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# Possible role of n-hexane as an endocrine disruptor in occupationally exposed women at reproductive age

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ABSTRACT

Human exposure to n-hexane has been associated with subfertility and, experimentally, with a decrease in follicular development. In order to assess occupational exposure to n-hexane on ovarian function and gonadotropic hormones, we studied Mexican women labouring in a leather shoe factory (n = 34). Individual environmental levels for seven solvents, n-hexane included, were measured; also, urinary 2,5-hexanedione (2,5-HD) was determined. For ovarian function and hormonal status, FSH, LH, oestradiol and anti-Müllerian hormone (AMH) levels were determined. We performed all determinations also in a reference group, administrative workers with no exposure to solvents (n = 32). Results: N-hexane and urinary 2,5-HD levels were higher in exposed group ($p < 0.001$). More cases of oligomenorrhea as well as longer time for getting pregnant were observed in exposed women compared with controls; a positive association was found between menstrual cycle length and "time for getting pregnant" ($p = 0.010$); significant associations between FSH serum levels and 2,5-HD urinary levels (post-shift sample) were observed in non-smokers participants presenting oligomenorrhea from exposed group. Also, we found a trend for lower oestradiol levels in exposed participants with current smoking habit ($p = 0.059$). Conclusions: 2,5-HD urinary levels are associated with decreased gonadotropins levels; hence, n-hexane should be considered an endocrine disruptor in reproductive-age women.

## 1. Introduction

Endocrine disrupting chemicals (EDC) have been described as a single chemical or mixture of chemicals interfering with hormone action. EDC are highly heterogeneous because of the nature of the chemical itself as well as their structural features. (Costa et al., 2014). Once EDC are in contact with the human body, they can exert either one or several effects: imitating or partially imitating an endogenous ligand (such as hormones) in the receptor complex, then producing overstimulation or, binding to the receptor of a hormone producing no effect (as antagonist). Studies in women exposed to EDC and their effects have been associated with damage in the reproductive system, some effects represented as irregularities in the menstrual cycle such as longer menstrual cycles, which may reduce fecundability (Kabir et al., 2015).

In women, menstrual cycle could be disrupted by several factors, including EDC exposure. EDC effects on the reproductive system might cause some disturbances that may be also associated with chronic diseases i.e.: short cycles have been associated with an increased risk of breast and lung cancer, whereas irregular or long cycles have been associated with pre- and post- menopausal fractures, breast cancer, myocardial disease, and risk of developing metabolic diseases (Cho et al., 2001; Thurston et al., 2000). Menstrual cycle could be affected by a large variety of chemical agents. It has been reported some adverse effects on the reproductive system by exposure to volatile organic compounds (VOC´s) or mixtures of VOC´s in occupational exposures. Some of the effects reported are: menstrual cycle length irregularities (either longer or shorter), spotting bleeding or irregular bleeding duration, as well as dysmenorrhea and/or amenorrhea (Cho et al., 2001; Burdof et al., 2006, Béranger et al., 2012). Another study in women occupationally exposed to low levels of benzene (one of the most studied VOC's) showed a shorter length of luteal phase and decreased FSH levels as well as impaired luteal progesterone levels (Chen et al., 2001). Also, menstrual disorders have been associated to

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occupational exposures to a mixture of compounds, as well as adverse pregnancy outcomes like premature birth or spontaneous abortion, besides other impairments related with fertility. (Sallmén et al., 2008; Hassani et al., 2014). In the former studies, there is no specification about the amount of exposure, nor the mean levels of exposure of participants to compounds or a mixture of compounds and also, they neither performed biomonitoring of the exposure through quantification of urinary levels of the metabolites derived from VOC´s exposure.

Biomonitoring is very important since, for some occupational exposures, the toxic effect is due to the metabolite itself, not to the parental compound; such is the case of n-hexane and its main active metabolite in human body: 2,5-hexanedione (2,5-HD). N-hexane is a VOC commonly used in several industries, shoe and leather industries included. This solvent is present in primers solutions, as well as in glue and lacquers used in this manufacturing processes, particularly in the footwear manufacturing (Jiménez-Garza et al., 2018; ATSDR, 1999). **N**-hexane exposure has been linked with longer periods for getting pregnant, which was considered an indicator of subfertility in women occupationally exposed (Sallmén et al., 2008, 2006b). It is important to mention that, in those studies, VOC exposure was reported only qualitatively, not quantitatively. On the other hand, 2,5-HD exposure has been associated with reproductive health damage in female experimental animals: it has been observed a decrease in the number of growing follicles, accompanied by an increase in apoptotic follicles, as well as an increase in oxidative stress and alterations in the expression of genes related to ovarian hormone production. (Zhang et al., 2013). Another report in experimental animals showed that 2,5-HD (administered in drinking water to female rats) was able to disrupt the secretion of gonadotropic hormones: animals with the highest levels of 2,5-HD showed increased levels of FSH and LH hormones, suggesting a role of this metabolite as an endocrine disruptor (Abolaji et al., 2014). Other studies performed in human ovarian granulosa cells exposed to 2,5-HD in vitro have shown necrosis, as well as an increased number of atretic follicles after 24 h of culture with this metabolite (Sun et al., 2012). Besides, inhalation of n-hexane by female rats induced a decrease in the number of mature follicles and promoted apoptosis in granulosa cells from growing follicles, with no affection in the gonadotropic hormone levels (Li et al., 2015; Liu et al., 2012).

To date, no studies in humans have been performed searching for an association between n-hexane or its metabolite, 2,5-HD exposure with biomarkers of ovarian function, as well with hormones from the hypothalamic-pituitary-ovary axis in occupationally exposed women.

