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A crossover–crossback prospective study of dibutyl-phthalate exposure from mesalamine medications and semen quality in men with inflammatory bowel disease

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Abstract

Background—Phthalates are widely used chemicals with ubiquitous exposure. Dibutyl-phthalate (DBP), a male reproductive toxicant in animals, is understudied in humans. Some mesalamine

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medications used to treat inflammatory bowel disease (IBD) have DBP in their coating, whereas other mesalamine formulations do not.

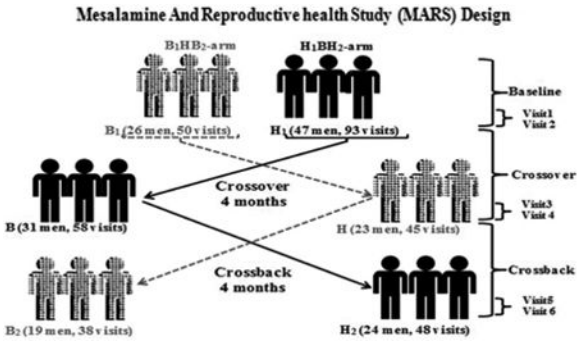
Objectives—Taking advantage of differences in mesalamine formulations, we investigated whether high-DBP exposure from mesalamine medications was associated with decreased semen parameters.

Methods—73 men with IBD taking mesalamine participated in a crossover-crossback prospective study. Men taking non-DBP containing mesalamine at baseline i.e., background exposure, crossed-over for four months to high-DBP mesalamine and then crossed-back for four months to their non-DBP mesalamine (B_1HB_2-arm;$Background_1$-$High$-$Background_2$) and vice versa for men taking high-DBP mesalamine at baseline (H_1BH_2-arm;$High_1$-$Background$-$High_2$). Men provided up to six semen samples (2: baseline, 2: crossover and 2: crossback).

Results—We estimated crossover, crossback and carryover effects using linear mixed models adjusted for abstinence time, age, season and duration on high-DBP mesalamine at baseline. Semen parameters in B_1HB_2-arm (26 men, 133 samples) decreased after high-DBP mesalamine exposure (crossover versus baseline), especially motility parameters, and continued to decrease further even after crossback to non-DBP mesalamine (crossback versus crossover).The cumulative carryover effect of high-DBP (crossback versus baseline) was a decrease of % total sperm motility by 7.61(CI:-13.1, -2.15), % progressive sperm motility by 4.23(CI:-8.05, -0.4) and motile sperm count by 26.0% (CI:-46.2%, 1.7%). However, H_1BH_2-arm (47 men, 199 samples) had no significant change during crossover or crossback.

Conclusions—Men newly exposed to high-DBP mesalamine for four months had a cumulative reduction in several semen parameters, primarily sperm motility, that was more pronounced and statistically significant even after exposure ended for four months.

Graphical abstract



Keywords

Phthalates; Mesalamine; Inflammatory Bowel Disease (IBD); Semen Quality

1. Introduction

Over the last several decades, accumulating evidence suggests a downward trend[1-3] and geographic variability in semen quality[4], a surrogate for male fertility. These trends raise

concern that lifestyle or environmental exposures may affect semen quality and male fertility[5]. One class of environmental chemicals for which there is concern about potential adverse male reproductive health effects are phthalates[6]. In experimental animal studies, several phthalates including dibutyl-phthalate (DBP) were anti-androgens and male reproductive toxicants, adversely affecting testicular function[7-11]. The most studied window of exposure is *in-utero* exposure which led to male reproductive tract malformations in rats[12-14]. Less well-studied are puberty and adulthood exposure. Studies in rats have shown effects of postnatal exposure to DBP on the male reproductive tract[15-18] and to butyl benzyl phthalate (BBzP)[19]. There are several epidemiologic studies in adult men that explored cross-sectional associations between background low-DBP environmental exposure, and other phthalates, with semen quality. Most of these studies were conducted in men recruited from infertility clinics[20-27], and although some studies found associations of DBP[22,24] and other phthalates[20,21,24,25,28] with lower semen quality, others did not[23,29,30].

In addition to widespread general population DBP exposure from personal care and consumer products[31,32], some medications such as specific mesalamine formulations have enteric coatings that contain DBP[31,33-36] despite the recent US Food and Drug Administration (FDA) recommendation against the use of phthalates in drug delivery vehicles[37]. Mesalamine or 5-aminosalicylic acid (5-ASA) is a commonly prescribed maintenance therapy for inflammatory bowel diseases (IBD), specifically ulcerative colitis (UC) and Crohn's disease (CD)[38]. Our research and others have shown that mesalamine medications with coatings that contain DBP contribute to high-DBP exposure as measured by concentrations of urinary monobutyl phthalate (MBP), the primary DBP metabolite[39,40]. Specifically, in individuals taking mesalamine medications that contain DBP, their urinary levels of MBP were approximately 1,000 times higher than the median levels reported for men in the US general population (National Health and Nutrition Examination Survey (NHANES))[41]. Therefore, patients with IBD taking DBP-containing mesalamine will have chronic high exposure to DBP because the medication is taken daily to treat IBD.

Mesalamine is the active ingredient in Asacol® and Asacol®HD and DBP is an excipient in their enteric coating[42]. Other mesalamine formulations such as Pentasa®, Lialda®, Apriso®, and Delzicol® do not contain DBP[36,43]. Asacol®, widely used to treat IBD in adults and children, was a first line of therapy for patients with UC and often used in pregnant women with IBD[43,44]. The aim of the study was to investigate the effect of high-DBP exposure on semen quality, taking advantage of the difference in mesalamine formulations to conduct a crossover-crossback prospective study in adult men with IBD.

2. Materials and Methods

2.1. Study population

We conducted a crossover-crossback prospective study in adult men with IBD (Mesalamine And Reproductive health Study (MARS)). Eligibility for participants in the MARS was 18 to 55 years of age and taking oral mesalamine for at least the past three months. All men must have had a mild IBD score on the simple clinical colitis activity index[45] (five or less for UC) or Harvey-Bradshaw index[46] (four or less for CD). Men were recruited from gastroenterology clinics at three Boston hospitals; Beth Israel Deaconess Medical Center

(BIMC), Brigham and Women's Hospital (BWH) and Massachusetts General Hospital (MGH) from October 2010 through October 2015. MARS was approved by the institutional review boards (IRBs) of Harvard T.H. Chan School of Public Health, BIMC, BWH and MGH. All men signed informed consents.

