Journal of Andrology, Vol. 25, No. 2, March/April 2004

The Relationship Between Environmental Exposure to Phthalates and Computer-Aided Sperm Analysis Motion Parameters

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ABSTRACT: The general population is exposed to phthalates through consumer products, diet, and medical devices. The present study explored whether phthalates, reproductive toxins in laboratory animals, were associated with altered sperm movement characteristics in men. Two-hundred twenty subjects provided a semen sample for computer-aided sperm analysis (CASA) and a urine sample for measurement of phthalate monoesters, monoethyl (MEP), monobenzyl (MBzP), mono-n-butyl (MBP), mono-2-ethylhexyl (MEHP), and monomethyl (MMP). Three CASA parameters, straight-line velocity (VSL), curvilinear velocity (VCL), and linearity (LIN), were used as measures of sperm progression, sperm vigor, and swimming pattern, respectively. There were suggestive dose-response relationships (shown as the predicted change in mean sperm motion parameter for the second and third tertiles compared with the first tertile; *P* value for trend) for MBzP with VSL (−2.36 μm/s, −2.81 μm/s; *P* = .09) and VCL (−1.67 μm/s, −2.45 μm/s; *P* = .4). There were suggestive negative associations between MBP and VSL (−3.07 μm/s, −2.87 μm/s; *P* = .08) and VCL (−3.25 μm/s, −3.46 μm/s; *P* = .2), and between MEHP with VSL (−1.09 μm/s, −2.73 μm/s; *P* = .1) and VCL (−0.29 μm/s, −2.93 μm/s; *P* = .3). In contrast to the other phthalates, MEP was positively associated with VSL and VCL but negatively associated with LIN. No consistent relationship was found for MMP and any sperm motion parameter. Although we did not find statistically significant associations, trends between CASA parameters, sperm velocity, and forward progression, and increased urinary levels of MBP, MBzP, and MEHP warrant further follow-up.

Key words: Environment, epidemiology, human phthalate.
J Androl 2004;25:293–302

Currently, scientific and public concern exists regarding whether several commonly used industrial chemicals, such as phthalates, are associated with male reproductive toxicity in humans. These concerns stem from studies showing that a large proportion of the general population in the United States is exposed to phthalates (Blount et al, 2000b; Centers for Disease Control and Prevention [CDC], 2003) as well as animal studies showing they are reproductive toxicants (Gangolli, 1982; Reel et al, 1984; Agarwal et al, 1985; Parmar et al, 1986; Akingbemi et al, 2001). Furthermore, although the data are not conclusive, several researchers have found declining sperm counts in several developed countries (Carlsen et al, 1992; Comhaire et al, 1996; Gyllenborg et al, 1999; Swan et al, 2000), and there is concern over whether industrial chemicals may be partly responsible (Sharpe and Skakkebaek, 1993).

In one of the first human studies to explore associations between environmental levels of phthalates and human semen parameters, we recently demonstrated a relationship between environmental levels of phthalates and traditional semen analysis parameters categorized by World Health Organization (1999) categories for count, motility, and morphology (Duty et al, 2003). Specifically, we found dose-response relations between tertiles of monobutyl phthalate (MBP) and sperm motility (OR per tertile: 1.0, 1.8, 3.0, *P* value for trend = .02) and sperm concentration (1.0, 1.4, 3.3, *P* value for trend = .07). There was

Supported by grants ES09718 and ES00002 from the National Institute of Environmental Health Sciences (NIEHS). The information herein is solely the responsibility of the authors and does not necessarily represent the official views of NIEHS. Dr S. Duty was supported by National Institutes of Health Training Grant T32 ES07069.

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Received for publication August 25, 2003; accepted for publication October 9, 2003.

also a dose-response relation between monobenzyl phthalate (MBzP) and sperm concentration. We also found limited evidence for an association between monomethyl phthalate (MMP) with poor sperm morphology. To further explore the relationship between phthalate exposure and sperm motility, we investigated in a larger data set whether computer-aided sperm analysis (CASA) motion parameters were associated with environmental exposure to phthalates. CASA parameters are not as easily interpretable as World Health Organization semen analysis categories but may offer additional insights into whether any particular aspect of sperm movement would be differentially affected.

Phthalates are used in many consumer products such as carpet backing, soaps, shampoos, paints, glues, hairsprays, nail polishes, insect repellents (Agency for Toxic Substances and Disease Registry, 1999), cosmetics, perfumes (Blount et al, 2000b), and medical products (Nassberger et al, 1987). Di(2-ethylhexyl) phthalate (DEHP), one of the more commonly used phthalates, leaches from blood products, and from intravenous and dialysate bags and tubing made with polyvinyl chloride (Nassberger et al, 1987).

There is consistent toxicological evidence of adverse developmental and reproductive effects of DEHP (Reel et al, 1984), butyl benzyl phthalate (BBzP) (Agarwal et al, 1985), and di-n-butyl phthalate (DBP) (Wine et al, 1997). Fewer live pups per litter, decreased live pup weights, and degeneration of the epididymis and testes have been found in pups after intrauterine DBP exposure (Wine et al, 1997). The National Toxicological Program (NTP) Center for the Evaluation of Risks to Human Reproduction (CERHR) Expert Panel recently concluded that there is a larger risk of DBP associated reproductive and developmental toxicity following gestation and lactation exposures as compared to adult exposures (NTP, 2000). This finding, together with the fact that animal studies are conducted with doses higher than exposures expected in the general population, prompted the NTP-CERHR Expert Panel to determine there was low concern for adverse reproductive effects in humans from adult exposure to DBP.

Currently, we know of only two other human studies on the possible relationship between phthalates and testicular function (Murature et al, 1987; Rozati et al, 2002). However, interpretation of these results is difficult because both measured the diesters in semen, and no information was provided to explain how contamination from diesters in laboratory equipment was avoided, and one study did not address potential confounders.

