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Transcript Profiling in the Testes and Prostates of Postnatal Day 30 Sprague-Dawley Rats Exposed Perinatally to 2-Hydroxy-4-methoxybenzophenone

Noriko Nakamura^{1,*}, Vikrant Vijay^{1,*}, Varsha G. Desai¹, Deborah K. Hansen¹, Tao Han¹, Ching-Wei Chang², Yu-Chuan Chen², Wafa Harrouk³, Barry McIntyre⁴, Paul M. Foster⁴, James C. Fuscoe¹, and Amy L. Inselman¹

¹Division of Systems Biology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States

²Division of Bioinformatics and Biostatistics, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States

³Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD 20993, United States

⁴National Toxicology Program, Research Triangle Park, NC 27709, United States

Abstract

2-hydroxy-4-methoxybenzophenone (HMB) is an ultraviolet (UV) light-absorbing compound that is used in sunscreens, cosmetics and plastics. HMB has been reported to have weak estrogenic activity by *in vivo* and *in vitro* studies, making it a chemical with potential reproductive concern. To explore if perinatal HMB exposure altered the gene expression profiling in the rat prostate and testis, we performed microarray gene expression profiling on the prostate and testis from Sprague-Dawley rat offspring fed various doses of HMB (0, 3000, or 30000 ppm) in 5K96 low phytoestrogen chow from gestational day (GD) 6 through postnatal day (PND) 21. Gene expression profiles of the prostate and testis were differentially affected by HMB dose and duration of exposure, and also indicated tissue-specific alterations. These genes that altered expression indicate a potential utility of candidate biomarker(s) for testicular or prostatic toxicity. Further studies are necessary to explore this potential.

Correspondence to: Noriko Nakamura, Ph.D., Division of System Biology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, United States, Phone: +1-870-543-7175; Fax: +1-870-543-7662;

Noriko.Nakamura@fda.hhs.gov.

* contributed equally

Conflict of interest

The authors declare no conflicts of interest.

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Keywords

2-hydroxy-4-methoxybenzophenone (HMB); prostate; testis; rats; gene expression profiling; perinatal exposure; mitochondria

1. Introduction

2-hydroxy-4-methoxybenzophenone (HMB) (CAS #131–57-7; oxybenzone or benzophenone-3) is an ultraviolet (UV) light-absorbing compound commonly used in cosmetic products, sunscreens, as well as plastics for preventing UV-induced photodecomposition [1–3]. Analysis of samples collected through the National Health and Nutrition Examination Survey (NHANES) revealed detectable levels of HMB in over 95% of human urine samples collected from adults [4,5]. Detectable levels of HMB have also been reported in urine from infants and in breast milk [6].

Previous studies in mice and rats have reported effects of HMB exposure on the male reproductive system [7–10]. Rats and mice exposed to 50000 ppm of HMB in the diet had significant declines in body weight and sperm production [9]. Reduced serum testosterone levels were also observed in postnatal day (PND) 23 rats treated with HMB; however, reductions were not dose-dependent [10]. We have previously reported on the estimated plasma HMB concentration in humans that were obtained from females exposed topically to 2 mg/cm² HMB for 4 days [10, 11]; where levels were comparable to those seen after exposure to 3000–10000 ppm HMB in rats. Numerous animal studies have shown that exposure to endocrine disrupting chemicals (EDCs) can alter gene expression profiles in the reproductive organs of males, especially those related to steroid hormone biosynthesis in the testis [12–14]. In addition, EDCs may increase the risk of prostate cancer development as demonstrated in primary prostate cultures treated with EDCs and in epidemiological studies in humans [15–17]. Furthermore, fetal exposure to high doses of estradiol or diethylstilbestrol in animal models has been shown to affect the development of the prostate [18], while neonatal exposure to EDCs leads to hyperplasia of the prostate intraepithelial cells, increasing the risk of cancer development [19]. Multiple *in vivo* and *in vitro* studies have demonstrated the weak estrogenic activity of HMB [20–22]. In this study, we examined whether perinatal exposure influenced gene expression in the prostate and testes of PND 30 rats perinatally exposed to 3000 or 30000 ppm doses of HMB in the diet by whole genome gene expression microarray analysis. Given that estrogen also regulates mitochondrial function [23], we also evaluated the effects of perinatal exposure on the expression of mitochondria-related genes and pathways. Global gene expression profiling and pathway analyses indicate tissue-specific and dose-related HMB effects on various nuclear and mitochondrial genes and pathways in the prostate and testis of PND 30 rats.

2. Materials and Methods

2.1. Materials:

Reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA), MP Biomedicals (Solon, OH, USA), or Acros Organics USA (Morris Plains, NJ, USA), unless otherwise indicated.

2.2. Animals and treatments:

Male rats used in the current study were part of a larger study sponsored by the National Toxicology Program (NTP) to evaluate the effects of HMB exposure on pre- and postnatal development. In brief, time-mated female Sprague-Dawley rats (11–13 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN, USA) and were received on gestational day (GD) 3 (day of vaginal plug detection was designated as GD 0) where they were housed individually under controlled conditions with a 12:12 h light–dark cycle, $23 \pm 3^\circ\text{C}$ room temperature, and $50 \pm 20\%$ relative humidity. From GD 6 until PND 21, the dams ($n = 25$, per group) were provided *ad libitum* low-phytoestrogen chow (Purina 5K96; Purina Mills LLC, St Louis, MO, USA) containing 0 (control), 3000, or 30000 ppm HMB (Catalog #HH13–026; Ivy Fine Chemicals, Cherry Hill, NJ, USA). In our previous study [10], we estimated typical human exposure to be similar in plasma HMB concentration in the female rats dosed prenatally with between 3000 and 10000 ppm in the diet. Dietary administration of HMB to the dams was terminated on PND 21, and male offspring were weaned on PND 28. The male offspring were dosed with HMB via chow and milk.

The animals were euthanized on PND 30. All animal procedures were approved by the NCTR Institutional Animal Care and Use Committee and adhered to the Guide for the Care and Use of Laboratory Animals prescribed by the National Research Council [24].

2.3 Sample collection:

Male offspring were euthanized on PND 30 by carbon dioxide asphyxiation, followed by cardiac exsanguination and collection of tissue samples. Body weight and the weights of reproductive organs [paired testes, paired epididymides, prostates (all lobes), and seminal vesicles] were recorded. Liver and paired kidney weights were also recorded. Blood was collected by cardiac puncture and transferred into blood collection tubes (BD, Franklin Lakes, NJ, USA) and serum was obtained by centrifugation at $3,000 \times g$ for 10 min at room temperature.

2.4. Measurement of serum testosterone levels:

Testosterone ELISA kits (Calbiotech Inc., Spring Valley, CA, USA) were used to determine serum testosterone levels, which were measured using a microplate reader (Spectra Max 190) and the accompanying software (SoftMax Pro 4.3.1 L, Molecular Devices, LLC, Sunnyvale, CA, USA).

2.5. Microarray analysis:

RNA samples from prostate and testis of PND 30 rats (1 male pup/litter; $N=5$ litters/group) were extracted using the miRNeasy Kit (Qiagen, Valencia CA, USA). After extraction, the

concentration of each RNA sample was determined using a NanoDrop 2000c spectrophotometer (version 3.0.1, Thermo Fisher Scientific Inc, Wilmington DE, USA). One-color microarray-based gene expression analysis using Agilent QuickAmp Kits was performed following the manufacturer's instructions (Agilent Technologies, Santa Clara CA, USA). The total RNA (~500 ng) was labeled with Cy3, and the labeled-cRNA was purified with an RNeasy Kit (Qiagen); the cRNA concentrations were determined with a Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific). Equal amounts (600 ng) of purified Cy3-labeled cRNA were hybridized to Agilent SurePrint G3 rat 8 × 60K microarrays (Agilent Technologies) for 17 hours at 65°C in a hybridization oven. After washing, hybridized microarray slides were scanned with an Agilent DNA Microarray Scanner C and the images were further analyzed using Agilent's Feature Extraction software (V10.7.3).

2.6. Analyses of microarray data:

The Agilent rat whole genome (8 × 60K) arrays contain 62,976 spots for probe localization. Data processing, normalization and statistical analyses were performed on all transcripts from the control, low and high dose groups from each organ (prostate or testis) using SAS 9.4 package (SAS, Cary, NC, USA). All 30 raw microarray data files and preprocessed normalized data (15 files each for prostate and testis) are accessible at NCBI's Gene Expression Omnibus (GEO) website and the GEO accession number is GSE111267 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111267>). A generalized linear model procedure (proc glm) was used to measure statistical significance ($p < 0.05$) between control and perinatally exposed groups. A relative fold change in the expression level of each gene transcript was calculated as a difference in average log₂ intensity values. Genes with greater than a 1.5-fold change in expression and less than a 0.05 p-value were considered differentially expressed (DEG) and these DEGs were further used for pathway analysis using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems Inc., Redwood City, CA, USA). To examine HMB's influence on the mitochondria in the testis and prostate, expression data for 860 mitochondria-related genes were extracted from the whole genome data set and categorized into 85 GO terms (biological processes or molecular functions) to perform Gene Ontology (GO) analysis. This analytical approach has proven useful in understanding drug effects on various mitochondria-related processes and pathways in other tissues [25–28]. GO analysis was performed to measure the overall significance of treatment effect on each GO term using a modified meta-analysis method, which combines p-values ($p < 0.05$) calculated for each gene while taking the inter-gene correlation structure into account within each GO term [29].

2.7. cDNA synthesis:

The cDNA for qPCR confirmation of gene expression changes was synthesized with an iScript Kit (Bio-Rad Laboratories, Hercules, CA, USA) or Super Script IV VILO Master Mix (Thermo Fisher Scientific Inc.) using 1 µg of RNA from the microarray samples.

2.8. Conventional RT-PCR:

To determine testis-specific gene expression, RT-PCR was carried out using the primer pairs shown in Table 1 and JumpStart RedTaq Readymix (Cat#: P0982; Sigma-Aldrich, St. Louis, MO, USA). The 20-µl reaction mixture was processed using an initial denaturation step at

95°C for 2 min, followed by 25 amplification cycles (95°C for 30 sec, 60°C for 30 sec, and 72°C for 10 sec). 18S ribosomal RNA (*Rn18s*) was used as an internal control. Products were separated on 1.6% agarose gels. The images were captured by MultiImage II and Alpha Imager HP software (Alpha Innotech Corp., Santa Clara, CA, USA).

