HHS Public Access

Author manuscript

Int J Impot Res. Author manuscript; available in PMC 2014 September 01.

Published in final edited form as:

Int J Impot Res. 2014 ; 26(2): 67–75. doi:10.1038/ijir.2013.37.

ORAL BISPHENOL A (BPA) GIVEN TO RATS AT MODERATE DOSES IS ASSOCIATED WITH ERECTILE DYSFUNCTION, CAVERNOSAL LIPOFIBROSIS, AND ALTERATIONS OF GLOBAL GENE TRANSCRIPTION

I Kovanecz[1,2], **R Gelfand**[1,3], **M Masouminia**[1], **S Gharib**[1], **D Segura**[1], **D Vernet**[1,3], **J Rajfer**[1,2], **DK Li**[4,5], **K Kannan**[6], and **NF Gonzalez-Cadavid**[1,2,3,#]

[1]Division of Urology, Department of Surgery, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, CA

[2]Department of Urology, David Geffen School of Medicine at UCLA, Los Angeles, CA

[3]Division of Endocrinology, Charles Drew University of Medicine and Science, Los Angeles, CA

[4]Department of Health Research and Policy, Stanford University, Stanford, CA

[5]Division of Research, Kaiser Permanente

[6]Wadsworth Center, New York State Department of Health, Albany, NY

Abstract

Introduction—Bisphenol A (BPA), a suspected reproductive biohazard and endocrine disruptor released from plastics is associated with erectile dysfunction (ED) in occupationally exposed workers. However, in rats, despite the induction of hypogonadism, apoptosis of the penile corporal smooth muscle, fat infiltration into the cavernosal tissue, and changes in global gene expression with the intraperitoneal administration of high dose BPA, ED was not observed.

Aims—We investigated whether BPA administered orally rather than intraperitoneally to rats for longer periods and lower doses will lead to ED.

Main Outcomes Measures—ED, histological, and biochemical markers in rat penile tissues.

Methods—2.5-month old rats were given drinking water daily without and with BPA at 1 and 0.1 mg/kg/day. Two months later, erectile function was determined by cavernosometry (DIC) and electrical field stimulation (EFS) and serum levels of testosterone (T), estradiol (E2), and BPA were measured. Penile tissue sections were assayed by Masson (smooth muscle (SM)/collagen), Oil Red O (fat), TUNEL (apoptosis), immunohistochemistry for Oct 4 (stem cells), and α-SM actin/ calponin (SM and myofibroblasts), applying quantitative image analysis. Other markers were assayed by western blots. DNA microarrays/microRNA assays defined transcription profiles.

[#]**Corresponding author**: Nestor F. Gonzalez-Cadavid, Ph.D., LABioMed at Harbor-UCLA Medical Center, Urology Research Laboratory, Building C3, 1124 West Carson Street, Torrance, CA, 90502 **Telephone**: 310-222-3824; **fax:** 310-222-1914; ncadavid@ucla.edu.

Results—Orally administered BPA did not affect body weight, but: 1) decreased serum T and E2; 2) reduced the EFS response and increased the DIC drop rate; 3) increased within the corporal tissue the presence of fat, myofibroblasts and apoptosis; 4) lowered the contents of SM and stem cells, but not nerve terminals; and 5) caused alterations of the transcriptional profiles for both mRNA and microRNAs within the penile shaft.

Conclusions—Long-term exposure of rats to oral BPA,caused a moderate corporal veno-occlusive dysfunction (CVOD), possibly due to alterations within the corporal tissue that pose gene transcriptional changes related to inflammation, fibrosis and epithelial/ mesenchymal transition (EMT).

Keywords

Erectile dysfunction; fibrosis; corporal veno-occlusive dysfunction; epithelial mesenchymal transition; microRNA

INTRODUCTION

Bisphenol A (BPA) is a suspected reproductive biohazard and endocrine disruptor that in experimental animals has been shown to affect multiple reproductive organs (testis, ovaries, prostate, etc), presumably because of its well documented “selective estrogen receptor modulator” properties (1). BPA poses potential environmental and occupational risks because of its widespread use in the manufacture of polycarbonate plastics, epoxy resins, dental sealants, and other plastics, and for lining food and beverage cans (2–4). It is released, particularly upon moderate heating, into our ecosystem, thus posing environmental risks as well as occupational risks for related workers, either by inhalation or oral/dermal contamination (5–7).