The use of CASA in the clinical andrology laboratory has become commonplace due to the speed of analysis and objectivity of measurements. However, technical problems exist for several reasons, including the dependence of parameter estimates on frame acquisition rate and the number of frames analyzed, as well as the variability in the depth of the counting chamber (European Society of Human Reproduction and Embryology, 1996; Kraemer et al, 1998). CASA generally underestimates sperm movement parameters in samples with sperm counts >100 million/mL and overestimates them in samples with low counts; however, CASA accurately measures samples with sperm counts ranging from 40 to 100 million/mL (Mortimer et al, 1995). Currently, no standard protocols exist for CASA operation, but there are recommendations from the European Society for Human Reproduction and Embryology Andrology Special Interest Group (European Society of Human Reproduction and Embryology, 1996) for optimizing the CASA instrument for the specific setting of its use. The predictive value of CASA parameters has been compared with conventional semen analysis in a donor insemination program; CASA was superior in predicting which ejaculate achieved pregnancy compared to the traditional semen analysis parameters (Macleod and Irvine, 1995).

Based on the findings from rodent toxicity studies and our previous human study on a small number of men, we hypothesized that MBP, MBzP, and possibly MEHP will be associated with sperm motion parameters, whereas MEP and MMP will not.

Materials and Methods

Recruitment

The study was approved by the Harvard School of Public Health (HSPH) and Massachusetts General Hospital (MGH) Human Subjects Committees and all subjects signed an informed consent. Subjects were male partners of a subfertile couple who were between 20 and 54 years of age and who presented to the Vincent Burnham andrology laboratory at MGH between January 2000 and October 2001 for semen analysis as part of an infertility workup. Individual men may or may not have been infertile. Men presenting for postvasectomy semen analysis were excluded. Height and weight were measured and a questionnaire was used to collect information on medical history and lifestyle factors.

Semen Sampling

Each subject produced a semen sample on-site by masturbation into a sterile, wide-mouthed, plastic specimen cup. The sample was allowed to liquefy at 37°C for 20 minutes prior to analysis. Subjects were instructed to abstain from ejaculation for 48 hours prior to producing the semen sample and to complete a questionnaire on health, personal habits, and the length of their sexual abstinence period. Each subject provided a single semen sample.

Computer-Aided Sperm Analysis

Sperm motion analysis and conventional semen analysis parameters were measured on the same semen sample. All analyses

were performed without knowledge of the subject's phthalate levels. Within 1 hour of collection, a 5-μL aliquot of fresh semen was loaded into a 10-μm-deep Makler chamber (Sefi Medical Instruments, Haifa, Israel), placed on a stage warmer set at 37°C, and evaluated using a Hamilton-Thorne Integrated visual optic system (HTM-IVOS Version 10, Beverly, Mass). Setting parameters and the definition of measured sperm motion parameters for the CASA were established by Hamilton-Thorne Company (frames acquired, 30; frame rate, 60 Hz; straightness [STR] threshold, 80.0%; medium average path velocity [VAP] cutoff, 25.0 μm/s; low VAP cutoff, 5.0 μm/s; count slow as motile, no; static head size, 1.0–2.9; static head intensity, 0.6–1.4; static elongation, 0–80; and the duration of the tracking time, 0.5 seconds). A minimum of 200 sperm from at least four different fields was analyzed for each specimen.

CASA outcomes include VAP, which is a mathematically smoothed velocity; straight line velocity (VSL); curvilinear velocity (VCL); amplitude of lateral head displacement (ALH), which corresponds to the mean width of the head oscillation as the cell swims; and beat cross frequency (BCF), which measures the frequency with which the cell track crosses the cell path in either direction. VAP, VSL, (STR = VSL/VAP × 100), and linearity (LIN = VSL/VCL × 100) are indicators of sperm progression, whereas VCL, ALH, and BCF are indicators of sperm vigor. STR and LIN are also used to describe sperm swimming pattern.

Seven CASA sperm motion parameters were measured, and as expected, many were strongly correlated with each other because they describe different aspects of the same movement. Measures of progression, VAP and VSL were highly correlated ($r = .96$: $P < .0001$), indicating they were likely measuring a similar characteristic of sperm movement. VSL was chosen over VAP as a measure of progression because it is a direct measurement as opposed to a mathematically smoothed value. VCL was chosen as a measure of vigor and was strongly and positively correlated with ALH ($r = .84$; $P < .0001$) but not correlated with BCF ($r = -0.06$; $P = .39$). The two measures of swimming pattern (LIN and STR) were strongly correlated ($r = .89$; $P < .0001$), indicating they were likely measuring a similar characteristic of sperm movement. LIN was chosen as a measure of swimming pattern because the other parameters chosen for this study (VSL and VCL) are components of LIN and not of STR. Therefore, a measure of progression (VSL), vigor (VCL), and swimming pattern (LIN) were chosen for in-depth statistical analyses. These three measures are also not as heavily dependent on the type of CASA instrument used, allowing for some comparison with results from other studies.

Urinary Phthalate Monoesters

Eight urinary phthalate monoesters were measured in a single spot urine sample collected at MGH at the same visit that the semen sample was collected. The sample was collected in a sterile specimen cup, aliquoted to cryovials, frozen at −20°C within 1 hour, and archived until shipment to the CDC for analysis. The analytical approach has been described in detail elsewhere (Blount et al, 2000a; Silva et al, 2003). Briefly, urinary phthalate metabolite determination involved enzymatic deconjugation of the metabolites from the glucuronidated form, solid-phase extraction, separation with high-performance liquid chromatography, and detection by tandem mass spectrometry. Detection limits were in the low nanogram-per-milliliter range (ng/mL). Reagent blanks and $^{13}C_4$-labeled internal standards were used along with conjugated internal standards to increase precision of measurements. One method blank, two quality control samples (human urine spiked with phthalates), and two standards were analyzed along with every 21 unknown urine samples. Analysts at the CDC in Atlanta, Georgia, were blind to all information concerning subjects. The monoester phthalate metabolites were measured because of potential sample contamination from the parent diester and because some of the metabolites are believed to be the active toxicant as opposed to the parent diester compounds (Peck and Albro, 1982; Li et al, 1998).