2.2. Study design

Eligible men were invited to participate in up to six visits; to account for within-person variability in semen parameters. Each man was asked to participate in two visits at baseline, after crossover and after crossback. At each of the two baseline visits, participants provided semen, urine and blood samples collected two weeks apart (visits 1 and 2). Men were then asked to crossover to another formulation of mesalamine; men who were taking non-DBP mesalamine at baseline crossed-over to DBP-containing mesalamine medication (i.e., Asacol®) and vice versa for men prescribed DBP-containing mesalamine at baseline crossed-over to non-DBP mesalamine. After crossover for four months, two sets of semen, urine and blood samples were collected two weeks apart (visits 3 and 4). Participants were then asked to crossback for four months to their original mesalamine medications after which two sets of semen, urine and blood samples were collected two weeks apart (visits 5 and 6) (Figure-1).

In brief, men who were prescribed non-DBP mesalamine with background exposure from other sources crossed-over to high-DBP mesalamine then crossed-back to non-DBP mesalamine (B_1HB_2-arm: $Background_1$-$High$-$Background_2$). Men who were prescribed high-DBP mesalamine crossed-over to non-DBP mesalamine then crossed-back to high-DBP mesalamine (H_1BH_2-arm: $High_1$-$Background$-$High_2$). The ‘wash-in’ and ‘wash-out’ periods between crossover and crossback were four months to extend beyond the 70 days average period of spermatogenesis[47]. Questionnaires about lifestyle factors, medical history and ejaculation abstinence time were administered at every visit.

Among the 47 men in the H_1BH_2-arm, 13 men participated only in a short protocol defined as up to four visits. These 13 men did not want to change medication but because the manufacturer was reformulating Asacol® to remove DBP, we anticipated that they would be ‘switched’ to a non-DBP mesalamine when this came to market. Warner Chilcott discontinued Asacol® in 2013 and introduced Delzicol® (non-DBP mesalamine) to the market. However, Asacol®HD (containing DBP) remained on the market. For 10 of the 13 men, their physician changed their medications to Asacol®HD, thus they never crossed-over to non-DBP mesalamine and only contributed to the baseline visits while three men changed medication to Delzicol® i.e. crossed-over. However, by design none of the men in the short protocol crossed-back.

Men were asked to abstain from ejaculation for 2-5 days before providing semen samples, collected by masturbation at the MGH andrology laboratory into a sterile container and analyzed using standardized clinical protocols and quality control (QC) as described previously[22,48]. Briefly, semen was allowed to liquefy at 37° C for 20 minutes. The physical properties of the semen were reported, including the sample volume, pH, color and viscosity. Ejaculate volume was measured using a graduated serological pipet. Sperm concentration and motility were assessed with a computer-aided semen analysis (CASA; 10HTM-IVOS,

Hamilton-Thorne Research, Beverly, MA) which is used for routine diagnostic applications[49]. For semen concentration and motility assessment, 5 μl of semen from each sample was placed into a pre-warmed (37°C) Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). Minimum of 200 sperm cells from at least four different fields were analyzed from each specimen. Sperm motility was expressed as the percentage of total motile (progressive + non-progressive) and percentage of progressive motile spermatozoa and defined as World Health Organization (WHO-2010) grade “a” sperm (rapidly progressive with a velocity ≥25 μm/sec) plus “b” grade sperm (slow/sluggish progressive with a velocity of ≥5 μm/sec but < 25 μm/sec)[49]. Sperm morphology was measured on two slides for each specimen (with at least 200 cells assessed per slide) with a microscope using an oil-immersion 100× objective (Nikon, Tokyo, Japan). Sperm morphology was assessed using Kruger's strict criteria [50]. To minimize variability, the laboratory followed a constant analysis set-up including play-back and QC plots if sperm counts < 20 or > 50 million/mL. Unusual values were re-evaluated. In addition to a quarterly competency technicians' evaluation, an outside evaluator performed proficiency testing, biannually. The technicians performing the semen assays were blinded to the study group.

2.3. Statistical Analysis

We created a six-level indicator variable cross-classifying each observation according to medication type (high-DBP or non-DBP mesalamine) and period (baseline, crossover and crossback) for the two study arms (H_1BH_2 and B_1HB_2). We considered for analysis, ejaculate volume, sperm concentration, motility (% motility and % progressive motility) and morphology. We also calculated total sperm count, motile sperm count, and morphologically normal sperm count. We used natural log-transformed sperm concentration, total sperm count, motile sperm count and morphologically normal count to satisfy model's normality assumption.

We performed descriptive statistics for participants' baseline and time-varying characteristics in both study arms. We also tested for any differences between the two arms using Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables.

We used linear mixed effects models (LMEM) with a random intercept to account for within-person correlation among longitudinal measures of a given outcome arising from person-to-person heterogeneity across the study population. We estimated the DBP mesalamine crossover, crossback and carryover effects on semen parameters as absolute mean differences for the non-transformed semen parameters and as percent change for the log-transformed parameters.

Selection of covariates was based on directed acyclic graphs (Supplementary Figure-2) and statistical considerations (>10 % change in the effect estimate). The final model included abstinence time (< 2 days, 2≤ days <4 and ≥ 4 days), age at baseline (continuous), the season of the sample collection (warm and cold) and duration on high-DBP containing mesalamine at baseline (continuous in years). There were two samples missing abstinence times and we imputed the category of abstinence time based on the other semen samples provided by the same man (both men had the same abstinence time category for all their other semen samples).

In our analysis, we also considered that the duration of IBD as a chronic inflammatory condition may affect semen quality. In preliminary models, we considered adjustment for duration of IBD, severity score of the disease, IBD condition (UC/CD), race, history of reproductive diseases or surgeries, BMI and smoking but they were not confounders and thus not retained in the final models.

We assessed model sensitivity to the covariance structure implied by the random intercept model, using empirical standard errors that are robust to misspecification of the covariance structure[51]. As a sensitivity analysis, we further adjusted for body mass index (BMI <18.5, 18.5 ≤BMI<25, 25≤BMI<30 or ≥30)[52] and smoking status (never, former and current) in addition to the above covariates. As a secondary analysis, we applied fixed effect models (FEM) that, rather than assume a random distribution for the person-specific intercepts, estimate these terms as ordinary fixed regression coefficients in the model. These models isolate the longitudinal within-person effect of exposure, adjusted for the same covariates as above[51].

