Phthalate Exposure in Assisted Reproduction Patients versus Healthy Couples: Comparison between Traditional Statistics and Chemometrics

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Abstract

Human exposure to phthalates is known to interfere with fertility. Five widely used phthalate metabolites were measured in the urine of 55 couples of patients of an Assisted Reproduction Center and 73 healthy couples (controls) by means of HPLC-MS/MS. Traditional statistical analysis revealed that 4 metabolites out of 5 have higher concentrations in the urine of patients than in the control's, and this is true also for males and females separately. PLS-DA (Partial Least Squares Discriminant Analysis) provided good results in term of classification of controls (more than 95%) but not for patients. Separating the group by gender, the classification improved both for patients and controls. In both cases a higher metabolites concentration in the patients is confirmed.

Introduction

Phthalates are a family of industrial compounds with a common chemical structure, dialkyl or alkyl/aryl esters of 1,2-benzenedicarboxylic acid: they are widely used in commercial products, mainly plastics, but also cosmetics and pharmaceuticals, and therefore they can be considered ubiquitous pollutants. They have been shown to have adverse effects on liver, kidney and the reproductive system because they can mimic sex hormones or antagonize their effects [1], and have been also associated with oxidative stress, cognitive disorders and obesity; for these reasons the use of phthalates is regulated in the UE, but only for what concerns the content of six phthalate diesters in toys and other children products, according to the reception of 79/769/EEC.

Materials & Methods

Phthalate metabolites are excreted in the urine conjugated with glucuronic acid, and therefore a hydrolysis is carried out by ß- glucuronidase enzyme before analysis to determine the total amount: the free fraction can be determined without performing this hydrolysis. The quantitative determination was carried out by HPLC-MS/MS API 4000 (AB Sciex) with isotopic dilution for the following metabolites: the monoethyl phthalate (MEP) for the diethyl phthalate (DEP), the mono (2-ethylhexyl) phthalate (MEHP) and mono (2-ethyl-5hydroxyhexyl) phthalate (MEHHP) for the di-ethyl-hexyl phthalate (DEHP), the mono butyl phthalate (MnBP) for the n-butyl phthalate (DNBP) and the mono benzyl phthalate (MBzP) for the butyl-benzyl phthalate (BBzP)(99% purity, CDN Isotopes, Quebec). A validated HPLC/MS/MS analytical method with isotopic dilution for the determination of these compounds in human urine described in a previous investigation [2] was used for the analysis of urine samples of 55 couples of patients of an Assisted Reproduction Center, both with and without enzymatic hydrolysis, and results were compared to those of 73 couples of the same mean age having one or more children (controls). Both classical statistical test (SPSS® 19.0, IBM® Corporation) and chemometrics (MATLAB release 2009b) have been used to analyze the results.

Results

Traditional statistical analysis: prior to performing any other statistical analysis, the normality of the distribution of the concentrations of the analytes was evaluated using the Shapiro–Wilk test. Results show a not normal distribution of the urinary concentration of the excreted metabolites: the Wilcoxon test reveals significant differences ($p<0.05$) in the total concentration of MEHP and MnBP between men and women, not verified for the excreted free fraction, revealing a gender difference in

the metabolic excretion pathway. Besides the total concentration of all the metabolites, except MBzP, is higher in the urines of the patients than in those of controls (Mann-Whitney $p < 0.05$).

Chemometrics: a PLS-DA classification model was built on the data set of the urinary total concentrations of the five metabolites in all subjects, after autoscaling, using cross-validation (10 cancellation groups) in order to select the optimal complexity of the latent variables model and to evaluate its predictive ability. Satisfactory results regards the classification of healthy individuals (more than 95%) were provided, whereas only 53% of patients were correctly classified. The model included three latent variables (LVs) and the projection of the samples onto these LVs is shown in Figure 1.