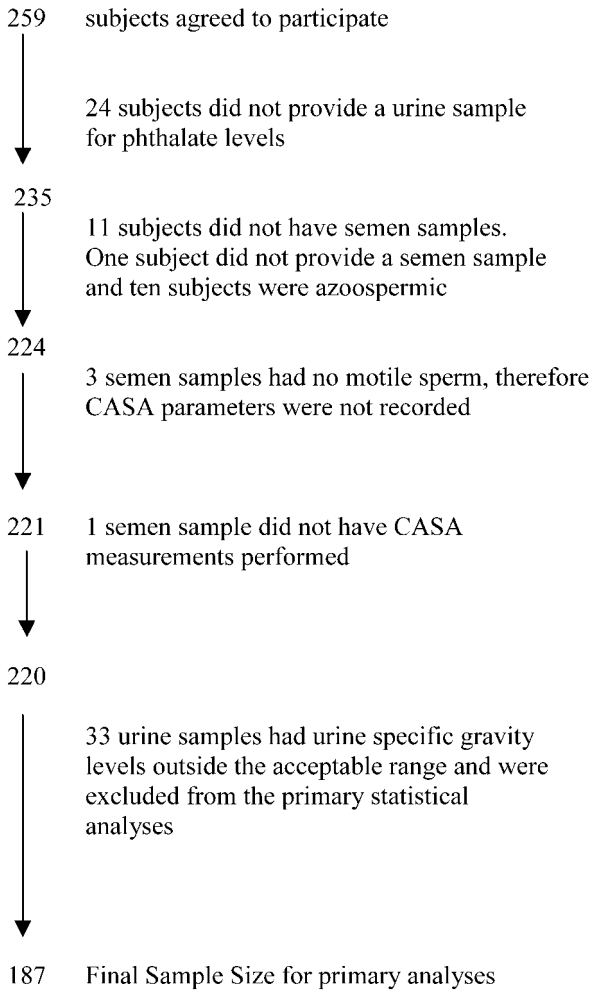
Urinary phthalate levels were normalized for dilution by specific gravity adjustment. In the primary analysis, we excluded samples that were considered unreliable, including samples with specific gravity less than 1.01 or greater than 1.03 (Teass et al, 1998). Specific gravity was measured using a hand-held refractometer (National Instrument Company, Baltimore, Md), which was calibrated with deionized water prior to each measurement. Phthalate concentrations were corrected for specific gravity by the following formula: $P_c = P\ ((1.024–1)/SG-1))$, where P_c is the specific gravity corrected phthalate concentration (ng/mL), P is the observed phthalate concentration (ng/mL), and SG is the specific gravity of the urine sample (Boeniger et al, 1993; Teass et al, 1998).

Statistical Methods

Statistical Analysis Software version 8.1 (SAS Institute Inc, Cary, NC) was used for data analysis. Descriptive and summary statistics were generated, outcomes were analyzed for normality, and predictors were explored for evidence of nonlinearity with the outcome. Pearson correlation coefficients were used to explore the relationships among the normally distributed CASA parameters and to determine which CASA parameters to explore in statistical analysis. Spearman correlation coefficients were used to explore the relationships among the non-normally distributed phthalate levels and traditional semen analysis parameters.

In preliminary analyses, scatter-plots and multiple linear regression analysis were used to explore the relationship among each CASA parameter and each phthalate metabolite, adjusting for appropriate covariates. In the primary analysis, phthalate levels were divided into tertiles and entered into the model as dummy variables to explore dose-response relationships. The use of tertile phthalate level cut points offers more flexibility for linear regression modeling and does not impose an assumption of linearity between exposure and outcome. In addition, the use of tertiles allowed individuals with nondetected values to be more easily included in the data analysis. Tertiles are also useful to explore nonlinear dose responses such as U-shaped responses or threshold responses.

Covariates considered for inclusion in the models included smoking status, race, age, body mass index, and abstinence time. Their inclusion in the multivariate models was based on statistical and biological considerations (Hosmer and Lemeshow, 1989). In addition, because CASA measurements are not inde-



Subject Exclusions.

pendent (they all measure different aspects of sperm motion in a single semen sample), covariates considered potential confounders in one model were included in all models. Age was modeled as a continuous independent variable after checking for appropriateness using a quadratic term. Abstinence time was modeled as an ordinal five-category variable (2 or fewer days, 3, 4, 5, and 6 or more days). Smoking status was included as a dummy variable (current and former versus never).

Results

Participants and Traditional Semen Parameters

Sixty-six percent of 392 men who were approached at their scheduled andrology clinic appointment agreed to participate in this study. The predominant reason for declining was insufficient time to participate in the study. Of the 259 recruited men, 220 had both urinary phthalate measurements and semen samples sufficient for performing CASA (Figure). Demographic characteristics and semen parameters are described in Table 1. Subjects were primarily white (79%), with a mean (SD) age of 36.3 years (5.6) and 72.3% had never smoked. Using the World Health Organization (1999) reference values for low sperm concentration (<20 million/mL), poor motility (<50% motile), and Kruger strict criteria (Kruger et al, 1988) for abnormal morphology (<4% normal), 29 men (13.2%) had low sperm concentration, 92 men (41.8%) had poorly motile sperm, and 49 men (22.3%) had less than 4% normally shaped sperm. Although the mean values for each of the traditional semen analysis parameters were above the WHO reference values, 51.8% of subjects had at least one parameter below WHO reference values. The semen parameter categories were not mutually exclusive. A man could contribute data to any one, two, or all three of the below-reference-value groups.

CASA Parameters

Correlation coefficients for CASA parameters and traditional semen parameters (count, motility, and morphology) are shown in Table 2. The strongest correlation between CASA and traditional semen analysis parameters was found for percent motility and both VCL ($r = .44$; $P < .0001$) and VSL ($r = .51$; $P < .0001$). The mean (SD) VSL, VCL, and LIN were 44.7 μm/s (9.6), 77.8 μm/s (16.8), and 58.2% (6.5), respectively. The distribution of CASA parameters is shown in Table 3.

