SUMMARY

Exposure to environmental endocrine disrupting chemicals and female ovarian reserve.

Introduction

At the beginning of the 21st century in many developed countries the number of couples seeking for infertility treatment has increased dramatically, approximately 15% of couples in the United States and other developed countries are infertile (Fritzi and Speroff, 2011), while in the 1960s it concerned 7-8% of couples. The World Health Organization defines infertility as a social disease. The definition of infertility was widely accepted as a lack of pregnancy despite regular sexual intercourse (minimum 3 during the week), maintained for over 12 months without using any contraceptive methods. This is influenced by many factors, such as the impact of lifestyle and late decision about procreation, but also increasingly often postulated impact of environmental factors such as exposure to modern civilization threats such as environmental endocrine disrupting chemicals (EDCs). The dramatic increase of the number of infertile couples caused that reproductive health, especially the impact of EDCs, became an important public health issue. Numerous studies indicate that the exposure to environmental contaminants called endocrine disruptors (EDCs) is widespread and may negatively affects animal and human reproductive health. Exposure to EDCs has been linked to several diseases including infertility (Colborn et al., 1993).

Increased global industrial activity has exposed humans to wide variety of modern chemical substances like: phthalates, parabens, bisphenol A, triclosan and many more. These compounds due to mass production are commonly found in the environment. Exposure occurs through contact with these compounds in food, water, air, through contact with plastics or cosmetics. Those chemicals are found in a variety of products used daily like: plastic bottles,

food cans, detergents, cosmetics, toys or pesticides. These compounds belong to a broad group called "endocrine disrupting chemicals" (ECDs) in the English literature. They are compounds that have the ability to interact with the endocrine system disrupting its normal function, leading to impaired synthesis, function, storage and / or metabolism of hormones and can have an adverse effect on female and male fertility (Colborn et al., 1993) and play a role in the pathogenesis of infertility (Crain et al., 2008; Mendola et al., 2008).

The potential mechanism of the influence of endocrine disrupting chemicals (EDCs) on female fertility is related to their similarity to natural ligands that have the ability to bind to receptors: aryl hydrocarbons (AHR) and estrogen receptors (ER) that are involved in modulation of ovarian reserve. These compounds affect both the initial ovarian reserve established during fetal life and modulate ovarian reserve in adult life (Richardson et al., 2014).

Objective

The aim of the study is to assess the impact of environmental exposure to endocrine disrupting chemicals (parabens, bisphenol A, triclosan, synthetic pyrethroids) on ovarian reserve.

The specific objectives relate to:

1). Evaluation of female ovarian reserve by testing:

a). the number of antral follicles (ACF-antral follicle count) evaluated in accordance with recommendations (Broekmans et al. 2010);

b). concentration of AMH (Anti-Müllerian Hormone);

c). hormone concentrations: FSH (follicle stimulating hormone) and estradiol measured between 2-4 days of the cycle.

2). Assessment of exposure to environmental factors - assessment in urine (two times)

a) parabens - (methyl, ethyl, propyl, buthyl, isobuthyl)

b) pyrethroids - selected pyrethroid metabolites (cis-DCCA (cis- (2,2-dichlorovinyl) -2,2-dimethylcyclopropane-1-carboxylic acid) (CDCCA), trans-DCCA (trans- (2,2-dichlorovinyl) -2,2-dimethylcyclopropane-1-carboxylic acis) (TDCCA), cis-DBCA (cis- (2,2-dibromovinyl) -2,2-dimethylcyclopropane-1-carboxylic), 3-PBA (3-phenoxybenzoic acid));

c) bisphenol A;

d) triclosan.

3) Assessment of lifestyle related factors (smoking, alcohol consumption, obesity, physical activity, diet, stress) and occupational exposure, which will be taken into account in the analysis as potential confounding factors.

