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ORIGINAL ARTICLE

Urinary phthalate metabolites and semen quality: a review of a potential biomarker of susceptibility

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Summary

Phthalates are a class of chemicals with widespread general population exposure. Some phthalates are reproductive and developmental toxicants in laboratory animals. Advances in the field of phthalate research in humans are dependent on the development and implementation of biomarkers to assess exposure and outcome, as well as potential markers that may be indicative of increased susceptibility. Recently, we incorporated a novel biomarker of potential 'susceptibility' into our study on the relationship of phthalates with semen quality and sperm DNA damage among men recruited from an infertility clinic. We measured urinary concentrations of three di(2-ethylhexyl) phthalate (DEHP) metabolites, mono(2-ethylhexyl) phthalate (MEHP) and two oxidative metabolites, mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP). We calculated the percent of DEHP excreted as the hydrolytic monoester (i.e., MEHP). We referred to this as %MEHP and considered it a phenotypic marker of the proportion of DEHP excreted in the urine as MEHP. In our sperm DNA study, we found novel results for the DEHP metabolites. Although MEHP was positively correlated with the oxidative metabolites, the association of sperm DNA damage with MEHP, as compared to MEHHP and MEOHP, were in opposite directions. We hypothesized that MEHP is the bioactive toxicant and further metabolism to MEHHP/MEOHP may lower internal burden of MEHP and thus be protective from sperm DNA damage. An alternative explanation may include that the relative percentage of DEHP excreted as MEHP was a surrogate for the function of phase I enzymes. Men with high %MEHP may have higher levels of sperm DNA damage because of poor metabolism (detoxification) of other genotoxic chemicals. Our hypothesis that %MEHP may represent a phenotypic marker of metabolism is novel but requires further exploration to confirm.

Introduction

Phthalates are a class of chemicals with widespread general population exposure. Some phthalates are reproductive and developmental toxicants in laboratory animals. High molecular weight phthalates [e.g., di(2-ethylhexyl) phthalate (DEHP)] are primarily used as plasticizers in the manufacture of flexible vinyl plastic which, in turn, is used in consumer products, flooring and wall coverings, food contact applications, and medical devices (David *et al.*, 2001; ATSDR, 2002). Manufacturers use low molecular weight phthalates (e.g., diethyl phthalate and dibutyl phthalate) in personal-care products (e.g., perfumes, lotions, cosmetics), as solvents and plasticizers for cellulose acetate, and in making lacquers, varnishes, and coatings, including those used to provide timed releases in some pharmaceuticals (ATSDR, 1995, 2001; David *et al.*, 2001).

As a result of the ubiquitous use of phthalates in personal care and consumer products, human exposure is widespread. Exposure through ingestion, inhalation and dermal contact are considered important routes of exposure for the general population (Adibi *et al.*, 2003; ATSDR, 1995, 2001, 2002; Rudel *et al.*, 2003). Parenteral

exposure from medical devices and products containing phthalates are important sources of high exposure to phthalates, primarily DEHP (ATSDR, 2002; Green *et al.*, 2005). Upon exposure, phthalates are rapidly metabolized and excreted in urine and faeces (ATSDR, 1995, 2001, 2002). The most common biomonitoring approach for investigating human exposure to phthalates is the measurement of urinary concentrations of phthalate metabolites.

Advances in the field of phthalate research in humans are dependent on the development and implementation of biomarkers to assess exposure and outcome, as well as potential markers that may be indicative of increased susceptibility. Biomarkers of exposure and outcome are relatively well developed and have been incorporated into a variety of health related studies. Recently, we incorporated a novel marker of potential 'susceptibility' into our study on the relationship between urinary levels of phthalate metabolites with sperm quantity and quality and sperm DNA damage. Study subjects consisted of male partners of subfertile couples that presented to an infertility clinic in MA, USA. The susceptibility marker that we incorporated was specific for DEHP, one of the biologically active phthalates in experimental studies on laboratory animals. In this mini-review, we describe the results from our studies using a novel biomarker of susceptibility and offer insights on its utility in epidemiologic investigations.

Semen quality studies: application of a biomarker of susceptibility

In our semen quality studies, we measured urinary concentrations of three DEHP metabolites, mono(2-ethylhexyl) phthalate (MEHP) and two oxidative metabolites, mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) (Hauser *et al.*, 2006, 2007). The percent of DEHP excreted as the hydrolytic monoester (i.e., MEHP) was calculated and referred to as %MEHP. It was considered a phenotypic marker of the proportion of DEHP excreted in the urine as MEHP. The greater the %MEHP, the larger the percentage of DEHP excreted as MEHP relative to the excretion of the two oxidative metabolites.