Phthalate Monoesters

Eight urinary phthalate metabolites, monoethyl (MEP), monomethyl (MMP), mono-2-ethylhexyl (MEHP), mono-n-butyl (MBP), monobenzyl (MBzP), mono-n-octyl phthalate (MOP), mono-3-methyl-5-dimethylhexyl phthalate (isononyl), (MINP), and monocyclohexyl (MCHP) phthalates were measured. Because more than 75% of the population had levels of MCHP, MOP, and MINP below the limit of detection, the results for these metabolites were not informative and are not included in the analysis. MEP was detected in 100% of subjects, whereas MBP and MBzP were detected in more than 95% of subjects, and 75% had detectable levels of MEHP and MMP. These five phthalates were used in all statistical analyses.

There was a wide distribution of both specific gravity-adjusted and unadjusted phthalate monoester levels (Table 4). The rank order of phthalate distribution is similar to the distribution found in the National Health and Nutrition Examination Survey (NHANES) 1999–2000 and III data (Blount et al, 2000b; CDC, 2003) with the highest levels (geometric mean) found for MEP (183.1 ng/mL), followed by MBP (18.0 ng/mL), MBzP (8.6 ng/mL), MEHP (7.0 ng/mL), and MMP (4.2 ng/mL). Thirty-three (15%) samples were excluded from primary analysis because of extreme specific gravity values (<1.010 or

Table 1. *Subject demographics and distribution of semen parameters (N = 220)*

Characteristic*	Mean (SD)	Number (%)
Age	36.3 (5.6)	
Body mass index	28.1 (4.6)	
Semen parameters		
Sperm concentration (million/mL)	115.6 (99.2)	
Subjects <20 million sperm/mL		29 (13.2)
Sperm motility (% motile)	52.2 (22.6)	
Subject <50% motile sperm		92 (41.8)
Sperm morphology† (% normal morphology)	7.4 (4.6)	
Subjects <4% normal morphology		49 (22.3)
Race		
White		173 (79.0)
Black/African American		14 (6.4)
Hispanic		14 (6.4)
Other		18 (8.2)
Smoking		
Never smoked		159 (72.3)
Ever smoked		61 (27.7)
Current smoker		21 (9.5)
Former smoker		40 (18.2)
Abstinence time		
<2 days		57 (26.0)
3 days		70 (32.0)
4 days		35 (16.0)
5 days		21 (9.1)
6 or more days		37 (16.9)
Previous exam for infertility		67 (30.5)

* One person missing race, body mass index, and abstinence time.
† Kruger Strict Criteria used for morphology determination.

>1.030) (Boeniger et al, 1993). The final sample size for statistical modeling was 187 men.

Covariates: Age, Race, Abstinence Time, and Smoking Status

There were suggestive relationships among smoking status and MEP, MBP, and MMP, as well as among race and MEP, MBzP, and MBP, and between abstinence time with MEP. For example, median MEP levels were higher in current smokers (235.9 ng/mL) and former smokers (199.4 ng/mL) than in men who had never smoked (138.5 ng/mL). This pattern was similar for MMP and MBP (data not shown). African American and Hispanic men had twofold to fourfold higher MEP phthalate levels

Table 2. *Spearman correlations and* P *values among CASA and semen parameters (N = 220)*

	VSL	VCL	LIN	SC	SM	SMPH*
VSL	1					
VCL	0.82	1				
	<0.0001					
LIN	0.30	−0.21	1			
	<0.0001	0.002				
SC	0.26	0.29	−0.09	1		
	0.0001	<0.0001	0.20			
SM	0.51	0.44	0.14	0.60	1	
	<0.0001	<0.0001	0.04	0.0001		
SMPH*	0.29	0.29	−0.0001	0.34	0.44	1
	<0.0001	<0.0001	1.00	0.0001	0.0001	

* Morphology measured by strict criteria. CASA indicates computer-aided sperm analysis; VSL, straight line velocity (μm/s); VCL, curvilinear velocity (μm/s); LIN, linearity (%); SC, sperm concentration in millions/mL; SM, percent motile sperm; and SMPH, percent of sperm with normal morphology.

Table 3. *Distribution of CASA motion parameters*

	Percentiles and Summary Statistics						
Parameter	Number	Minimum	25th	50th	75th	Maximum	Mean (SD)
VSL (μm/s)	220	20.3	38.3	45.5	51.1	67.5	44.7 (9.6)
VCL (μm/s)	220	37.9	67.3	77.9	88.0	135.7	77.8 (16.8)
LIN (%)	220	39	54	58	62	78.0	58.2 (6.5)

CASA indicates computer-aided sperm analysis; VSL, straight-line velocity; VCL, curvilinear velocity; LIN, linearity; and SD, standard deviation.

(505.5 and 347.0 ng/mL, respectively) than either white men (151 ng/mL) or those of other races (116.5 ng/mL). This pattern was also observed for MBzP (data not shown). In addition, men with abstinence times greater than 5 days on average had lower MEP levels (112 ng/mL) than those with less than 5 days of abstinence (187 ng/mL). In contrast to these data, the median and mean CASA parameter values were nearly identical across all levels of these covariates.

Age was related to some of the phthalate levels and sperm motion parameters. For instance, for each 1-year increase in age, VSL, VCL, and LIN decreased −0.27 μm/s (95% CI: −0.51, −0.03), −0.38 μm/s (95% CI: −0.80, 0.04), and −0.06 % (95% CI: −0.23, 0.11), respectively. In addition, for every year increase in age, MEP, MBzP, MBP, MEHP, and MMP increased 38.5 ng/mL (95% CI: 9.12, 67.90), 1.1 ng/mL (95% CI: −0.06, 2.16), 1.4 ng/mL (95% CI: −3.89, 6.61), 0.7 ng/mL (95% CI: −0.97, 2.37), and 0.01 ng/mL (95% CI: −0.33, 0.36), respectively.