## 2. Materials and methods

### 2.1. Study design and population

This research was conducted in a leather shoe factory, as well as in a food-distribution company, both from the city of León, Guanajuato, México. Sixty-six women aged 18–37, clinically healthy, were recruited in a cross-sectional design. We separated participants in two groups: a) exposed group to VOC´s mixture (EG, n = 34, women working in the pre-finishing area from a shoe factory, with direct contact with VOC´s and without personal protection equipment), and b) control group (CG, without exposure to any chemical, n = 32), working in a food distribution company, with no solvent exposure. People from control group were matched by age and BMI, according to the findings in exposed group. Characteristics of former occupational exposure to VOC´s such as history of exposure in a previous job, labour seniority in the current job, and cumulated time of exposure were investigated.

For both groups, we did not include participants with any antecedent or current signs of the following: thyroid disorders, polycystic ovary syndrome, endocrine disease history in the previous six months, hysterectomy, oophorectomy, pregnancy, breast feeding at time of current study, and clinical evidence of chronic disease or current infectious disease. Also, we did not include participants having current treatment with anxiolytics, antidepressants, β-blockers, $Ca^{++}$ channel blockers, hypnotic medication or hormone replacement therapy, as well as use of hormonal contraceptives. We neither included people with antecedents of chemotherapy and/or radiotherapy.

We also asked for gynecologic/obstetric history: age at menarche, menstrual history in the last year, date of last menstrual period, age at first pregnancy and current day of the ovarian cycle, pregnancy history, outcome birth, menstrual cycle length (self-reported, 21–35 or > 35 days) in the last year, bleeding duration and also dysmenorrhea presence in the last year. For the “time for getting pregnant” variable, we asked to participants in a face-to-face interview at the workplaces, to tell us what was the period they last to get pregnant during the time they were working at the factory with n-hexane exposure. In this interview, we collected all obstetric and gynaecologic antecedents.

We also collected other variables such as: schooling in years, smoking habit (yes or no) and coffee consumption at least one drink/day (yes or no). Height and weight were determined with indoor clothing, without shoes; body mass index (BMI) ($kg/m^2$) was calculated. We also obtained a measure of adiposity by electrical bioimpedance with a TANITA BF 522W (Tokyo, Japan). Total cholesterol, triglycerides, fasting glucose and lipid profile were determined using conventional methods.

Study protocol was approved by the University of Guanajuato's Bioethics Committee (Code registration: CIBIUG-P-36-201). All the participants signed a written informed consent to participate in the study before being included. Also, we explained to each of them about the purposes of the study.

### 2.2. Individual exposure assessment

Individual exposure for 7 different VOC´s were determined: acetone, benzene, ethylbenzene, n-hexane, methylethylketone (MEK), toluene and xylenes, using personal diffusive samplers containing an active carbon Cartridge (Radiello®, Supelco, Bellefonte PA, USA). VOC's were extracted with carbon disulphide from the cartridges and were analysed by a PerkinElmer Gas Chromatograph-mass spectrometer (GC–MS) equipped with a PONA column (50 m, id 0.2 mm, film 0.5 µm) (Agilent Technologies, Santa Clara, California, USA) under the following conditions: oven was heated at 50 °C for 15 min; 10 °C were added/min up to 150 °C for1 min; and 15 °C were added/min up to 220 °C for 1 min. The MS operated in electron impact (EI) mode monitoring total ions from 40 to 399 m/z.

### 2.3. Biomonitoring

Participants from EG were asked for two urine samples: one before starting their daily duties (pre-shift sample) and the other sample eight hours after the first one (post shift sample); for CG we only asked for post-shift sample, considering that CG participants were not exposed to any VOC's. Samples were collected at the end of labour week (Friday) for both groups. Urine samples were stored at −20 °C until analysis. The objective was to measure in those urine samples the amount of 2,5-HD as a biomarker of n-hexane exposure. Briefly, 2,5-HD urinary and cyclohexanone (internal standard, SI) levels were determined by gas chromatography coupled with mass spectroscopy (GC/MS) after extraction from urine by diatomaceous earth columns and dichloromethane. The organic solution was concentrated to a smaller volume by evaporation using p-cymene as a keeper. The concentrated extract was then transferred to autosampler vials and analysed by GC/MS EI/SIM (Perkin Elmer, Waltham, Massachusetts, USA) equipped with a column PONA 50 m, id 0.2 mm, film 0.5 microns (Agilent Technologies, Santa Clara, California, USA). The detection limit of the analytical procedure was 12.0 µg/l.

### 2.4. Hormone levels determination

8 mL peripheral blood (forearm) was collected in tubes without anticoagulant. Plasma was separated, and Serum follicle-stimulating hormone (FSH) (IMMULITE® FSH kit), LH (IMMULITE® LH kit) and estradiol (17β-estradiol, $E_2$) (IMMULITE® estradiol kit) were determined in plasma from samples collected at follicular phase (from day 1 to day 7 of menstrual cycle for each participant) for participants of both groups; all measures were performed by chemiluminescence (IMMULITE® 1000 System, Siemens), following the manufacturer's instructions. All analyses were carried out by duplicate. Minimum detectable concentrations were: FSH: 0.7 mIU/mL-130 mIU/mL; LH: 0.7 mIU/mL-170 mIU/m land estradiol: 15 pg/mL, respectively. For AMH measurement, serum was obtained the same day of the environmental level's determination, regardless of woman's menstrual cycle day.

For AMH quantification, a commercial ELISA kit was used (Elabscience, Human AMH ELISA kit, E-ELh0317; WuHan, China), according to manufacturer´s indications. Range for AMH serum levels was: 93.75–6000 pg/ml, sensivity: 56.25 pg/mL.

