FOLLICULAR FLUID CONCENTRATIONS OF PHTHALATES AND THEIR METABOLITES AMONG WOMEN AFTER IN VITRO FERTILIZATION

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Abstract: *The aim of the study was to identify phthalates and their metabolites from follicular fluid in patients who underwent assisted reproduction procedure (in vitro fertilization), as well as possible factors influencing their concentrations. It was demonstrated the presence and accumulation of phthalates in the follicular fluid during procedures of sampling and transportation prior to be analyzed due to the transfer of these chemicals from the medical devices used in the in vitro fertilization procedure. The developed and tested analysis methodology by gas chromatographic technique coupled with mass spectrometry allows reaching an appropriate quantification with the posibility of its application as method of toxicological diagnosis. Our study is the first in Romania to use the follicular fluid as matrix for the human biomonitoring to further explore phthalate exposure and human health consequences.*

INTRODUCTION

Experimental "in vivo" and "in vitro" studies and, concurent human studies suggest that environmental pollutants play an important role in female infertility. The concern for phthalate diesters, bisphenol A and benzophenones as risk factors for female infertility is increasing, due to their ubiquitous presence, frequent exposure and significant experimental evidences as endocrine disruptors.(1) Exposure to phthalates has been associated with altered serum thyroid levels and sex hormone levels during pregnancy, increased risk of low birth weight, and low sperm count and a decline in semen quality in men.(2-4) In addition to being classified as endocrine disruptors, phthalates have also been associated with obesity, cardiovascular disease and diabetes.(5) Moreover, DEHP is considered to be a probable carcinogenetic agent in humans.(6) As endocrine disruptors, the effects of phthalates (PHTs) have already been reported on folliculogenesis and ovocyte development (7), even very low levels of phthalates metabolites have to be considered in the risk assessment.(8) In the case of reproductive function assessment, including the "in vitro" fertilization procedure, a number of studies focused on the determination of xenobiotics in the seminal fluid and follicular fluid as exposure biomarkers that may have implications in the clinical and epidemiological studies.(9) It is a fact that a transfer or accumulation of PHTs occurs in the preovulatory phase of the ovocyte during infertility treatments underwent by women, and this concentration is inversely associated with the success of assisted human reproductive procedures.(10)

Only a few studies have determined phthalates metabolites in the follicular fluid. Modern equipments and methods of chemical analysis (Gas Chromatography, Liquid Chromatography, High Performance Liquid Chromatography, Micellar Electrokinetic Capillary Chromatography) created the possibility to study the etiology of female idiopathic infertility with focus on very low levels of exposure to non-persistent organic pollutants.(7)

In Romania, the impossibility to diagnose female infertility of idiopathic cause following ubiquitous exposure to organic pollutants requires the development and implementation of an analysis method of phthalates and their metabolites (MPHT) in the follicular fluid (FF) during "in vitro" fertilization procedure (IVF). It addresses the forms of transfer, transport, preservation and analysis from FF of four PHTs (they allow identification of contamination during sampling, transport and preservation until analysing) and their metabolites (they allow identification of contamination within maximum 48 hours before the IVF procedure.

The sampling procedure of the follicular aspirate and then isolation of the oocyte involves a time frame when the biological fluid comes into contact with teflon and plastic materials (polystyrene/polypropylene). In addition, due to the rapid transformation of phthalates in the external environment, accelerated by light and high temperature, by the possibility of contamination during sampling and post-sampling, by intermediate steps from the moment of egg pick-up and obtaining the FF until its analysis in the laboratory are essential in obtaining high accuracy results.

Our study is the first in Romania to use FF as matrix for the human biomonitoring to further explore phthalate exposure and human health consequences. There are only two studies previously published referring to the drinking water and PHT in Romania. The study of Dumitrascu et al., (11) was focused on the determination of PHT from drinking water, while Sulentic et al., (12) approached in addition the biomonitoring of some PHT and their metabolites by measuring these compounds in the urine of adolescents.

PURPOSE

The aim of the study was the identification of PHTs and their metabolites from FF in order to be implemented in the specific procedures of biotoxicological analyses. The specific objectives were a) to test the experimental model for sampling,

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preservation and transport of FF within the IVF procedure until analysis steps, b) to test and validate an optimized method of gas chromatographic analysis coupled with mass spectrometry (GC-MS) of PHTs and their metabolites from a less used matrix and c) to analyze the variability of such compounds concentration in FF of patients who underwent assisted reproduction procedure (IVF) and possible factors influencing them.

MATERIALS AND METHODS

Study protocols were approved by the Scientific Research Ethical Committee at "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania, in the frame of the research project PN-II-P2-2.1-CI-2017-0058 "Gas chromatographic method for the determination of phthalates in follicular fluid of patients following assisted reproductive treatments - in vitro fertilization".