Multiple Regression Analyses

Presented in Table 5 are the regression coefficients, their 95% confidence intervals, and test for trend *P* values for individual CASA parameters regressed on tertile of specific gravity-adjusted phthalate monoester levels. These models are adjusted for age, abstinence time, and smoking. Overall, although not statistically significant, MBP, MBzP, and MEHP had negative relationships with VSL, VCL, and LIN. No consistent relationship was found for MMP and any sperm motion parameter, and unexpectedly, a generally positive relationship was observed between MEP and both VSL and VCL. There were dose-response relationships (shown as the predicted change in mean sperm motion parameter for the second and third tertiles compared to the first tertile, followed by the *P* value for trend) for MBzP with VSL (−2.36 μm/s, −2.81 μm/s, *P* value for trend .09) and VCL (−1.67 μm/s, −2.45 μm/s, *P* value for trend .4), although neither reached statistical significance. A general decrease in LIN was also evident for higher MBzP levels. MBP was associated with a decline in VSL (−3.07 μm/s, −2.87 μm/s, *P* value for trend .08), VCL (−3.25 μm/s, −3.46 μm/s, *P* value for trend .2), and LIN (−1.60%, −1.00%, *P* value for trend .4). For MEHP there was evidence of dose-response relationships with VSL (−1.09 μm/s, −2.73 μm/s, *P* value for trend .1), VCL (−0.29 μm/s, −2.93 μm/s, *P* value for trend .3), and LIN (−0.96%, −1.30%, *P* value for trend .3), but none reached statistical significance. In contrast to the other phthalates, for MEP there were positive relationships with VSL (1.17 μm/s, 2.73 μm/s, *P* value for trend .1) and VCL (3.16 μm/s, 6.36 μm/s, *P* value for trend .03), but negative associations with LIN (−0.43 %, −0.65%, value for trend .6).

Table 4. *Urinary phthalate monoester concentrations unadjusted and adjusted for specific gravity (ng monoester/mL urine)*

			Percentile						
Phthalate Monoester	Number	Minimum	5th	25th	50th	75th	95th	Maximum	Geometric Mean
Unadjusted									
Ethyl (MEP)	220	8.2	23.6	58.5	152.7	461.3	2416.7	9475.6	174.2
Benzyl (MBzP)	220	<LOD	1.1	4.0	9.9	18.3	49.7	450.2	8.3
Butyl (MBP)	220	<LOD	2.5	10.1	17.8	34.4	90.0	3169.9	17.5
2-Ethylhexyl (MEHP)	220	<LOD	<LOD	2.6	6.1	20.6	147.9	520.2	7.2
Methyl (MMP)	220	<LOD	<LOD	1.7	4.6	11.0	32.6	451.9	4.7
Specific gravity adjusted									
Ethyl (MEP)	220	9.8	28.1	64.2	157.1	466.6	2002.1	11 370.7	183.1
Benzyl (MBzP)	220	<LOD	1.6	4.6	9.4	17.0	46.8	540.2	8.6
Butyl (MBP)	220	<LOD	4.0	11.3	18.0	31.6	73.9	2623.4	18.0
2-Ethylhexyl (MEHP)	220	<LOD	<LOD	2.9	6.3	19.6	130.9	445.9	7.0
Monomethyl (MMP)	220	<LOD	<LOD	2.1	4.5	10.5	32.3	278.1	4.2

LOD indicates limits of detection. LOD for unadjusted phthalate monoesters (ng/mL) are as follows: MEP, 1.0; MBzP, 0.8; MBP, 0.6; MEHP, 1.2; and MMP, 0.71. Note: Specific gravity–adjusted results did not exclude samples with extreme specific gravity.

Table 5. *Adjusted regression coefficients for changes in sperm motion parameters associated with tertiles of phthalate monoester levels* (N† = 187)*

		Sperm Motion Parameter‡		
Phthalate Monoester	Tertile	Straight Line Velocity§ Coefficient# (CI)	Curvilinear Velocity‖ Coefficient# (CI)	Linearity¶ Coefficient# (CI)
Ethyl (MEP)	2	1.17 (−2.04, 4.38)	3.16 (−2.39, 8.70)	−0.43 (−2.76, 1.89)
	3	2.73 (−0.53, 5.99)	6.36 (0.74, 11.99)	−0.65 (−3.01, 1.71)
	P for trend	0.1	0.03	0.6
Benzyl (MBzP)	2	−2.36 (−5.56, 0.83)	−1.67 (−7.27, 3.92)	−1.57 (−3.87, 0.74)
	3	−2.81 (−5.99, 0.37)	−2.45 (−8.02, 3.11)	−1.59 (−3.88, 0.71)
	P for trend	0.09	0.4	0.2
Butyl (MBP)	2	−3.07 (−6.24, 0.10)	−3.25 (−8.80, 2.30)	−1.60 (−3.90, 0.69)
	3	−2.87 (−6.09, 0.35)	−3.46 (−9.09, 2.18)	−1.00 (−3.34, 1.33)
	P for trend	0.08	0.2	0.4
2-Ethylhexyl (MEHP)	2	−1.09 (−4.32, 2.14)	−0.29 (−5.92, 5.35)	−0.96 (−3.29, 1.37)
	3	−2.73 (−5.93, 0.48)	−2.93 (−8.52, 2.66)	−1.30 (−3.61, 1.02)
	P for trend	0.1	0.3	0.3
Methyl (MMP)	2	0.40 (−2.82, 3.62)	−0.63 (−6.23, 4.97)	0.83 (−1.47, 3.14)
	3	0.81 (−2.46, 4.09)	−1.27 (−6.97, 4.42)	1.88 (−0.46, 4.23)
	P for trend	0.6	0.7	0.1

* Specific gravity-adjusted urinary phthalate monoester concentration (ng/mL urine).
† Thirty-three of 220 subjects were excluded because the specific gravity value was out of the acceptable range (<1.010 or >1.030).
‡ Hamilton Thorne Integrated Visual Optic System version 10 software was used to measure sperm motion parameters.
§ Straight line velocity (μm/s) is a measure of sperm progression, coefficient units are μm/s per tertile.
‖ Curvilinear velocity is a measure of sperm vigor, coefficient units are μm/s per tertile.
¶ Linearity (%) is a measure of sperm swimming pattern, coefficient units are percent per tertile.
# Adjusted for age (continuous), and smoking (current, former versus never) and abstinence time (≤2 days, 3, 4, 5, and 6+ days).