To calculate %MEHP, MEHP, MEHHP and MEOHP were converted to nanomoles per millilitre. MEHP (nmol/mL) was divided by the sum of MEOHP (nmol/mL), MEHHP (nmol/mL) and MEHP (nmol/mL), and multiplied by 100. Urine samples below the limit of detection (LOD) for MEHP, MEHHP or MEOHP were assigned a value of ½LOD. To our knowledge, the use of %MEHP as a phenotypic marker of DEHP metabolism and excretion was novel and had not been used in human health studies.

We did not find evidence of dose-response relationships of MEHP, MEHHP and MEOHP with the three measured semen parameters (sperm concentration, motility and morphology) when they were modelled individually (Hauser *et al.*, 2006). For reduced sperm motility, the odds ratio for each %MEHP quartile compared with the reference (adjusted for age, abstinence, and smoking) was 1.0, 1.3, 1.6, 1.5; p for trend 0.27. This may indicate that individuals who excrete DEHP primarily as MEHP (i.e., have a lower ability to metabolize MEHP to the oxidative metabolites) may be at increased risk for low sperm motility as compared with individuals who primarily excrete DEHP as oxidative metabolites (i.e., MEHHP and MEOHP). We recognize that there are additional DEHP metabolites that were not measure (Koch *et al.*, 2005; Silva *et al.*, 2006a,b), thus %MEHP was not comprehensive, although MEHP, MEHHP and MEOHP account for about 50% of the DEHP dose (Koch *et al.*, 2004, 2005). Currently, additional DEHP metabolites are being measured, including mono-(2-ethyl 5-carboxy pentyl) phthalate, and will be incorporated into future calculations of %MEHP.

We also recently published data on the relationship between urinary phthalates and sperm DNA damage, measured with the neutral comet assay (Hauser *et al.*, 2007). The neutral comet assay has been previously described (Singh & Stephens, 1998; Duty *et al.*, 2003). Please see Box 1 for details.

The DEHP metabolites had interesting and unexpected associations with sperm DNA damage. MEHP was positively associated with Tail% (3.06%, 95% CI: 1.33, 4.79; p-value = 0.0006). Because MEHHP and MEOHP were strongly correlated ($r = 0.98$), they were not included in the same statistical model. Furthermore, because of their strong correlation their relationships with comet assay parameters were nearly identical. For instance, after adjusting for age and smoking status, for an interquartile range (IQR) increase in specific-gravity-adjusted MEHHP and MEOHP concentrations, comet extent (CE) decreased 8.5 and 8.6 μm, respectively, and tail distributed moment (TDM) decreased 4.5 and 4.4 μm, respectively. For MEHHP these results represented a 6.6 and 8.0% decrease in the study population median CE and TDM, respectively. Despite a positive correlation of the oxidative metabolites with MEHP (for MEHHP the r was 0.76, p-value < 0.0001; and for MEOHP the r was 0.73, p-value < 0.0001), the associations of sperm DNA damage with MEHP as compared to MEHHP and MEOHP were in opposite directions.

We hypothesized that MEHP is the bioactive toxicant and further metabolism to MEHHP and MEOHP may lower internal burden of MEHP and thus be protective from sperm DNA damage. To test this hypothesis, we

included MEHP and MEHHP or MEOHP in the same regression model. Although MEHP was moderately correlated with MEHHP and MEOHP, we did not detect collinearity when both were included in the same model. When MEHHP or MEOHP were included in the model, MEHP was more strongly associated with sperm DNA damage than when modelled alone. For an IQR increase in MEHP, this represented increases in the study population median (95% CI) of 17.3% (8.7–25.7%) for CE; 14.3% (6.8–21.7%) for TDM, and 17.5% (3.5–31.5%) for tail%. MEHHP and MEOHP were more strongly 'protective' of sperm DNA damage; the study population comet assay parameter medians (95% CI) decreased 19.9% (−11.6 to −2.8%) for CE and 19% (−26.3 to −11.8%) for TDM. In other words, for a given urinary concentration of MEHP, the higher the concentration of MEHHP or MEOHP, the lower the level of sperm DNA damage. Alternatively, for a given urinary concentration of MEHHP or MEOHP, the higher the urinary level of MEHP, the higher the level of sperm DNA damage.