The study protocol consisted of the following:

- Recruitment of the patients and obtaining the Informed Consent (25 patients);
- Collection of follicular content in 2 test tubes each, made of 2 different materials (polystyrene and glass) for each patient (50 samples resulted);
- Separation of oocytes and FF;
- Transport of samples to the Laboratory of the Environmental Health Center;
- Analysis of FF samples after 2 hours following sampling;
- Interpretation of results.

Potential participants were identified from the Human Assisted Reproduction Department "Prof. Dr. I.V. Surcel" in Cluj-Napoca, Romania. The participants were contacted during their visits at the department and were given further information on the study purpose and procedures and asked permission to be recruited by signing the informed consent form.

The FF sampling was done by using a needle puncture - sterile, with cutting tip (V-Tip™) made of stainless steel, marked for eco-guidance at tip, attached to the Teflon tubing connected to the washing system and to two test tubes - borosilicate glass, reusable and polystyrene for collection of the follicular aspirate. Each test tube was covered with acetone pre-washed aluminum foil and then the cap was fixed. Once collected, the follicular aspirate was slopped into polystyrene Petri dishes, minimizing the contact time of the FF with these. Samples were transported to the laboratory in an isothermal container at a temperature between 1-4ºC no later than 2 hours after sampling and immediatelly extracted for the further analyze of PHTs.

Analyzes of four PHTs with their metabolites were performed for the following:

- Di(2-ethylhexyl) phthalate - DEHP with metabolites: mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP) and mono(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP);
- Benzyl butyl phthalate-BzBP with metabolite monobenzyl butyl phtalate-MBzBP;
- Dibutyl phthalate-DBP with metabolite monobutyl phthalate-MBP;
- Diisobutyl phthalate-DiBP with metabolite mono-isobutyl phthalate-MiBP.

The analytical method of PHTs and metabolites consisted in conditioning with phosphoric acid and ammonium acetate solution of 2 ml of F from each sample. Internal standard and β –glucuronidase solution were added, then the sample was sealed and incubated at 37ºC for 2.5 hours. After enzymatic hydrolysis the sample was extracted with solvent for 30 minutes in an ultrasonic bath. The organic phase was died with sodium sulphate and evaporated to dryness under nitrogen stream. Phthalate metabolites were dissolved in ethyl acetate, the derivatization agent (N,O-bis(trimethylsilyl) trifluoroacetamide cu 1% trimethylchlorosilane (BSTFA cu 1%TMCS)) was added and let for 1h in the thermoreactor. The sample was transferred into 1.5 ml vials, covered with aluminium foil before placing the vial cap and then analysed on GC-MS with a TG-5MS column, 30 m long, 0.25 mm diameter, 0.25 µm of phase. A blank and a laboratory control sample was analyzed for every 10 FF samples analyzed. Phthalates were not detected in any of the blank samples, indicating that no phthalate contamination occurred during sample analysis. The values for the control were all confirmed to be between the limits of a Shewhart Control Chart.

We analyzed the distribution of phthalate concentrations in FF samples and we found that data is slightly skewed, giving a higher mean value. So, to eliminate the influence of extreme values, we calculated the descriptive statistics using the median value and the interquartile range (IQR).

RESULTS

The study was conducted on a group of 25 patients who underwent assisted reproduction procedure (IVF) at the mentioned specialized department in Cluj-Napoca.

Phthalates

Three phthalates were identified in the analyzed samples (Di(2-ethylhexyl) phthalate-DEHP, Dibutyl phthalate-DBP and Diisobutyl phthalate-DiBP), and their concentration registered a great individual variability, dependent on the sampling and storage parameters. The concentration of benzyl butyl phthalate-BzBP was analyzed below the detection limit of the method (<0.2 µg/l) in all samples, regardless of the test tube type. The total measured PHTs concentrations in FF of the patients (n= 25), as well as the rage of concentration of analyzed compounds are presented in table no. 1. All the compounds registered a larger range of concentrations in the polystyrene test tubes compared to those measured in samples collected in glass test tubes.

Table no. 1. The range of PHTs concentrations in follicular fluid

		Glass sampling tubes	Polystyrene sampling tubes
PHTs total	µg/l	0,67-2,78	0,58-3,04
DBP	µg/l	0,31-2	0,27-2,05
DiBP	µg/l	0,02-0,35	0,03-0,40
DEHP	µg/l	0,2-0,85	0,18-1,05

The median concetration of total identified PHTs (table no. 2.) was noticed to be higher for the samples collected in polystyrene test tubes compared to those collected in glass test containers. The analysis of DBP and DiBP showed a higher median concentrations in the samples collected in the polystyrene test tubes than those in FF collected in the glass ones at 2 hours following the FF sampling. In the case of DEHP a similar median concentrations between glass and polystyrene sampling tubes was observed.