Sensitivity Analysis

Sensitivity analyses were conducted to explore the impact of using specific gravity for exclusionary purposes only and not for adjustment of phthalate levels. In a reanalysis, the urinary data were not specific gravity–adjusted, but 33 unreliable samples, those with urine specific gravity levels outside the acceptable range (1.01–1.03), were excluded. The interpretation of the results was similar to the primary analyses. As in the primary analyses, VSL, VCL, and LIN generally declined with phthalate exposure for each phthalate except MEP. MEP again was associated with increased VSL and VCL, but with a minor decrease in LIN. For MBzP and MBP, the magnitude of the dose-response relationships with CASA parameters became weaker; however, for MEHP, the dose-response relationship became stronger.

We also explored the use of quintile rather than tertile cut points, and despite the small sample size in each quintile, the results were consistent with the use of tertile cut points. Finally, we explored the relationship between CASA parameters and continuous phthalate concentrations. Overall, the interpretation of the continuous analysis data was similar and showed suggestive inverse dose-response relationships among CASA parameters with MBzP, MBP, and MEHP, and a positive dose-response relationship for MEP with VSL and VCL.

Discussion

In the present study, we explored both linear and nonlinear relationships between CASA parameters and phthalate metabolites. Multiple linear regression analyses, in which phthalates were used as both a continuous measure and categorized into both tertiles and quintiles, were performed. This allowed us to explore threshold dose responses and U-shaped responses (Calabrese and Baldwin, 2001). Although they were not statistically significant, we found an overall pattern of decline in CASA parameters VSL, VCL, and LIN for phthalate monoesters MBP, MBzP, and MEHP. The absence of consistent statistically significant dose-response relationships may reflect a lack of power due to the relatively small number of subjects. Toxicological studies, primarily in rats, consistently show that select phthalate monoesters, including MEHP and MBP, are male reproductive and developmental toxicants (Parmar et al, 1986; Srivastava et al, 1990; Wine et al, 1997).

In laboratory animals, several researchers have explored the relationship between CASA parameters and chemical exposures, including epichlorohydrin and its metabolite, alpha-chlorohydrin (Slott et al, 1990, 1997). Epichlorohydrin, after 4 hours of inhalational exposure, transiently decreased path velocity despite no significant change in the percentage of motile sperm (Slott et al,

1990). Similarly, alpha-chlorohydrin given to male hamsters for 4 days resulted in a significant dose-dependent decline in VCL, VAP, and VCL_{select} despite no change in the percentage of motile sperm. In addition, alpha-chlorohydrin exposure was associated with a nonlinear impairment in in vitro fertilizing ability, which exhibited a threshold-like response (Slott et al, 1997). These studies suggest that CASA parameters may serve as a more sensitive maker of reproductive toxicity than semen parameters (Perreault and Cancel, 2001). One mechanism whereby sperm motion may be impaired includes oxidative stress and the production of reactive oxygen species and subsequent lipid peroxidation of sperm plasma membrane (Aitken, 1997; Storey, 1997; Armstrong et al, 1999).

Although CASA parameters may prove to be sensitive biomarkers of reproductive toxicity in humans, they are difficult to compare across studies because of the use of different CASA instruments and settings (Davis et al, 1992). Despite this limitation, human studies have shown that CASA parameters can be used to predict fertility (Aitken et al, 1982) and pregnancy (Macleod and Irvine, 1995; Larsen et al, 2000). Furthermore, there are also epidemiologic studies using CASA parameters as a marker of altered semen quality. Selevan et al (2000) examined the association between air pollution levels and VSL, VCL, and LIN and found that medium levels of air pollution adversely affected VCL but improved LIN. High air pollution levels, however, improved VSL and VCL but unexpectedly decreased LIN. The inference is that although the sperm traveled faster, the pattern was more erratic, and therefore, forward progression actually decreased.

The strengths of the present study include the availability of a reliable biomarker of phthalate exposure instead of relying on self-reported exposures. Biomarkers have the potential to quantify exposures to chemicals from all routes of exposure, including oral, dermal, inhalation, and ingestion (Teass et al, 1998). Furthermore, by measuring the monoester phthalate (ie, the metabolite), we avoided difficulties resulting from contamination from plastic products, such as the urine specimen collection cup (Blount et al, 2000a). One limitation in the present study was that if phthalates are associated with a complete lack of sperm motility, this study is not able to detect this, because by definition, we were able to explore motion parameters only on motile sperm.

Evidence of widespread exposure of the general population to phthalates comes from a recent study on phthalate metabolite levels in urine collected for the Second National Report on Human Exposure to Environmental Chemicals, NHANES 1999–2000 (CDC, 2003). The NHANES survey collected biological samples and information about the health and diet of people in the United States (National Center for Health Statistics, 2001). Four phthalate metabolites, MEP, MEHP, MBP, and MBzP were present in more than 75% of US subjects sampled (CDC, 2003). In the present study, 100% of subjects had measurable MEP, 95% had detectable MBP and MBzP, whereas 75% had detectable MMP and MEHP levels.