An association of BPA exposure in women with the development of polycystic ovary syndrome was recently reported (8), as well as a series of studies in men, specifically workers in Chinese factories employing BPA, that show a correlation between the concentration of BPA in the urine and the development of erectile dysfunction (ED) and semen deterioration (9–12). These studies on ED, however, did not ascertain the etiology of the ED, i.e. was it due to an effect of the BPA on the hypothalamic pituitary axis, the cavernosal nerves, and/or a direct effect of the BPA on the cavernosal tissue itself.

In the rabbit, a high dose of BPA given intraperitoneally for 2 weeks reduced the in vitro relaxation of corpora cavernosa strips and induced lipofibrotic infiltration of the corpora smooth muscle, but other histopathological markers or erectile function in vivo were not measured (13). In our previous study (14) in young male rats (1.5 months old) exposed for 3 months to intraperitoneal administration of BPA at relatively high levels (25 mg/kg/day), but only 1/3 of those in the aforementioned rabbit study and ½ of the Lowest Observed Adverse Effect Level (LOAEL) for BPA (15), we found a) a corporal histopathology normally associated with ED, b) hypogonadism, and c) parallel changes in global gene expression that would be consistent with ED, Although this exposure in the rats led to urinary excretion of BPA that was 7-fold higher than that in the occupationally exposed workers who were found to have ED, no frank ED by EFS or corporal veno-occlusive dysfunction (CVOD) could be

detected in our animals. This posed the question as to whether oral administration of BPA for longer periods, which is more representative of human exposure, and with different BPA kinetics and metabolism than by the intraperitoneal route may eventually exacerbate the underlying corporal pathology when applied to older rats that are more prone to ED. This could occur to a degree that ED as measured by EFS and cavernosometry may manifest itself.

The elucidation of the significance of the BPA-induced changes on the penile corporal pathology also needs further molecular characterization beyond what was shown in our previous rat study, where DNA microarrays were used to define alterations in the transcriptional signature profile for mRNAs. A complementary approach is based on the global profile of microRNAs (miRs) are novel key regulators of transcriptional and translational processes whose alterations are involved in a number of diseases and also affect key biological processes (16,17). There is increasing evidence that environmental chemicals like BPA and estrogen, may affect the miR profile and target genes whose inactivation may be potentially noxious. In the case of BPA, there is still very limited information, from only a few systems (18,19), and none from the penile corpora cavernosa. Our knowledge of whether BPA exposure alters the miR profile in this tissue may help to understand the mechanism of the histopathology and of the ED potentially induced by BPA.

Therefore, in the current work we investigated whether a prolonged oral exposure of BPA to adult rats; a) leads dose-proportionally to BPA accumulation and reduces the levels of sex steroids in serum; b) induces loss of smooth muscle cells and lipofibrosis in the corpora cavernosa and changes in the expression of genes related to corporal compliance which may underlie a potential ED; c) alters the miR profile for penile tissue RNA in a manner suggestive of potential pathological effects on corporal tissue compliance and d) causes ED as determined by EFS and DIC.

MATERIALS AND METHODS

Animal procedures

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, National Academy Press, Washington, DC, USA, revised 1996) and was approved by the IACUC at LABioMed. All 2.5-month old male Fischer 344 rats were given daily drinking water containing either BPA at 10 or 1 mg/l (corresponding to approximate doses of 1 or 0.1 mg/kg/day of BPA), or no addition (controls) (n=8/group). Treatments were interrupted 3 days before completion (washout) at 4.5 months, and erectile function was measured as follows:

Dynamic Infusion Cavernosometry—It was performed as previously described (20,21). Briefly, basal intracavernosal pressure (ICP) was recorded, and 0.1ml papaverine (20 mg/ml) was administered through a cannula into the corpora cavernosa. The ICP during tumescence was recorded as “ICP after papaverine” 5 minutes after the injection. Saline was then infused through another cannula, increasing infusion rate by 0.05 ml/min every 10 seconds, until the ICP reached 100 mmHg (“infusion rate”), then the infusion rate was

adjusted to maintain a steady ICP level just above 100 mmHg ("maintenance rate"). The "drop rate" was determined by recording the fall in ICP within the next 1 minute after the infusion was stopped.

