these lead to a decrease in the chance to select the best embryos before transferred (Cd and Pb).

INTRODUCTION

Human reproductive toxicity associated with a high dose and occupational exposure to mercury (Hg), cadmium (Cd), and lead (Pb) has been reported in terms of reduced fecundity and fertility (Rzymski et al., 2015). Contamination of the environment, especially air, water, and soil with heavy metals such as heavy metals, leads to an increase in heavy metals in food so that the presence of heavy metals in food is therefore inevitable. Plants and plant products, such as bread and cereals, fish and meat, are the most susceptible to contamination, Pb and Cd are the most recognized reproductive toxins human exposure to occupational and environmental pollutants, contributing to adverse effects on chromatin integrity (Wdowiak et al., 2018).
Previous studies have documented that the relationship between persistent inorganic contaminants (such as As-Cd and Pb) and increased oxidative stress, cell apoptosis, endocrine disruption, and epigenetic harm may adversely affect the outcomes of Intracytoplasmic Sperm Injection (ICSI) patients by determining their concentrations in body tissues, so that controlling exposure to such toxic elements could be of interest to treat couples with reduced fertility, and increase the likelihood of success in assisted reproduction techniques (García-Forteà et al., 2016).

Toxic metals may be transported to the embryos directly into the oocyte during folliculogenesis and maturation, and the presence of contaminants in follicular fluid may compromise oocyte quality and there have been significant negative correlations between blood Pb levels and the number of metaphase II (MII) oocytes, implantation, clinical pregnancy, and ongoing pregnancy rates. (Tolunay et al., 2016).

On the other hand, Sharma and Ercal (Sharma et al., 2014 and, Ercal et al., 2001) they demonstrated that heavy metals possess a potent oxidative-stress-inducing potential in body cells through lipid membrane disintegration and that gametes are, to a certain extent, prone to oxidative stress, this may be caused by the weakening of cellular-based defensive mechanisms.

In addition to heavy metal concentrations such as Pb and Cd have been shown to be negatively associated with the In vitro fertilization (IVF) outcome; however, recent toxicology tests have not thoroughly evaluated the clinical impacts of heavy metals on the IVF process outcome (Bloom et al., 2012).

Heavy metals occur more at lower concentrations in the follicular fluid than in urine, follicular fluid bathes oocytes and is, therefore, a reasonable biomarker of the local micro follicular environment, the consistency of which can have an effect on oocyte competence, embryo quality, implantation and live birth (Ingle et al., 2016).

### MATERIALS AND METHODS

#### Patient Selection:

The exclusion criteria were being ever smoker, The couples were exposed to pollution by smoking or other passive smoking depends on their lifestyle, the woman being 40 years or younger, presence of polycystic ovary syndrome, endometriosis documented by laparoscopy, hydrosalphinx, abnormal uterine cavity documented by hysterosalpingography (HSG), and having other endocrine disorders. Patients were followed up and samples were collected throughout the ART procedure and pregnancy at Dr. Faris Medical center for Infertility and Human Reproduction located on Heliopolis, Cairo, Egypt. Patients were then categorized into two groups high level of heavy metals concentration and low level of heavy metals concentration (Performing annually approximately 75 IVF cycles).

#### Stimulation Protocols:

1. **Down-Regulation Protocol (the Standard Long Protocol):**

Patients administered oral combination pills during the stimulation cycle. The pituitary down-regulation with Gonadotrophin-Releasing Hormone (GnRH) agonist decapeptyl 0.1 was initiated at a daily subcutaneous dose of 0.1 mg daily until the day of Human Chorionic Gonadotropin (HCG) on day 21 of the preceding cycle (Tehraninejad et al., 2017 and Zegers-Hochschild et al., 2017).

2. **Short Stimulation Protocol:**

In the short or 'flare-up' protocol, GnRH analog therapy is initiated on the first or second day of menses and FSH is started 2 or 3 days later. This protocol takes advantage of the initial release of gonadotropins on the first days of agonist administration and pituitary suppression at the time of follicular aspiration (Zegers-Hochschild et al., 2017).

3. **Ultra-Short Stimulation Protocol:**

The ultra-short flare Gonadotropin-releasing hormone agonist / Gonadotropin-releasing hormone Antagonist (GnRHa / GnRHant) protocol has recently been introduced to the controlled ovarian
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hyperstimulation (COH) armamentarium protocols (Orvieto, 2015).