The NHANES 1999–2000 survey contains data on monoester levels stratified by gender or age (6–11 years, 12–19 years, and ≥20 years). The NHANES report did not include cross-stratification by age and gender (eg, the report did not present data for a substratum consisting entirely of adult males age ≥20 years). Therefore, the NHANES male stratum included men from 6 to >60 years of age, whereas the age strata included both men and women. This lack of cross-stratification by age and gender makes the NHANES male stratum not entirely comparable to the present study on adult men because of trends in phthalate monoesters with age. For instance, children (6–11 years) and adolescents (12–19 years) had higher median levels of MBP, MBzP, and MEHP than adults (≥20 years). In contrast, median levels of MEP were lower for the age group 6–11 years than for the other two age groups. It is unknown whether age and gender differences in monoester concentrations reflect differences in exposure, body-size relationships, or metabolism. Because of the absence of a stratum of adult men in the NHANES report, the comparisons made between the present study and NHANES are meant as qualitative guidance but should not be used to determine precise quantitative differences. The samples in the present study and the NHANES samples were both analyzed by the same CDC laboratory.

In the present study, unadjusted median MEP levels (152.7 ng/mL) were similar to median levels in NHANES men (154 ng/mL), whereas MEHP was higher in the present study (6.1 ng/mL) as compared to NHANES (3.40 ng/mL). In contrast, in the present study, median MBP (17.8 ng/mL) and MBzP (9.9 ng/mL) levels were lower than in the NHANES data set (23.1 ng/mL and 17.7 ng/mL, respectively). The inclusion in the NHANES male stratum of children and adolescents with higher MBP and MBzP levels than adults may account for the higher median levels of MBP and MBzP in NHANES as compared to the present study. In contrast, the high levels of MEHP found in the present study would be even higher than NHANES levels if children and adolescents, with higher MEHP levels than adults, were excluded from the NHANES male stratum. MMP was not measured in the NHANES data set.

There is controversy about the best way to correct for urine volume when using a single spot urine sample (Boeniger et al, 1993; Teass et al, 1998). In our sensitivity analysis, we used specific gravity criteria to exclude 33 samples and analyzed the data without adjusting the remaining phthalate levels. The results were consistent with

the primary analysis, although the relationship between MBP and MBzP became weaker, while the relationship for MEHP and both VSL became stronger. This may indicate that the additional measurement error introduced by not using specific gravity adjustment biased the results toward the null hypothesis, making relationships more difficult to detect.

It is interesting to note that study subjects were exposed to several phthalates simultaneously, which raises the issue of how to explore relationships between semen parameters and exposures to multiple phthalates that may act via similar mechanisms. Gray and colleagues (2000) suggested that risk assessments for phthalate-induced reproductive toxicity should consider phthalates as a group and include exposures from multiple sources (Gray et al, 2000). Although they provide preliminary phthalate ester toxic equivalency factors (PE-TEFs) for reproductive toxicity induced in utero, they do so to stimulate discussion and further research about how we should estimate cumulative and aggregate risk to phthalates. Additional dose-response studies are necessary before we can apply PE-TEFs in both toxicological and epidemiological studies.

In conclusion, although we did not find statistically significant associations between CASA parameters and adult exposure to phthalates, there were trends that warrant further follow-up. These data extend the results of our previous study (Duty et al, 2003) that found an association between MBP and lower sperm motility. Although intriguing, these results are preliminary and should be explored in larger, as well as different study populations.

Acknowledgments

The authors thank Dr Dana Barr, Centers for Disease Control and Prevention, the staff of the Vincent Memorial Obstetrics and Gynecology Service Andrology Laboratory and In Vitro Fertilization Unit at Massachusetts General Hospital, our research nurse, Ms Linda Godfrey-Bailey, and research assistants Ms Ana Trisini and Mr Ramace Dadd. We also thank the reviewers for their insightful comments.

References

Agarwal DK, Maronpot RR, Lamb JC IV, Kluwe WM. Adverse effects of butyl benzyl phthalate on the reproductive and hematopoietic systems of male rats. *Toxicology.* 1985;35:189–206.

Agency for Toxic Substances and Disease Registry. Di-n-butyl Phthalate, 1999. Available at: www.atsdr.cdc.gov/toxprofiles. Accessed March 28, 2002.

Aitken RJ. Molecular mechanisms regulating human sperm function. *Mol Hum Reprod.* 1997;3:169–173.

Aitken RJ, Best FS, Richardson DW, Djahanbakhch O, Mortimer D, Templeton AA, Lees MM. An analysis of sperm function in cases of unexplained infertility: conventional criteria, movement characteristics, and fertilizing capacity. *Fertil Steril.* 1982;38:212–221.

Akingbemi BT, Youker RT, Sottas CM, Ge R, Katz E, Klinefelter GR, Zirkin BR, Hardy MP. Modulation of rat leydig cell steroidogenic function by Di(2-Ethylhexyl)Phthalate. *Biol Reprod.* 2001;65:1252–1259.

Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ, Sikka SC. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Med.* 1999;26:869–880.

Blount BC, Milgram KE, Silva MJ, Malek NA, Reidy JA, Needham LL, Brock JW. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. *Anal Chem.* 2000a;72:4127–4134.

Blount BC, Silva MJ, Caudill SP, et al. Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect.* 2000b;108:979–982.

Boeniger MF, Lowry LK, Rosenberg J. Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. *Am Ind Hyg Assoc J.* 1993;54:615–627.

Calabrese EJ, Baldwin LA. U-shaped dose-responses in biology, toxicology, and public health. *Annu Rev Public Health.* 2001;22:15–33.

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *Br Med J.* 1992;305:609–613.

Centers for Disease Control and Prevention. Second National Report on Human Exposure to Environmental Chemicals. NCEH publication 02–0716. National Center for Environmental Health Web site. Available at: www.cdc.gov/exposurereport. Accessed February 4, 2003.

Comhaire F, Van Waeleghem K, De Clercq N, Schoonjans F. Declining sperm quality in European men. *Andrologia.* 1996;28:300–301.

Davis RO, Rothmann SA, Overstreet JW. Accuracy and precision of computer-aided sperm analysis in multicenter studies. *Fertil Steril.* 1992;57:648–653.

Duty SM, Silva MJ, Barr DB, et al. The relationship between environmental exposure to phthalates and human semen parameters. *Epidemiology.* 2003;14:269–277.

