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Phthalate Exposure and Human Semen Quality in Shanghai: A Cross-sectional Study[1]

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Objective To monitor the level of phthalates in human semen samples and to analyze the relationship between phthalate levels and semen parameters. **Methods** Concentrations of three kinds of commonly used phthalates (di-ethyl phthalate, DEP; di-n-butyl phthalate, DBP; di-2-ethylhexyl phthalate, DEHP) were measured using reversed-phase HPLC. Semen parameters were measured by computer aided sperm analysis (CASA). **Results** The three phthalates were detected in most of the biological samples, with median levels of 0.30 mg/L (0.08-1.32 mg/L) in semen specimens. There was a significant positive association between liquefied time of semen and phthalate concentrations of semen. The correlation coefficient was 0.456 for DEP, 0.475 for DBP, and 0.457 for DEHP, respectively. There was no significant difference between phthalate concentrations of semen and sperm density or livability, though the correlation coefficients were negative. **Conclusion** These results suggest that people who reside in Shanghai are exposed to phthalates, especially to DBP and DEHP. Although the level of phthalates is relatively mild, an association of phthalate levels and reduced quality of human semen has been shown in the present study.

Key words: Environmental endocrine disruptor; Phthalates; Reversed-phase HPLC; Biological samples

INTRODUCTION

Phthalate is a class of chemical compounds widely used in industrial activities. In the whole world, about twenty kinds of phthalates are used as plasticizers, such as dimethyl phthalate (DMP), diethyl phthalate (DEP), di-iso-propyl phthalate (DiPP), dipropyl phthalate (DPP), di-iso-butyl phthalate (DiBP), di-n-butyl phthalate (DBP), n-butyl benzyl phthalate (BBP), di-2-ethylhexyl phthalate (DEHP), di-n-octyl phthalate (DOP), *etc*. They are mainly used as plasticizers for polyvinyl chloride (PVC) resins, adhesives, and cellulose film coating (about 85% of the whole production), and less applied in cosmetics, insect repellents, insecticide carriers and propellants. The amount of phthalates used as plasticizers accounts for about 40%-50% in plastics industry. In China, the total amount of phthalate production was 263 000 tons in 1998, of which about 45% was DEHP.

Phthalate can readily be released into the environment through volatilization and leaching from plastics and during the process of production of plastics and plastic materials or after their disposal. Organisms (including plants, microorganisms, aquatic, amphibian, and terrestrial animals) can bio-accumulate phthalate compounds from lower level organisms to higher level ones, and consequently the adverse effects could be biomagnified higher by hundreds of times[1-3].

In recent years, phthalate has been considered as an environmental endocrine disruptor, which could affect reproduction and development of organisms by disturbing hormone synthesis. Animal data suggest that a broad spectrum of health outcomes are associated with phthalate exposure including developmental toxicity (cleft palate, decreased pup weight, testicular damage), endocrine disruption (testicular toxicity, decreased sperm motility, decrease fertility, decreased milk synthesis), and carcinogenicity[1,4-6].

For these reasons, the United States Environmental Protection Agency (USEPA) and several other agencies or countries have classified the most commonly used phthalates as priority pollutants. In 2000, the National Program's Center for the Evaluation of the Risks to Human Reproduction evaluated seven phthalates (including DEHP, DBP,

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DEP, BBP, DINP, DIDP and DIBP) for their potential ability to cause reproductive toxicity to humans. However, up to the present, no causal relationship between phthalate exposure and human adverse effects has been established. One of the main reasons is that there is no sufficient information on human phthalates exposure or subsequent adverse effects. More data and research are needed in this area.

In this study, the concentrations of three kinds of commonly used phthalates in human biological samples were measured by reverse-phase high-performance liquid chromatography (HPLC). All the human semen samples were collected from people living in Shanghai. These data provide some background information on phthalate exposure, which may be helpful for further assessing the effects of phthalates on human health.

MATERIALS AND METHODS

Chemicals

DEHP, DBP, and DEP were purchased from Shanghai Reagent Co. of China National Pharmaceutical Group Corporation and used as analytical standards without further purification. HPLC-grade methanol and acetonitrile were purchased from Tianjin Siyou Biomedical Technique Ltd. and from Shanghai Wujing Chemical Engineering Factory, respectively. Skellysolve B (analytical grade) was obtained from China Medical Corporation. The pure water used in the whole study was redistilled. All the other reagents used were of analytical grade.

Study Subjects

Participants residing in Shanghai were surveyed in 2002. Semen species of 52 men (aged from 23 to 48 years) were collected from outpatients of Shanghai Institute of Planned Parenthood Research. All collected samples were stored at -20℃ and in the dark until use.