### 2.5. Statistical analysis

Data normality was assessed by the Kolmogorov-Smirnov test. We compared groups of women (EG vs CG) using Student's t test or the Mann–Whitney U test for independent variables, as appropriate. We examined correlations of n-hexane with levels of hormones as well as 2,5-hexanodione with levels of hormones. Same correlations were performed with biochemical molecules and anthropometric data. Also, we examined each VOC present in the mixture analyzed separatelly with hormones; using simple correlation and Pearson´s correlation test. To evaluate odds ratio of "long menstrual cycle" variable (longer than 35 days) and odds ratio for "time to get pregnant" variable and another obstetric history in both groups, Chi square test, performing an *ODDS RATIO* test were used. After the former analysis, we performed a bivariate regression analysis, in order to adjust for variable "age". Further, a simple linear regression analysis was performed between "time to getting pregnant" variable and "menstrual cycle length" variable. We performed a bivariate logistic regression analysis, setting as predictors variables: VOC´s levels (including the seven VOC's measured), and as a dependent variables "oligomenorrhea" (presence or absence) and "time for getting pregnant" (less than 6 months and more than 6 moths). After the bivariate analysis, we conducted a multivariate model with the same dependent and independent variables.

Finally, we used a linear regression model to analyse a possible association between levels of AMH, FSH, LH and oestradiol with all VOC's environmental levels and also for 2,5-HD urinary levels. A general lineal model (GLM) with covariates, searching for association between all VOC´s exposure levels, and levels or urinary 2,5-HD and hormones levels (FSH, LH, AMH and oestradiol) was performed; this model was adjusted by condition of oligomenorrhea, and smoking status from participants in the exposed group. In this GLM model, we validated according to regression diagnostic tests, which included linearity, homoscedasticity, multicollinearity and normality of residuals. Significance was set at $p < 0.05$. For analysis we used SPSS 21TM software as well as Stata version 11.1(Stata Corp. LP, College Station, TX). For logistic regression, we used NCSS 12.

## 3. Results

When comparing EG vs CG differences regarding age, BMI as well as for another variables such as glucose, total cholesterol and triglycerides, and categorical variables, we did not find any statistical difference. Results are summarized in Table 1.

**Table 1**
Sociodemographic and clinic characteristics of study population.

| Variable | Exposed Group Mean ± sd n = 34 | Control Group Mean ± sd n = 32 | *p* |
|---|---|---|---|
| Age (years) | 28.9 ± 5.3 | 27.3 ± 5.0 | 0.1 |
| Weight (Kg) | 70.5 ± 17.7 | 62.7 ± 11.0 | 0.4 |
| Height (m) | 1.5 ± 0.05 | 1.5 ± 0.05 | 0.7 |
| BMI | 28.4 ± 6.2 | 24.4 ± 3.7 | 0.3 |
| Schooling (years) | 11.2 ± 1.9 | 19.8 ± 2.8 | 0.2 |
| *Age at menarche | 12 (9 − 17) | 12 (10 − 14) | 0.5 |
| *Gravidity | 0 (0 − 4) | 0 (0 − 3) | 0.1 |
| *Abortions | 0 (0 − 2) | 0 (0 − 1) | 0.4 |
| Glucose (mg/dl) | 91.8 ± 24.6 | 88.1 ± 12.9 | 0.9 |
| Tryglicerides (mg/dl) | 120.9 ± 58.4 | 114.3 ± 67.3 | 0.6 |
| Total Cholesterol (mg/dl) | 173.3 ± 32.5 | 165.8 ± 28.7 | 0.3 |
| HDL (mg/dl) | 39.8 ± 8.8 | 42.1 ± 9.2 | 0.3 |
| Current smoking (yes) | 3 | 2 | |
| Coffe consumption (yes) | 29 | 25 | |

n = 66 *Student t* test, *Mann–Whitney Test; median (rank).

### 3.1. N-hexane individual exposure levels and urinary biological monitoring

N-hexane was the compound with the highest exposure levels (mean 49.7 mg/m$^3$) whereas ethylbenzene showed the lowest levels (mean 0.13 mg/m$^3$) (Fig. 1). Table S1 shows levels of seven compounds between both groups compared with a reference value. We observed statistically significant differences (highest levels in the exposed group, $p = < 0.001$)

### 3.2. Biomonitoring

Urinary 2,5-HD was higher (Fig. 2A) in EG for the post-shift sample (224. 4 ± 173.2 μg/L, $p < 0.001$) compared with CG (42.8 ± 41.92 μg/L) ($p < 0.001$). Additionally, in EG we observed a significant difference among pre-shift (110 μg/L) and post-shift (224.4 μg/L) urinary samples ($p = < 0.001$, Fig. 2B).

### 3.3. Antecedents related with fecundity and fertility

According to the gynaecologic/obstetric history, in exposed group 79 % (27 out of 34) of participants reported long menstrual cycles (> 35 days), and 35.2 % of women in the same group reported menstrual cycles longer than 45 days (12 out of 34). In this group, 20 % of women reported more than 7 days of bleeding, and 23 % of women from EG reported dysmenorrhea. We found significant differences for menstrual cycle length between groups: 79 % of exposed women showed longer cycles compared with 20 % of control group as described in Table 2. OR for menstrual cycle length was 1.26 (95 % CI = 1.07 − 1.50). Also, in 70.6 % of exposed woman we found longer time for getting pregnant compared with 5.8 % of women in control group, as described in Table 3. OR for time to get pregnant was 6.25(95 % CI = 1.24 − 31.27) for both analyses we adjusted by age, and significant differences were maintained. After this observation we performed a second analysis with the cases who reported "intention to get pregnant" from the time they started to work in the shoe factory. A positive correlation was found in exposed group between "time to get pregnant" and menstrual cycle length (r = 0.507, $p = 0.010$).