Although not a primary aim of our study, we explored the cross-sectional differences between the two arms at baseline by restricting the data to the average of the first two visits, applying linear regression adjusted for the covariates above. We further explored the effect of the duration under high-DBP mesalamine at enrollment on baseline semen parameters in the H_1BH_2-arm.

We conducted all statistical analyses using SAS version 9.4 (SAS Institute Inc., Cary, NC) and considered two-sided alpha <0.05 as statistically significant.

3. Results

Out of 215 confirmed eligible men, 73 agreed to participate (34%) (Supplementary Figure-1) and provided 332 semen samples with an average of 4.6 semen samples per man [range: 1 to 6]. The B_1HB_2-arm included 26 men (133 semen samples) with an average 5.1 samples per man. The H_1BH_2-arm included 47 men (199 samples) with an average of 4.9 samples per man. The two study arms had comparable baseline fixed and time-varying characteristics (Table-1). The semen parameter distributions are presented (Table-2). Men enrolled in the short protocol provided 31 samples (26 baseline and 5 after crossover samples).

Among the 60 full-protocol enrolled men, 51 (85%) men crossed-over medications, 23 (89%) in B_1HB_2-arm and 28 (82%) in H_1BH_2-arm; 43 (72%) men crossed-back medications, 19 (73%) in B_1HB_2-arm and 24 (71%) in H_1BH_2-arm. During follow-up, 9 (15%) men dropped out, 3 (12%) in B_1HB_2-arm and 6 (18%) in H_1BH_2-arm (p-value= 0.64). The median period between the second baseline visit (visit-2) and collection of crossover semen samples at visit-3 and similarly between visit-4 and visit-5 for crossback was 119 days. The median time period between visits on the same medication (visits-1&2), (visits-3&4) and (visits-5&6) was 16 days. The H_1BH_2-arm men were on their high-DBP mesalamine medication at entry for median 2.9 years and up to 24.1 years. The B_1HB_2-arm

men were on their non-DBP mesalamine medication at baseline for median 1.0 year and up to 10.3 years.

None of the semen samples were azoospermic (Table-2); 188 (55%) samples were above the reference limits of the World Health Organization (WHO-2010)[49] with regard to sperm concentration, total count, percent motile and percent normal morphology.

Although not statistically significant, in a cross-sectional analysis of the baseline visits, sperm motility and morphology parameters for men in H_1BH_2-arm were higher than among men in B_1HB_2-arm (Figure-2). However, among men in the H_1BH_2-arm at the baseline, there was a negative association between the number of years on high-DBP containing mesalamine and semen parameters, steeper in the early years as compared to the later years (data not shown).

In the adjusted analyses (Table-3 & Figure-2), on average among men in the B_1HB_2-arm who were newly-exposed to high-DBP mesalamine after crossover had decreased (though non-statistically significant) semen parameters, apart from ejaculate volume and % normal morphology. Their % total sperm motility decreased by 4.19 (95% confidence interval (CI): -9.25, 0.86), % progressive sperm motility by 1.76 (CI: -5.29, 1.78) and motile sperm count by 13.7% (CI: -35.7%, 15.8). After crossback to their non-DBP mesalamine, all semen parameters (except sperm concentration) continued to decrease *further*. The % total sperm motility further decreased by 3.41 (CI: -8.69, 1.86), % progressive sperm motility by 2.47 (CI: -6.16, 1.22) and motile sperm count by 14.3% (CI: -36.9%, 16.5%). The *cumulative carryover* effect of high-DBP exposure (i.e., crossback compared to baseline) was a decrease in *all* semen parameters; % total sperm motility decreased by 7.61 (CI: -13.1, -2.15), % progressive sperm motility by 4.23 (CI: -8.05, -0.4) and motile sperm count by 26.0% (CI: -46.2%, 1.7%). In contrast, in the H_1BH_2-arm, semen parameters did non-significantly change after crossover or crossback.

We found consistent results from the sensitivity analyses using empirical standard errors in LMEMs and after further adjustment for BMI and smoking (data not shown) and from FEMs which fully adjust for both observed and unobserved factors that do not change within a person across visits[51] (Supplementary Table-1).

4. Discussion

In our crossover-crossback study, men who were on non-DBP mesalamine at baseline (B_1HB_2-arm) and newly exposed for four months (crossover period) to high-DBP had lower semen parameters, primarily % total sperm motility, % progressive sperm motility and motile sperm count. This decrease was more pronounced and became statistically significant even after crossback for four months to their original non-DBP mesalamine. Thus, the effect of high-DBP on sperm motility parameters was not reversible after four months. Among men who were on high-DBP at baseline (H_1BH_2-arm), there was no statistically significant change in semen parameters after crossover to non-DBP mesalamine or crossback to their original high-DBP mesalamine. Therefore, in both arms, the four month wash-out period was *not* long enough to reverse the effect of high-DBP exposure.

In the cross-sectional analysis of baseline semen characteristics, unexpectedly, men who were on high-DBP mesalamine at baseline (H_1BH_2-arm) had non-significantly higher percent sperm motility and normal morphology than men who were on non-DBP mesalamine at baseline (B_1HB_2-arm). However, among men in the H_1BH_2-arm, there was a downward trend in all semen parameters associated with increased duration on high-DBP mesalamine. This negative association with years on high-DBP mesalamine among men in H_1BH_2-arm argues against a 'protective' effect of high-DBP and suggests chance sampling variability at baseline due to non-randomization at the baseline and cautions against cross-sectional analyses. These results highlight the power of the study that by design removes subject to subject variability from the comparison of interest, thus avoiding the purely cross-sectional analysis that is likely to suffer from this random variability.

There is an accumulating literature on the association of background low environmental exposure to phthalates with semen quality. A recent meta-analysis that included 14 publications on phthalates and semen quality concluded that higher urinary MBP concentrations were associated with reduced sperm concentration and straight line velocity[53]. All 14 publications were cross-sectional with 11 conducted in infertility clinics. Two recent US studies on fertile men not included in the meta-analysis[29,30] did not find significant associations. Our study differs in many respects from the earlier cross-sectional studies. We used a more powerful crossover-crossback prospective design and therefore had repeated semen samples from men during both high and background DBP exposure. This allowed for within-person comparisons and was by design adjusted for non-time varying covariates. Furthermore, our design allowed us to determine the carryover effect that was not observable in the earlier cross-sectional designs.

Experimental studies, primarily during postnatal exposure[19,54], have shown decreased serum testosterone concentration and increased FSH and LH concentrations in male rats exposed to BBzP which suggested tubular atrophy and loss of the germinal epithelium as a result of Sertoli cell injury.