We considered these results as evidence that oxidative metabolism of DEHP may impart differences in risk for sperm DNA damage. We also noted that %MEHP (percentage of DEHP excreted as MEHP relative to total measured metabolites) varied across individuals (from less than 1 to 60%) and was strongly associated with increased sperm DNA damage. An IQR increase in %MEHP was associated with an 11.7% increase relative to the study population median CE (95% CI: 6.2–17.2%), a 9.8% increase relative to the study population median TDM (95% CI: 4.9–14.7%), and an 11.4% increase relative to the study population median Tail% (95% CI: 2.4–20.5%).

Interpretation of %MEHP results

Our hypothesis that %MEHP was a phenotypic marker of DEHP metabolism to 'less toxic' metabolites is consistent with our understanding of the metabolism of DEHP (Fig. 1) which hydrolyzes first to MEHP and subsequently metabolizes to MEHHP and MEOHP, among other oxidative metabolites (Koch *et al.*, 2005; Silva *et al.*, 2006a,b). These DEHP oxidative metabolites are more easily excreted in urine than MEHP. Therefore, oxidation of MEHP could effectively decrease internal body burden of MEHP, which in turn may have a protective effect if MEHP is the bioactive metabolite. Inter-individual variability in the percentage of MEHP and of oxidative metabolites that are excreted in the urine has been observed (Becker *et al.*, 2004; CDC 2005; Silva *et al.*, 2006a). Therefore, as the proportion of urinary excretion of DEHP as MEHP varies across individuals, urinary concentrations of MEHP alone do not represent total body burden of DEHP exposure. The inclusion of oxidative metabolites, as shown by our results may explain the lack of a relationship between MEHP and DNA damage in our earlier study for which measurements of MEHHP and MEOHP were not available (Duty *et al.*, 2003).

An alternative explanation may include that the relative percentage of DEHP excreted as MEHP was a surrogate for the function of cytochrome P450 phase I enzymes. If other genotoxicants requiring phase I enzymes for detoxification were associated with sperm DNA damage, men with high %MEHP (which represents low functionality of phase I enzymes) may also be poor metabolizers of other genotoxic chemicals. Thus men with high %MEHP have higher levels of sperm DNA damage because of poor metabolism (detoxification) of other genotoxic chemicals.

Currently, our hypothesis that %MEHP may represent a phenotypic marker of metabolism is novel but requires further exploration in similar and dissimilar study settings. We are not aware of published studies using %MEHP and would hope that this mini-review will lead to its application in epidemiologic studies on phthalates and health endpoints. Health endpoints of interest range from semen quality to pregnancy outcomes.

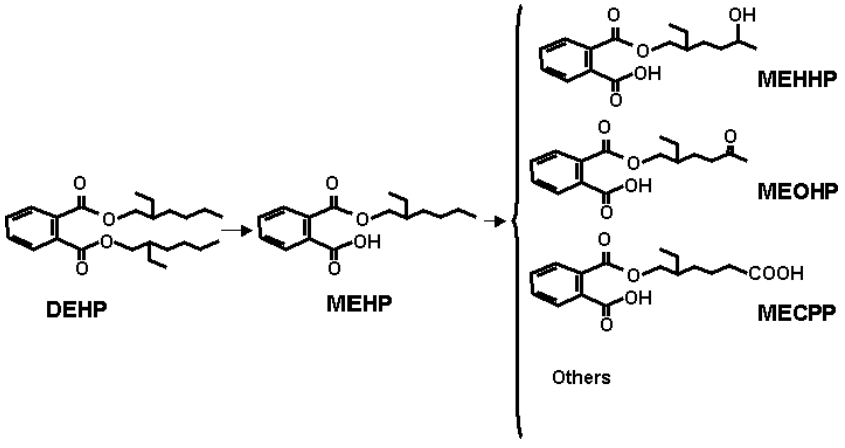

Figure 1 Schematic metabolic diagram of DEHP. Abbreviations: Di(2-ethylhexyl) phthalate (DEHP); mono(2-ethylhexyl) phthalate (MEHP); mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP); mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), two oxidative metabolites; mono-(2-ethyl 5-carboxy pentyl) phthalate (MECPP).