Pretreatment

Defrosted semen samples were centrifuged (2000 g for 5 min) and 2 mL of the upper aliquot layer was added to skellysolve B (5 mL) and acetonitrile (1 mL) in a glass tube. The mixture was vortexed (5 min) and then centrifuged (2000 g for 5 min). The upper organic layer was transferred into a clean glass tube. The aqueous phase was extracted again with 5 mL skellysolve B as mentioned above. The organic phases were combined and evaporated to dryness by air pump. After evaporation, the residue was dissolved in 0.2 mL of skellysolve B saturated with ethanol. Twenty μL of the mixture was injected into the HPLC for UV detection.

Chromatographic Analysis

A HP-1100 HPLC (Hewlett-Packard, Japan) equipped with a diode array UV detector operating at 228 nm was used in this study. Separation was achieved by using a 5 μm Inertsil®ODS-3 column (250mm × 4.6mm) (GL Sciences Inc., Japan). The column was operated at 35℃. A linear gradient mobile phase ranging from 90% methanol and 10% water to 100% methanol over 4 minutes was used and maintained at 100% methanol for 12 minutes, and then back to 90% methanol and 10% water over 4 minutes. The running time was 20 minutes at a flow-rate of 0.8 mL/min.

The retention time of phthalates was defined through the use of appropriate phthalate standards. The external standard method was used in the qualitative and quantitative measurement of samples. The peak area was calculated for each sample and the amount of each kind of phthalate was determined using the calibration curves obtained during the validation of the respective methods (Standard curve was prepared for each of the following phthalates: DEP, DBP, and DEHP. The retention time and peak area were determined for each sample, and the quantification was performed by comparison to the standard curves).

Precautions

Because phthalates are ubiquitous environmental contaminants, all glass wares used in the study were washed carefully and then rinsed with skellysolve B. The containers were rinsed with redistilled water twice before being used. No plastic products were used in this study.

Measurement of Semen Parameters

After liquefied at 37℃ and the pH determined with test paper, 10 μL fresh semen sample was diluted with 190 μL dilution buffer (Shanghai Baiyulan Biotech Co.). Ten μL of the dilution was dripped on blood cell counting chamber and parameters of semen specimens were detected by computer aided sperm analysis (CASA).

Statistical Method

Spearman correlation analysis was employed in statistical analysis of the data.

RESULTS

Standard Curves and Detection Limits of Chemical Standards

We detected three commonly used phthalates (DEP, DBP and DEHP) in biological samples using reverse-phase HPLC. The external standard method was used to precisely determine the phthalate levels in biological samples. The linear equation of concentration-peak area standard curves from different concentrations of each phthalate standard (0.2, 0.4, 4, 8, and 12 μg/L) was y= -6.27+51.68x (r=0.9973) for DEP, y=39.80+36.15x (r=0.9920) for DBP and y=8.23+28.70x (r=0.9993) for DEPH. The detection limit of ODS-3 column to DEP, DBP, and DEHP was 0.3 ng each.

Calibration Curves

Extra-labeled method was used in this study. Each sample added with different concentrations of chemical standards was pretreated as mentioned above. Twenty μL of pretreated samples was injected into the chromatograph and detected by an UV detector. The calibration curves were obtained for peak-area difference versus chemical standard concentration. The linear equation of calibration curves was y = -31.37 + 24.99x (r = 0.9816) for DEP, y = -14.60 + 45.98x (r = 0.9962) for DBP and y = -13.30+28.50x (r = 0.9921) for DEHP in human semen samples.

Measurements of Phthalate Levels and Semen Parameters

The concentrations of three commonly used phthalates in human semen samples and conventional indexes of semen samples are presented in Table 1. The data showed that phthalates could be detected in virtuely all the biological samples, with median levels of 0.30 mg/L (0.08-1.32 mg/L) in semen specimens. However, there were no significant differences in concentrations of the three phthalates in semen samples.

Spearman Correlation Analysis

Nonparametric procedure was employed in statistical analysis of the data because of the sample size, variability of the data, and uncertainty about the underlying distribution. In this study, Spearman correlation was used to explore the relationship between phthalate concentrations and semen parameters. The statistical results are summarized in Table 2.

TABLE 1

Average Phthalate Levels and Parameters of Men's Semen (*n*=37)

	DEP (mg/L)	DBP (mg/L)	DEHP (mg/L)	Age	Spermatozoa Density ($\times 10^6$/mL)	Volume (mL)	Liquefied Time (min)	pH	Rate of Malformation (%)	Vitality (%)
Range	0.13-1.32	0.09-0.57	0.08-0.98	23-48	0-130	0.8-4.8	20-35	7.0-7.5	16-52	30-84
Mean	0.47	0.16	0.28	31.6	45.53	2.85	29	7.2	26	64

Note. ND: not detected. Dw: dry weight. Ww: wet weight.

