

## Heavy Metals in Semen of Oligospermic Patients without Known Etiologic Factors

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### Abstract

Environmental contaminants, heavy metals and pesticides, could be a cause for oligoasthenospermia. Analyses of the five heavy metals (Pb, Cd, Hg, Ni, and Cu) have showed values that are above the normal range in oligospermic patients in a selected group. We used blood values to ascertain reference values for heavy metal concentrations, as there is a lack of baseline values for seminal plasma in previously published papers. Three recent research projects in the same area of southern Italy ("Land of fires" in Campania and Sicily) have shown correlation between environmental pollution from heavy metals and oligospermia. At the Zygote Center in Salerno (Campania, Italy), a group comprising 200 couples with fertility problems was monitored over a 2-year period. A group of eight oligospermic men was selected according to the following criteria: (1) absence of genetic factors; (2) absence of cryptorchidism, epididymitis, and varicocele. The patient sample group is small, because selection criteria are very strict. Tests were conducted on a group of 20 normospermic patients, as a control sample. A specific amount of seminal fluid was first centrifuged and then analyzed using atomic absorption spectrophotometry, which is used to determine the presence of heavy metals. Subsequently, the High Performance Liquid Chromatography-Mass Spectrometry technique was applied to detect a panel of 500 pesticides. Traces of heavy metals in seminal plasma have been found in 4 out of 8 in the sample group and in 2 of the control patients. The difference was statistically significant. No pesticides were found either in control or in oligospermic patients.

**Keywords:** Pollution and male infertility; Sperm quality and pollution; Heavy metals in seminal fluid; Pesticides in seminal fluid; Pb, Cd, Ni, Cu in seminal fluid; "Land of fires"

### Introduction

It has been ascertained that pollution can cause serious problems to human male fertility: in areas with a high concentration of heavy metals, a high level of male sterility has been observed [1]. There is a clear and strong correlation between abnormal concentrations of antimony (Sb) and lead (Pb), and poor sperm quality. Moreover, men who live in areas highly contaminated by these elements (Sb, Pb) have lower quality semen [2].

In addition to heavy metals, pesticides in fruit and vegetables have been shown to have adverse effects on male fertility [3]. The pesticides, along with heavy metals, radioactivity, and toxic fumes from organic chemicals, have been shown to be the cause of the majority of problems regarding erectile dysfunction as well as for the drop in male fertility [4].

In this paper, we evaluated, through various methods (Atomic Absorption Spectroscopy and HPLC-MS/MS), the possibility of pesticides in the seminal plasma (a panel of 500 pesticides—UNI EN 15662: 2009) and heavy metals (Pb, Cd, Cu, Ni, and Hg), which were potentially capable of altering the parameters of spermatozoa in a group of eight men with non-identified cause for genetic factors such as Y chromosome microdeletions [5], aneuploidies [6], chromosomal translocations [7], and heterochromatic polymorphisms. In addition, in this group, cryptorchidism, epididymitis, and varicocele were eliminated. Therefore, pollution remains the most likely cause of impaired spermatogenesis in these patients. The same analysis to detect

contaminants (pesticides and heavy metals) was conducted on a group of 20 normospermic patients, as a control sample.

### Materials and Methods

All laboratory research was conducted after patients had been informed of what the trial involved and had given their consent.

#### AZF microdeletions detection of the Y chromosome

##### Extraction

The genomic DNA was extracted from the biological sample using a commercially available kit (HP PCR template preparation), following the manufacturer's instructions (Roche Diagnostics).

##### Amplification

DNA is amplified by PCR multiplex. Using appropriate primer pairs, it is possible to amplify different Y-STSs \* (sY84, sY86, sY127, sY134, sY254, sY255), that is, DNA sequences corresponding to specific markers included in the AZF $\alpha$ , AZF $\beta$ , and AZF $\gamma$  regions of the Y chromosome. In addition, primers for ZFY/ZFX gene and SRY gene are present.

##### Result

The amplification products obtained are detected by reverse-dot blot on a strip, on which allele-specific probes (KIT YChromStrip OPEGEN by OPERON) are deposited.

##### Quality assurance

In addition to the sample, ZFX/ZFY is present as the internal check of the PCR (the absence of this band is indicative of a PCR failure). The

band for the SRY gene will appear every time the DNA sample contains specific sequences of this gene (e.g., in males 46, XX).

### Detection of metals and pesticides on seminal fluid

To detect environmental contaminants, a specific amount of semen was centrifuged at 3000 rpm/min for a minute to exclude the cellular component from the analysis conducted on the seminal plasma. The determination of metals was conducted using the determination technique with atomic spectrophotometry (System quality UNI CEI EN ISO/IEC 17025).