2.9. Quantitative PCR (qPCR):

The qPCR analyses were performed using the PikoReal Real-Time PCR System (Thermo Fisher Scientific Inc.) with the Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific Inc.) or the ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific Inc.) per the standard operating procedures prescribed by the manufacturer. The cDNA, synthesized as described above, served as a template in 20- μ l reaction mixtures. The reaction conditions were as follows for the Luminaris Color HiGreen qPCR Master Mix and the PowerUp SYBR Mix: initial denaturation steps at 50°C for 2 min and 95°C for 10 or 2 min, followed by 40–45 amplification cycles (95°C for 15 sec, 60°C for 1 min), respectively. The relative steady-state transcript levels were calculated using threshold cycle (C_t) values and the following equation: relative quantity = 2^{-C_t} [30]. The expression levels were normalized using *Rn18s* as an internal control for each sample. The relative ratios of the transcript levels in each sample were calculated setting the values for controls at one. The qPCR reactions for each sample were performed in triplicate (1 male pup/litter; N=4–5 litters/group). The specific primer pairs used in this study are shown in Table 1. Genes selected for analyses included distal-less homeobox 3 (*Dlx3*) and mutated in colorectal cancer (*Mcc*). Both have previously been reported to have altered levels of expression in prostate cancer patients [31–35].

2.10. Statistical analysis:

Data are presented as the mean $\pm$ SEM. Exposure group means were obtained using a single pup from each litter. To determine statistical significance for body weight, organ weights and hormone analysis, a one-way ANOVA followed by Tukey's test was performed. qPCR data was analyzed using a *t*-test with Bonferroni adjustment [36,37]. A p-value of 0.05 was considered statistically significant.

3. Results

3.1. Effect of perinatal HMB exposure on body and male reproductive organ weights in PND 30 rats

Daily observation of male offspring did not reveal any clinical observations related to perinatal HMB exposure. At necropsy on PND 30, body weights were 22% lower in the 30000 ppm HMB exposure group (Table 2) than controls. Rats exposed perinatally to 30000 ppm also displayed significantly lower weights of the paired-testis, paired-epididymis, and prostate; weights were lower in 30000 ppm exposed males relative to controls (26.0%, 17.6% and 18.5%, respectively). The paired-testis weight to body weight ratio was also significantly lower for the 30000 ppm HMB group; however, there were no changes in the relative weights of the paired epididymis and prostate for the 30000 ppm HMB exposure group. Rats exposed to HMB did not display any differences in seminal vesicle weight. The

liver and paired-kidney weights were lower in a dose-dependent manner with the 30000 ppm HMB exposure group attaining statistical significance; however, relative liver and paired-kidney weights were similar to controls.

3.2. Effect of HMB on serum testosterone levels in PND 30 rats

Serum testosterone levels in rats perinatally exposed to 3000 and 30000 ppm HMB were 13.5% and 28.3% lower than controls, with statistical significance obtained in the 30000 HMB group (Table 2).

3.3. Differential effects of HMB on gene expression in PND 30 prostate and testis

Microarray analyses of prostate gene expression patterns of rats perinatally exposed to 0, 3000 or 30000 ppm HMB identified differential significant expression of 334 and 689 genes in the 3000 and 30000 ppm HMB exposure groups, respectively, when compared to controls ($p < 0.05$, fold change (FC) > 1.5). Seventy-six genes overlapped between the two HMB exposure groups in the prostate. Microarray analyses of testis-gene expression patterns identified 239 and 1,159 genes that were significantly altered in the testis of the 3000 and 30000 ppm HMB perinatally exposed groups, respectively. Between the two HMB exposure groups, 220 genes overlapped in expression profile in the testis.

3.4. Principal component analysis (PCA)

PCA was conducted by comparing the overall expression profiles of the control prostate versus the control testis (Figure 1A), the control prostate versus the prostate from the 3000 and 30000 ppm HMB exposure groups (Figure 1B) and the control testis versus the testis from the 3000 and 30000 ppm HMB exposure groups (Figure 1C) to determine the association among the five groups. Principal component 1 (PC1) captured 88.2%, PC2 8.6% and PC3 2.2% of the variance in the data of the control prostate and testis groups (Figure 1A); together 99.0% of the variance in these samples were captured. Similarly, PC1 captured 45.0% and 64.0%, PC2 18.4% and 11.4%, and PC3 7.8% and 5.1% of the variance in the prostate and testis samples, respectively (Figure 1B, C). Together 71.2% and 80.5% of the total variance in the prostate and testis samples, respectively, were captured by the first three components. The PC1 represents that a treatment-related distinct separation between control and HMB exposure groups exist.

3.5. Ingenuity Pathway Analysis (IPA)

IPA was used to determine the gene pathways affected in the prostate and testis following perinatal exposure to HMB. A total of nine canonical pathways in the prostate were significantly altered in the 3000 ppm HMB exposure group, while eight pathways were affected in the 30000 ppm exposure group (Tables 3–4; Supplemental Tables 1–2). In the testis, 20 canonical pathways were affected in the 3000 ppm exposure group, while 15 were significantly altered in the 30000 ppm HMB exposure group (Tables 5–6; Supplemental Tables 3–4). The significantly altered pathways (i.e. those with p -values < 0.05) were different between the two tissue types examined. Cell cycle-related pathways (control of chromosomal replication and G2/M DNA damage checkpoint regulation) were the top pathways affected in the prostate of animals perinatally exposed to 30000 ppm HMB (Table

4) whereas sperm motility and inositol pyrophosphates biosynthesis were two of the pathways significantly affected within the testis (Table 6). Overall, there were very few pathways commonly affected by HMB exposure with respect to dose and tissue type (Tables 3–6).

3.6. Estrogen-related genes

The expression of estrogen-related and estrogen-response genes were also examined in the prostate and testis with both groups being altered (Supplemental Table 5) [38]. In the testis, the expression of estrogen receptor 1 (*Esr1*) and estrogen receptor 2 (*Esr2*) were lower in the 30000 ppm exposure group than the control group. This exposure group also displayed lower expression of the Fos protooncogene, AP-1 transcription factor subunit (*Fos*) and prolactin (*Pr1*); however, the changes did not attain statistical significance. In the prostate, the expression of the tumor protein p53 (*Tp53*) gene was the only gene significantly decreased by exposure to 30000 ppm of HMB. Other genes [e.g. *Esr2*, cytochrome P450 family 17 subfamily A member 1 (*Cyp17a1*), NAD(P)H quinone dehydrogenase 1 (*Nqo1*) and BRCA1, DNA repair associated (*Brca1*)] were altered; however, none of the changes were significant relative to control expression levels. There were also no significant differences when expression of *Esr1*, *Esr2* and *Cyp17a1* were examined by qPCR in both the testis and prostate (Figure 2). The transcript levels of *Cyp17a1*, in general, were increased 4-fold in the prostates of 30000 ppm HMB perinatally exposed rats. However, due to the large variability among animals, the increase was not statistically significant.

3.7. Gene expression validation

3.7.1. Prostate—IPA identified biologically significant HMB exposure-related changes in cell cycle pathways including G2/M checkpoint regulation in the prostate. To confirm the changes seen in the cell cycle pathways were occurring in prostate and testis tissues of perinatally exposed HMB animals, ADP-ribosylhydrolase-like 1 (*Adprh1*) and DNA polymerase mu (*Polm*) were selected as marker genes for qPCR assessment (Supplemental Table 6A). Although *Dlx3* and *Mcc* were not identified in the IPA categories, microarray analysis indicated the transcript levels of both genes were significantly lower in HMB exposed groups of only prostate (Supplemental Tables 6A and 7). Because changes in expression of *Dlx3* and *Mcc* genes previously shown to be lowered in prostate cancer [30–34] and *Mcc* gene is also previously shown change abnormal heterozygosity in germ cell tumors in the testis [39], they were also selected for qPCR assessment. The transcript levels of *Adprh1* was significantly lower in the prostates of both the 3000 and 30000 ppm HMB exposure groups as determined by qPCR and microarray analysis (Figure 3; Supplemental Tables 6A and 7). *Polm*, *Dlx3* and *Mcc* transcript levels in the prostate were also lower in HMB perinatally exposed animals by qPCR and microarray analysis. Although microarray analysis showed significant differences in the 3000 and 30000 ppm HMB groups, only the decrease of three genes observed in the 3000 ppm HMB exposure group was statistically significant by qPCR when compared to control group. Both methods found these genes were down-regulated in the 3000 and 30000 ppm HMB treated groups. In contrast to the prostate, the transcript levels for all four genes (*Adprh1*, *Polm*, *dlx3*, *Mcc*) were higher in the testis of the HMB exposure groups via qPCR. The higher expression pattern was, however, not different between the control and HMB groups via qPCR, and no significant changes were

observed by microarray analysis (Figure 3). The exception, however, was for that of *Adprhl1* where the increased transcript levels in the HMB 30000 ppm exposure group was significantly higher than controls. For all four genes validated by qPCR, there was a significant difference in the transcript levels in the testis of the 30000 ppm HMB perinatally exposed animals (Figure 3) when compared to the 30000 ppm HMB exposure group for the prostate.

3.7.2. Testis—IPA identified that the top ten genes down-regulated in the testis of the 30000 ppm HMB exposure group were all testis-specific genes (Table 7). This was, in contrast to the changes observed in the testis of the 3000 ppm exposure group, where ubiquitously expressed genes were impacted (Table 8). Among the top 100 significantly down-regulated genes in the microarray analysis, 42 genes were observed to be testis-specific genes with higher fold changes ($FC > 5.0$) in the 30000 ppm HMB group, 18 of which were expressed abundantly in the testis. In contrast, these genes were not significantly changed in the 3000 ppm HMB group. To confirm this observation, genes related to the sperm motility pathway from IPA were selected and validated by qPCR; these included Cystatin-like 1 (*CstII*), Protamine 1 (*Prm1*), Protamine 2 (*Prm2*), and IQ motif containing F6 (*Iqcf6*). IPA identified the oxidative stress/molecular chaperone pathway to be affected including DnaJ heat shock protein family (Hsp40) member B7 (*Dnajb7*) gene was selected to be used in the qPCR as a confirmation for the change seen for this pathway [40]. As these genes (*CstII*, *Prm1*, *Prm2*, *Iqcf6*, *Dnajb7*) were not expressed in the prostate (Supplemental Figure 1), we examined the transcript levels only in the testis (Figure 4). Interestingly, the transcripts levels of these genes were significantly reduced in the 30000 ppm HMB exposure group via qPCR as well as via microarray analysis (Supplemental Table 6B); there were no significant decreases in expression between the 3000 ppm HMB exposure group and control animals.