In the bivariate logistic regression analysis, we did not find association between VOC´s levels and oligomenorrhea. However, we observed significant, positive correlations: acetone and n-hexane with "time for getting pregnant" as shown in Table 4.

### 3.4. Hormone levels

Mean levels for serum hormones were: AMH (4.1 ng/mL), FSH (6.4 mUI/mL), LH (7.2 mUI/mL) and estradiol (35.4 pg/mL) for EG

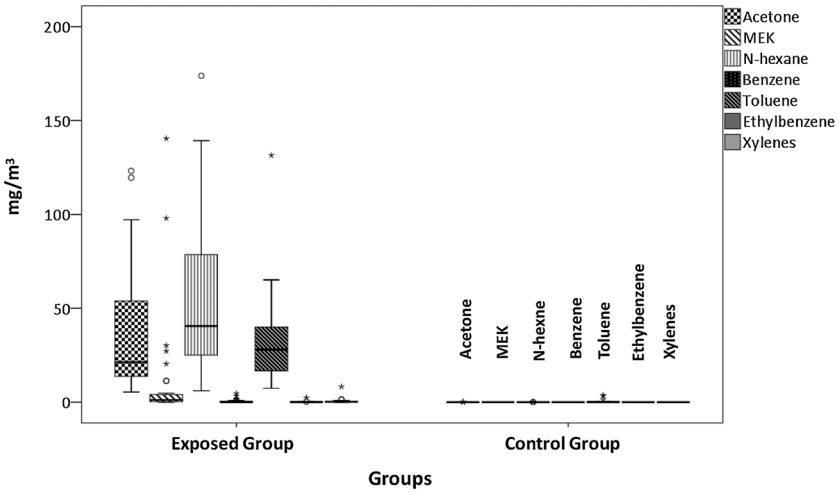


**Fig. 1.** Volatile organic compounds levels (mg/m$^3$) between groups.
★== *atypical value (a value 3 times higher, above maximum value, ○=atypical value found 1.5–3 times higher, above maximum value). Mann-Whitney U test. All compound levels where higher in the exposed group ($p < 0.001$).

group; while for EG group were: AMH (4.2 ng/mL); FSH (5.0 mUI/mL); LH (3.6 mUI/m) and estradiol (34.7 pg/mL). We did not find statistically significant differences for serum concentrations of these hormones between groups ($p > 0.05$) as shown in Table 5. However, we found a marginal decrease in oestradiol levels ($p = 0.059$) in participants from EG presenting oligomenorrhea and smoking habit Fig. 3.

When performing simple linear regression, we found the following statistically significant correlations: negative correlation between FSH levels and n-hexane levels (Fig. 4A, r= −0.443, p = 0.014)); and also a negative correlation of FSH levels and 2,5-HD urinary levels (Fig. 4B, r= −0.364, p = 0.044).

Fig. 4. **A.** Correlation between FSH serum concertation and n-hexane levels in exposed group. (r= -0.443, $p = 0.014$, n=32) **B.** Correlation between serum concertation FSH and 2,5-hexanodione urinary levels (post-shift sample) in exposed group (r= −0.364, $p = 0.044$, n=32).

Further, we carried out an analysis in order to examine a possible contribution of other solvents on level hormones, in participants from EG. From a generalized linear model with covariates, the we observed an association between 2,5-HD urinary levels vs. FSH serum levels, however this effect was only observed in non-smoker women reporting oligomenorrhea (n = 23, Table 6).

**Table 2**
Menstrual cycle length between groups.

| Group | menstrual cycle > 35 days | | Total |
|---|---|---|---|
| | Yes | No | |
| Non-exposed group | 0 | 32 | 32 |
| Exposed group | 27 | 7 | 34 |
| | | | 66 |
| | | $X^2 = 7.370$ | $p = 0.007$ |
| | **Value** | **CI** 95 % | ***P*** |
| Adjusted OR | 1.26 | 1.07 − 1.50 | 0.007 |

**Table 3**
Time for getting pregnant between groups.

| Group | Time to get pregnant > 6 months | | Total |
|---|---|---|---|
| | Yes | No | |
| Non-exposed group | 2 | 30 | 32 |
| Exposed group | 24 | 10 | 34 |
| | | | 66 |
| | | $X^2 = 5.945$ | $p = 0.015$ |
| | **Value** | **CI** 95 % | ***P*** |
| Adjusted OR | 6.25 | 1.24 − 31.27 | 0.015 |

## 4. Discussion

This cross-sectional study is, to the best of our knowledge, the first study in humans investigating a direct effect of n-hexane and its metabolite (2,5-HD) on female reproductive system, considering this solvent as a possible endocrine disruptor. These assumptions are based on results from two different correlation models where n-hexane exposure levels as well as 2,5 HD urinary levels showed significant differences on FSH levels (negative correlation, Fig. 4, Tables 5,6) and positive correlation with n-hexane and time for getting pregnant (Table 4).