Materials and Methods

Study population

To complete the adopted research goals, 511 women aged 24-39 were included in the study. The study involved women who attended to the infertility treatment clinic for the diagnosis and treatment of infertility (ie. the absence of pregnancy despite regular sexual intercourse (minimum 3 per week), maintained over 12 months without using any contraceptive methods) from December 2014 to June 2016 year. Only women with regular and ovulatory menstrual cycles were qualified for the research. Women who had in the past three miscarriages, over three performed in vitro fertilization cycles, were excluded from the study. Exclusion criteria were conditions that may lead to iatrogenic or spontaneous reduction of ovarian reserve, such as previous surgical treatment of the ovaries, chemotherapy or pelvic radiotherapy, the presence of cysts in the ovaries including endometrial (excluding functional cysts), chronic diseases that could reduce ovarian reserve (e.g., adrenocortical insufficiency, abnormal karyotype, fragile X syndrome) and conditions leading to anovulatory cycles such as: polycystic

ovary syndrome, premature ovarian insufficiency, hypogonadotropic hypogonadism, hyperprolactinemia. Women who agreed to participate in the study and signed the inform consent to participate in the study were asked to complete the questionnaire. Information obtained from the interview was included in the analysis as potential confounding factors.

The study obtained the consent of the Bioethics Committee operating at the Institute of Occupational Medicine in Lodz - Resolution No. 23/2014 of 04/11/2014.

Data obtained from the questionnaire

The interview included data on socio-demographic characteristics, lifestyle: smoking, alcohol consumption, diet (based on a questionnaire on the frequency of consumption of selected products - Food Frequency Questionnaire), physical activity (based on the Seven Day Physical Activity Recall questionnaire on physical activity in recent years 7 days to calculate energy expenditure (Metabolic Equivalent Task-MET)), stress (Cohena-stress questionnaire, questionnaire for Subjective Work Characteristics Questionnaire - occupational stress)), past diseases and other exposures occurring in the environment of residence or work (the questionnaire of exposures and professional work prepared at the Institute of Occupational Medicine in Łódź).

Based on anthropometric measurements, the Body Mass index (BMI) and Waist-to-Hip Ratio (WHR) were calculated.

Anthropometric measurements

In order to assess the occurrence of obesity in the examined women, the following measurements were made: 1) body weight with an accuracy of 0.1 kg - using SECA electronic personal weight (model 8801321009, SECA UK Ltd, Birmingham, UK); 2) height, with an accuracy of 1 mm - using the stadiometer (Leicester Height Measure, SECA UK Ltd), 3) waist and hip circumference, with an accuracy of 1 mm – using a measuring tape.

Biological material was collected from the study subjects: blood and urine. Urine was collected twice (from 3 to 6 months apart) to verify the exposure to nonpersistant environmental compounds.

Ovarian reserve assessment

Ovarian reserve was evaluated using:

1) the number of antral follicles (ACF - antral follicle count),

2) hormone concentration:

a) AMH (anti-Müllerian hormone)

b) FSH (follicle-stimulating hormone) (performed between day 2 and day 4 of the cycle)

c) Estradiol (performed between day 2 and day 4 of the cycle)

The study of the amount of antral follicles was carried out in accordance with the recommendations (Broekmans et al. 2010). The tests were carried out only by certified doctors in the field of ultrasound examination in gynecology, trained in the assessment of AFC. All studies were performed at the beginning of follicular phase, most often between 2-4 days of the cycle in which the menstrual period occurred spontaneously or bleeding from withdrawal of bicomponent hormonal contraception was achieved. Performing the assessment of the number of antral follicles in the early follicular phase is recommended to reduce AFC fluctuation in the cycle resulting from the presence of a cyst or a corpus luteum. Follicles with dimensions of 2 to 10 mm were considered for the assessment.

Concentration of AMH in serum was determined by enzyme-linked immunosorbent assay (ELISA) using recommended by manufacturer commercial kits (The AMH Gen II ELISA kit and The Inhibin B Gen II ELISA kit; Beckman Coulter, Inc., USA).

The concentations of FSH and estardiol in the blood serum were evaluated by chemiluminescence immunoassay using commercial sets for the VITROS Eci system according to manufacturer's instructions (Ortho-Clinical Diagnostics Johnson & Johnson, UK).

Clinical part of the study (evaluation of ovarian reserve and performance of hormone levels tests) and the assessment of the clinical status of the recruited patients were carried out in the infertility treatment clinic or in cooperating units.