Postnatal exposure to other anti-androgenic phthalates (di(2-ethylhexyl)phthalate) caused germ cell apoptosis with increased membrane localization of Fas[55,56] or germ cells detachment from the seminiferous epithelium probably due to cellular adhesion loss between Sertoli cells[57]. Also, studies suggested that in older animals DBP preferentially targets Sertoli cells [58,59].

Studies during fetal development also have shown that DBP is anti-androgenic. It decreases Leydig cell testosterone production by interfering with steroidogenesis by down-regulating gene and/or protein expression important in the steroidogenic pathway, cholesterol transport and metabolism[60-66], while increasing oxidative stress along with reactive oxygen species[67].

Several studies have identified numerous medications as a source of phthalate exposure[34-36,39,40,43] Phthalate levels in medications may not be openly displayed, due to proprietary formulations[36]. Medications with phthalates in the coating include mesalamine, didanosine, omeprazole, theophylline[34] and other medications including over-the-counter

preparations[31,35,36]. Our research and others have shown that mesalamine medications with DBP contribute to high-DBP exposure as measured by urinary MBP concentrations[34,39,40].

The human daily DBP intake from the maximum recommended dose of Asacol® is 21 mg[42,68]. Therefore, the estimated maximum daily DBP intake is 350 μg/kg/day based upon 60 kg body weight. Similarly, the human DBP daily intake from the maximum recommended dose of Asacol®HD is 48 mg[69] with the estimated maximum daily DBP intake of 800 μg/kg/day. These intakes are orders of magnitude higher than the estimated average DBP human intake (0.84-5.22 μg/kg/day) [70], several times higher than the US Environmental Protection Agency (EPA) reference dose (100 μg/kg/day)[39] and orders of magnitude higher than the European tolerable daily intake (10 μg/kg/day)[71].

We considered measuring urinary MBP concentrations in the urine sample collected at each study visit. However, due to the unique crossover-crossback design and the very high exposure from Asacol® (1,000 fold higher than urinary levels when not taking Asacol®), we concluded that urinary MBP concentrations would not add significant information beyond using medication as an indicator of high and low DBP exposure. We offer three primary justifications for not measuring urinary MBP concentrations. First, clear documentation that Asacol® contributes to very high exposure to DBP [31,34-36,39,40,42,68,69]. Second, the study is considered interventional in which we assigned participants to the different medications. Therefore, the data was appropriately analyzed based on the assigned medication (as an intention to treat analysis) for our interventional design. Third, from a statistical perspective, continuous urinary data would essentially dichotomize exposure given the very large differences in urinary MBP when taking Asacol® and we would use the same modeling approach. Specifically, the estimates of exposure response, even if we had the continuous urinary MBP concentrations, would have been completely dominated by the medication type (1,000 times difference when taking Asacol®) with no overlap in urinary MBP concentrations between those visits when taking Asacol® and those visits when not taking Asacol®.

Our study had several potential limitations including non-randomization of mesalamine at baseline which may have resulted in chance sampling variability at the baseline. However, there is no reason to believe that the prescribing practices of physicians were associated with the presence or absence of DBP enteric coating. To assign high-DBP exposure, we relied on self-reported use of mesalamine medications as prescribed over the study period[72] rather than measuring urinary MBP concentrations which only represent recent exposure (i.e., past several hours) due to the short DBP half-life. Although the sample size for this study was not very large due to the innovative study and length of participation, the power of the study came from the unique design that removes subject to subject variability from the comparison of interest avoiding the purely cross-sectional analyses that likely to suffer from this random variability. We also conducted a post-hoc power analysis and the study had a sufficient number of participants to provide 80% power for detecting at least 23% decrease in sperm concentration and an absolute mean difference of at least 0.4 ml of ejaculate volume, 1% fewer morphologically normal sperm, 6% fewer motile sperm and 4% fewer progressive motile sperm, which is reasonable for a study of this design. Finally, although semen quality

is an imperfect predictor of fertility[73], semen analysis is considered the cornerstone of the laboratory evaluation for infertile men[74].

Our study had several important strengths. We implemented a unique innovative study design that is rarely performed in environmental epidemiology. We were able to compare, within the same men, their semen parameters during periods of high-DBP to background-DBP exposure and vice versa accounting for confounding by measured and unmeasured non-time-varying characteristics[75]. This is a major strength as compared to previous cross-sectional studies. We were able to explore whether there was a carryover effect of high-DBP exposure. Our study was not restricted to infertile men as most of the previous literature. The means of all semen parameters were comparable to those for fertile US adult men[4]. Although there may be a concern of generalizing results from men with IBD, which has a significantly increasing temporal trend in incidence worldwide especially in industrialized countries[76], these results raise concern about high-DBP exposure and poor semen quality. In addition, there is *no* evidence that IBD or mesalamine is linked to male infertility[77-81]. Asacol® and its mesalamine alternatives for treating IBD have the same active ingredients, therefore confounding by indication was unlikely.

Compared to environmental background exposure, we were able to explore high-DBP exposure (1,000 times background). A recent gastroenterology consensus report has recommended switching to non-DBP mesalamine in pregnant women or who may contemplate pregnancy[33], without any recommendations for men of reproductive age. Therefore, our results will be informative for future recommendations of use of non-DBP mesalamine formulations among men. To our knowledge, no previous human studies investigated effects of such *high*-DBP exposure on semen parameters.

5. Conclusions

High-DBP exposure from Asacol® (mesalamine) had adverse effects on semen parameters in adult men, with the most robust effects on sperm motility. Most importantly, the effect of DBP carried-over even after removing the exposure suggesting that high-DBP had longer term effects on spermatogenesis. Further studies are needed to explore whether the carryover effect is potentially reversible after a wash-out period longer than four months. Finally, studies of the effect of high-DBP exposure on sperm epigenetics and reproductive and non-reproductive hormones would add additional insights. Attention should be given to such high-exposure sources and our study and further research will provide more guidance on further regulation of DBP coating of medications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DBP	dibutyl phthalate
FDA	Food and Drug Administration
5-ASA	5-aminosalicylic acid
IBD	Inflammatory Bowel Disease
UC	ulcerative colitis
CD	Crohn's disease
MBP	monobutyl phthalate
NHANES	National Health and Nutrition Examination Survey
MARS	Mesalamine And Reproductive health Study
BIMC	Beth Israel Deaconess Medical Center
BWH	Brigham and Women's Hospital
MGH	Massachusetts General Hospital
IRB	institutional review board
B_1HB_2	$Background_1$-High-$Background_2$ DBP exposure
H_1BH_2	$High_1$-Background-$High_2$ DBP exposure
QC	quality control

BMI	body mass index
Kg	Kilogram
m	meter
SD	standard deviation
LMEM	mixed effects models
FEM	fixed effect models
N	number of men
WHO	World Health Organization
EPA	Environmental Protection Agency

Highlights

- Mesalamine with coating containing dibutyl phthalate(DBP) impart high-DBP exposure.
- Men newly exposed to DBP containing mesalamine had a reduction in semen quality.
- The most robust effect of high-DBP exposure from mesalamine was on sperm motility.
- High-DBP effect on sperm motility persisted for 4 months after removing exposure.