**Oocyte Retrieval:**

The patient reports to the assisted conception unit on the morning of the procedure. She should be fasting for at least five hours; she is usually advised not to have any food or drink from the preceding midnight. She is prepared in the ward and taken to the procedure room of the unit. Various forms of anesthesia can be used, but short-acting intravenous sedatives and narcotics are popular such as propofol combined with alfentanil. The vagina can be cleansed with antiseptics and irrigated with normal saline to remove traces of the antiseptic. There is however no guarantee that the irrigation will remove all traces of the antiseptic which if it comes into contact with the oocytes may exert toxic effects. Alternatively, the vagina can be wiped of all mucus with gauze swabs soaked in normal saline and no increase in pelvic infection has been noted with this cleaning method especially if this is performed in conjunction with antibiotic prophylaxis. A transvaginal ultrasound probe can be used to carry out and oocytes are aspirated from follicles in both ovaries through a needle that is used to pierce the vaginal wall and puncture the follicle. Each tube of aspirated fluid is examined under stereo (Leica M80 Germany) magnification to identify the oocyte which is then removed and washed in a clean culture medium. The retrieved oocytes are placed in culture dishes.

**Serum Collection:**

Venous blood samples were obtained following the standard procedure, using tubes with a clotting activator (S-Monovette, Sarstedt, No: 04.1905) for serum preparation. Serum samples were centrifuged at 2000xg for 15 min to separate the serum from the plasma. Specimens were immediately frozen (-80°C) until further use for assessment of levels of different heavy metals in the lab of Regional Center for Fungi and its Application- Faculty of Science, Al Azhar University.

**Follicular Fluid Preparation And Freezing:**

The follicular fluid was obtained from the largest follicle (> 18 mm) visualized on ultrasound before using any flushing medium and only consisted of fluid from one follicle. This follicle was aspirated using a transvaginal ultrasound probe with a 17 gauge, 35 cm oocyte aspiration needle (Cook® Double Lumen Aspiration Needle, USA) mounted on a needle guide directly attached to the probe. Between 0.5 mL and 5.0 mL FF was aspirated from a single large follicle in each ovary. The follicular fluid was transferred to a sterile Petri dish, and after the oocyte was removed, the fluid was placed into a 15 mL conical tube centrifuged for 5 minutes at 1500 g, distributed into 1.8 mL polypropylene cryovials, and stored at 80°C until further use (Bloom et al., 2012).

**Trace Elements Determination:**

All heavy metal analyses were performed at once after all samples were collected and transferred to the laboratory (Fungi Research Center, Faculty of Science, Al Azhar University) on dry ice. Follicular fluid sample of 1 mL was digested in Teflon vessels with 9 mL HNO3 in a microwave. After cooling, the clear supernatant was transferred to polypropylene tubes and diluted to 20 mL with deionized water. 5 mL of this solution was mixed with 5 mL of concentrated HCl and 1 mL of 50% KI in polypropylene tubes for analysis. Samples had been submitted for analyze (pb) by Atomic Absorption (Flame Method). The data are expressed in mg/L (ppm) using Atomic Absorption Spectrophotometer (Model: GBC932AA) at RCMB.

**Digestion of Samples:**

Ultra-pure water was used to prepare all solutions. The trace elements were determined after digestion with nitric acid 0.5% then 1mL of Sample added to 4mL of Mix Acid (nitric + perchloric) 1:1 then Heat Mix with sample until dryness (about 15 Min) finally Add 2 mL Milli-Q (0.5 % nitric)
Condition of Pb:
By technique Graphite Furnace

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</tr>
<tr>
<td>Atomization temperature</td>
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Condition of Cd:
By technique Graphite Furnace

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Condition of As:
By technique Flame

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<tr>
<td>Atomization temperature</td>
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Statistical Analysis:
Statistical analysis was performed using SPSS version 24. Parametric (normally distributed) variables were expressed as mean ± standard deviation (SD), while non-parametric variables were expressed as median (inter-quartile range). A Chi-square test was used to compare means.

RESULTS
1- Effect of Normal And High Levels Of Cadmium (mg/L) on Oocytes Yield, Quality of Oocyte, Fertilization Rate and IVF Outcome In Females Undergoing (ICSI):

According to table 1, there is no significant difference between the outcome of oocyte (P= 0.893) or maturation rate (P=0.65). After ICSI the fertilization rate of oocytes obtained from patients with a high level of Cd did not has statistically difference in comparison with the fertilization rate of oocytes obtained from low levels of Cd concentrations (P=0.415). Although there was no significant difference between the cleavage rate of oocytes obtained from patients with a high level of Cd and normal level (P=0.109), there was a significant difference in embryos quality; Grade (A) embryos in normal level concentration group higher than high-level group (P=0.01611), on the other hand, the percentage of pregnancy rate of normal level concentration group higher than high-level concentration group, however, there was no significant difference in pregnancy rate of normal Cd concentration group in comparison of high-level concentration group (P=0.501).