TABLE 2

Correlation Coefficient for Phthalate Concentrations and Human Semen Parameters (*n*=52)

		Age	Spermatozoa Density	Volume	Liquefied Time	pH	Rate of Malformation	Livability
DEP	Correlation Coefficient	0.034	–0.247	–0.102	0.456[a]	0.162	0.185	–0.130
	Sig (2-tailed)	0.861	0.146	0.552	0.005	0.344	0.279	0.451
DBP	Correlation Coefficient	0.090	–0.259	–0.388[b]	0.475[a]	0.308	0.289	–0.247
	Sig (2-tailed)	0.642	0.128	0.020	0.003	0.068	0.087	0.147
DEHP	Correlation Coefficient	0.119	–0.231	–0.372[b]	0.457[a]	0.225	0.363[b]	–0.312
	Sig (2-tailed)	0.539	0.176	0.025	0.005	0.187	0.029	0.064

Note. [a]Correlation is significant at the 0.01 level (2-tailed). [b]Correlation is significant at the 0.05 level (2-tailed).

The data showed that there was a significant positive association between liquefied time of semen and phthalate concentrations of semen. The correlation coefficient was 0.456 for DEP, 0.475 for DBP, and 0.457 for DEHP, respectively. No significant difference was found between phthalate concentrations of semen and sperm density or livability, though the correlation coefficients were

negative. Negative correlation coefficients between semen phthalate levels and sperm vitality grades suggested that phthalates could significantly affect sperm movement ability.

DISCUSSION

Since Carlsen and co-workers reported that the quality of male sperm declined to 40%-50% in the past fifty years, much attention has been paid to potential biological effects related to environmental endocrine disruptors (EEDs) such as hypospadias, testicular maldescent, *etc*.[7] Phthalate, an important EED, is widely used as plasticizer in the production of plastics because of its advantages of low cost, more variety and high production volume. During the process of plastic softening, phthalates could not really polymerize with macromolecular carbon-chain of plastics. As time goes by, phthalates can gradually be released from plastics, thus entering into the environment and affecting organisms.

There have been many studies on phthalate exposure. From available exposure data, phthalates have been found in many kinds of environmental specimens, including air, ground water, soil, sediment, seafood, *etc*. The concentrations of phthalates in these samples range from 10^{-9} to 10^{-1} mg/L (mg/kg) [1-3,8-10]. However, there are few data on phthalate concentrations in human biological samples, which could reflect the actual levels of human inner exposure to phthalates. In this study, phthalate levels in biological specimens (semen) collected from people residing in Shanghai were measured. The results showed that phthalates could be detected in nearly all the biological samples, with median levels of 0.30 mg/L (0.08-1.32 mg/L) in semen specimens, which coincide with the few data published in other literatures.

As a kind of environmental endocrine disruptors, the reproductive toxicity of phthalates showed in animal experiments is considered as disrupting effects on animal's endocrine system[1,12-15]. The concentration of phthalates in human semen could directly reflect the exposure status of target organ because the male reproductive system is the main target organ of phthalates. But there are few reports on the association between phthalate levels in semen and semen quality. In a report of ATSDR, the investigators could not find a quantitative association between DBP concentrations in semen and spermatozoa density[1]. In this study, there was a positive association between liquefied time of semen and phthalate concentrations of semen and a negative correlation between semen volume and concentrations of DBP or DEHP. There was also a positive association between the rate of sperm malformation and DEHP concentrations, but no significant differences were found between phthalate concentrations of semen and age, sperm density, pH value or sperm livability. After the sperm vitality was graded into A to D (increasing vitality order), there was a significant positive correlation between grade B sperm vitality and DBP concentrations and a negative correlation between grade C sperm vitality and concentrations of DBP or DEHP.

To evaluate the sperm quality, parameters of spermatozoa and plasma of sperm should be measured. Sperm plasma is excreted by accessory sex glands, such as epididymis, seminal vesicle, prostate gland, bulbo-urethral gland, *etc*. The main components of sperm plasma include fructose, creatoxin, citromalic acid, acid phosphatase of prostate, *etc*, and its main function is to provide substances that are necessary for sperm movement and maturation. Prolonging liquefied time of semen could change sperm plasma components and affect accessory sex gland function, which coincides with the results in other reports that exposure to phthalates could damage the experimental animal accessory sex gland. Semen originates not only from accessory sex gland but also from seminiferous tubule fluid. In previous studies, method of ligating efferent is used to evaluate the excrete function of sertoli cells[16]. The phenomenon that sertoli cell excretion is completely inhibited after exposing adult rats to phthalates for 1 h suggests that the rapid inhibition of seminiferous tubule fluid after exposure is a significant characteristic of testis damage induced by phthalates, and can also explain the declined sperm volume found in this study. Phthalates could affect spermatocytogenesis and induce testis atrophy in animal experiments, which could explain some results of this study, such as increased malformed spermatozoa, declined sperm livability and reduced sperm volume.

In this study, phthalates were commonly detected in human biological samples and could affect the quality of human semen. Thus, the potential hazard of phthalates affecting human beings and their offspring can not be ignored. More studies on exposure determination and reproductive toxicity of phthalates are needed in the future.

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