Limited studies have investigated toxic effects derived from organic solvent exposure on the reproductive system and the few of them have some methodologic limitations, i.e., how the VOC's exposure levels were determined. Consistently, in those studies, exposure was reported as environmental exposure, and not as individual one. Also, those

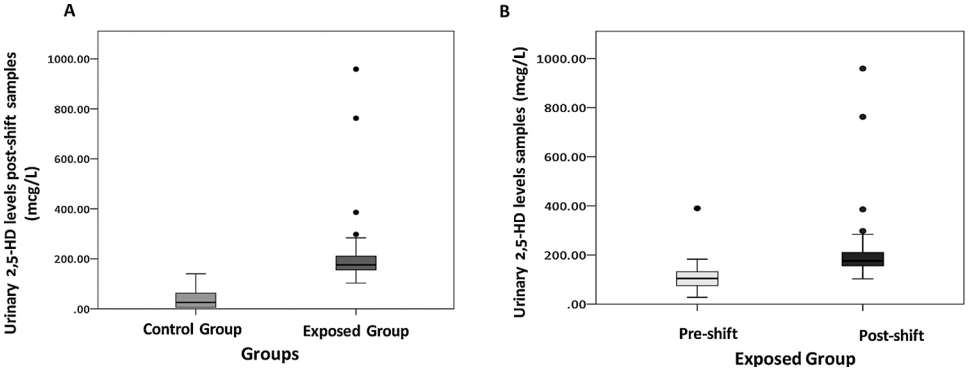


**Fig. 2.** Urinary 2,5-hexanedione levels as biomarker of n-hexane exposure.
**A.** 2,5-hexanedione levels between control group vs exposed groups in post-shif sample ($p < 0.001$)(n = 34) **B.** 2,5-hexanedione levels in exposed group in pre and post-shift samples ($p = < 0.001$)(n = 34) ●= atypical value found 3 times higher, above maximum value. U Mann–Whitney test.

**Table 4**
Association between "time for getting pregnant" and VOC´s levels in exposed group.

| Compound | β (IC 95 %) | *P* |
|---|---|---|
| Acetone | 0.04 (0.0002 – 0.0086) | 0.04 |
| N-hexane | 0.007 (0.002–0.011) | 0.02 |

n = 34.

**Table 5**
Hormone levels between groups.

| Hormone | Exposed Group Mean (rank) n = 34 | Control Group Mean (rank) n = 32 | *P* | *Reference value |
|---|---|---|---|---|
| AMH (ng/mL) (rank) | 4.1 (1.9 – 6.0) | 4.2 (1.6 – 5.8) | 0.76 | 1 – 4 |
| FSH (mUI/mL) | 6.4 (0.9 – 35.8) | 5.0 (2.1 – 8.4) | 0.18 | 4.7 – 21.5 |
| LH (mUI/mL) | 7.2 (1.2 – 31.3) | 3.6 (0.8 – 7.2) | 0.38 | 1.68 – 15 |
| Oestradiol (pg/mL) | 35.4 (16.4 – 87.1) | 34.7 (15 – 144) | 0.68 | 15 – 350 |

n = 64, *U Mann-Whitney* test, p < 0.05. *According with ESHRE guidelines.

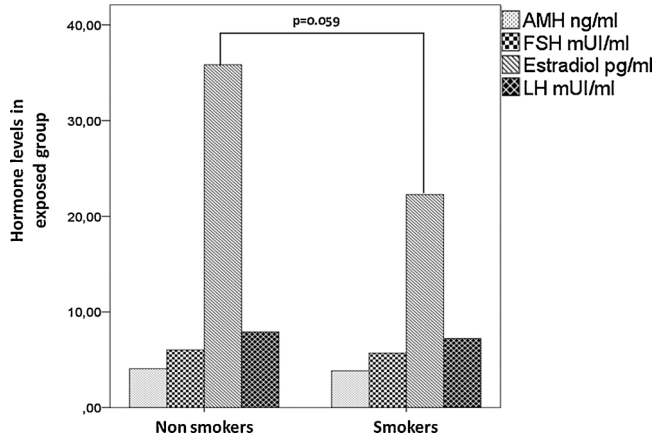


**Fig. 3.** Hormone levels in participants with oligomenorrhea and smoking habit in EG.n = 23; smokers n = 3, non-smokers = 20 *U Mann–Whitney* test, $p = 0.059$.

reports did not show the levels of urinary metabolites, neither the levels of biologic markers related to reproductive disfunction, such as serum hormones, like the report from Sallmén et al., where women in a shoe factory were studied. Population studied in that report were allegedly exposed to a mixture of eight solvents; among those solvents, it was

**Table 6**
Association between 2,5HD µg/L urinary levels (post-shift sample) and FSH serum levels in participants with oligomenorrhea and without smoking habit in exposed group (n = 23).

| **Variable** | | |
|---|---|---|
| $R^2$ | 0.2163 | |
| R adjusted | 0.1789 | |
| Prob > F | 0.0254 | |
| **Independent variable** | β (95 % IC) | *p* |
| 2,5HD µg/L | −0.00391 (−0.0073 to 0.0005) | 0.025 |
| Constant | 6.9645 (5.9029–8.0261) | 0.000 |

n = 23.

reported the presence of n-hexane, nonetheless they did not specify if they were environmental or individual exposure levels. In that same report, findings related with solvent exposure were longer menstrual cycles and longer time to get pregnant among shoe factory's workers compared with controls. Our results are in line with the previous report, finding the presence of oligomenorrhea and a longer time to get pregnant; notwithstanding, our results showed a positive correlation between "time for getting pregnant" with n-hexane and acetone. This could indicate that high levels of these solvents have to do with a longer time to get pregnant. It is worth to mention that, in the present study we were able to associate, for the first time, a specific solvent contribution for the delay in time to get pregnant, since we measured individual exposure levels (Table S1 and Table 4).

In another study about women occupationally exposed to a VOC mixture, which was performed in a pharmaceutical industry (Hassani et al., 2014), it was reported an environmental exposure to a mixture of VOC´s (including n-hexane but not the BTEX, namely benzene, toluene, ethylbenzene and xylenes). Authors in that report found environmental mean exposure levels for n-hexane of 72.92 mg/m$^3$; consistently, it was reported an increase in menstrual disorders associated with occupational exposure, including the following: longer menstrual cycle length, irregular cycles, and bleeding or spotting between periods which were more frequent in exposed group compared with controls. Although the study of women in the pharmaceutical industry reveals the presence of n-hexane associated with prolonged menstrual cycles, in the present study we reported lower levels of individual exposure (mean 49.7 mg/m$^3$, Fig. 1) compared to women in the pharmaceutical company, and our findings are similar: presence of oligomenorrhea in women exposed to a VOC´s mixture including n-hexane, as well as a longer time to get pregnant in the exposed group (Tables 2,3).