Table 1: Effect of normal and high levels of cadmium (mg/L) on Yield, quality, fertilization rate of oocyte, and IVF outcome in females undergoing ICSI.

<table>
<thead>
<tr>
<th>Cadmium</th>
<th>Ova</th>
<th>MII</th>
<th>Fertilization</th>
<th>Cleaved</th>
<th>EmbryoGrading</th>
<th>Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G (A)</td>
<td>G (B)</td>
</tr>
<tr>
<td>Normal level Cd</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(N=16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>High level Cd</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>106&lt;sup&gt;b&lt;/sup&gt;</td>
<td>142&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(N=59)</td>
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<tr>
<td>P-Value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.893</td>
<td>0.65</td>
</tr>
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</table>
2- Effect of Normal And High Levels Of Arsenic (mg/L) on Oocyte Yield, Quality of Oocyte, Fertilization Rate, and IVF Outcome in Females Undergoing (ICSI):

According to table 2, there is no significant difference between the outcome of oocyte (P= 0.414) or maturation rate (P=0.880). After ICSI the fertilization rate of oocytes obtained from patients with a high level of As did not have statistically different in comparison with the fertilization rate of oocytes obtained from low levels of As concentrations (P=0.815). Although the results show cleavage rates didn’t affect by the concentration of As, there was no significant difference between cleavage rate of oocytes obtained from patients with a high level of As and normal level (P=0.526), although no significant difference in embryos quality; Grade (A) embryos in normal level concentration (P=0.15), on the other hand, the percentage of pregnancy rate of normal level concentration group higher than high-level concentration group, however, there was no significant difference in pregnancy rate of normal As concentration group in comparison of high-level concentration group (P=0.833).

Table 2: Effect of normal and high levels of Arsenic (mg/L) on Yield, quality, fertilization rate of oocyte and IVF outcome in females undergoing ICSI:

<table>
<thead>
<tr>
<th>Arsenic</th>
<th>Ova</th>
<th>MII</th>
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<th>Cleaved</th>
<th>EmbryoGrading</th>
<th>Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal level As (N=32)</td>
<td>391a</td>
<td>298a</td>
<td>240a</td>
<td>201a</td>
<td>49a</td>
<td>65a</td>
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<tr>
<td>High level As (N=43)</td>
<td>640a</td>
<td>495a</td>
<td>409a</td>
<td>359a</td>
<td>115a</td>
<td>121a</td>
</tr>
<tr>
<td>P-Value</td>
<td>0.414</td>
<td>0.880</td>
<td>0.815</td>
<td>0.526</td>
<td>0.15</td>
<td>0.815</td>
</tr>
</tbody>
</table>

3- Effect of Normal And High Levels Of Lead (mg/L) on Oocytes Yield, Quality Of Oocyte, Fertilization Rate and IVF Outcome in Females Undergoing (ICSI):

The obtained results from the study of the effect of normal and high levels of Pb on Yield, quality and fertilization rate of oocyte of females undergoing IVF (ICSI) showed that illustrated in (Table 3). There is no significant difference between the outcome of oocyte (P= 0.664) or maturation rate (P=0.641). After ICSI the fertilization rate of oocytes obtained from patients with a high level of Pb was not different in comparison with the fertilization rate of oocytes obtained from low levels of cadmium concentrations (P=0.563), cleavage rates didn’t get affected by the concentration of Pb, there was no significant difference between cleavage rate of oocytes obtained from patients with high level of Pb and normal level (P=0.444), but there was significant difference in embryos quality; GI embryos in normal level concentration group higher than high level group (P=0.00021), significant difference in embryos quality and GIII embryos was significance in high level concentration of Pb than low level (P=0.0043) , from other hand the percentage of pregnancy rate of normal level concentration group higher than high level concentration group however there was no significant difference in pregnancy rate of normal Pb concentration group in comparison of high level concentration group (P=0.489).
Table 3: Effect of normal and high levels of Lead (mg/L) on Yield, quality, fertilization rate of oocyte, and IVF outcome in females undergoing ICSI:

<table>
<thead>
<tr>
<th>Lead</th>
<th>Ova</th>
<th>MII</th>
<th>Fertilization</th>
<th>Cleaved</th>
<th>EmbryoGrading</th>
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<td>Normal level Pb (N=31)</td>
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<td>G (A)</td>
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</tr>
<tr>
<td>454 a</td>
<td>386 a</td>
<td>326 a</td>
<td>283 a</td>
<td>110 a</td>
<td>91 a</td>
<td>82 a</td>
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<tr>
<td>High level Pb (N=44)</td>
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<td></td>
<td></td>
<td>G (B)</td>
<td></td>
</tr>
<tr>
<td>580 a</td>
<td>414 a</td>
<td>329 a</td>
<td>279 a</td>
<td>55 b</td>
<td>95 a</td>
<td>129 b</td>
</tr>
<tr>
<td>P-Value</td>
<td>0.664</td>
<td>0.641</td>
<td>0.563</td>
<td>0.444</td>
<td>0.00021</td>
<td>0.735</td>
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<td></td>
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**DISCUSSION**