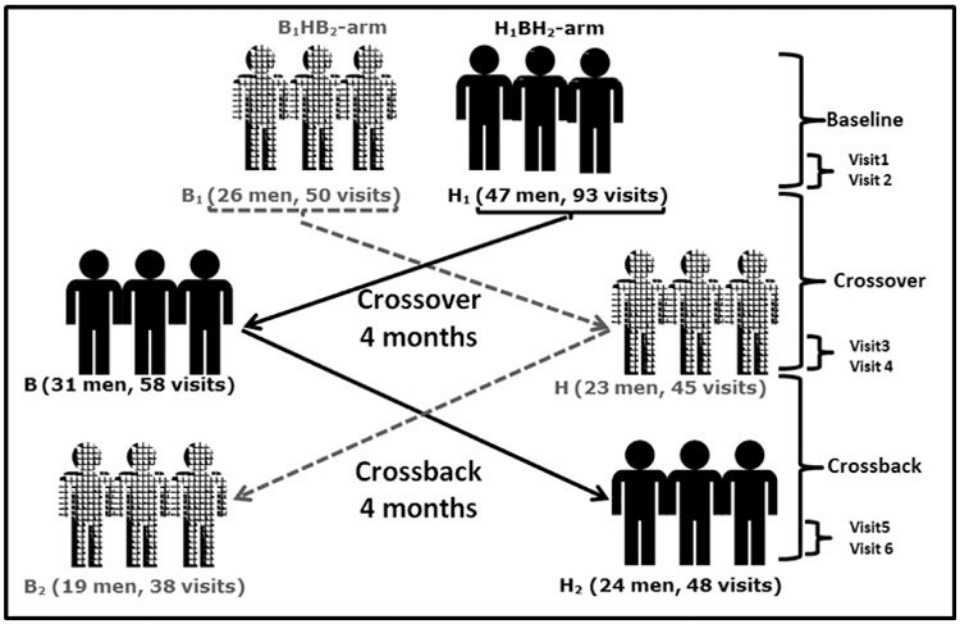
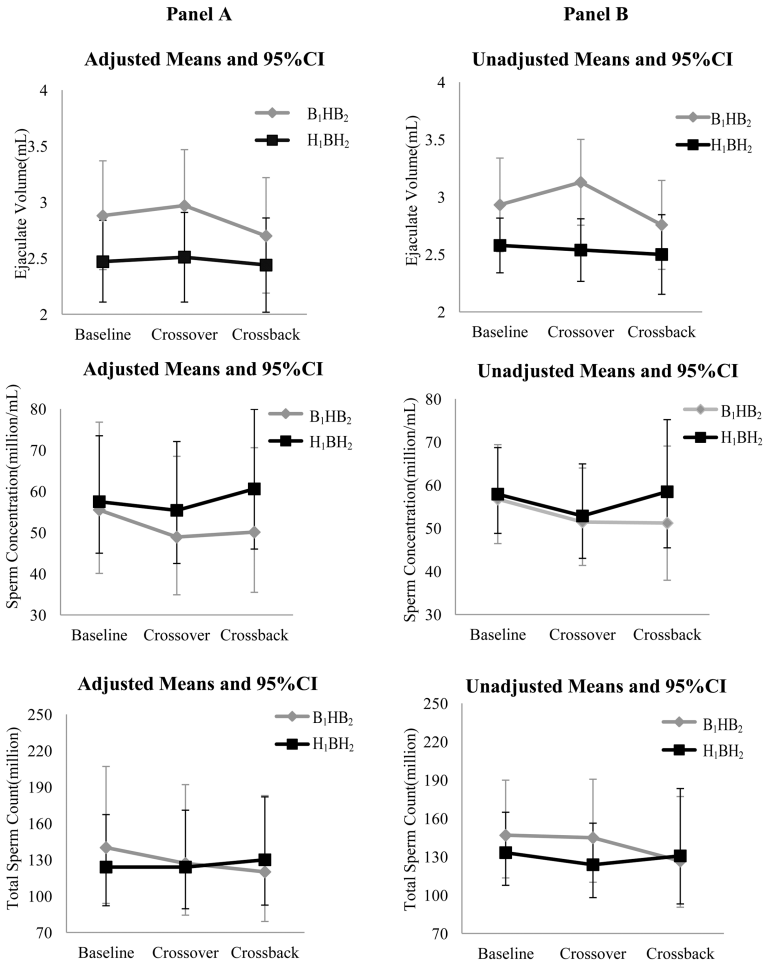
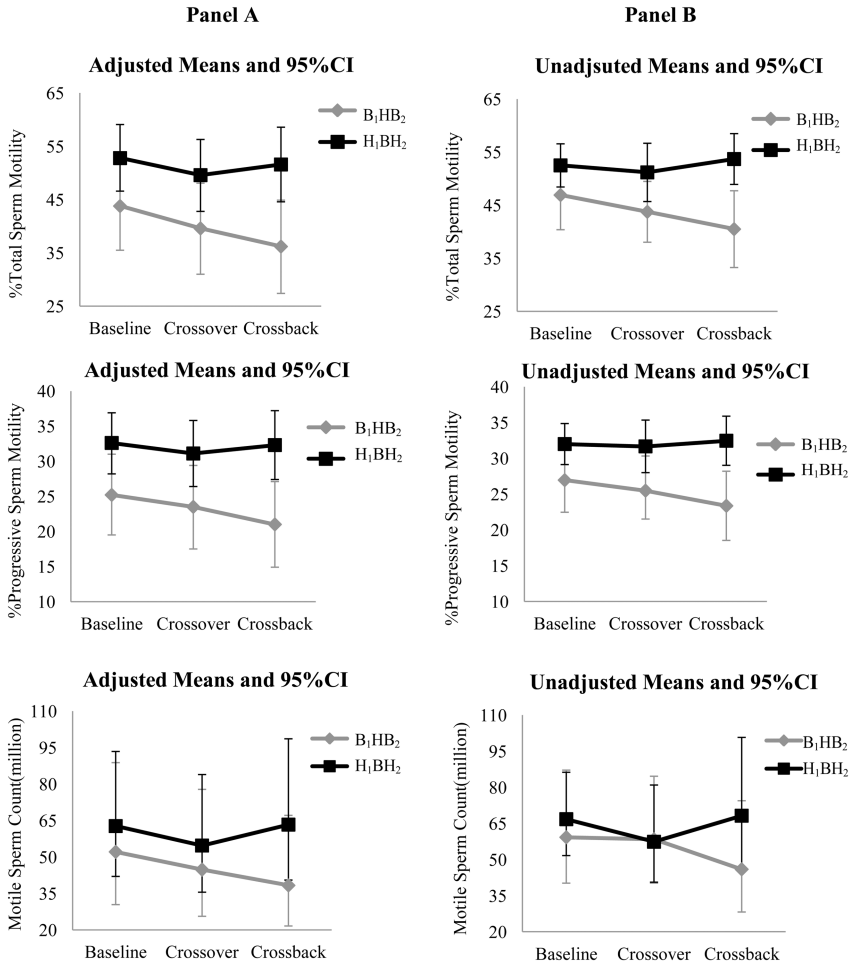
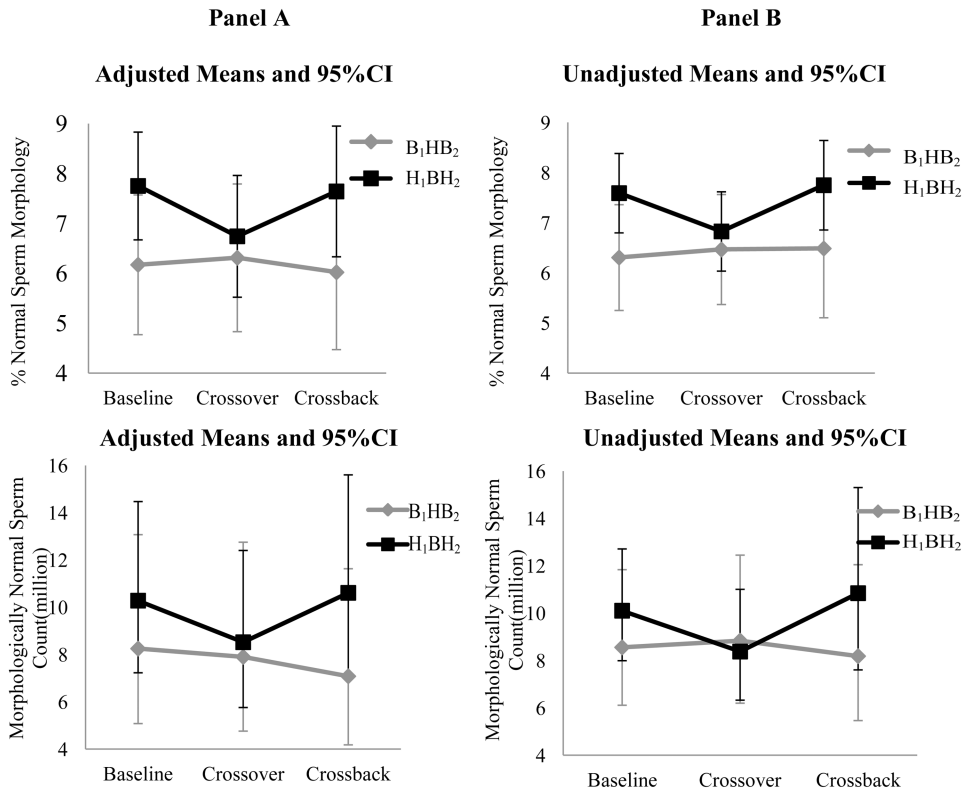