On the other hand, Chen et al. (2001), reported significant associations between menstrual disorders with exposure to a VOC's mixture: length of luteal phase was shorter in women with occupational exposure to toluene and benzene. Mean benzene levels of exposure in that

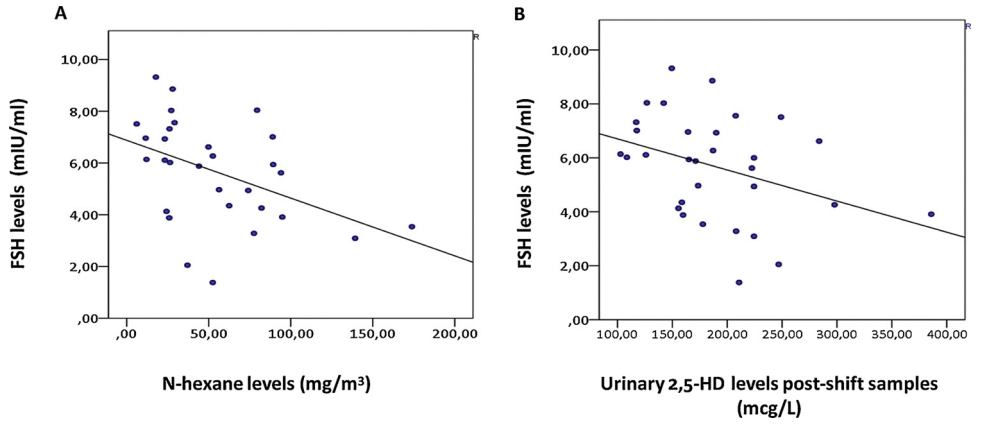


**Fig. 4.** Associations of n-hexane and 2,5-HD over FSH levels.

study were 8.88 mg/m$^3$, those levels were higher compared with our results (mean levels of 0.049 mg/m$^3$, Fig. 1). In another study in women occupationally exposed to low-levels of benzene, it was found an association with menstrual disorders increasing up to 53 % the probability of oligomenorrhea and a decrease in FSH serum secretion (Cho et al., 2001), according with our results: we report presence of oligomenorrhea in EG exposed to a VOC mixture, including n-hexane and low levels of benzene (Tables 3 and 4). Our results concur with the previous report regarding oligomenorrhea, however, we did not find statistical significance between benzene exposure levels and FSH levels.

In addition, cumulated time of exposure to aromatic compounds at low-levels has shown an increment in the risk of developing menstrual disorders (Burdorf et al., 2006); women in the present study had a cumulative average exposure time of 7.8 years (94 months), so we also think that menstrual disorders could be the effect of long-term exposure to VOC; however, we did not find a statistical significant relationship when performing linear regression with variable "cumulated time of exposure".

On the other hand, according to WHO and the ESHRE Capri workshop group (ESHRE, 1995) 80 % of women referring oligomenorrhea or amenorrhea have normal serum gonadotropin and normal or low levels of oestradiol, notwithstanding of this fact, they are considered as presenting an anovulatory disfunction state (Lie Fong et al., 2015). Such is the case of our study group: we did not find significant differences in ovarian and gonadotropins hormones serum levels in exposed group compared with controls, which can be classified by the mentioned organisms as anovulatory dysfunctions. Anovulatory disfunctions are the most common cause of infertility or subfertility, whose etiology is not well defined and may be due to an abnormality of intra-ovarian regulation, which we think is not the case, because we did not find differences in AMH serum levels between groups.

According with Table 5, all hormone levels in exposed group where between the range considered as "normal"; this means that correlations found in this work may be considered as not clinically significant, however, we consider that, since this is the first insight in elucidating a possible contribution of n-hexane (and its metabolite) in deleterious effects in the reproductive female system, supporting what was found in experimental animals, then these correlations deserve to be released.

Based on our results, we think that the possible cause of affection we observed is due to an interference exerted by n-hexane in the endocrine axis (hypothalamic- pituitary-ovary). Our results seem different from the results obtained by Abolaji et al., in rats exposed to 2,5-HD in drinking water, where they found increases in LH and FSH, as well as a reduction in oestrogens. However, we could say that our results are similar to those obtained by Liu et al., where they found no differences in gonadotropic serum levels in a model of inhalation exposure to n-hexane in mice. It is worth to mention that, in the report from Abolaji et al., exposure was to 2,5 HD directly, with exposure levels considered very high. If we put together the data obtained in Fig. 4, along with the data from Tables 2–4, and, considering that gonadotropic hormones LH and FSH are the primary endocrine factors that regulate apoptosis in ovarian follicles in granulosa cells (Tilly et al., 1995; Hattori et al., 2018; Regan et al., 2018) we propose that the FSH reduction exerted by 2,5-HD is causing an increased apoptosis in growing follicles at the ovaries from EG participants. We think that, results from the statistical analysis, reported in Table 6, supports this toxicological mechanism. In this case, 2,5-HD is involved with oligomenorrhea (Table 6), affecting FSH levels, that may cause a prolonged follicular phase, and then this could explain longer menstrual cycles (Table 2); since it has been proposed menstrual cycle length as a prognostic factor of subfertility in premenopausal and healthy women (Geloven et al., 2013). This latter fact supports our findings regarding an association between n-hexane levels and "time for getting pregnant" (Table 4), it means that, these are successive phenomena: a longer menstrual cycle could predict a longer time for getting pregnant in women exposed (classified as subfertility). Then, according to our results, it is possible that the exposure to n-hexane in women exerts an effect of interference on the endocrine axis. However, these preliminary results should be confirmed in a larger population.