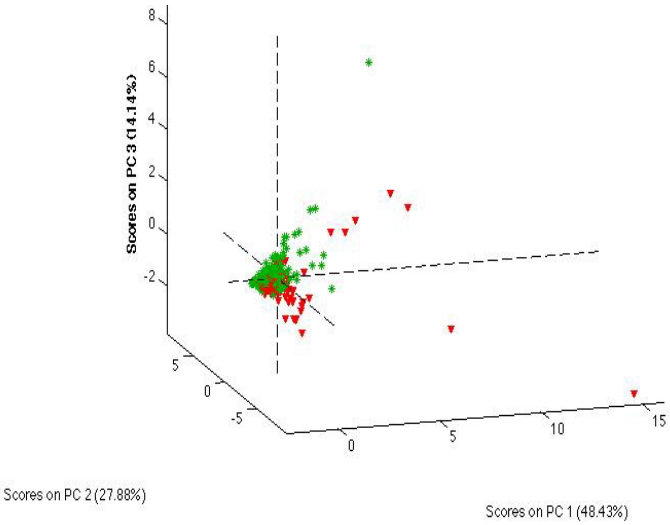

Figure 1: PLS-DA on the entire data set. Projection of samples onto the first three LVs of the PLS model. (*) Controls; (▼)Patients.

From these results, to verify whether patients' gender could be a source of additional variability that partially masked the differences between patients and controls, PLS-DA analysis was carried out on the two matrices resulting by considering men and women separately. PLS-DA model built on men data set, after autoscaling, included two LVs and was able to correctly classify in cross validation 65.3% and 85.2% of patients and controls respectively, while PLS-DA model on women data set, after autoscaling, included two LVs and resulted in 71% (patients) and 98.5% (healthy women) predictive ability in cross-validation (Figure 2). In both cases, a significant improvement in the discrimination between patients and controls was shown and, in accordance to traditional statistical analysis, an increase of urinary concentrations of four of the five monitored metabolites and a decrease of MBzP concentration in patients is confirmed.

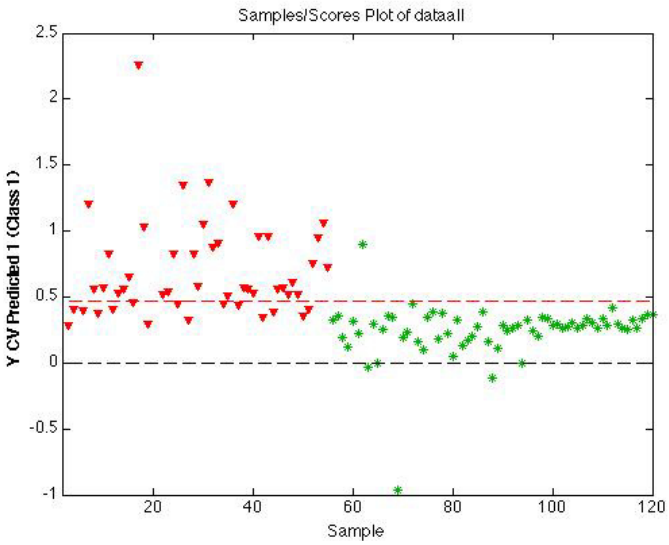

Figure 2: PLS-DA on women data set. Graphical representation of the cross-validated predictions. (*) Controls; (▼)Patients. Dashed red line indicates the classification threshold.

Conclusions

Both traditional statistical and chemometric analyses lead to the same conclusions, that there is a higher urinary total concentration of MEP, MEHP, MEHHP and MnBP in the couples with infertility than in the controls, but not of MBzP. A gender difference in the conjugation with glucuronic acid of the monoesters has been also shown, that can be interpreted as an slower excretion/increased susceptibility of men to MEHP and MnBP.

These results enhance the importance of controlling and reducing the environmental and occupational exposure of individuals in the fertile age to diethyl phthalate, di-ethyl-hexyl phthalate and n-butyl phthalate.

References

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