3.8. Differential effects of HMB on mitochondria-related and drug metabolism gene ontology (GO)

Because of a significant HMB-mediated reduction in testis weight (Table 2) and down-regulation of a number of testis-specific genes (Table 7), the effects of perinatal HMB exposure on the expression of mitochondria-related genes was also examined (Table 9). It has been shown that testosterone regulates mitochondrial function [41] and a decrease in testosterone production in Leydig cells is associated with an increase in mitochondrial reactive oxygen species [42]. Mitochondria-related genes have been categorized into 85 GO terms (molecular function/biological processes) for better biological interpretation of complex gene expression data [24, 28]. While only 3 GO terms (intracellular protein transport, microtubule-based process, and mitochondrial fusion) were significantly altered in the prostate after exposure to 30000 ppm HMB, the testis had 12 GO terms which were significantly affected at 3000 ppm HMB and 41 GO terms at 30000 ppm HMB (Supplemental Table 8). GO terms related to apoptosis, energy metabolism (oxidative phosphorylation, fatty acid metabolism, Krebs cycle), mitochondrial biogenesis, and steroid biosynthesis/metabolism were some of the mechanisms significantly affected by HMB exposure only in the testis with a greater effect at 30000 ppm compared to controls and 3000 ppm (Table 9). The effect of HMB exposure on the expression level of individual genes

associated with these GO terms in the prostate and testis are presented in the Supplemental Table 9.

Fifty-one of the 75 genes (68%) evaluated for apoptosis had higher expression following 30000 ppm HMB exposure in the testes compared to controls; many of which were pro-apoptotic genes. The BCL2-associated X apoptosis regulator (*Bax*) that encodes a protein responsible for inducing cell death by releasing cytochrome C from the mitochondria [43] was 1.2-fold significantly up-regulated. More than 60% of GO terms genes evaluated for energy metabolism and mitochondrial biogenesis also showed higher expression in the testis of rats exposed to 30000 ppm HMB as compared to controls; a significant effect was seen in complex I, III, IV, and V at 30000 ppm in the testes. Mitochondrial biogenesis is responsible for increasing mitochondrial mass and copy number in cells for generation of ATP in response to greater energy demand. The expression of nuclear respiratory factor 1 (*Nrf1*), gamma (PPARG) coactivator 1 alpha (*Ppargc1a*), and transcription factor A, mitochondrial (*Tfam*) was 1.1- to 1.4-fold higher in HMB-exposed rats compared to controls; however, the effect was statistically significant only in *Nrf1* expression. Conversely, 30000 ppm HMB dose resulted in a decline in the expression of all nine genes interrogated for steroid biosynthesis/metabolism category in the testes compared to controls. The 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hmgcs2*), hydroxysteroid dehydrogenase like 2 (*Hsd12*), and translocator protein (*Tspo*) genes were significantly down-regulated by more than 1.23-fold. The expression of steroidogenic acute regulatory protein (*Star*) and cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*), which encode proteins catalyzing the rate-limiting step in steroid biosynthesis, was declined by 1.6- and 1.2-fold, respectively, although this effect was not statistically significant. In addition to mitochondria-related genes, drug metabolism (phase I and II) GO terms were affected in both the prostate and testis exclusively in the 30000 ppm HMB dose. Of 48 genes evaluated for phase I, 26 genes (54%) in testis and 22 genes (46%) in prostate had higher expression compared to controls (Supplemental Table 9). Of 48 genes evaluated for phase II, 31 genes (65%) expressed in testis and 21 genes (44%) expressed in the prostate had lower expression levels compared to controls. In the testis, transcript levels of cytochrome P450, family 4, subfamily f, polypeptide 18 (*Cyp4f18*), and cytochrome P450, family 4, subfamily f, polypeptide 17 (*Cyp4f17*) genes in phase I were 1.13- to 1.49-fold significantly higher, whereas the expression of glutathione S-transferase alpha 2 (*Gsta2*) and glutathione S-transferase alpha 2 (*Gsta5*) genes of phase II were significantly lower by 1.39- to 1.71-fold compared to controls. In the prostate, cytochrome P450, family 21, subfamily a, polypeptide 1 (*Cyp21a1*) and cytochrome P450, family 4, subfamily a, polypeptide 3 (*Cyp4a3*) of phase I were significantly down-regulated by 1.34- to 3.15-fold, and sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 2 (*Sult2a2*) and UDP glucuronosyltransferase 2 family, polypeptide A3 (*Ugt2a3*) of phase II were significantly down-regulated by 1.33- to 1.5-fold, compared to controls.

3.8.1. Mitochondria-related gene expression validation—To confirm the changes observed in the GO analysis, genes which showed a significant HMB effect were selected from the steroid biosynthesis, energy metabolism (oxidative phosphorylation, fatty acid metabolism, Krebs cycle) and apoptosis GO categories and were verified by qPCR (Figure

5). Genes included BCL2-associated agonist of cell death (*Bad*), NADH dehydrogenase (ubiquinone) flavoprotein 3, mitochondrial (*Ndufv3*), aconitase 2 (*Aco2*), *Tspo*, and *Hmgcs2*. Additionally, transcript levels of *Cyp4f18* and *Gsta2* associated with drug metabolism (phase I and II) were examined (Figure 5). Transcript levels of *Aco2* in the prostate were significantly reduced in the 3000 ppm HMB exposure group compared to controls, while the expression of *Ndufv3* in the testis was significantly increased in the 30000 ppm HMB exposure group. The other genes examined were slightly altered but did not reach statistical significance as a result of HMB exposure. Similarly, the transcript levels of *Aco2* and *Gsta2* in the HMB exposure groups were also slightly increased in the testis compared to the prostate but no significant differences in gene expression were noted between the two tissues.

4. Discussion

HMB has previously been reported to have weak estrogenic activity and was suspected of acting as a reproductive toxicant negatively impacting development of the reproductive system impairing fertility. In this study, we focused on the potential effects of perinatal HMB exposure (via dietary exposure to 3000 or 30000 ppm in a low phytoestrogen chow) on the developing male reproductive system from GD 6 through PND 21 to confirm whether exposure to HMB results in adverse effects on sperm production and testosterone levels as was previously reported [7–9]. Male pups were euthanized at PND 30 and tissues were collected to examine global gene expression profiles. We determined 1) the effects of perinatal exposure to HMB on gene expression profiles of the prostate and testis, 2) identified genes/pathways that were differentially affected between the tissue types, and 3) determined the effect of HMB dose on expression levels.

4.1. Body and organ weights and serum testosterone levels

Perinatal exposure to 3000 ppm HMB did not result in any effects. Perinatal exposure to 30000 ppm HMB via the diet resulted in adverse findings as evidenced by lower weights of the paired testes, epididymis and prostate. However, the mean pup weight at necropsy was substantially lower in the HMB-treated group than the control group. This finding was not accompanied by maternal weight loss [10]. Rats perinatally exposed to 30000 ppm HMB also exhibited lower absolute liver and paired-kidney weights, but relative organ weight to body weight ratios of these tissues were similar to controls. As the testicular weight during development is independent of body weight [44,45], the highest perinatal dose of HMB may be delaying testis and prostate development. The body and organ weight findings observed in this study are consistent with previous studies [8–10] examining the effects of HMB exposure. Additionally, rats perinatally exposed to 30000 ppm of HMB displayed lower testosterone levels, and is consistent with both growth retardation and/or a direct effect on testicular function.

4.2. Transcriptional profiling of the prostate and testis

The present study found that perinatal HMB exposure had different effects on the transcriptional profiles of the prostate and testis of PND 30 rats. Although prostate weight was lower in the 30000 ppm HMB group, suggesting a delay in development, transcript

levels of key prostate developmental genes [e.g. homeobox B13 (*Hoxb13*), NK-3 transcription factor, locus 1 (*Nkx3-1*), bone morphogenetic protein 4 (*Bmp4*)] were not altered. Prins et al. [46] previously demonstrated that high doses of estrogen impair prostate growth in the rat. This absence of an “estrogenic response”, may be the result of HMB’s purported weak estrogenic activity. This is consistent with adverse effects on estrogen-dependent gene expression being related to their potency in inducing estrogen receptor-mediated responses [47]. Most altered gene expression/pathways in the prostate and testis were found in the 30000 ppm HMB exposure group. This group also had reduced body and organ weights, suggesting the altered gene expression in the 30000 ppm HMB dose group may, in part, be due to the delay of growth. Prostate weight was not significantly reduced in the 3000 HMB dose group; however, cell cycle-related genes were found to be altered. Therefore, these data suggest that gene changes occurring at perinatal HMB exposure levels of 3000 ppm are independent of growth retardation. Nonetheless, further investigation is needed to clarify the potential direct effects of HMB on the reproductive organ function/maturation from those secondary to nonspecific general toxicity effects resulting in delayed organ growth and maturation.

Researchers have previously reported altered expression levels of genes related to steroidogenesis [e.g. *Star*, *Cyp11a1*] when examined in the testes of late gestational or prepubertal aged animals following *in utero* or prepubertal exposures to known EDCs including ethinyl estradiol, genistein, bisphenol A, dibutyl phthalates and di-(2 ethylhexyl) phthalate [12,14,48]. In the present study, however, changes in steroidogenic enzyme gene expression was not statistically significant via microarray analysis or qPCR (Supplemental Figure 2; Supplemental Table 9). In addition, no significant differences were observed on the gene expression profiling of the estrogen-related or androgen-dependent genes (Supplemental Table 10). Taken together, these results suggest that perinatal HMB exposure may not be acting as an EDC on postnatal development of the male reproductive system. However, it is important to note that exposure to HMB ended two days prior to tissue collection which may have impacted the gene expression profile of the testis and prostate captured at the time of necropsy. Testosterone levels are low at PND 30 as the animals are still prepubescent [49] and the Leydig cells are just starting to transition from fetal to adult cells [50]. These results in the lower expression of genes related to steroidogenesis which may hinder detection of meaningful changes between the control and HMB dose groups.