The quantitative determination of 500 pesticides (EEC/EU n°839:2008 regulations) was conducted using the technique (UNI EN 15662: 2009; MI 293 Rev 1, 2010) of high performance liquid chromatography-mass spectrometry (HPLC-MS/MS).

#### Metals detect (Cd, Ni, Cu, Pb)

When the concentration range in samples is from 0.01 to 10 mg/kg of metal, the following procedure should be used: for concentrations above 10 mg/kg, it is possible to return to the previously mentioned range by using a diluted sample. The parameter to be determined is the metal, and the measuring range is from 0.01 to 10 mg/kg. The test must be conducted in a working environment at a temperature between 18 and 25°C.

#### Equipment and Reagents

- Atomic absorption spectrophotometer with electromagnetic atomization (Shimadzu GFA 2500)
- Hollow-cathode lamp capable of emitting the spectrum of the element under examination
- Class A glassware

Reference samples and reference materials required

Reagents must be of recognized quality and analytical purity

- The water used must be ultrapure for trace metal analysis
- 65% pure Nitric Acid, 70% pure perchloric acid, 98% pure sulfuric acid for trace metal analysis, standard reference solutions of 1000 mg/l.

The software starts checking for instrument component verification. Concentration of metal (mg/kg) =  $(A - B) * V/M$

A = concentration (mg/L) of metal in the sample; B = concentration (mg/L) of metal in white; V = final volume (mL) of digested solution; M = sample weight (g). The results are entered directly on the Eurosoft management program

#### Hg detection

By using an appropriate mineralized sample, using the cold vapors method, the mercury determination by atomic absorption spectrophotometry is effectively described in detail in the following procedure. The procedure is applicable to samples in the concentration range of 0.01–10 mg/kg of metal, whereas for concentrations above 10 mg/kg, it is possible to return to that range by diluting the sample. The parameter to be determined is Mercury (Hg), and the measuring range from 0.01 to 10 mg/kg.

$$\text{Concentration of metal (mg/kg)} = C * V/M$$

C = mercury content expressed in pg/ml in the sample solution; V = volume of solution expressed in ml; M = portion of the sample used for the analysis, expressed in grams.

#### Pesticides determination (EEC/EU n°839:2008 regulations)

The new European Plant Variety Control Regulation, which has been in place since September 2008, harmonizes maximum residue limits (LMRs) of pesticides in all EU countries and regulates their process of fixing in plant and animal origin products to human consumption. The 500 pesticides, analyzed on semen, are the most widespread used for which the European Regulation requires determination on foods for human consumption. The list of 500 pesticides (1° is 2,4 D, 2° is 2,4 DB...16° is 2-Nitroaniline...311° is Hexachlorobenzene,...499° is Zeta-Cypermethrin, 500° is Zoxamide) was not attached for brevity.

The analytic procedure is suitable for concentrations above 0.01 mg/kg.

The chromatographic system used in the laboratory for the separation of active ingredients in pesticides is an UltraFast Liquid Chromatography (UFLC) XR-Prominence Shimadzu.

To calculate the final concentration obtained, the formula used is as follows:  $X: 50 = y: 1000$ . X = weighted quantity, 50 = volume of the flask.

The quantitative analysis is based on the calibration line obtained with known concentration standards, because the standard volume injected in the chromatograph is equal to the sample volume being injected for the assay, that is, 5 µl. The value of x (p.a. concentration) is calculated as follows:

$$\text{Sample area: } X = \text{Standard area: conc. Standard}$$

### Selection of the Oligospermic and Normospermic Samples

A group of approximately 200 couples with fertility problems was studied at the Zigote center in Salerno Campania, Italy, over a 2-year period. Eight oligozoospermic patients were selected from this sample group, for which known genetic and non-genetic factors for male sterility could be excluded.

#### Oligospermic patients

The patients, aged between 28 and 44 years, underwent genetic-clinical visit, genetic analyses, and semen collection for seminogram. For the purposes of this study, the following investigations were performed: (a) conventional seminogram [8] (according to WHO 2010 criteria), (b) molecular-genetic investigations (karyotype, microdeletion Y, and cystic fibrosis of peripheral blood).