Toxic, mechanistic pathways that could explain how VOC's affect the menstrual cycle are unclear, but there are two proposed pathways: first, it could exist an interference from VOC's in the endocrine axis (hypothalamus-pituitary-ovary); in this case, the VOCs could affect GnRH secretion from hypothalamus, which controls the secretion of gonadotropins (Maqbool et al., 2016). The second pathway that could explain the role of VOC´s in menstrual disorders could be an impairment in enterohepatic metabolism affecting the circulation of steroids, since the cytochrome p450 family is essential in the metabolic control of steroids and highly sensitive to environmental exposures (Abolaji et al., 2014). Previously, it has been reported a decreased activity in CYP2E1 in workers exposed to toluene (Jiménez-Garza et al., 2012); this is important since this enzyme (CYP2E1) is the mean responsible for the biotransformation of most of the VOC's, benzene and n-hexane included. Lastly, our results shown in Fig. 3, showing a trend for decreased oestradiol levels in exposed smokers, can be possibly attributed to a synergistic effect due to a concomitant exposure to one or several VOC's plus cigarette smoke, since smoking habit *per se*, has not shown a significant change in levels of oestradiol (Marom-Haham, Shulman, 2016); we think that, if we had had a larger number of exposed smokers, we would observe a significant difference. Quantification of individual levels (and not environmental ones) of n-hexane and other six compounds, as well as urinary determination of n-hexane's metabolite are definitely, important strengths of this study, since other reports did not show exposure levels of n-hexane or either 2,5-HD urinary levels. It is worth to mention that, mean levels for n-hexane are below occupational exposure limits in humans; besides, results of measured individual levels here reported corresponds to only one day of work, which means that, in another given day, this levels could be higher; we can not then rule out that in another day n-hexane levels could be higher than the permissible levels, which means that higher levels of n-hexane could have more devastating effects.

As judged by the results shown in Table 4, acetone and n-hexane have to do with a longer time for getting pregnant, however, acetone did not show any significant association with gonadotropin hormones (as n-hexane and 2,5-HD did), thus we cannot rule out acetone contribution with subfertility; but we cannot attribute to acetone an endocrine disrupting effect since we did not find any statistical significance between acetone levels with any of the hormones measured. In fact, a previous study showed that women exposed to high levels of acetone presented shorter menstrual cycles (Lin et al., 2013), a result totally opposite to all the previous studies in women exposed to VOC's (Hassani et al., 2014; Sallmén et al., 2006, 2008, Cho et al., 2001) including the results found here.

In addition to the facts mentioned above, our study group had more than 3 year of cumulative exposure, an estimated time enough to cause menstrual disturbances (Cho et al., 2001). Also, the present study used hormone levels as biomarkers of ovotoxicity in two pathways: paracrine (AMH) and endocrine (FSH, LH and oestradiol).

Some limitations of present study were the number of participants of study, and also, we missed to determine other biomarkers of possible endocrine disruption in women, such as measurement of prolactin, inhibin B and progesterone; and also we did not measured another solvent's metabolites other than 2,5-HD. We think that a longitudinal approach would have offered a better understanding of the claimed damage to women exposed on fertility and menstrual disturbances.

## 5. Conclusions

Results obtained here supports previous evidence where occupational exposure to VOCs, specially to n-hexane, has been shown as a cause of subfertility and menstrual disorders; also, given the associations observed for n-hexane individual exposure and FSH levels, as well

as the association between 2,5-HD and FSH levels in exposed women presenting oligomenorrhea, we propose that n-hexane is, possibly, one of the mean VOC´s that can act as an endocrine disruptor. More studies are needed for elucidating if other VOC metabolites different to 2,5-HD are involved in endocrine disrupting effects, since in this work, statistical analyses were performed only with the parent compounds of another VOC's.

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## Declaration of competing interest

None.

## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2020.04.022.

## References

Abolaji, A.O., Adedara, I.A., Soladogun, A., Salau, V., Oguaka, M., Farombi, E.O., 2014. Exposure to 2,5-hexanedione is accompanied by ovarian and uterine oxidative stress and disruption of endocrine balance in rats. Drug Chem. Toxicol. 545, 1–8. https://doi.org/10.3109/01480545.2014.974265.

Agency for toxic substances and disease registry, 1999. Public Health Service. Toxicological Profile for n-hexane. pp. 1–26. . Consulted: February 27th, 2019. https://www.atsdr.cdc.gov/toxprofiles/tp113.pdf.

Béranger, R., Hoffmann, P., Christin-Maitre, S., Bonneterre, V., 2012. Occupational exposures to chemicals as a possible etiology in premature ovarian failure: a critical analysis of the literature. Reprod. Toxicol. 33, 269–279. https://doi.org/10.1016/j.reprotox.2012.01.002.

Burdorf, A., Figà-Talamanca, I., Jensen, T.K., Thulstrup, 2006. Effects of occupational exposure on the reproductive system: core evidence and practical implications. Occup. Med. 56, 516–520. https://doi.org/10.1093/occmed/kql113.

Chen, H., Wang, X., Xu, L., 2001. Effects of exposure to low-level benzene and its analogue on reproductive hormone secretion in female workers. Chin. J. Prev. Med. 35, 83–86.

Cho, S.I., Damokosh, A.I., Ryan, L.M., Chen, D., Hu, Y.A., Smith, T.J., et al., 2001. Effects of exposure to organic solvents on menstrual cycle length. J. Occup. Environ. Med. 43, 567–575 PMID: 11411330.