European Society of Human Reproduction and Embryology. Consensus workshop on advanced diagnostic andrology techniques. ESHRE Andrology Special Interest Group. *Hum Reprod.* 1996;11:1463–1479.

Gangolli SD. Testicular effects of phthalate esters. *Environ Health Perspect.* 1982;45:77–84.

Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci.* 2000;58:350–365.

Gyllenborg J, Skakkebaek NE, Nielsen NC, Keiding N, Giwercman A. Secular and seasonal changes in semen quality among young Danish men: a statistical analysis of semen samples from 1927 donor candidates during 1977–1995. *Int J Androl.* 1999;22:28–36.

Hosmer DW Jr, Lemeshow S. Model building strategies and methods for logistic regression. In: Hosmer DW Jr, Lemeshow S, eds. *Applied Logistic Regression.* New York: John Wiley & Sons; 1989:82–134.

Kraemer M, Fillion C, Martin-Pont B, Auger J. Factors influencing human sperm kinematic measurements by the Celltrak computer-assisted sperm analysis system. *Hum Reprod.* 1998;13:611–619.

Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Mata JF, Oehninger S. Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril.* 1988;49:112–117.

Larsen L, Scheike T, Jensen TK, et al. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. The Danish First Pregnancy Planner Study Team. *Hum Reprod.* 2000;15:1562–1567.

Li L-H, Jester WF, Orth JM. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured sertoli cells and gonocytes from neonatal rats. *Toxicol Appl Pharmacol.* 1998;153:258–265.

Macleod IC, Irvine DS. The predictive value of computer-assisted semen

analysis in the context of a donor insemination programme. *Hum Reprod.* 1995;10:580–586.

Mortimer D, Aitken RJ, Mortimer ST, Pacey AA. Workshop report: clinical CASA—the quest for consensus. *Reprod Fertil Dev.* 1995;7:951–959.

Murature DA, Tang SY, Steinhardt G, Dougherty R. Phthalate esters and semen quality parameters. *Biomed Environ Mass Spectrom.* 1987;14: 473–477.

Nassberger L, Arbin A, Ostelius J. Exposure of patients to phthalates from polyvinyl chloride tubes and bags during dialysis. *Nephron.* 1987;45:286–290.

National Center for Health Statistics. National Health and Nutrition Examination Survey Web site. Available at: www.cdc.gov/nchs/nhanes.htm. Accessed December 4, 2001.

National Toxicology Program. Toxicology and carcinogenesis studies of butyl benzyl phthalate (CAS No. 85-68-7) in F344/N rats (feed studies). Report NTP TR 458, NIH Publication 97-3374. Rockville, Md: U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health; 1997.

National Toxicology Program. NTP-CERHR expert panel report on Di-n-butyl phthalate. Research Triangle Park, NC: U.S. Department of Health and Human Services, Center for the Evaluation of Risks to Human Reproduction; 2000.

Parmar D, Srivastava SP, Seth PK. Effect of di(2-ethylhexyl)phthalate (DEHP) on spermatogenesis in adult rats. *Toxicology.* 1986;42:47–54.

Peck CC, Albro PW. Toxic potential for the plasticizer di(2-ethylhexyl) phthalate in the context of its disposition and metabolism in primates and man. *Environ Health Perspect.* 1982;45:11–17.

Perreault SD, Cancel AM. Significance of incorporating measures of sperm production and function into rat toxicology studies. *Reproduction.* 2001;121:207–216.

Reel JR, Tyl RW, Lawton AD, Jamb JC. Diethylhexyl phthalate (DEHP): Reproduction and fertility assessment in CD-1 mice when administered in the feed PB84-181734. Research Triangle Park, NC: National Toxicology Program; 1984.

Rozati R, Reddy PP, Reddanna P, Mujtaba R. Role of environmental estrogens in the deterioration of male factor fertility. *Fertil Steril.* 2002;78:1187–1194.

Selevan SG, Borkovec L, Slott VL, Zudova Z, Rubes J, Evenson DP, Perreault SD. Semen quality and reproductive health of young Czech men exposed to seasonal air pollution. *Environ Health Perspect.* 2000;108:887–894.

Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract. *Lancet.* 1993;341: 1392–1395.

Silva MJ, Malek NA, Hodge CC, et al. Improved quantitative detection of 11 urinary phthalate metabolites in humans using liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry. *J Chromatogr B.* 2003;789:393–404.

Slott VL, Jeffay SC, Dyer CJ, Barbee RR, Perreault SD. Sperm motion predicts fertility in male hamsters treated with α-chlorohydrin. *J Androl.* 1997;18:708–716.

Slott VL, Jeffay SC, Suarez JD, Barbee RR, Perreault SD. Synchronous assessment of sperm motility and fertilizing ability in the hamster following treatment with α-chlorohydrin. *J Androl.* 1995;16:523–535.

Srivastava S, Singh GB, Srivastava SP, Seth PK. Testicular toxicity of di-*n*-butyl phthalate in adult rats: effect on marker enzymes of spermatogenesis. *Ind J Exp Biol.* 1990;28:67–70.

Storey BT. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol Hum Reprod.* 1997;3:203–213.

Swan SH, Elkin EP, Fenster L. The question of declining sperm density revisited: an analysis of 101 studies published 1934–1996. *Environ Health Perspect.* 2000;108:961–966.

Teass AW, Biagini RE, DeBord G, Hull RD. Application of biological monitoring methods. In: Eller PM, ed. *NIOSH Manual of Analytical Methods.* 4th ed. Cincinnati, Ohio: U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Physical Sciences and Engineering; 1998:52–62.

Wine RN, Li L-H, Barnes LH, Gulati DK, Chapin RE. Reproductive toxicity of Di-*n*-butylphthalate in a continuous breeding protocol in Spraque-Dawley Rats. *Environ Health Perspect.* 1997;105:102–107.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction.* New York: Cambridge University Press; 1999.