The patients in this study provided a semen sample to assess the initial conditions of their semen according to the parameters considered normal in accordance with the WHO guidelines, to determine the causes of infertility. As patients, on the basis of data obtained from the semen analyses, resulted in being oligozoospermic, teratozoospermic, and/or asthenozoospermic, we proceeded to screening through the molecular-genetic investigations to identify potential genetic causes (Y microdeletion, translocations, inversions and duplications, and numerical sex chromosome abnormalities, Cystic Fibrosis). In total, eight oligoastenospermic patients, whose average age was 33.9 and for whom the cause of their infertility could not be identified,

were recruited: molecular-genetic investigations were normal and cryptorchidism, epididymitis, varicocele, and so on were eliminated after clinical and instrumental examination.

### Normospermic patients

The control group comprised 20 males with normal fertility (presence of one or two children without assisted fertilization procedures) aged between 30 and 42 years. Their geographical origin was the same as that of the group of oligospermic patients. The seminal parameters of the control group were normal according WHO standards. In the control group, heavy metals and pesticides were studied as in the oligospermic group.

## Results

### The oligospermic sample

Table 1 shows the data collected during the semen analyses of the eight pathological patients.

A quantity of semen sample (seminal plasma) was used to detect the levels of Pb, Cd, Hg, Cu, and Ni (mg/kg) and for the analysis of the panel of 500 pesticides.

For the evaluation of heavy metals in seminal plasma, as threshold values were assumed to be the hematic values of the heavy metals, we used these reference values for evaluation of our samples: Pb  $\leq$  0.400 mg/kg; Cd  $\leq$   $5 \times 10^{-3}$  mg/kg; Hg  $\leq$   $5 \times 10^{-3}$  mg/kg; Ni  $<$  0.04 mg/kg, Cu  $<$  1.4 mg/kg.

The data that emerged from the analysis to determine the presence of tested heavy metals are grouped in the following table (Figure 1).

In Figure 1, above patients are marked with the same Arabic numerals of Table 1. The boxes with heavy metal abnormal values have their edges highlighted. The boxes with an asterisk, while being lower than the blood threshold values, are significant because the blood-testicular barrier must be considered.

### The control sample

Traces of Pb in the seminal fluid at concentration  $>$  0.01 mg/kg (0.02 mg/kg and 0.03) were found only in the 2/20 control group subjects.

### Statistical analysis

Applying the Chi-square test to the percentage of subjects with heavy metals in the semen (2/20 in the normospermic sample and 4/8 in the oligospermic one), a value of 3.73 is obtained that makes the difference statistically significant ( $p <$  0.05).