Costa, E., Spritzer, P., Hohl, A., Bachega, T., 2014. Effects of endocrine disruptors in the development of the female reproductive tract. Arq. Bras. Endocrinol. Metab. 58, 153–161 doi:10.1590.0004-2730000003031.

Geloven, N., Van der Veen, F., Bossuyt, P.M.M., Hompes, P., Zwinderman, A., Mol, B., 2013. Can we distinguish between infertility and subfertility when predicting natural conception in couples with an unfulfilled child wish? Hum. Reprod. 28 (3), 658–665. https://doi.org/10.1093/humrep/des428.

Hassani, S., Namvar, M., Ghoreishvandi, M., Attarchi, M., Golabadi, M., Seyedmehdi, S.M., 2014. Menstrual disturbances and hormonal changes in women workers exposed to a mixture of organic solvents in a pharmaceutical company. Med. J. Islam. Repub. Iran 28, 1–10.

Hattori, K., Orisaka, M., Shin, F., Kimihisa, T., Yamazaki, Y., Mizutani, T., Yoshida, Y., 2018. Luteinizing hormone facilitates antral follicular maturation and survival via thecal paracrine signaling in Cattle. Endocrinology 159, 2337–2347.

Jiménez-Garza, O., Márquez-Gamiño, S., Albores, A., Caudillo-Cisneros, C., Carrieri, M., Bartoloucci, G., Manno, M., 2012. CYP2E1 phenotype in mexican workers ocupationally exposed to low levels of toluene. Toxicol. Lett. 210, 254–263. https://doi.org/10.1016/j.toxlet.2012.01.021.

Jiménez-Garza, O., Guo, L., Hyang-Mín, B., Carrieri, M., Bartoloucci, G., Barrón-Vivanco, B., Bacarelli, A., 2018. Aberrant promoter methylation in genes related to hematopoietic malignacy in workers exposed to VOC mixture. Toxicol. Appl. Pharmacol. 339, 65–72. https://doi.org/10.1016/j.taap.2017.12.002.

Kabir, E., Rahman, M., Rahman, I., 2015. A review on endocrine disruptors and their possible impacts on human health. Environ. Toxicol. Pharmacol. 40, 241–258. https://doi.org/10.1016/j.etap.2015.06.009.

Li, H., Zhang, C., Ni, F., Guo, S., Wang, W., Liu, J., 2015. Gestational N-hexane inhalation alters the expression of genes related to ovarian hormone production and DNA methylation states in adult female F1 rat offspring. Toxicol. Lett. 239, 141–151. https://doi.org/10.1016/j.toxlet.2015.09.018.

Lie Fong, S., Schipper, I., Valkenburg, O., de Jong, F.H., Visser, J.A., Laven, J.S., 2015. The role of anti-Müllerian hormone in the classification of anovulatory infertility. Eur. J. Obstet. Gynecol. Reprod. Biol. 186, 75–79. https://doi.org/10.1016/j.ejogrb.2015.01.00.

Lin, C., Huang, C., Hwang, J., Wang, J., Weng, S., Shie, R., Chen, P., 2013. Shortened menstrual cycles in LCD manufacturing workers. Occup. Med. 63, 45–52. https://doi.org/10.1093/occmed/kqs172.

Liu, J., Huang, H.L., Pang, F., Zhang, W.C., 2012. The effect of n-hexane on the gonad toxicity of female mice. Biomed. Environ. 25, 189–196.

Maqbool, F., Mostafalou, S., Bahadar, H., Abdollahi, M., 2016. Review of endocrine disorders associated with environmental toxicants and possible involved mechanisms. Life Sci. 145, 265–273. https://doi.org/10.1016/j.lfs.2015.10.022.

Marom-Haham, L., Shulman, A., 2016. Cigarette smoking and hormones. Curr. Opin. Gynecol. 28 (4), 230–235. https://doi.org/10.1097/GCO.0000000000000283.

Regan, S.L., Knight, P.G., Yovich, J.L., Stanger, J.D., Leung, Y., Arfuso, F., Almahbobi, G., Dharmarajan, A., 2018. The effect of ovarian reserve and receptor signalling on granulosa cell apoptosis during human follicle development. Mol. Cell. Endocrinol. 470, 219–227.

Sallmén, M., Baird, D.D., Hoppin, J.A., Blair, A., Sandler, D.P., 2006. Fertility and exposure to solvents among families in the Agricultural Health Study. Occup. Environ. Med. 63, 469–475.

Sallmén, M., Neto, M., Mayan, O.N., 2008. Reduced fertility among shoe manufacturing workers. Occup. Environ. Med. 65, 518–524.

Sun, Y., Lin, Y., Li, H., Liu, J., Sheng, X., Zhang, W., 2012. 2,5-Hexanedione induces human ovarian granulosa cell apoptosis through BCL-2, BAX, and CASPASE-3 signalling pathways. Arch. Toxicol. 86, 205–215.

The ESHRE Capri Workshop Group, 1995. Anovulatory infertility. Hum. Reprod. 10, 1549–1553.

Thurston, S.W., Ryan, L., Christiani, D.C., Snow, R., Carlson, J., You, L., 2000. Petrochemical exposure and menstrual disturbances. Am. J. Ind. Med. 38, 555–564.

Tilly, J.L., Tilly, K.I., Kenton, M.L., Johnson, A.L., 1995. Expression of members of the Bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased Bax and constitutive Bcl-2 and Bcl-x long messenger ribonucleic acid levels. Endocrinology 136, 232–241.

Zhang, W., Huang, L., Kong, C., Liu, J., Luo, L., Huang, H., 2013. Apoptosis of rat ovarian granulosa cells by 2,5-hexanedione in vitro and its relevant gene expression. J. Appl. Toxicol. 33, 661–669.