4.2.1. Prostate specific down-regulated genes—While genes related to steroidogenesis, androgen-dependent genes, or estrogen-related pathways were not affected by perinatal HMB exposure, significant effects were observed in cell cycle and prostate cancer-associated genes in the prostate. Expression of *Adprhl1*, *Polm*, *Dlx3* and *Mcc* were significantly decreased in the prostate rats exposed to HMB perinatally relative to controls as was observed by microarray and qPCR. In contrast, transcripts levels of the same genes increased slightly in the testis via qPCR. However, the increase in the testis was not significantly different from control group. The qPCR data (fold changes) were different from those observed by microarray. It is considered that the changes of the expression levels of these genes in the testis were too slight to demonstrate a true difference between qPCR and microarray.

Dlx3 is a member of the distal-less homeobox (DLX) family with a known function in vertebrate and invertebrate development [51]. In addition to development, DLX family members have been associated with cancer (especially, DLX2, DLX4 and DLX7) [51,52]. While expression of *Dlx3* in prostate cancer or benign prostate hyperplasia patients has not been reported, changes in its expression levels have been associated with cell cycle alterations in keratinocytes [35]. *Dlx3* has been shown to activate genes involved in proliferation (i.e. mitogen-activated protein kinase 1 signaling) and has been found to either be absent (e.g., human skin cancer), or present at low levels in keratinocytes. However, to our knowledge, no studies have investigated the relationship between the expression of *Dlx3* in the prostate of patients with cancer or benign prostate hyperplasia. Additional studies are needed to determine the role of *Dlx3* in prostate disease.

MCC has previously been shown to act as a tumor suppressor in prostate cancer [32]. Additionally, examination of *Mcc* transcript levels from prostate cancer patients revealed a reduction in *Mcc* levels in 50% of the prostate samples analyzed [31]. In this study, prostate tissue from rats perinatally exposed to both doses of HMB resulted in lower levels of *Mcc* expression (albeit only the low HMB exposure level was significant, due to the large variance). Further studies are, however, needed to confirm the significance of *Mcc* expression level changes in response to perinatal HMB exposure.

In this study, we report that *Adrp11* gene expression levels were altered in the prostates following perinatal exposure to HMB. *Adrp11* belongs to the ADP-ribosylhydrolase protein family whose primary function is to remove ADP-ribose from ADP-ribosylated proteins [54]. *Adrp11* is also reported to play an important role in the outgrowth of the heart chamber [55], and has been reported to act as a tumor suppressor in studies of knockout animals lacking the gene [56]. It is possible that *Adrp11* may suppress tumor growth. However, the function of *Adrp11* in the prostate is not well understood.

We identified another gene, *Polm*, which was overexpressed following perinatal exposure to HMB relative to control rats. *Polm* is a member of the X family of DNA polymerases whose primary function is to prevent tumorigenesis by a repair of DNA damages [57,58]. Previous studies have reported that *polm* may also act to repair DNA damage in the prostate [58]. Similar to the genes discussed above, *Polm*'s function in the prostate has not been well understood.

The decreased expression of genes related to tumor suppression in the prostate and the slight albeit increased expression, of the same genes in the testis, suggests that tissue specific differences exist in response to perinatal exposure to HMB. Further experiments are needed to determine if the results observed in this study are related specifically to HMB exposure or are part of a generalized response to chemical insult to the prostate and testis during a sensitive window of development. Another question that remains to be answered is whether the altered gene expression observed in the prostate and testes of PND 30 rats following perinatal exposure to HMB persists into adulthood. If the change is development-specific, the gene expression profile in peripubertal animals might be a useful biomarker for predicting the risk of prostate disease or cancer development.

4.2.2. Testis-specific gene expression—In the testis, perinatal exposure to 30000 ppm of HMB resulted in down-regulation of testis-specific genes. Of the top ten genes down-regulated following HMB exposure, all (ten) are specifically expressed in the testis (Table 7). However, it has not been reported if these genes play a function related to cell cycle or proliferation. In contrast, exposure to the 3000 ppm dose of HMB significantly altered transcript levels of many ubiquitously expressed genes, as opposed to the testis-specific genes affected by the higher (30000 ppm) dose of HMB. Genes or splice variants with expression limited to the testis generally play important roles in spermatogenesis [59–62]. Both *Prm1* and *Prm2* have spermatogenic cell-specific and time-specific expression patterns [63]. As perinatal exposure to 30000 ppm HMB delayed body growth and reduced testis weights the timing and expression of the testis-specific genes such as *Prm1* and *Prm2* may also be altered. A previous NTP study [9] found reduced sperm numbers in adult rats dosed with 50000 ppm HMB. Previous observations for dibutyl phthalate (a known EDC) have been reported where a reduced sperm number was seen in rats exposed with higher EDCs [64]. It is possible that altered expression of these testis/spermatogenic specific-genes may be responsible for the reduced sperm numbers observed in previous studies with the highest dose of HMB or other EDCs. However, due to significant declines in body weights observed as a result of exposure to 50000 ppm HMB, the reduction observed in sperm number may possibly be due to overt toxicity. It is highly likely that the gene expression differences in the 30000 ppm HMB exposure group are due to developmental delay associated with growth retardation.

It is important to note that the findings in this study appear limited to the highest dose of 30000 ppm HMB exposure group. As previously discussed [10], exposure to doses of 10000 ppm HMB was not associated with any adverse effects on the prostate or testis. Likewise, we determined that human equivalent exposure levels in plasma were achieved with a 10000 ppm HMB dose exposed to rats perinatally [10,65]. Thus, the associated gene changes observed in this study were only observed at concentrations which are more than typical human exposures. Additionally, we have not determined if these findings in testis-specific gene expression are exclusively obtained with HMB exposure, whether they are secondary to growth retardation, or are induced by other reproductive or testicular toxicants.

The summary of the testis results for this study is shown in Figure 6. Genes related to tumor suppression, such as *Mcc* or *Dlx3*, had increased levels of expression in response to perinatal HMB exposure, with higher (30000 ppm) levels producing larger increases in expression. Changes in testis-specific gene expression were also dependent upon dose. However, unlike the gene expression changes observed in the tumor suppressor gene sets, changes in testis-specific gene expression were restricted to the 30000 ppm HMB exposure group. The full function of the down-regulated testis specific genes (about 100 genes) that was observed by microarray analysis has not been determined. Our findings suggest several possible scenarios: 1) a dose-dependent HMB response is obtained in the testis; 2) a more sensitive response to the 30000 ppm HMB dose in the testis compared to the prostate secondary to growth retardation induced by HMB. In either scenario, testis-specific genes may be used as endpoints to measure toxicity or growth retardation caused by chemicals. However, this

hypothesis will need to be tested more rigorously in future studies with HMB and other chemicals.

4.3. Mitochondrial related and drug metabolism gene expression

HMB is reported to be metabolized in phase I and II reactions [66,67]. However, information regarding the specific enzymes involved in phase I and II HMB metabolism is sparse. In this study, we found a significant effect of HMB exposure on gene sets associated with drug metabolism (phase I and II) in both the testis and prostate of PND 30 rats perinatally exposed to 30000 ppm of HMB. More than 50% of the 48 genes evaluated for phase I metabolism were up-regulated in the testis and down-regulated in the prostate, whereas 65% and 44% of the 48 genes evaluated for phase II metabolism were down-regulated in the testis and prostate of 30000 ppm HMB perinatally exposed animals, respectively. The differential effects of HMB exposure on drug metabolism in the prostate and testis is unclear. In general, drugs are changed to water-soluble active or inactive metabolites during the process of the phase I and II metabolism. Given that greater up-regulation of phase I genes and down-regulation of phase II genes are observed in the testis than the prostate may signal that more reactive HMB metabolites are formed in the testis and may in part contribute to the underlying differential effects seen in the two organs [66].

Several key mitochondrial pathways represented by GO categories were also significantly affected by perinatal exposure to HMB, but the effects were limited to the testis with more pronounced effects at 30000 ppm HMB dose. In the prostate, changes in mitochondrial DNA are associated with prostate cancer and disease [68]. However, it is still unknown what role(s) the mitochondria have in regulating prostate function. In the testis, mitochondria are known to act as a needed energy source for spermatogenesis and sperm motility [60,69]. In addition, mitochondrial function is vital in regulating apoptosis [70]. Germ cell death within the testis is triggered by factors such as reduced testosterone and estradiol levels or drugs [71] and ATP is required for induction of cell death [72]. Mitochondria produce ATP needed for apoptosis via oxidative phosphorylation within the spermatocytes and spermatids. As the Sertoli cells and spermatogonia utilize glycolysis as their primary energy source, they are protected from cell death [73,74]. A recent investigation has shown this to be the reason why apoptosis is observed in spermatocytes following exposure to EDCs [75]. The present study also supports previous studies where we observed an up-regulation of the majority of genes associated with mitochondrial biogenesis, energy metabolism (oxidative phosphorylation, Krebs cycle, and fatty acid metabolism), and apoptosis. Down regulation of genes involved in steroid biosynthesis was also observed; however, since the rats used in this study were prepubescent, the implications for effects on the testis and prostate of mature males cannot be drawn. These results also suggest a potential underlying mode of action for HMB exposure on growth retardation induced by 30000 ppm HMB perinatal exposure and may explain the tissue-specific responses observed (Figure 7). In this study, we were unable to confirm the significant differences observed in the mitochondria and drug metabolism pathways by microarray analysis using a second technique, the qPCR assay. This discrepancy could be due to a number of factors including: 1) changes in expression levels of the mitochondria-related genes was too small for verification by qPCR, 2) not all genes are fully expressed in PND 30 rats, especially those in the testis (e.g. glutathione-S-

transferase A5), and 3) a relatively small sample number used for data analysis. Therefore, further experiments are necessary to confirm the role of mitochondria and drug metabolism-related changes in gene expression and to elucidate the potential mechanism(s) underlying perinatal exposure to HMB.