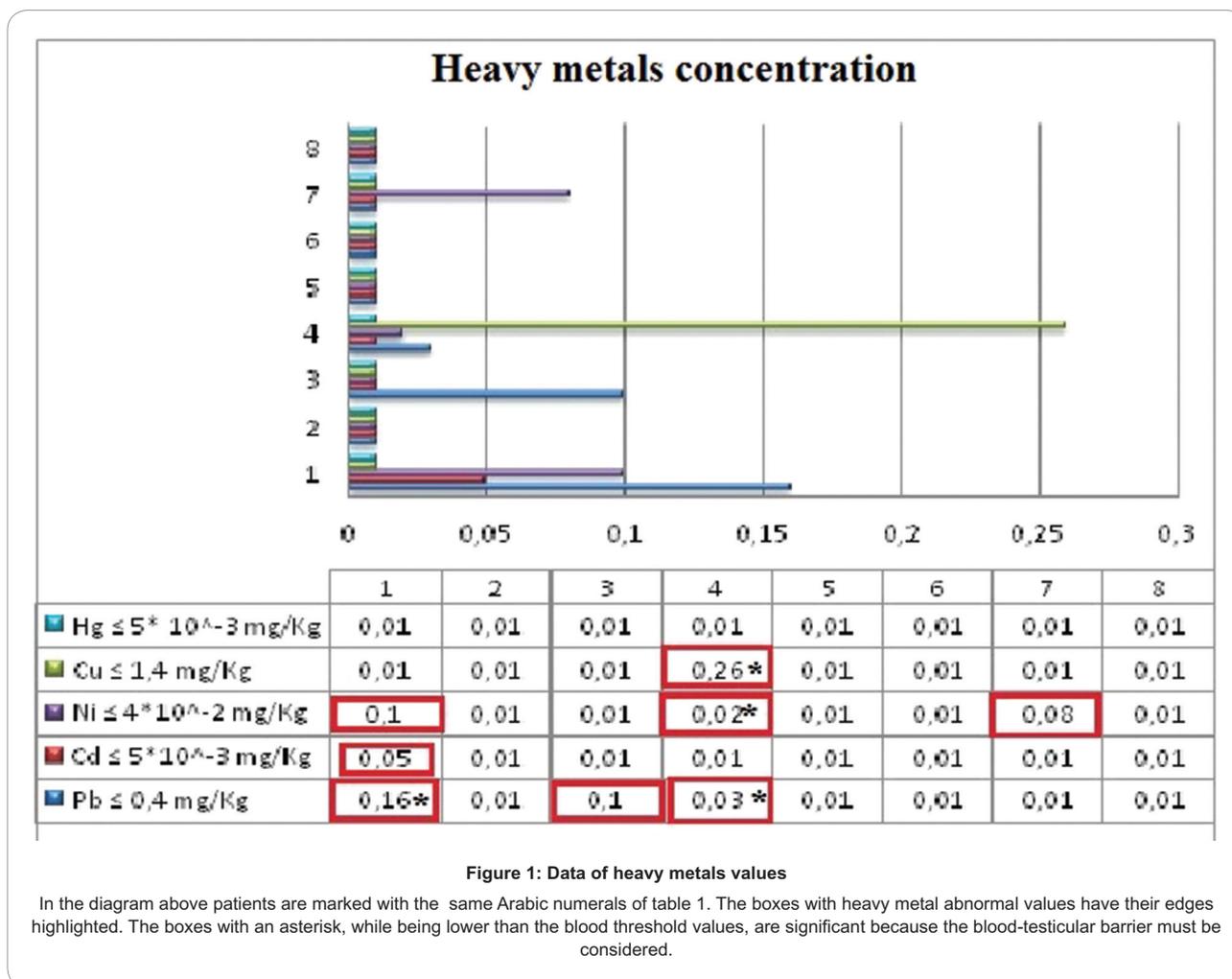
### Discussion

The function of heavy metals such as “endocrine disruptors” (EDs) has been widely documented in previously published research and papers. Over the years, numerous in vitro and in vivo studies have shown the harmful effect of the following heavy metals Pb, Cd, Hg, Cu, and Ni on male reproductive health and/or sperm parameters [2]. A major toxicity mechanism of many trace elements is their ability to produce high levels of reactive oxygen species (ROS), resulting in an unbalanced reduction–oxidation (RedOx) status affecting biological molecules, including lipid peroxidation [9]. As there has been a significant increase in the incidence of male infertility observed in several countries around the world, it has become a recurring theme in the international scientific literature [10, 11]. Low dose exposure is a ubiquitous phenomenon that occurs as a result of contamination of food, water, and air; so attention is not only to the percentage of people who are exposed to metals for professional reasons, but to ordinary consumers. Clinical and experimental studies provide strong evidence that environmental pollutants can cause damage to spermiogenesis through epigenetic alterations and high degree sperm DNA fragmentation [12].

A recent paper “Human semen as an early, sensitive biomarker of environmental exposure Preliminary results of the ECOFOODFERTILITY Project” [13] investigating the human semen from areas of the Campania region (Italy) with an environmental pollution owing to the illegal disposal of toxic waste has detected higher Zn, Cu, and Cr as well as reduced sperm motility and higher sperm DNA Fragmentation Index (DFI). In addition, young male residents in areas with high environment exposure had a significantly increased telomere length in sperm [14]. Our sample of oligospermic patients belongs to the same geographical area (Campania, Italy) of the ECOFOODFERTILITY project, although not all of our patients came from high environmental impact areas. An important differential criterion is that, while in the ECOFOODFERTILITY Project, the

	Color and aspect	Volume (ml)	Viscosity	Fluidification (minute)	pH	N° sperm/ml Million/ml	N° total sperm	Motility after 1 h (%)	Progressive motility (%)	Diagnostic conclusions
1	Light yellow	7	Normal	Complete 60'	7.0	20.2	141.4	40	30	Light oligo-asthenoterathospermia
2	Ivory	5	Normal	Complete 40'	8.0	Rare	–	20	20	Severe oligo-asthenospermia
3	Ivory	6	Normal	Complete 60'	7.0	6.3	37.8	50		Medium-light oligospermia
4	Light yellow	3	Normal	Incomplete 60'	8.0	181	543	46	30	Mild asthenospermia
5	Light yellow	4.5	Normal	Complete 60'	8.0	8	36	50	45	Medium/severe oligospermia
6	White milk	5.5	Normal	Complete 60'	8.0	17.2	94.6	50	40	Light oligospermia
7	Gray opalescent	2.5	Normal	Complete 60'	8.0	8.6	21.5	20	15	Oligo-teratoasthenospermia severe
8	Gray	3.0	Increased	Incomplete 60'	7.0	2.8	8.4	1	–	Severe oligoasthenospermia

Table 1: Semen analyses of the eight pathological patients



patients were selected solely on the origin area (high and low pollution impact areas), our oligospermic sample was selected by excluding the known causes of oligospermia: this criterion makes the finding of heavy metals in semen even more significant. The results of our study confirm the negative action of heavy metals on seminal fluid.