Future directions of phthalate research

There remain many unanswered questions in the area of research on health effects of phthalates. Further study is necessary because of the consistent toxicological data on adverse effects as well as widespread general population exposure. Suggested research needs include studies that target populations with high exposure to phthalates. This includes adult men with occupational exposure, pregnant women exposed through the use of medications containing phthalates, as well as individuals exposed through the use of a variety of phthalate containing products. Research on early life exposure is also needed, ranging from pre-natal to neonatal to pubertal exposure windows. These life stages likely represent critical windows of vulnerability to exposure to phthalates.

We also need to identify susceptibility factors that may increase risk of adverse effects following exposure to phthalates. Genetic factors may modify the exposure-dose relationship by altering the metabolism or excretion of phthalates. Additionally, genetic factors may modify dose-response relationships by altering the biological response to a given internal dose. Examples of these factors are limited but may include differences in hormone receptors or cellular responses to a given dose. More research in this area of susceptibility is critical to our understanding of human health risks.

We need to develop methods to better study mixtures of chemicals, such as exposure to multiple phthalates at different levels and how they may act additively or synergistically, or even antagonistically. Statistical methods need to incorporate the biological activity of the different phthalate metabolites, both the monoesters and oxidative metabolites. To inform the statistical models, we need to collect data on how mixtures behave in biological systems. Finally, we need to also develop methods to determine interactions between phthalates and other environmental chemicals that may have similar modes of action and thus further interact with phthalates.

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Box 1

Principle of the comet assay:

Comet assay

The comet assay is a simple electrophoresis technique for analysing and quantifying DNA damage in individual mammalian cells. It is based on the principle that during electrophoresis, damaged or broken DNA migrates away from the cell nucleus. The images obtained and analysed look like a 'comet' with a distinct head, comprised of intact DNA and a tail, consisted of damaged or broken pieces of DNA. The extent of DNA liberated from the head of the comet is proportional to the degree of DNA damage.

Comet extent (CE): a measure of the total comet length from the beginning of the head to the last visible pixel in the tail.

Tail%: a measure of the proportion of total DNA that is present in the tail.

Tail distributed moment (TDM): an integrated value that takes into account both the distance and intensity of comet fragments:

$$\mathrm{TDM} = \Sigma(\mathrm{I} * \mathrm{X})/\Sigma\mathrm{I}$$

where ΣI is the sum of all intensity values that belong to the head, body, or tail, and X is the x-position of the intensity value.

It appears from the definition of these three parameters, that the higher the parameter value, the more DNA damage.

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Panel discussion

R.J. Aitken

The Comet assay for DNA damage is dependent on chromatin dispersal. Any xenobiotics that cause cross linking of chromatin, whether this is protein/protein, DNA/DNA, or DNA/protein cross linking, will appear to be protecting the chromatin from DNA damage, when this is not really the case. Under these circumstances there is just a different type of DNA damage occurring which involves cross links that are not detected in the Comet assay. Have you looked at DNA adducts rather than the Comet assay as a monitor DNA damage?

R. Hauser

I agree that the Comet assay has its limitations and is a non;specific marker of DNA damage. Currently, we have not measured DNA adducts.

T.K. Jensen

The difference between the infertile US males and the Swedish conscripts may be due to increased susceptibility to chemicals in the US group. Have you any information on the difference in exposure levels to phthalates between the 2 groups?

R. Hauser

I agree that one potential explanation is that the subfertile US males may be more susceptible to phthalates. The differences in exposure levels between the US and Swedish study were relatively minor. For instance, the Swedish conscripts had higher levels of monobutylphthalate (MBP) in their urine but this is not likely to account for the differences in the association of MBP with semen quality across studies.

N.E. Skakkebæk

It is stated that the Comet assay measures DNA damage, but what exactly is it telling us? We know that there is something wrong with the DNA, but abnormalities are seen more commonly when there is a higher percentage of abnormal sperm in which circumstance everything is abnormal. What is the significance of an abnormal result? Are we detecting mutations and inheritable defects? The expression ''DNA damage'' is not specific.

R. Hauser

The Comet assay does not measure mutations or adducts, but detects strand breaks in chromatin which are unlikely

to be inheritable defects. It is known that abnormalities in the Comet assay or sperm chromatin structure assay are associated with reduced male fertility, and lower pregnancy rates following intracytoplasmic sperm injection (ICSI), and therefore of predictive value.

H. Leffers

MEHP is a charged particle and therefore should not be able to enter into cells unless by a receptor mechanism, and we have never detected MEHP within cells. Can you explain its mechanism of action? We would expect that it has to enter the sperm head in order to cause DNA damage.

R. Hauser

I do not think the mechanism of action at a molecular level is known.