Evaluation of exposure to environmental factors

Two urine samples were used to evaluate environmental chemicals concentration such as:

1) metabolites of synthetic pyrethroids: cis-DCCA (cis- (2,2-dichlorovinyl) -2,2-dimethylcyclopropane-1-carboxylic acid (CDCCA), trans-DCCA (trans-(2,2-dichlorovinyl) -2,2-dimethylcyclopropane-1-carboxylic acid (TDCCA), cis-DBCA (cis- (2,2-dibromovinyl) -2, 2-dimethylcyclopropane-1-carboxylic acid) (DBCA), 3-PBA (3-phenoxybenzoic acid));

2) total concentration of parabens metabolites (sum of free and combined with glucuronic acid parabens: methyl (MP), ethyl (EP), propyl (PP) and buthyl (BP) and isobuthyl (iBuP) parabens),

3) bisphenol A (BPA)

4) triclosan (TCL)

Isolation of the analytes from the matrix was carried out using semi-automatic micro-extraction to the solid phase (Micro-Extraction by Packed Syringe - MEPS), the extracts were converted into a derativates and analysed using gas chromatography with tandem mass spectrometry (GC-MS / MS).

Analytical methods have been subjected to internal quality control (use of reference materials) and external laboratory control through participation in international quality control

programs (G-EQUAS-The German External Quality Assessment Scheme for Analyzes in Biological Materials).

Environmental factors have been identified in the Department of Toxicology, Medical University of Gdansk.

Confounding factors and statistical analysis

In the analysis of the impact of the selected environmental compounds on the parameters of the ovarian reserve of women in accordance with the adopted research objectives, a number of confounding factors were taken into account. These factors were selected based on the literature and analyzes based on the study. The statistical analysis was performed using the statistical package R. The first stage of the analysis was the identification of interfering factors. The next stage included linear or logistic regression. The final multifactor model included confounding factors and all relevant factors in a one-factor model at the significance level of 0.1. Exposure is presented as a continuous variable as well as in the relevant subgroups that characterize exposure quartiles.

Results

Study population

In the study 511 patients of the infertility treatment clinic were recruited and agreed to participate in the study. Most of the recruited women had higher education (75%) and secondary education (21%). People with vocational education accounted for around 4% of the respondents. The average age of the surveyed women was 33 years, 93% of them were married, 92% were professionally active, only 8% reported to be unemployed. Analyzing the infertility period of the examined couples referring to the clinic, it was found that the duration of infertility was in most cases was 3-5 years (29.55%) and above 5 years (35.23%). 81% of the surveyed

women consumed coffee, most often every day (70.67%). History illness concerned a small number of women surveyed and were not related to partner infertility. Only 15% of respondents declared one of the diseases mentioned in the questionnaire (diabetes, hypertension, heart defects, epilepsy). Only 15% of respondents declared one of the diseases mentioned in the questionnaire (diabetes, hypertension, heart defects, epilepsy). Most of the examined women had normal body weight (59%, BMI 18.5-24.9 kg / m2) and were no cigarette smokers (92.17%). The examined women were physically active, 68% of respondents declared some kind of physical activity. 81% of the surveyed women consumed coffee, most often every day (70.67%). Occupational stress measured by the Subjective Work Characteristics Questionnaire was moderate (mean= 95). The level of life stress as measured by the Perceived Stress Scale was also medium- 22 points.

Evaluation of the ovarian reserve

The number of antral follicles in the examined women was 12.73 ± 8.94 and it was within the normal range, because <4 follicles is associated with a significant reduction in the chance of pregnancy and that ovarian response to ovulation stimulation will be not satisfactory (Radwan and Wołczyński, 2011). The concentration of AMH was 1.17 ± 1.46 ng / ml and was slightly higher than the norm range. In the case of FSH and estradiol the mean concentration was 6.38 ± 2.18 mIU /ml and 93.74 ± 16.63 respectively and within the norms for these hormones.