The fact that Cu can be toxic to sperm (present in the seminal fluid of an oligospermic subject of our sample) can also be deduced from the anti-fecondative action of the Cu intrauterine spirals in use for decades as a contraceptive method. Cu toxicity has been demonstrated in vitro also on other cell types such as embryonic neurons [15].

Hg was not present in the 22 elements tested in the ECOFOOD project. Hg is a very toxic substance that represents a serious and global threat to human health, in the form of methyl mercury in fish and seafood, ecosystems, and wildlife. Most Hg emissions and exposure hazards derive from anthropic activities, such as primary extraction and Hg transformation, Hg use in industrial products and processes, extraction and processing of artisan gold, coal combustion, and Hg waste management. The Seventh Environment Action Program adopted by Decision no. 1386/2013/EU of the European Parliament and of the Council declares that action must be taken to ensure a reduction of the significant adverse effects of chemical substances such as Hg on health human beings and environment by 2020. Mercury has been tested in our samples of oligospermic and normal subjects. A positive finding

is the absence of Hg in the tested samples, likely as a result of the effectiveness of European regulations that strongly restrict its use.

An additional difference compared to the ECOFOODFERTILITY project is that 500 pesticides were analyzed in the semen. Pesticides present in fruit and vegetables also have harmful effects on male fertility. Along with heavy metals, radioactivity, and toxic fumes of organic chemicals, pesticides are largely responsible for erectile dysfunction and fall in fertility in the male sex [4].

In our study, the determination analysis of the 500 pesticides panel gave a negative result; no trace of active principles was found in the seminal plasma of patients analyzed.

In our sample, the pesticides seem to be not responsible for the decrease in quality (concentration, motility, morphology) of semen, although the sample size is considerably small to draw conclusions. It is possible that the blood-testicular barrier is more effective for pesticides than for heavy metals. Another possible hypothesis is that exposure to heavy metals is ubiquitous, while exposure to pesticides may only affect certain specific work activities.

The following data emerge from this research carried on a selected group of oligospermic patients: a possible cause of parameters outside the normal range, by means of semen analysis (analyzed according to WHO 2010 criteria), could be found in the heavy metals values.

An analysis to detect the five analyzed heavy metals (Pb, Cd, Hg, Ni, and Cu) revealed positive values. Abnormal values were found for Pb, Cd, Ni, and Cu. The values of Pb and Cu, although being lower than the threshold value for blood, are significant data, because there are higher than threshold value of sensitivity and also because there is no previous data relating to analyzed biological matrix (seminal plasma) in this study.

Only in 2 out of 20 members of the control group, the Pb concentration was found to be  $> 0.1$  mg/kg.

Although not having the reference values in this biological matrix (seminal plasma), the high concentration of heavy metals measured in pathological patients according to our initial premise is noteworthy.

## Conclusion

Altered spermatogenesis, owing to heavy metal contamination, emerges from data analyses of our group of oligospermic patients, which were selected because they showed no other known cause of oligospermia. The absence of pesticides in the sample of patients analyzed (i.e., levels below the threshold value) has highlighted that, in our present study, the latter cannot be considered as contaminants responsible for sperm parameters below the norm. The results of our analyses have therefore led us to focus on the presence of significant levels of heavy metals in semen: Pb, Cd, Ni, Cu, and Hg. The presence of heavy metals in seminal samples in infertile patients where genetic causes have already been excluded is an indication of a correlation between their presence and infertility.

The presence of heavy metals in the semen has revealed that the cause of their infertility may be related to environmental factors such as water, soil, and air pollution, and food contamination.

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## Authors' roles

Ph.D. biologist Anna Flavia Rispoli contributed considerably to the designing of this project, laboratory analysis, and writing the article. Gynecologist Guglielmo Stabile Ph.D. contributed by providing gynecological counseling to infertile couples. Biologist Carminia Marina Ingenito Ph.D. was instrumental in semen analyses and genetic testing. Dr. Mariano Stabile, Medical Doctor in Genetics, was responsible for designing the project, correlating the findings of it, as well as having final approval of the version to be published.

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