5. Conclusion

In this study, we observed differential effects on gene expression in the prostate and testis of PND 30 rats exposed perinatally from GD 6-PND 21 to HMB. The gene expression changes seen in this study were only observed at concentrations which exceed typical human exposure to HMB. In addition, we found that different levels of HMB exposure affected various pathways and genes in both the prostate and testis. Differential effects of perinatal exposure to HMB were seen in the mitochondria GO categories of steroid biosynthesis, energy metabolism (oxidative phosphorylation, fatty acid metabolism, Krebs cycle) and apoptosis. However, these effects were predominantly found in the testis of rats dosed perinatally with 30000 ppm HMB. In addition to effects on pathways, expression levels of individual genes (e.g. *Mcc*, *Dkk3*, *Adphrl1*, *Polm*) were also altered in a tissue-specific manner. Perinatal exposure to 30000 ppm HMB affected the cell cycle or proliferation pathway in the prostate. Some testis-specific expressed genes (e.g. *Prm1*, *Prm2*, *Iqcf6*, *Cstll* etc.) were significantly reduced only in the testis of rats dosed perinatally with 30000 ppm HMB, while they were not significant in the testis of rats dosed perinatally with 3000 ppm HMB. These findings suggest that the genes identified in this study may be used as potential candidates of biomarker(s) for testicular and prostatic toxicity or growth retardation. Additional studies are needed to determine whether the changes observed in this study are specific responses to HMB exposure or are more general changes which are observed in response to growth retardation induced by HMB during the growth phase of prepubescent male rats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Kamogawa H, Responsive polymers. V. Preparation of some polymeric phenolic ultraviolet absorbers, *Kogyo Gijutsuin Sen'i Kogyo Shikensho Kenkyu Hokoku* 86 (1969) 95–96.
- [2]. Klein K, Encyclopedia of UV absorbers for sunscreen products, *Cosmetic & Toiletries*. 107 (1992) 45–64.

- [3]. Dormán G, Prestwich GD, Benzophenone photophores in biochemistry, *Biochemistry*. 33 (1994) 5661–5673. [PubMed: 8180191]
- [4]. Calafat AM, Wong L-W, Ye X, Reidy JA, Needham LL, Concentrations of the sunscreen agent benzophenone-3 in residents of the United States: National Health and Nutrition Examination Survey 2003–2004, *Env. Health. Perspect* 116 (2008) 893–897. [PubMed: 18629311]
- [5]. Calafat AM, Weuve J, Ye X, Jia LT, Hu H, Ringer S, Huttner K, Hauser R, Exposure to bisphenol A and other phenols in neonatal intensive care unit premature infants, *Env. Health. Perspect* 117(2009) 639–644. [PubMed: 19440505]
- [6]. Schlumpf M, Kypke K, Wittassek M, Angerer J, Mascher H, Mascher D, Vökt C, Birchler M, Lichtensteiger W, Exposure patterns of UV filters, fragrances, parabens, phthalates, organochlor pesticides, PBDEs, and PCBs in human milk: correlation of UV filters with use of cosmetics, *Chemosphere*. 81 (2010) 1171–1183. [PubMed: 21030064]
- [7]. Lewerenz HJ, Lewerenz G, Plass R, Acute and subacute toxicity studies of the UV absorber MOB in rats, *Food. Cosmet. Toxicol* 10 (1972) 41–50.
- [8]. Christian M, Final report on the safety assessment of benzophenones-1, -3, -4, -5, -9, and -11, *J. Am. College. Toxicol* 2 (1983) 35–73.
- [9]. National Toxicology Program, NTP technical report on toxicity studies of 2-hydroxy-4-methoxybenzophenone (CAS number: 131–57-7) administered topically and in dosed feed to F344/N rats and B6C3F₁ mice, NIH Publication No 92–3344, 1992.
- [10]. Nakamura N, Inselman AL, White GA, Chang C-W, Trbojevich RA, Sephr E, Voris KL, Patton RE, Bryant MS, Harrouk W, McIntyre BS, Foster PM, Hansen DK, Effects of maternal and lactational exposure to 2-hydroxy-4-methoxybenzone on development and reproductive organs in male and female rat offspring, *Birth. Defects. Res. B. Dev. Reprod. Toxicol* 104 (2015) 35–51. [PubMed: 25707689]
- [11]. Janjua NR, Kongshoj B, Andersson AM, Wulf HC, Sunscreens in human plasma and urine after repeated whole-body topical application, *J. Eur. Acad. Dermatol. Venereol* 22 (2008) 456–461. [PubMed: 18221342]
- [12]. Liu K, Lehmann KP, Sar M, Young SS, Gaido KW, Gene expression profiling following in utero exposure to phthalate esters reveals new gene targets in the etiology of testicular dysgenesis, *Biol. Reprod* 73 (2005) 180–192. [PubMed: 15728792]
- [13]. Anway MD, Skinner MK, Transgenerational effects of the endocrine disruptor vinclozolin on the prostate transcriptome and adult onset disease, *Prostate*. 68 (2008) 517–529. [PubMed: 18220299]
- [14]. Vo TT, Jung EM, Dang VH, Yoo YM, Choi KC, Yu FH, Jeung EB, Di-(2 ethylhexyl) phthalate and flutamide alter gene expression in the testis of immature male rats, *Reprod. Biol. Endocrinol* 7 (2009) 104. doi: 10.1186/1477-7827-7-104. [PubMed: 19781091]
- [15]. Prins GS, Endocrine disruptors and prostate cancer risk, *Endocr. Relat. Cancer* 3 (2008) 649–656.
- [16]. Diamanti-Kandarakis JP, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC, Endocrine-disrupting chemicals: an Endocrine Society scientific statement, *Endocr. Rev* 30 (2009) 293–342. [PubMed: 19502515]
- [17]. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, Toppari J, Zoeller RT, EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals, *Endocr. Rev* 6 (2015) E1–E150.
- [18]. vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, Dhar MD, Ganjam VK, Parmigiani S, Welshons WV, Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses, *Proc. Natl. Acad. Sci. U S A* 94(1997) 2056–2061. [PubMed: 9050904]
- [19]. Soto AM, Sonnenschein C, Environmental causes of cancer: endocrine disruptors as carcinogens, *Nat. Rev. Endocrinol* 6 (2010) 363–370. [PubMed: 20498677]
- [20]. Nakagawa Y, Suzuki T, Metabolism of 2-hydroxy-4-methoxybenzophenone in isolated rat hepatocytes and xenoestrogenic effects of its metabolites on MCF-7 human breast cancer cells, *Chem. Biol. Interact* 139 (2002) 115–128. [PubMed: 11823001]

- [21]. Gomez E, Pillon A, Fenet H, Rosain D, Duchesne MJ, Nicolas JC, Balaguer P, Casellas C, Estrogenic activity of cosmetic components in reporter cell lines: parabens, UV screens, and musks., *J. Toxicol. Environ. Health A* 68 (2005) 239–251. [PubMed: 15799449]
- [22]. Kunz PY, Galicia HF, Fent K K, Comparison of in vitro and in vivo estrogenic activity of UV filters in fish *Toxicol. Sci.* 90 (2006) 349–361.
- [23]. Klinge CM, Estrogenic Control of Mitochondrial Function and Biogenesis, *J. Cell Biochem* 105 (2008) 1342–1351. [PubMed: 18846505]
- [24]. National Research Council, Guide for the care and use of laboratory animals, National Academy Press, Washington, D.C, 2011.
- [25]. Desai VG, Lee T, Delongchamp RR, Leakey JE, Lewis SM, Lee F, Moland CL, Branham WS, Fuscoe JC, Nucleoside reverse transcriptase inhibitors (NRTIs)-induced expression profile of mitochondria-related genes in the mouse liver, *Mitochondrion* 8 (2008) 181–195 [PubMed: 18313992]
- [26]. Joseph A, Lee T, Moland CL, Branham WS, Fuscoe JC, Leakey JE, Allaben WT, Lewis SM, Ali AA, Desai VG, Effect of (+)-usnic acid on mitochondrial functions as measured by mitochondria-specific oligonucleotide microarray in liver of B6C3F1 mice, *Mitochondrion* 9 (2009) 149–158. [PubMed: 19460291]
- [27]. Lee T, Manjanatha MG, Aidoo A, Moland CL, Branham WS, Fuscoe JC, Ali AA, Desai VG, Expression analysis of hepatic mitochondria-related genes in mice exposed to acrylamide and glycidamide, *J. Toxicol. Environ. Health A* 75 (2012) 324–339. [PubMed: 22480170]
- [28]. Vijay V, Moland CL, Han T, Fuscoe JC, Lee T, Herman EH, Jenkins GR, Lewis SM, Cummings CA, Gao Y, Cao Z, Yu LR, Desai VG, Early transcriptional changes in cardiac mitochondria during chronic doxorubicin exposure and mitigation by dexrazoxane in mice, *Toxicol. Appl. Pharmacol* 295 (2016) 68–84. [PubMed: 26873546]
- [29]. Lee T, Desai VG, Velasco C, Reis RJ, Delongchamp RR, Testing for treatment effects on gene ontology, *BMC Bioinformatics* 9 Suppl 9 (2008) S20.
- [30]. Livak KJ, Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ Ct method, *Methods* 25 (2000) 402–408.
- [31]. Gao X, Zacharek A, Grignon D, Liu H, Sakr W, Porter A, Chen Y, Honn K, High-frequency of loss of expression and allelic deletion of the apc and mcc genes in human prostate-cancer, *Int. J. Oncol* 6 (1995) 111–117. [PubMed: 21556510]
- [32]. Gao X, Porter AT, Honn KV, Involvement of the multiple tumor suppressor genes and 12-lipoxygenase in human prostate cancer. Therapeutic implications, *Adv. Exp. Med. Biol* 407 (1997) 41–53. [PubMed: 9321930]
- [33]. Shukla R, Upton KR, Muñoz-Lopez M, Gerhardt DJ, Fisher ME, Nguyen T, Brennan PM, Baillie JK, Collino A, Ghisletti S, Sinha S, Iannelli F, Radaelli E, Dos Santos A, Rapoud D, Guettier C, Samuel D, Natoli G, Carninci P, Ciccarelli FD, Garcia-Perez JL, Faivre J, Faulkner GJ, Endogenous retrotransposition activates oncogenic pathways in hepatocellular carcinoma, *Cell* 153 (2013) 101–111. [PubMed: 23540693]
- [34]. Edwards SK, Baron J, Moore CR, Liu Y, Perlman DH, Hart RP, Xie P, Mutated in colorectal cancer (MCC) is a novel oncogene in B lymphocytes, *J. Hematol. Oncol* 7(2014) 56. [PubMed: 25200342]
- [35]. Palazzo E, Ilett M, Cataisson C, Gormley A, Bible PW, Pietroni V, Radoj N, Hwang J, Blumenberg M, Yuspa SH, Morasso MI, The homeoprotein DLX3 and tumor suppressor p53 co-regulate cell cycle progression and squamous tumor growth, *Oncogene* 35 (2016) 3114–3124. [PubMed: 26522723]
- [36]. Hochberg Y, A sharper Bonferroni procedure for multiple tests of significance. *Biomtrika* 75 (1988) 800–802.
- [37]. Shaffer JP, Multiple hypothesis testing, *Annu. Rev. Psychol* 46 (1995) 561–584.
- [38]. Ikeda K, Horie-Inoue K K, Inoue S, Identification of estrogen-responsive genes based on the DNA binding properties of estrogen receptors using high-throughput sequencing technology, *Acta Pharmacol. Sin* 36 (2015):24–31. [PubMed: 25500870]
- [39]. Peng HQ, Bailey D, Bronson D, Goss PE, Hogg D, Loss of heterozygosity of tumor suppressor genes in testis cancer, *Cancer Res.* 55 (1995) 2871–2875. [PubMed: 7796415]