The concentration of selected environmental chemicals

The results of the study showed that participants of the study were exposed to the environmental factors that interfere with the endocrine function: parabens, synthetic pyrethroids, bisphenol A and triclosan. The mean concentration of parabens in the urine of the

subjects in the first sample was: buthyl 4.70 ± 2.96 ng / ml, ethyl 11.2 ± 7.0 ng / ml, methyl 92.68 ± 4.28 ng / ml, propyl 16.20 ± 6.33 ng / ml and isobuthyl 3.16 ± 2.55 ng / ml and was similar to the level of parabens in other studies among both women attending the infertility treatment clinic for diagnostic purposes as well as women from the general population. In the case of synthetic pyrethroid metabolites, the geometric mean ± SD was: CDCCA 0.22 ± 2.40 ng / ml TDCCA 0.50 ± 2.62 ng / ml, DBCA 0.26 ± 2.42 ng / ml, 3-PBA 0.31 ± 2.60 ng / ml. The mean concentration of triclosan and bisphenol A was 2.78 ± 7.17 ng / ml, 1.38 ± 2.34 ng / ml, respectively.

In the second urine sample, the concentrations of the tested compounds (geometric mean ± SD) in the case of synthetic metabolites of pyrethroids were: CDCCA 0.23 ± 0.30 ng / ml, TDCCA 0.27 ± 0.33 ng / ml, DBCA 0.30 ± 0, 90 ng / ml, 3-PBA 0.25 ± 0.33 ng / ml. The concentration of parabens (geometric mean ± SD) were: buthylparaben 3.99 ± 9.90 ng / ml, ethylparaben 5.71 ± 44.97 ng / ml, methylparaben 49.13 ± 105.39 ng / ml, propylparaben 9.14 ± 38.58 ng / ml. IBuP paraben was not detected in the second urine sample. The concentration of triclosan and bisphenol A in the second urine sample was 1.67 ± 30.34ng / ml and 1.27 ± 1.71 ng / ml, respectively.

Correlations between the studied environmental chemicals

In the first urine sample the tested chemicals strongly correlated with each other. A statistically significant correlation was observed between the synthetic pyrethroid metabolites: CDCCA, TDCCA, DBCA, 3-PBA ($p < 0.001$). Bisphenol A (BPA) and buthyl paraben (BP) significantly statistically correlated with all tested compounds. Triclosan correlated significantly statistically correlated with all tested compounds, except propylparaben (PP) ($p = 0.28$). Ethylparaben (EP), methylparaben (MP), propylparaben (PP), isobuthylparaben

(iBuP) correlated with all tested compounds except some of the synthetic pyrethroid metabolites.

Due to the fact that the second urine test was conducted among a smaller number of women (N = 120), the correlations between the tested compounds looked different than in the first analysis. The tested compounds were correlated in a smaller number of cases. Three of the synthetic pyrethroid metabolites (CDCCA, TDCCA and 3-PBA) correlated with each other at the significance level of $p < 0.001$. In contrast, the DBCA metabolite did not significantly correlate with CDCCA and TDCCA, but correlated with 3PBA. This was due to the fact that it was only detected in 30% of the samples tested. 3-PBA significantly correlated with MP, PP, BP, TCL and BPA. The studied parabens (EP, PP, MP, BP) also correlated with each other, and no statistically significant correlation was found between EP and BP and PP and BP. Additionally no correlations were observed between MP and BP and BPA and all evaluated parabens and triclosan.

The concentrations of parabens (MP, EP, PP) and triclosan correlated with each other in two urine samples. However, in the case of bisphenol A, synthetic metabolites of pyrethroids and buthyl paraben the concentrations in the I and II samples were different.

The association between exposure to selected environmental chemicals and ovarian reserve

When the exposure variable were analyzed as continuous variables, it was shown that the concentration of propyl and buthyl paraben resulted in a decrease in the number of antral follicles ($p=0.028$ and $p=0.04$ respectively). Also, exposure to bisphenol A had a negative effect on the number of antral follicles ($p=0.03$). There was no relationship between exposure to other parabens tested (ethylparaben, methylparaben, isobuthylparaben), triclosan and synthetic pyrethroids and the number of antral follicles. The concentration of bisphenol A in the urine correlated with the decreased concentration of the AMH ($p=0.02$). Exposure to

propyl parabens correlated positively with the concentration of FSH (p=0.03) and negatively with estradiol (p=0.048). Also, ethyl paraben reduced the estradiol concentration (p=0.01).