Figure 1. Mesalamine And Reproductive health Study (MARS) Design

B_1HB_2-arm: B_1 represents background-exposure at baseline, **H** represents high-DBP exposure after crossover and **B_2** represents background-exposure after crossback; included 26 men taking mesalamine that did not contain DBP at baseline. **H_1BH_2-arm: H_1** represents high-DBP exposure at baseline, **B** represents background-exposure after crossover and **H_2** represents high-DBP exposure after crossback; included 47 men taking mesalamine-containing DBP at baseline. **Abbreviations: MARS**, Mesalamine And Reproductive health Study; **DBP,** dibutyl phthalate.







Figures 2. Adjusted and unadjusted means (95%CI) of semen parameters at baseline, crossover and crossback for the 2 arms-MARS

Panel A shows the adjusted means and 95% CI and **Panel B** shows the unadjusted means and 95% CI [adjusted for abstinence time (categorical), age (continuous), season (warm versus cold) and duration on DBP-containing mesalamine medication at baseline (in years)]. For the log-transformed outcomes (sperm concentration, total sperm count, motile sperm count and morphologically normal sperm count), the geometric means and 95% CI are presented. **B_1HB_2-arm, B_1** represents background-exposure at baseline, **H** represents high-DBP exposure after crossover and **B_2** represents background-exposure after crossback; included 26 men taking mesalamine that did not contain DBP at baseline. **H_1BH_2-arm, H_1** represents high-DBP exposure at baseline, **B represents** background-exposure after crossover and **H_2** represents high-DBP exposure after crossback; included 47 men taking mesalamine-containing DBP at baseline. **Abbreviations: MARS**, Mesalamine And Reproductive health Study; **95% CI**, 95% Confidence Interval; **DBP**, dibutyl phthalate.