Table no. 2. The median concentrations and IQR of PHTs in follicular fluid

		Glass sampling tubes	Polystyrene sampling tubes
PHTs total	µg/l	1,37 (0,73)	1,46 (0,92)
DBP	µg/l	0,81 (0,43)	0,86 (0,69)
DiBP	µg/l	0,10 (0,13)	0,13 (0,13)
DEHP	µg/l	0,45 (0,26)	0,44 (0,24)

The influence of sampling/storage conditions on the

concentrations of total measured PHTs and identified compounds are presented in the figures no. 1-4.

Figure no. 1. The influence of sampling/ storage conditions on the concentrations of total measured PHTs (µg/l)

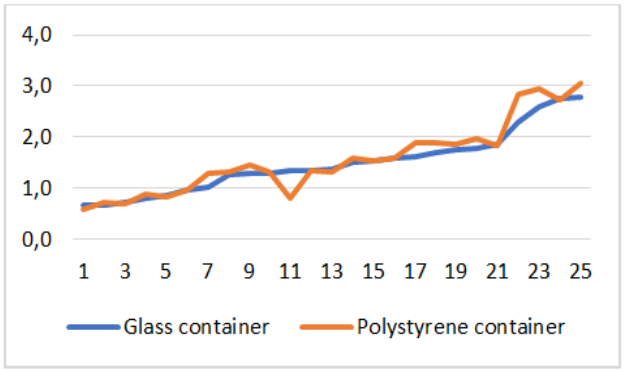


Figure no. 2. The influence of sampling/ storage conditions on the concentrations of DBP (µg/l)

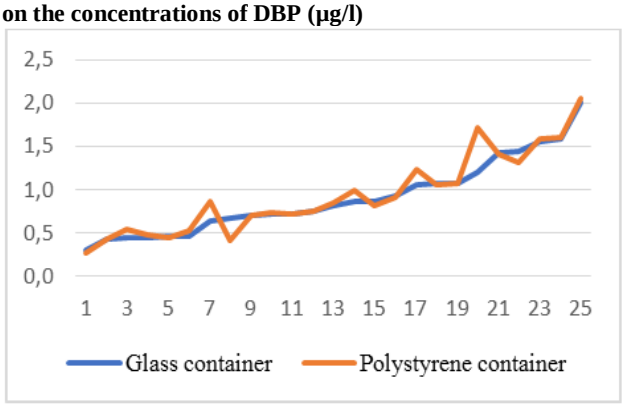
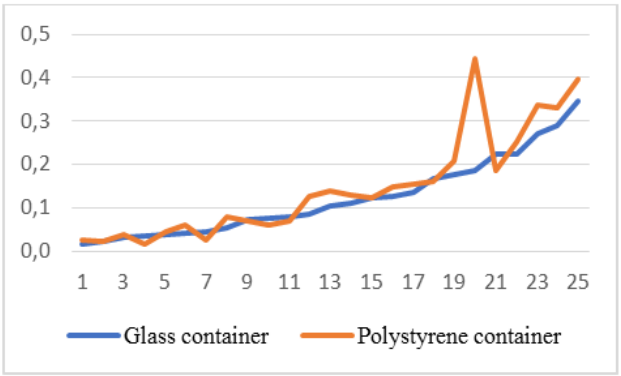


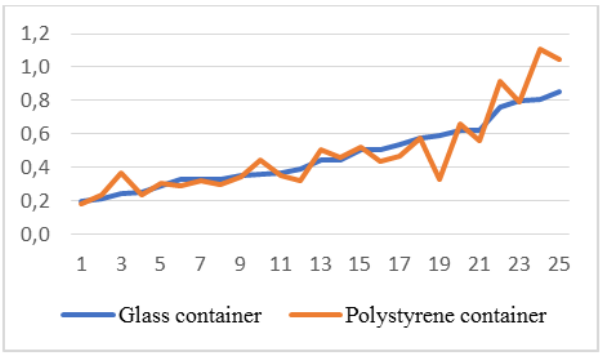
Figure no. 3. The influence of sampling/ storage conditions on the concentrations of DiBP (µg/l)



It becomes obvious that while the samples stored in glass containers have concentrations distributed liniarly, especially between Q_1 (7^{th} value) and Q_3 (19^{th} value), the samples stored in polystyrene containers showed a high variability with an unpredictable relation to the latter (having either positive or negative deviations). Approximately, these deviations vary from half to more than double, higher variability being observed, in general, at higher concentrations, especially in the last 25% of data (above Q_3).