- [40]. Ohtsuka K, Hata M, Molecular chaperone function of mammalian Hsp70 and Hsp40--a review. *Int. J. Hyperthermia* 16 (2000) 231–245. [PubMed: 10830586]
- [41]. Velarde MC, Mitochondrial and sex steroid hormone crosstalk during aging, *Longev. Healthspan* 3 (2014) 2. [PubMed: 24495597]
- [42]. Chen H, Cangello D, Benson S, Folmer J, Zhu H, Trush MA, Zirkin BR, Age-related increase in mitochondrial superoxide generation in the testosterone-producing cells of Brown Norway rat testes: relationship to reduced steroidogenic function?, *Exp. Gerontol* 36 (2001) 1361–1373. [PubMed: 11602210]
- [43]. Sawai H, Domae N, Release of cytochrome c from mitochondria precedes Bax translocation/activation in Triton X-100-induced apoptosis, *Leuk. Res* 32 (2008) 445–453. [PubMed: 17689609]
- [44]. O'Connor JC, Davis LG, Frame SR, Cook JC, Evaluation of a Tier I screening battery for detecting endocrine-active compounds (EACs) using the positive controls testosterone, coumestrol, progesterone, and RU486, *Toxicol. Sci* 54 (2000) 338–54. [PubMed: 10774816]
- [45]. Rehm S, White TE, Zahalka EA, Stanislaus DJ, Boyce RW, Wier PJ, Effects of food restriction on testis and accessory sex glands in maturing rats, *Toxicol. Pathol* 36 (2008) 687–694. [PubMed: 18648097]
- [46]. Prins GS, Huang L, Birch L, Pu Y Y, The role of estrogens in normal and abnormal development of the prostate gland, *Ann. NY. Acad. Sci* 1089 (2006) 1–13. [PubMed: 17261752]
- [47]. Schug TT, Janesick A, Blumberg B, Heindel JJ, Endocrine Disrupting Chemicals and Disease Susceptibility, *J. Steroid Biochem. Mol. Biol* 127 (2011): 204–215. [PubMed: 21899826]
- [48]. Naciff JM, Hess KA, Overmann GJ, Torontali SM, Carr GJ, Tiesman JP, Foertsch LM, Richardson BD, Martinez JE, Daston GP, Gene expression changes induced in the testis by transplacental exposure to high and low doses of 17{alpha}-ethynyl estradiol, enistein, or bisphenol A, *Toxicol. Sci* 86 (2005) 396–416. g [PubMed: 15901920]
- [49]. Lee VW, de Kretser DM, Hudson B, Wang C, Variation in serum FSH, LH and testosterone in male rats from birth to sexual maturity, *J. Reprod. Fertil* 42(1975) 121–126. [PubMed: 1110463]
- [50]. Chen H, Stanley E, Jin S, Zirkin BR, Stem Leydig cells: from fetal to aged animals. *Birth Defects Res C Embryo Today*. 90 (2010) 272–283. [PubMed: 21181888]
- [51]. Panganiban G, Rubenstein JL Developmental functions of the Distal-less/Dlx homeobox genes, *Development*. 129 (2002) 4371–4386. [PubMed: 12223397]
- [52]. Samuel S, Naora H, Homeobox gene expression in cancer: insights from developmental regulation and deregulation, *Eur. J. Cancer* 41 (2005) 2428–2437. [PubMed: 16199152]
- [53]. Schwartz AM, Man YG, Rezaei MK, Simmens SJ, Berg PE, BP1, a homeoprotein, is significantly expressed in prostate adenocarcinoma and is concordant with prostatic intraepithelial neoplasia, *Mod. Pathol* 22 (2009) 1–6. [PubMed: 18931648]
- [54]. Takada T, Okazaki IJ, Moss J, ADP-ribosylarginine hydrolases, *Mol. Cell. Biochem* 138 (1994) 119–122. [PubMed: 7898453]
- [55]. Smith SJ, Towers N, Saldanha JW, Shang CA, Mahmood SR, Taylor WR, Mohun TJ, The cardiac-restricted protein ADP-ribosylhydrolase-like 1 is essential for heart chamber outgrowth and acts on muscle actin filament assembly, *Dev. Biol* 416 (2016) 373–388. [PubMed: 27217161]
- [56]. Kato J, Zhu J, Liu C, Stylianou M, Hoffmann V, Lizak MJ, Glasgow CG, Moss J, ADP-ribosylarginine hydrolase regulates cell proliferation and tumorigenesis, *Cancer Res* 71 (2011) 5327–5335. [PubMed: 21697277]
- [57]. Daley JM, Laan RL, Suresh A, Wilson TE, DNA joint dependence of pol X family polymerase action in nonhomologous end joining, *J. Biol. Chem* 280 (2005) 29030–29037. [PubMed: 15964833]
- [58]. Escudero B, Herrero D, Torres Y, Cañón S, Molina A, Carmona RM, Suela J, Blanco L, Samper E, Bernad A, Polμ deficiency induces moderate shortening of P53^{-/-} mouse lifespan and modifies tumor spectrum, *DNA Repair (Amst)*. 54 (2017) 40–45. [PubMed: 28460268]
- [59]. Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM, Haploinsufficiency of protamine-1 or -2 causes infertility in mice, *Nat. Genet* 28 (2001) 82–86. [PubMed: 11326282]

- [60]. Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA DA, Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc. Natl. Acad. Sci. U S A* 101 (2004) 16501–16506. [PubMed: 15546993]
- [61]. Chau KM, Cornwall GA, Reduced fertility in vitro in mice lacking the cystatin CRES (cystatin-related epididymal spermatogenic): rescue by exposure of spermatozoa to dibutyl cAMP and isobutylmethylxanthine, *Biol Reprod* 84 (2011) 140–152. [PubMed: 20811015]
- [62]. Harris TP, Schimenti KJ, Munroe RJ, Schimenti JC, IQ motif-containing G (Iqcg) is required for mouse spermiogenesis, *G3 (Bethesda)* 4 (2014) 367–372. [PubMed: 24362311]
- [63]. Eddy EM, The scaffold role of the fibrous sheath, *Soc. Reprod. Fertil Suppl.* 65 (2007) 45–62.
- [64]. National Toxicology Program, NTP technical report on toxicity studies of dibutyl phthalate (CAS number: 84–74-2) administered in feed to F344/N rats and B6C3F1 mice, NIH Publication No 95–3353, 1995.
- [65]. Mutlu E, Pierfelice J, McIntyre BS, Cunny HC, Kissling GE, Burbach B, Waidyanatha S, Simultaneous Quantitation of 2-Hydroxy-4-Methoxybenzophenone, a Sunscreen Ingredient, and its Metabolites in Harlan Sprague Dawley Rat Plasma Following Perinatal Dietary Exposure. *J. Anal. Toxicol* 41 (2017) 744–754. [PubMed: 28977387]
- [66]. Okereke CS, Abdel-Rahman MS, Friedman MA, Disposition of benzophenone-3 after dermal administration in male rats, *Toxicol. Lett* 21 (1994) 788–791.
- [67]. Wang L, Kannan K, Characteristic profiles of benzophenone-3 and its derivatives in urine of children and adults from the United States and China, *Environ. Sci. Technol* 47 (2013) 12532–12538. [PubMed: 24073792]
- [68]. Grupp K, Jedrzejewska K, Tsourlakis MC, Koop C, Wilczak W, Adam M, Quaas A, Sauter G, Simon R, Izbicki JR, Graefen M, Huland H, Schlomm T, Minner S, Steurer S, High mitochondria content is associated with prostate cancer disease progression. *Mol. Cancer* 12 (2013) 145. [PubMed: 24261794]
- [69]. Gupta G, Srivastava A, Setty BS, Activities and androgenic regulation of kreb cycle enzymes in the epididymis and vas deferens of rhesus monkey, *Endocr. Res* 20 (1994) 275–290. [PubMed: 7995257]
- [70]. Erkkila K, Kytanen S, Wikstrom M, Taari K, Sinha Hikim AP, Swerdloff RS, Dunkel L, Regulation of human male germ cell death by modulators of ATP production, *Am. J. Physiol. Endocrinol. Metab* 290 (2006) E1145–E1154. [PubMed: 16403780]
- [71]. Shaha C, Tripathi R, Mishra DP, Male germ cell apoptosis: regulation and biology, *Philos. Trans. R. Soc. Lond. B Biol. Sci* 365 (2010) 1501–1515. [PubMed: 20403866]
- [72]. Ricci JE, Waterhouse N, Green DR, Mitochondrial functions during cell death, a complex (I-V) dilemma, *Cell Death. Differ* 10 (2003) 488–492. [PubMed: 12728246]
- [73]. Robinson R, Fritz IB, Metabolism of glucose by Sertoli cells in culture, *Biol. Reprod* 24 (1981) 1032–1041. [PubMed: 6268203]
- [74]. Grootegeed JA, Jansen R, Van der Molen HJ, The role of glucose, pyruvate and lactate in ATP production by rat spermatocytes and spermatids, *Biochim. Biophys. Acta* 767 (1984) 248–256. [PubMed: 6498180]
- [75]. Alam MS, Kurohmaru M, Butylbenzyl phthalate induces spermatogenic cell apoptosis in prepubertal rats, *Tissue. Cell* 48 (2016) 35–42. [PubMed: 26747412]
- [76]. Tong W, Cao X, Harris S, Sun H, Fang H, Fuscoe J, Harris A, Hong H, Xie Q, Perkins R, Shi L, Casciano D, ArrayTrack--supporting toxicogenomic research at the U.S. Food and Drug Administration National Center for Toxicological Research, *Environ. Health. Perspect* 111 (2003) 1819–1826. [PubMed: 14630514]

Highlights

- HMB exposure differentially affected transcript levels in rat prostate and testis.
- Gene expression changes in response to HMB were dose and tissue type dependent.
- Drug metabolism pathways were altered by HMB exposure in the prostate and testis.
- HMB exposure affected apoptosis and energy metabolism pathways in the testis.