The concentration of the synthetic pyrethroid metabolites (CDCCA, TDCCA, DBCA, 3-PBA) did not significantly influence the concentration of any of the hormones tested (AMH, FSH, estradiol). Also, exposure to methylparaben, buthylparaben, isobuthylparaben and triclosan was not significantly related to the concentration of hormones tested.

When the model was controlled for potential confounders (age, BMI and smoking) exposure to propyl paraben and bisphenol A decreased the antral follicles count (p=0.04 and p=0.03 respectively). Regarding the concentration of tested hormones, exposure to bisphenol A had a negative effect on the concentration of AMH (p=0.02), exposure to propylparaben increased FSH (p=0.028) and decreased the estradiol concentration (p=0.04). There was no relationship between exposure to other parabens tested (methylparaben, ethylparaben, buthylparaben and isobuthylparaben), triclosan and synthetic pyrethroids, and the antral follicles count of and the concentration of hormones tested.

It was observed that the concentration of propyl paraben in the urine in the second and third percentile ((25-50] and (50-75] percentile) decreased the number of antral follicles (p = 0.03 and p = 0.03 respectively). Also the concentration of bisphenol A and buthyl paraben in the third and fourth quartiles ((50-75] and > 75th percentile) decreased the antral follicles count (p=0.04, p=0.04 and p=0.028, p=0.03 respectively). Concentration of bisphenol A in the fourth quartile negatively affected the concentration of AMH (p=0.04) and the concentration of propyl of paraben in the second and third quartiles increased the FSH concentration (p=0.03 and p=0.04 respectively).

In the case of estradiol, there was a decrease in the concentration of this hormone in the second and third quartiles of ethylparaben exposure (p=0.031 and p=0.026) and in the second quartile of propylparaben exposure (p=0.049). There was no relationship between the antral

follicles count and the concentration of analyzed hormones and exposure to other tested parabens (methyl, butyl and isobutyl), triclosan or synthetic pyrethroids in the second, third or fourth quartile of exposure.

When the model was adjusted for potential confounding factors such as age, smoking, BMI, exposure to propyl paraben in the third quartile ((50-75] percentile) resulted in the reduction of the antral follicles count (p=0.048), decreased estradiol concentration (p=0.03) and increased FSH concentration (p=0.026). Also exposure to bisphenol A in the fourth quartile (> 75th percentile) decreased the number of antral follicles (p = 0.028) and decreased the AMH concentration (p = 0.03).

There was no statistically significant relationship between the exposure to parabens: methyl, ethyl, butyl, isobutyl, triclosan and tested synthetic pyrethroid metabolites (CDCCA, TDCCA, DBCA and 3-PBA) and the analysed parameters of ovarian reserve: antral follicles count and hormone concentrations: AMH, FSH, estradiol.

Conclusions

1. The results of the study showed that women participating in the study were environmentally exposed to the examined endocrine disrupting chemicals: parabens, synthetic pyrethroids, bisphenol A and triclosan confirmedby the results of exposure measurements using biological monitoring methods.

2. Exposure to parabens and bisphenol A negatively influenced ovarian reserve parameters. Exposure to propyl paraben and bisphenol A reduced the number of antral follicles. Regarding the concentration of tested hormones, bisphenol A exposure negatively affected AMH concentration, propyl paraben exposure increased FSH concentration and decreased estradiol concentrations.

3. There was no relationship between exposure to other paraben (methyl, ethyl, buthyl and isobuthyl), triclosan or synthetic pyrethroids and the antral follicles count and the concentration of hormones tested.

4. Women in reproductive age should be comprehensively informed through the mass media about the influence of exposure to common environmental chemicals, especially those disrupting endocrine functions and the ovarian reserve, particularly bisphenol A and propyl-paraben.

5. Women planning pregnancy and pregnant should receive information from their physicians about justified contact restrictions with these chemicals and information in which products contain them.