Table 1
Demographics of 73 men contributing 332 visits in the MARS Study by arm

	B_1HB_2-arm (26 men, 133 visits)	H_1BH_2-arm (47 men, 199 visits)	Total (73 men, 332 visits)	
	N(%)/Mean (SD),[Range]	N(%)/Mean (SD),[Range]	N(%)/Mean (SD),[Range]	P-value[b]
Baseline characteristics (at Visit 1), # men (N)[a]				
Age (Years)	33.9 (8.97), [20.2, 54.6]	34.9 (9.66), [19.6, 55.7]	34.6 (9.37), [19.6, 55.7]	0.82
Race				0.27
Caucasian N (%)	25 (96%)	38 (81%)	63 (86%)	-
Black/African American	1 (4%)	2 (4%)	3 (4%)	-
Asian	0	4 (9%)	4 (6%)	-
Other	0	3 (6%)	3 (4%)	-
BMI (Kg/m2)	25.3 (3.56), [19.4, 32.7]	26.4 (6.39), [19.5, 54.0]	26.0 (5.54), [19.4, 54.0]	0.88
BMI-categories N (%)				0.99
Normal weight (18.5 ≤BMI<25)	14 (54%)	24 (51%)	38 (52%)	-
Overweight (25≤BMI<30)	9 (35%)	16 (34%)	25 (34%)	-
Obese (BMI≥30)	3 (11%)	7 (15%)	10 (14%)	-
Education N (%)				0.82
Below college	3 (14%)	6 (14%)	9 (14%)	-
College graduate	10 (48%)	16 (38%)	26 (41%)	-
Graduate degree	8 (38%)	20 (48%)	28 (45%)	-
Smoking status N (%)				0.009
Never smoker	22 (85%)	36 (77%)	58 (80%)	-
Former smoker	1 (4%)	11 (23%)	12 (16%)	-
Current smoker	3 (11%)	0 (0%)	3 (4%)	-
Warm season at baseline[c]	5 (19%)	20 (43%)	25 (34%)	0.07
IBD diagnosis N (%)				0.61
Ulcerative colitis	16 (62%)	32 (68%)	48 (66%)	-
Crohn's disease	10 (38%)	15 (32%)	25 (34%)	-
IBD score[d]	1.65 (1.38), [0, 4]	1.19 (1.35), [0, 5]	1.36 (1.37), [0, 5]	0.13
Time-varying characteristics, #visits[a]				
Warm season [c]	61 (46%)	92 (46%)	153 (46%)	0.99
Ejaculation abstinence time				0.84
< 2 Days	10 (8%)	19 (10%)	29 (9%)	-
2≤Days<4	115 (86%)	169 (85%)	284 (85%)	-
≥4 Days	8 (6%)	11 (5%)	19 (6%)	-

B_1HB_2-arm, B_1 represents background-exposure at baseline, **H** represents high-DBP exposure after crossover and **B_2** represents background-exposure after crossback; included 26 men taking mesalamine that did not contain DBP at baseline; 16 men (62%) on Lialda®, 8 men (31%) on Pentasa®, one man (4%) on Apriso® and one man (4%) was not on mesalamine medication at the time of recruitment.

H_1BH_2-arm, H_1 represents high-DBP exposure at baseline, **B** represents background-exposure after crossover and **H_2** represents high-DBP exposure after crossback; included 47 men taking mesalamine-containing DBP at baseline; 24 men (51%) on Asacol® and 23 men (49%) on Asacol®HD.

[a]:N (%) for categorical/binary variables and mean (SD), [Range] for continuous variables

[b]:P-values are based on Fisher exact test for categorical variables and Kruskal Wallis test for continuous variables.

[c]:Season of sample collection, Warm: April Through September
10 men missing information on education; for 2 semen samples missing abstinence times, we imputed values for their categories based on the other samples given by the same man (both men had the same abstinence time category for all their other samples).

[d]:BD score: simple clinical colitis activity index, includes bowel frequency and urgency, presence of blood in the stool and general wellbeing. Mild IBD score: 5 or less for UC and 4 or less for CD.

Abbreviations: MARS, Mesalamine And Reproductive health Study; BMI, body mass index; Kg, Kilogram; m, meter; SD, standard deviation; IBD, Inflammatory Bowel Disease; UC, ulcerative colitis; CD, Crohn's disease; N, number of men; DBP, dibutyl phthalate; B_1HB_2, Background$_1$-High-Background$_2$ DBP exposure; H_1BH_2, High$_1$-Background-High$_2$ DBP exposure.

Table 2
Semen quality parameters for 73 men (332 semen samples) in the MARS study

Semen quality parameters	Mean (SD)	Median [Range]	n (%) < WHO lower referencelimits[a]
Ejaculate volume (mL)	2.71 (1.21)	2.5 [0.23, 7.10]	45 (14%)
Sperm concentration (million/mL)	74.4 (68.2)	59.8 [2.7, 670]	22 (7%)
Total sperm count (million)	192 (151)	168 [0.62, 828]	35 (11%)
%Total sperm motility	49.0 (20.6)	51.0 [0, 88]	111 (33%)
%Progressive sperm motility	29.4 (14.2)	30.0 [0, 77]	177 (53%)
Motile sperm count (million)[b]	111 (115)	74.3 [0, 621]	-
% Normal sperm morphology	7.01 (3.61)	7 [0, 23]	93 (28%)
Morphologically normal sperm count (million)[c]	15.6 (17.7)	9.63 [0, 131]	-

[a]Binary semen quality parameters less than WHO lower reference limits (2010)[49]**:** ejaculate volume<15 ml; sperm concentration <15 million/ml; total sperm count< 39 million; total motile sperm<40%; progressive motile sperm <32% and normal sperm morphology< 4% using “strict” Tygerberg method; **n:** # semen samples. There were 2 samples missing morphology **(n=330).**

[b]Motile count= Total Sperm Count × % Motile

[c]Morphologically normal count =Total Sperm Count × % normal morphology

Abbreviations: MARS, Mesalamine And Reproductive health Study; WHO: World health organization.

Table-3.A

Adjusted effect (95%CI) of crossover, crossback and carryover on semen parameters among men starting on mesalamine medication not containing dibutyl phthalate (B_1HB_2-arm)[a]

B_1HB_2-arm [Background (B_1)- High (H)- Background (B_2) dibutyl phthalate exposure]: 26 men, 133 semen samples