Figure 1. Gene expression and functional/pathway profiling in prostate and testis of PND 30 rats Principal component analysis (PCA) mapping are shown. PCA was performed with ArrayTrack software [76]. (A) Comparison of control group prostate and testis samples. Blue indicates the control prostate samples, while red indicates the control testis samples. (B) PCA among the control and HMB perinatally exposed groups for the prostate. (C) PCA among the control and HMB perinatally exposed groups for the testis.

Prostate

Testis

Figure 2. Relative transcript levels of estrogen-related genes (*Esr1*, *Esr2* and *Cyp17a1*) in the prostate (left) and testis (right) of PND 30 males perinatally exposed to HMB
Expression levels were determined by qPCR. Data are expressed as the mean fold change $\pm$ standard error of the mean (n=4–5 per group).

Figure 3. Relative transcript levels of cell cycle (*Adprh1*, *Polm*) and prostate cancer-related (*Dlx3* and *Mcc*) genes in the prostate (left) and testis (right) of PND 30 males exposed perinatally to HMB

Expression levels were determined by qPCR. Data are expressed as the mean fold change $\pm$ standard error of the mean (n=4–5 per group). **p < 0.01 compared to the control group for the prostate. *p < 0.05 compared to the control group for the prostate or testis. ^a p < 0.05 compared to the 30000 ppm HMB group for the prostate.

Figure 4. Relative transcript levels of *Prm1*, *Prm2*, *Cst1*, *Iqcf6*, and *Dnajb7* in the testis of PND 30 males exposed perinatally to HMB

Expression levels were determined by qPCR. Data are expressed as the mean fold change $\pm$ standard error of the mean (n=4–5 per group). *p < 0.05 compared to the control group.

Figure 5. Relative transcript levels of *Aco2*, *Tspo*, *Hmgcs1*, *Cyp4f18*, *Bad*, *Gsta2* and *Ndufv3* in the prostate and testis of PND 30 males exposed perinatally to HMB

Expression levels were determined by qPCR. Data are expressed as the mean fold change $\pm$ standard error of the mean (n=4–5 per group). *p < 0.05 compared to the control group in the prostate or testis.

	3000 ppm HMB	30000 ppm HMB
Genes that ubiquitously expressed in somatic cells
Prostate cancer-related genes (tumor suppressors) (i.e. <i>Mcc</i> , <i>Dlx3</i>)
Testis/spermatogenic cell-specific genes	$\pm$ or

Figure 6. Summary of gene expression changes in the testis of PND 30 rats exposed perinatally to HMB

Blue arrows indicate down-regulation; red arrows indicate up-regulation.

Figure 7. Proposed mechanism of HMB-induced effects in the prostate and testis of PND 30 rats

Transcript levels of genes related to drug metabolism in the prostate and testis were differentially affected by perinatal exposure to 30000 ppm HMB. The 30000 ppm HMB dose only influenced key mitochondrial pathways, represented by GO terms, in the testis. The increased energy (ATP) demand for execution of apoptosis might be met by up-regulation of genes involved in mitochondrial biogenesis and energy metabolism (i.e. oxidative phosphorylation, Krebs cycle, and fatty acid metabolism). As another possible pathway, down-regulation of genes involved in steroid biosynthesis (gray box) may lead to a decline in testosterone levels, which could induce expression of pro-apoptotic genes to promote cell death within the testis. Altogether, these transcriptional changes may lead to testicular toxicity at PND 30 following HMB exposure. The up-arrow indicates increased activity of the pathway (GO term) and the down-arrow indicates decreased activity of the pathway (GO term).

Table 1.

RT-PCR and qPCR primer pairs

Genes	Primer sequences	Amplified size (bp)	GenBank Accession #
<i>rAdppl1</i>	For: 5'-AGG AAC TGG AAC ACA AAG GCA Rev: 5'-TAG GGG ACG CTC TAA GCC TG	145	NM_001013054.1
<i>rPolm</i>	For: 5'-GCA GGG CTG CAG TAT TAC CA Rev: 5'-AAC TTC CCC CTT CGG AAA CC	146	NM_001011912.1
<i>rDlk3</i>	For: 5'-ACA GTC CCA ACA ACA GCG AT Rev: 5'-TAG TTG GGG GAG GCA CTG TA	139	NM_001105832.1
<i>rMcc</i>	For: 5'-GTC CAG CAC CGA CGA GGA G Rev: 5'-TCA TTC CAG CTG ATG AAG CCA T	72	NM_001170534.1
<i>rCyp4f18</i>	For: 5'-GCA TTA AGG AGA GCC TGC GA For: 5'-CGG CCA TCT GGG AGC ATA AT	85	NM_0010033686.1
<i>rAco2</i>	For: 5'-CCG TGG GCA TCT GGA TAA CA For: 5'-CAT TGC GCA CAG AAT TGG CT	86	NM_024398.2
<i>rHmgcs2</i>	For: 5'-AGG AGG CCA ATC CAT ACA ACC For: 5'-AGA TCC TAT GGG GTC GCT GT	128	NM_173094.2
<i>rTspo</i>	For: 5'-GAG CCT ACT TTG TGC GTG GT For: 5'-CGA ATA CAG TGT GCC CCA GA	110	NM_012515.2
<i>rGsta2</i>	For: 5'-CCT TTT CTG GCT GCT GGA GGA For: 5'-CCT CAA GAG AAG CAT GCT AAG GAT A	70	NM_012796.2
<i>rBad</i>	For: 5'-CTT GAG GAA GTC CGA TCC CG For: 5'-GCT CAC TCG GCT CAA ACT CT	113	NM_022698.1
<i>rNdufv3</i>	For: 5'-GTT CCG AGG AGA ACT GGC TT For: 5'-CAG TGT GGG CTC TTT GAA CAC	112	NM_001101011.2
<i>rCstl1</i>	For: 5'-CAA CGC AAG CAA TGA CTC CT For: 5'-GGA GGC TTT CTT CGT CTC GT	142	NM_001106522.1
<i>rPrm2</i>	For: 5'-GGC TTC ACA GGA TCC ACA AGA For: 5'-ATA GTG CCA CCT GCA TTT CCT	143	NM_012873.1
<i>rlqc16</i>	For: 5'-AGA TAC AGT CAT GGT GGC GAG For: 5'-ATC TTG GTT TGC ATT GAC CGC	106	NM_001109362.1
<i>rPrm1</i>	For: 5'-ATG GCC AGA TAC CGA TGC TGC For: 5'-CTA AAG GTG TAT GAG CGG CG	140	NM_001002850.1
<i>rDnajb7</i>	For: 5'-CCT TAT GTT TCT CGG GGG CA For: 5'-ATA GTT GCC CAC CCC AGG AT	78	NM_001130510.1
<i>Rn18s</i>	For: 5'-GAC CCG GGG AGG TAG TGA CGA Rev: 5'-GGA GCT GGA ATT ACC GCG GCT	141	M11188.1

Table 2.

Body and reproductive organs weights and serum testosterone levels in PND 30 male rats dosed perinatally with HMB

	Control 0 ppm	HMB (ppm)	
		3000	30000
Body weights (BW) (g)	89.17 ± 1.75 (n=20)	85.20 ± 1.07 (n=24)	69.72 ± 2.03* (n=21)
Paired testis weights (g)	0.849 ± 0.019 (n=20)	0.804 ± 0.011 (n=23) ^a	0.624 ± 0.024* (n=21)
Relative paired testis weights (g/BW10g)	0.096 ± 0.001 (n=20)	0.095 ± 0.001 (n=23) ^a	0.089 ± 0.001* (n=21)
Paired epididymis weights (g)	0.113 ± 0.003 (n=20)	0.114 ± 0.007 (n=23) ^a	0.093 ± 0.003* (n=21)
Relative paired epididymis weights (g/BW10g)	0.013 ± 0.0003 (n=20)	0.013 ± 0.0008 (n=23) ^a	0.013 ± 0.0003 (n=21)
Prostate weights (g)	0.054 ± 0.002 (n=20)	0.053 ± 0.002 (n=24)	0.044 ± 0.002* (n=21)
Relative prostate weights (g/BW10 g)	0.006 ± 0.0002 (n=20)	0.006 ± 0.0002 (n=24)	0.006 ± 0.0003 (n=21)
Seminal vesicle weights (g)	0.057 ± 0.003 (n=20)	0.054 ± 0.002 (n=24)	0.050 ± 0.002 (n=21)
Relative seminal vesicle weights (g/BW10g)	0.006 ± 0.0003 (n=20)	0.006 ± 0.0002 (n=24)	0.007 ± 0.0003 (n=21)
Liver weights (g)	3.836 ± 0.151 (n=20)	3.629 ± 0.072 (n=24)	3.006 ± 0.105* (n=21)
Relative liver weights (g/BW10g)	0.428 ± 0.010 (n=20)	0.427 ± 0.006 (n=24)	0.430 ± 0.005 (n=21)
Paired kidney weights (g)	0.837 ± 0.018 (n=20)	0.820 ± 0.013 (n=24)	0.680 ± 0.021* (n=21)
Relative paired kidney weights (g/BW10g)	0.094 ± 0.001 (n=20)	0.097 ± 0.001 (n=24)	0.098 ± 0.001 (n=21)
Serum testosterone levels (ng/mL)	0.385 ± 0.095 (n=10)	0.333 ± 0.094 (n=11)	0.276 ± 0.060* (n=10)

Values are mean ± SEM of 20–24 litters per group, except for serum testosterone measurements, where fewer animals were analyzed. BW10g = Body weight (BW)/10.

^aMissing testis and epididymis weights for one animal (litter).

* p < 0.05 compared to the control group.

Ingenuity canonical pathways in the prostate significantly altered by exposure to 3000 ppm HMB ($p < 0.05$; FC > 1.5)

Table 3.