The results of the present study revealed the effect of the normal and high levels of Cd, As and Pb concentration of infertile patient and ICSI outcomes. Several environmental factors affect oocyte maturation and embryo quality and may therefore play a role in decreasing fertility and infertility associated with heavy metals, particularly at high levels of exposure of heavy metals to the human body through the gastrointestinal system, the respiratory system and/or the skin. (Gerhard et al., 1889, Jensen et al., 2006 and Younglai et al., 2002). Bloom et al., (2012) reported clinical pregnancy decreased with increased Cd blood levels. Cd affects the reproduction of females, impairs the synthesis/regulation of hormones and deteriorates the pregnancy rate or its outcome even at lower doses (Kumar and Sharma 2019) and the (Agarwal et al., 2012) illustrated how Cd induces pregnancy-related abortions by effects on endocrine pathways or by fostering oxidative stress associated with adverse reproductive health. Exposure to Cd adversely impacted oocyte maturation and caused chromosome aberrations in IVF (Nandi et al., 2010).

As regards the concentration of Cd in follicular fluid, the present study indicated that there was no significant difference in the concentration of Cd in follicular fluid between oocyte results, maturation rate, oocyte fertilization rate, oocyte cleavage rate and this result agreement with Bloom et al., (2012) who noted that the association between the concentration of heavy element follicular fluid and oocyte maturation was defined, but with conflicting results with limited sample size, did not find an association between the concentration of follicular fluid Cd and oocyte maturation. On the other hand, embryos quality and development show a significant difference in embryos quality; Grade (A) embryos in normal level concentration group higher than high-level group (Shen et al., 2000).

However, in the present study, there was no significant difference in the pregnancy rate of the normal Cd concentration group compared to the high concentration group and this result is consistent with Bloom et al., (2012) who did not demonstrate any significant association between follicular fluid (FF) metals and the clinical pregnancy rate. Al Saleh et al., (2008) found a negative association with Pb blood concentration; a positive association for Cd in FF. Younglai et al., (2002) it has been shown that there is an inverse correlation between the blood Pb concentration and the ongoing pregnancy rate. However, this study has shown that the percentage of pregnancy in the normal concentration group is higher than the high concentration group, however; there was no significant difference in the pregnancy rate of the normal lead concentration group compared to the high-level concentration group. Although Silberstein et al., (2006) investigated Pb levels in 44 follicles of 9 patients and found that Pb concentrations in FF can exceed those in blood and are inversely associated with pregnancy. Regarding the concentration of Pb in follicular fluid, the present study showed that cleavage rates didn’t get affected by the concentration of Pb, but there was significant difference in embryos quality; GI embryos in normal level concentration group higher than high level group, and there is significant
difference in embryos quality and GIII embryos was significance in high level concentration of Pb than low level and these result agree with The Harun et al., (2016) who illustrated that there is significant negative correlations were found between blood Pb levels and number of MII oocytes, implantation, clinical pregnancy and ongoing pregnancy rates, So that he conclude that blood concentration of Pb and follicular fluid have significant impacts on ICSI outcome. This author Bloom et al., (2012) described that the probability of recovering an immature oocyte in case of the increase in the concentration of Pb in blood (Al-Saleh et al., 2008). In this study there is no significance difference between outcomes of oocyte or maturation rate.

Altered neurotransmitter levels like norepinephrine, dopamine, and serotonin are also related to As exposure (Tripathi et al., 1997). Arsenic-induced toxicity to the female reproductive system includes suppression in ovarian steroidogenesis, prolonged distrust, degeneration in cells associated with the female reproductive system (ovarian, follicular and uterine cells), (Navarro et al., 2004 and Wan et al., 2006). In this study, no statistically difference in fertilization rate of oocytes and cleavage rates didn’t affect by the concentration of As from patients with high level of arsenic and normal level, although no significant difference in embryos quality ; Grade (A) embryos in normal level concentration, from other hand the percentage of pregnancy rate of normal level concentration group higher than high level concentration group however there was no statistical significant difference in pregnancy rate of normal As concentration group in comparison of high level concentration group.

**Conclusion**

From the previous results, it can be concluded that a high level of heavy metal concentration in follicular fluid has a harmful effect on the grading of embryos and these lead to a decrease in the chance to select the best embryos before transferred (Cd and Pb).

**Conflict of interest statement:**

The authors of this article report no conflict of interest.

**Acknowledgment:**

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