	Fitted Means (95%CI)			Comparisons					
Semen quality parameter	Baseline (B_1)	Crossover (H)	Crossback (B_2)	Crossover effect (95%CI) H-B1	P value	Crossback effect (95%CI) B_2-H	P value	Carryover effect (95%CI) B_2-B_1	P value
Ejaculate volume (mL)	2.88 (2.4, 3.37)	2.97 (2.46, 3.47)	2.70 (2.19, 3.22)	0.09 (-0.22, 0.39)	0.57	-0.27 (-0.58, 0.05)	0.10	-0.18 (-0.51, 0.15)	0.29
Sperm concentration (million/mL)[b]	55.5 (40.1, 76.8)	48.9 (34.9, 68.5)	50.1 (35.5, 70.6)	-11.9% (-27.6%, 7.23%)	0.21	2.36% (-16.6%, 25.6%)	0.82	-9.8% -27.0%, 11.5%)	0.34
Total sperm count (million)[b]	140 (94, 207)	127 (84.3, 192)	120 (79.1, 183)	-8.96% (-28.7%, 16.2%)	0.45	-5.45% (-26.7%, 22.0%)	0.67	-13.9% (-33.9%, 12.1%)	0.26
%Total sperm motility	43.8 (35.5, 52)	39.6 (31, 48.1)	36.2 (27.4, 44.9)	-4.19 (-9.25, 0.86)	0.10	-3.41 (-8.69, 1.86)	0.20	-7.61 (-13.1, -2.15)	0.007
%progressive sperm motility	25.2 (19.5, 31)	23.5 (17.5, 29.4)	21.0 (14.9, 27.1)	-1.76 (-5.29, 1.78)	0.33	-2.47 (-6.16, 1.22)	0.19	-4.23 (-8.05, -0.4)	0.03
Motile sperm count (million)[b]	52.1 (30.4, 88.8)	44.8 (25.6, 77.8)	38.3 (21.6, 67.1)	-13.7% (-35.7%, 15.8%)	0.32	-14.3% (-36.9%, 16.5%)	0.32	-26.0% (-46.2%, 1.7%)	0.06
%Normal sperm morphology	6.17 (4.77, 7.57)	6.31 (4.83, 7.79)	6.02 (4.47, 7.56)	15.8%) 0.13 (-1.06, 1.33)	0.82	-0.29 (-1.54, 0.96)	0.65	-0.16 (-1.45, 1.13)	0.81
Morphologically normal sperm count (million)[b]	8.25 (5.08, 13.1)	7.90 (4.76, 12.8)	7.08 (4.18, 11.6)	-3.78% (-25.9%, 24.9%)	0.77	-9.14% (-30.8%, 19.4%)	0.49	-12.6% (-34.2%, 16.1%)	0.35

Table-3.B

Adjusted effect (95%CI) of crossover, crossback and carryover on semen parameters among men starting on mesalamine medication containing dibutyl phthalate (H_1BH_2-arm)[a]

H_1BH_2-arm[High (H_1)-Background (B) - High (H_2)] dibutyl phthalate exposure]: 47 men, 199 semen samples									
	Fitted Means (95%CI)			Comparisons					
Semen quality parameter	Baseline (H_1)	Crossover (B)	Crossback (H_2)	Crossover Effect (95%CI) B-H_1	P value	Crossback Effect (95%CI) H_2-B	P value	Carryover Effect (95%CI) H_2-H_1	P value
Ejaculate volume (mL)	2.47 (2.11, 2.84)	2.51 (2.11, 2.91)	2.44 (2.02, 2.86)	0.04 (-0.22, 0.29)	0.77	-0.07 (-0.35, 0.21)	0.62	-0.03 (-0.32, 0.25)	0.81
Sperm concentration (million/mL)[b]	57.5 (45, 73.5)	55.4 (42.5, 72.1)	60.6 (46, 79.9)	-3.7% (-18.4%, 13.6%)	0.65	9.49% (-8.77%, 31.4%)	0.33	5.43% (-12.1%, 26.5%)	0.57
Total sperm count (million)[b]	124 (92.1, 167)	124 (89.6, 171)	130 (92.6, 182)	-0.31% (-18.9%, 22.5%)	0.98	4.86% (-16.5%, 31.6%)	0.68	4.54% (-16.7%, 31.1%)	0.70
%Total sperm motility	52.8 (46.6, 59.1)	49.6 (42.8, 56.3)	51.6 (44.6, 58.6)	-3.28 (-7.54, 0.97)	0.13	2.02 (-2.68, 6.72)	0.40	-1.26 (-5.95, 3.42)	0.60
%progressive sperm motility	32.6 (28.2, 36.9)	31.1 (26.4, 35.8)	32.3 (27.4, 37.2)	-1.49 (-4.46, 1.49)	0.33	1.2 (-2.09, 4.49)	0.47	-0.29 (-3.56, 2.99)	0.86
Motile sperm count (million)[b]	62.7 (42, 93.4)	54.7 (35.5, 83.9)	63.3 (40.5, 98.6)	-12.6% (-31.8%, 12.1%)	0.29	15.4%(-12.2%, 51.8%)	0.30	0.91% (-23.2%, 32.7%)	0.95
%Normal sperm morphology	7.75 (6.67, 8.83)	6.74 (5.52, 7.96)	7.64 (6.33, 8.95)	-1.01 (-2.0, -0.02)	0.04	0.9 (-0.21, 2.01)	0.11	-0.11(-1.19, 0.97)	0.84
Morphologically normal sperm count (millionb)[b]	10.3 (7.23, 14.5)	8.52 (5.76, 12.4)	10.6 (7.12, 15.6)	-15.6% (-32.2%, 5.05%)	0.13	22.0% (-4.23%, 55.3%)	0.11	2.95% (-19.1%, 31%)	0.81

[a]:Adjusted for abstinence time (categorical: < 2 days, 2≤days<4 and ≥ 4 days), age (continuous), season (warm (April through September) vs cold) and period (# years on DBP-containing mesalamine medication at baseline) in the mixed effect model.

[b]:Sperm concentration, total sperm count, motile sperm count and morphologically normal sperm count are presented as percentage change by exponentiating the log-transformed Beta coefficient. The sum of the percent changes for crossover and cross back do not equal the total carryover effect for the log-transformed variables because they are on the multiplicative scale. For the variables that are not log-transformed, the sum of the percent changes for crossover and cross back equal the total carryover effect as they represent the absolute difference on the additive scale

Motile sperm count (million) and morphologically normal sperm count (million) were log-transformed after adding 1 due to few zero values, they were back transformed and 1 was subtracted from the means.

B_1HB_2-arm: B_1 represents background low-DBP exposure at baseline, H represents high-DBP exposure after crossover and B_2 represents background low-exposure after crossback.

H_1BH_2-arm: H_1 represents high-DBP at baseline, B represents background low-DBP exposure after crossover and **H_2** represents high-DBP after crossback.

Note: 2 samples missing morphology; negative sign means a decrease or % decrease for the log-transformed variables compared to the measure in the previous period.

Abbreviations: DBP, dibutyl phthalate; B_1HB_2: Background$_1$-High-Background$_2$ DBP exposure; H_1BH_2; High$_1$-Background-High$_2$ DBP exposure; 95%CI, 95% Confidence Interval.