Ingenuity Canonical Pathways	p-Value 3000 ppm
FXR/RXR Activation	0.0003
LPS/IL-1 Mediated Inhibition of RXR Function	0.004
Nicotine Degradation II	0.013
Extrinsic Prothrombin Activation Pathway	0.020
Hepatic Cholestasis	0.025
Cysteine Biosynthesis/Homocysteine Degradation	0.030
Nicotine Degradation III	0.042
Thyronamine and Iodothyronamine Metabolism	0.044
Thyroid Hormone Metabolism I (via Deiodination)	0.044

Table 4.

Ingenuity canonical pathways in the prostate significantly altered by exposure to 30000 ppm HMB ($p < 0.05$; FC > 1.5)

Ingenuity Canonical Pathways	p-Value 30000 ppm
Cell Cycle Control of Chromosomal Replication	1.77×10^{-8}
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0.0003
DNA Double-Strand Break Repair by Homologous Recombination	0.005
Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	0.019
Small Cell Lung Cancer Signaling	0.022
Estrogen-mediated S-phase Entry	0.023
Role of MAPK Signaling in the Pathogenesis of Influenza	0.030
Dermatan Sulfate Biosynthesis	0.046

Table 5.

Ingenuity canonical pathways in the testis significantly altered by exposure to 3000 ppm HMB ($p < 0.05$; FC > 1.5)

Ingenuity Canonical Pathways	p-Value 3000 ppm
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	0.003
Granulocyte Adhesion and Diapedesis	0.004
TNFR1 Signaling	0.005
NF- κ B Signaling	0.009
Toll-like Receptor Signaling	0.017
TNFR2 Signaling	0.019
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	0.021
Uracil Degradation II (Reductive)	0.023
Thymine Degradation	0.023
Agranulocyte Adhesion and Diapedesis	0.026
Graft-versus-Host Disease Signaling	0.026
Inhibition of Angiogenesis by TSP1	0.026
Tec Kinase Signaling	0.030
PPAR α /RXR α Activation	0.036
OX40 Signaling Pathway	0.041
Dolichyl-diphosphooligosaccharide Biosynthesis	0.045
T Cell Receptor Signaling	0.046
Cdc42 Signaling	0.047
Fc Epsilon RI Signaling	0.049
LXR/RXR Activation	0.049

Table 6.

Ingenuity canonical pathways in the testis significantly altered by exposure to 30000 ppm HMB (p < 0.05; FC > 1.5)

Ingenuity Canonical Pathways	p-Value 30,000 ppm
Inositol Pyrophosphates Biosynthesis	0.002
Uracil Degradation II (Reductive)	0.009
Thymine Degradation	0.009
Sperm Motility	0.010
Netrin Signaling	0.012
Calcium-induced T Lymphocyte Apoptosis	0.020
Atherosclerosis Signaling	0.030
Protein Kinase A Signaling	0.031
Inhibition of Angiogenesis by TSP1	0.039
Nur77 Signaling in T Lymphocytes	0.039
Flavin Biosynthesis IV (Mammalian)	0.039
CD28 Signaling in T Helper Cells	0.040
Neuroprotective Role of THOP1 in Alzheimer's Disease	0.043
Phospholipases	0.045
Graft-versus-Host Disease Signaling	0.047

Table 7.

Top 10 up-regulated and down-regulated genes in the testis of PND 30 males exposed perinatally to 30000 ppm HMB

Gene Symbol	Description	Fold Changes	Expression	Reference
Up-regulated				
<i>Lsmem1</i>	Leucine rich single-pass membrane protein 1	4.922	Muscle, heart mainly	https://www.ncbi.nlm.nih.gov/gene/680810
<i>Il19</i>	Interleukin 19	3.31	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/681145
<i>Olf607</i>	Olfactory receptor 607	2.67		https://www.ncbi.nlm.nih.gov/gene/546989 (mouse)
<i>Cited4</i>	Chp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 4	2.641		https://www.ncbi.nlm.nih.gov/gene/114491
<i>Apoc3</i>	Apolipoprotein C3	2.396	Liver mainly	https://www.ncbi.nlm.nih.gov/gene/24207
<i>V1ra14</i>	Vomerolateral 1 receptor A14	2.169		https://www.ncbi.nlm.nih.gov/gene/?term=V1ra14 (297442)
<i>Serpinc1</i>	Serpin family C member 1	2.121	Liver	https://www.ncbi.nlm.nih.gov/gene/304917
<i>Cybrd1</i>	Cytochrome B reductase 1	2.101	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/295669
<i>Mage12</i>	Melanoma antigen, family L2	2.096		https://www.ncbi.nlm.nih.gov/gene/679875
<i>Cyp2d6</i>	Cytochrome P450 family 2 subfamily D member 6	2.030	Liver, intestine	https://www.ncbi.nlm.nih.gov/gene/1565 (human)
Down-regulated				
<i>Affa-as1</i>	AFF1 antisense RNA 1	-13.882	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/?term=C4orf36+(LOC498330)
<i>CstII</i>	Cystatin-like 1	-13.018	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/296220
<i>I700080E11Rik</i>	RIKEN cDNA 1700080E1	-11.800	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/?term=I700080E11Rik (mouse)
<i>Pss37</i>	Protease, serine 37	-10.733	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/362346
<i>Pss52</i>	Protease, serine 52	-10.724	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/73382
<i>Fam71f2</i>	Family with sequence similarity 71 member F2	-10.174	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/500060
<i>C10orf120</i>	Chromosome 10 open reading frame 120	-10.157	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/399814 (Human)
<i>Iqcf6</i>	IQ motif containing F6	-9.651	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/440956
<i>Fam71b</i>	Family with sequence similarity 71 member B	-9.170	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/153745
<i>LOC102552669</i>	Uncharacterized LOC102552669	-8.789	Testis-specific	https://www.ncbi.nlm.nih.gov/gene/?term=C1orf234+Rattus+

Table 8

Top 10 up-regulated and down-regulated genes in the testis of PND 30 males exposed perinatally to 3000 ppm HMB

Gene Symbol	Description	Fold Changes	Expression	Reference
Up-regulated				
<i>Thpprs4</i>	Transmembrane protease, serine 4	3.270	Uterus, lung mainly	https://www.ncbi.nlm.nih.gov/gene/367074
<i>Hykk</i>	Hydroxyllysine kinase	2.709	Kidney mainly	https://www.ncbi.nlm.nih.gov/gene/300723
<i>Loc102550987</i>	LOC102550987	2.525	Kidney mainly	https://www.ncbi.nlm.nih.gov/gene/?term=Loc102550987
<i>Shank1</i>	SH3 and multiple ankyrin repeat domain 1	2.467	Brain mainly	https://www.ncbi.nlm.nih.gov/gene/78957
<i>Amy2b</i>	Amylase 2b	2.315	GI mainly	https://www.ncbi.nlm.nih.gov/gene/545562 (mouse)
<i>Loc103690551</i>	RNA-binding motif protein, Y chromosome, family 1 member F/J-like	2.297	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/?term=Loc103690551
<i>Sstr3</i>	Somatostatin receptor 3	2.269	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/171044
<i>Ntn5</i>	Netrin 5	2.219	Testis mainly	https://www.ncbi.nlm.nih.gov/gene/308587
<i>Apol2</i>	Apolipoprotein L2	2.206	Spleen mainly	https://www.ncbi.nlm.nih.gov/gene/315111
<i>Phf24</i>	PHD finger protein 24	2.179	Brain mainly	https://www.ncbi.nlm.nih.gov/gene/500446
Down-regulated				
<i>Wfdc21</i>	WAP four-disulfide core domain 21	-8.183	Liver mainly	https://www.ncbi.nlm.nih.gov/gene/360228
<i>Ccl21</i>	C-C motif chemokine ligand 21	-5.132	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/298006
<i>Krt18</i>	Keratin 18	-4.026	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/294853
<i>Fam180a</i>	Family with sequence similarity 180 member A	-3.819	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/362336
<i>Upk1b</i>	Uroplakin 1B	-3.059	Lung, uterus mainly	https://www.ncbi.nlm.nih.gov/gene/303924 expression changed by urothelial carcinogenesis
<i>Fzfb</i>	Frizzled-related protein	-3.044	Adrenal gland mainly	https://www.ncbi.nlm.nih.gov/gene/295691
<i>Aqp1</i>	Aquaporin 1	-3.012	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/25240
<i>Rein</i>	Resistin	-2.845	Thymus mainly	https://www.ncbi.nlm.nih.gov/gene/246250 (inflammation)
<i>Cxpm2</i>	Carboxypeptidase X, (M14 family), member 2	-2.708	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/293566
<i>Medag</i>	Mesenteric estrogen dependent adipogenesis	-2.577	Ubiquitous	https://www.ncbi.nlm.nih.gov/gene/360757

Table 9.

Effect of perinatal HMB exposure on select GO terms

Gene Ontology (GO) Terms (molecular function/biological processes)	Number of genes in a GO term	Prostate		Testis	
		3000 ppm p-Value	30000 ppm p-Value	3000 ppm p-Value	30000 ppm p-Value
Mitochondria-related GO Terms					
Apoptosis (intrinsic and extrinsic pathways)	75	0.978	0.169	0.158	0.013
Oxidative Phosphorylation	91	0.684	0.664	0.016	0.013
Complex I	38	0.612	0.537	0.040	0.050
Complex II	4	0.607	0.517	0.585	0.156
Complex III	8	0.428	0.061	0.167	0.001
Complex IV	22	0.940	0.855	0.037	0.003
Complex V	19	0.455	0.834	0.076	0.024
Fatty Acid Metabolism	60	0.759	0.765	0.047	0.014
Intracellular protein transport	3	0.898265	0.042505	0.76258	0.02296
Krebs Cycle	16	0.903	0.188	0.179	0.010
Microtubule-based process	2	0.487523	0.016644	0.556202	0.261776
Mitochondrial Biogenesis	4	0.258	0.232	0.934	0.049
Mitochondrial Fusin	4	0.434722	0.01984	0.164771	0.043014
Steroid Biosynthesis/Metabolism	8	0.392	0.518	0.194	0.002
Drug Metabolism					
Phase-1	48	0.071	0.045	0.312	0.002
Phase-2	48	0.656	0.040	0.055	0.005

Shown are the number of genes associated with select GO categories and the p-values for each category arranged by tissue type and dose. Highlighted p-values indicate a significant ($p < 0.05$) HMB effect.