Whether in the case of samples collected and preserved in polystyrene test tubes versus the glass ones, the growth rate was low after 2 hours (12%). However, the results showed that independently of the individual variability of PHTs concentrations, they can be measured in the FF (contamination marker secondary to follicular content collection procedure), the sampling and preservation methods until the moment of analysis influencing them directly.

Figure no. 4. The influence of sampling/ storage conditions on the concentrations of total measured of DEHP (µg/l)



Metabolites

In all 25 patients, all the analyzed metabolites of the parent compounds DBP, DiBP, and DEHP had concentrations below the quantification limit of the method (2.5 ng/ml), in both glass and polystyrene test tubes. The only unidentified metabolite was MIBP. Regardless of the test tube material, 5-OH-MEHP, the secondary metabolite of DEHP was identified in all FF analyzed samples, unlike 5-oxo-MEHP, the tertiary metabolite of DEHP which was identified in smaller proportion (96%) after 2 hours in the polystyrene test tube. A special mention must be made for MBzP which was identified in proportion of 100%, even though the parent BzBP was not identified above the quantification limit of the method in any sample. MBP and MEHP (primary metabolite of DEHP) were identified in smaller proportions in patients' FF: 76 % in samples from glass containers and 80% in samples from polystyrene containers for MBP and 92% in samples from both containers for MEHP. With regards to metabolites concentration, as in the case of PHT parent compounds, there was a great individual variability, as well as related to the test tube material even though the concentrations were below the quantification limit of the method. The highest median concentrations of metabolites, in decreasing order, for samples preserved in both types of test tubes were those of MEHP, 5-oxo-MEHP, MBP, 5-OH-MEHP and MBzP (table no. 3). Only for 5-OH-MEHPthe median concentration was higher in FF sampled in polystyren tubes comtared to the glass ones.

Table no. 3. The median concentrations with uncertainty of PHTs metabolites in follicular fluid

Metabolite (ng/ml)	Glass sampling tubes	Polypropylene sampling tubes
MBP	0,45±0,22	0,64±0,32
MBzP	0,12±0,06	0,11±0,05
MEHP	0,97±0,48	0,97±0,48
5-OH-MEHP	0,38±0,19	0,38±0,19
5-oxo-MEHP	0,87±0,44	0,86±0,43

It should be mentioned that although DBP had the highest median concentrations in FF, its metabolite ranged as level of median concentration below the primary and tertiary metabolites of DEHP.

DISCUSSIONS

Phthalates are used as plasticizers and are found in many products including medical tubing.(13) They are not chemically bound to the polymer, allowing for them to be easily released into the environment from plastic products.(14)

Intrafollicular microenvironment is crucial for the

health of the oocyte (15), and endocrine disruptor substances can broke its balance.(16) DEHP, an estrogen-like compound and a probable carcinogen in human, alters folliculogenesis and the oocyte development in mice, depending on the exposure age and duration.(17) The studies on the PHTs concentrations in FF reports various data. If Krotz et al., (18) showed that phthalates do not accumulate in FF of women undergoing infertility treatments in concentrations over the minimum levels to cause in vitro reproductive toxicity in animal models. Anyway, the PHTs and their metabolites could be measured in FF and Du et al., (7) did find high frequencies of PHTs metabolite median concentration over the used method limits of detection; the median concentrations were higher compared to the present study for MEHP and lower for MBzP and 5-OH-MEHP compared the FF concentrations measured in our patients.

CONCLUSIONS

By its results, the study contributes to using FF for diagnosis as a result of IVF procedure, being considered its waste. At the same time, it was conclusively demonstrated the presence and accumulation of phthalates in the FF as markers of contamination during procedures of sampling and transportation until the moment of analysis. On the other hand, the results showed that phthalate metabolites can also be identified in FF allowing to highlight the recent exposure of the patient, within up to 48 hours prior to the follicular aspirate collection procedure.

The obtained results clearly show the possibility of phthalates variable transfer from the medical devices used in the IVF procedure, becoming necessary to change them in order to limit to the maximum the oocytes' exposure to toxics after sampling.

The developed and tested analysis methodology by gas chromatographic technique coupled with mass spectrometry (GC-MS) allows reaching a similar detection limit and particularly appropriate from a biotoxicological point of view compared to high performance liquid chromatography coupled with mass spectrometry with triplecuadrupole.

Based on the research results and their application as methods of toxicological diagnosis, preventive behaviors may be induced related to patient's general exposure to PHTs and development of measures in order to increase the success rate of the assisted reproductive procedure and to achieve healthy pregnancies.

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