Electrical field stimulation of the cavernosal nerve (EFS)—EFS was performed preceding cavernosometry as previously described (22,23). Briefly, under anesthesia, the cavernosal nerve was exposed, and hooked by a bipolar platinum electrode. Systemic arterial and intracavernosal pressure measurements were obtained by simultaneous intrafemoral artery and cavernosal catheterization, respectively. EFS was applied at increasing voltages and a frequency of 15 Hz for 60 s, separated by 1-min intervals, with a Lab-Trax-4/24T data acquisition device with build in stimulator (WPI Inc. Sarasota, FL, USA). Intra-arterial and intracavernosal pressures were simultaneously recorded, and values were expressed in mmHg. The ratio between the maximal intracavernosal pressure (MIP) and the mean arterial pressure (MAP) at the peak of erectile response were calculated, to normalize for variations in systemic arterial blood pressure.

BPA and hormonal assays

BPA was assayed in blood serum, urine and fresh penile tissue by HPLC–ESI–MS/MS (24). Testosterone and estradiol were assayed by applying validated LC-MS/MS methods (25).

Determinations in tissue sections

After cavernosometry, animals were sacrificed and aliquots of the skin-denuded penile shafts were fixed overnight in 10% buffered formalin, washed, and stored in alcohol (70%) at 4°C until processed for paraffin embedded tissue sections (6–8 μm). Adjacent tissue sections were used for (20–23): a) Masson trichrome staining for collagen (blue) and SM cells (red); b) apoptotic index by the TUNEL reaction with the Apoptag kit (Millipore, Billerica, MA, USA); c) immunodetection with monoclonal antibodies against α-smooth muscle actin (α-SMA) as a SM and myofibroblast marker (Sigma kit, Sigma Diagnostics, St Louis, MO); calponin mouse monoclonal, 1:100 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) as marker for SM only, and d) Oct 4 mouse monoclonal, 1:100 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as stem cell marker. For immunodetection, sections were then incubated with biotinylated anti-Mouse IgG, followed by ABC complex (Vector labs, Temecula, CA) and AEC chromogen peroxidase substrate (Sigma). Sections were counterstained with hematoxylin. Negative controls in the immunohistochemical detections were done by replacing the first antibody with IgG isotype.

Aliquots of the penile shaft were alternatively embedded in OCT and used for obtaining frozen tissue sections that were subjected to Oil Red O staining for detecting fat droplets (26).

Quantitative image analysis (QIA) was performed by computerized densitometry using the ImageProPlus 5.1.1 program (Media Cybernetics, Silver Spring, MD), coupled to an Olympus BHS microscope equipped with an Spot RT color digital camera (20–23). For Masson, α-SMA, Calponin and Oil Red O staining, 40x magnification pictures were taken comprising the whole cross section of the penile shaft. For TUNEL, 12 fields at 200x were

photographed. For all determinations, only the corpora cavernosa and the tunica albuginea were analyzed in a computerized grid and expressed as % of positive area vs. total area. In all cases at least 3 matched sections per animal and 8 animals per group were analyzed.

Determinations in fresh tissue

Western blots (20–23)—Penile tissue homogenates (about 50 mg fresh tissue stored at −80 °C until use) were obtained using Bullet Blender Storm 24 (Next Advance, Inc, NY) using one scoop of SSP14B (1.4 mm) beads and 4 SSB32 beads (3.2 mm) in boiling lysis buffer consisting in 1% SDS, 1.0 mM sodium orthovanadate, 10 mM Tris pH 7.4 and protease inhibitors (3 μM leupeptin, 1 μM pepstatin A, 1mM phenyl methyl sulfonyl fluoride), cutting the tissue into small pieces, adding the beads and the lysis buffer, and then running the blender for 5 min at speed 8 for 3 times and centrifuging at 16,000 g for 5 min. The supernatant proteins (20–30 μg) were subjected to western blot analysis. Following a 7–10 % Tris-HCl polyacrylamide gel electrophoresis (PAGE) (Bio-Rad, Hercules, CA) proteins were transferred overnight at 4°C to nitrocellulose membranes then the non-specific binding was blocked with blocking solution (5% non-fat dried milk, 0.1% (v/v) Tween 20 in PBS) for 1 hour at room temperature. After several washes the membranes were incubated with the primary antibodies for 1 hour at room temperature. The monoclonal antibodies used were as follows: calponin 1 (Calp 1) mouse monoclonal (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) 1:500; nNOS rabbit monoclonal (Abcam, Cambridge Ms), 1:750, and, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference housekeeping protein mouse monoclonal (Millipore, Billerica, MA) 1:1500. Other antibodies were as detailed for immunohistochemistry.

The membranes after several washes were incubated for 1 hour at room temperature with a secondary antibody linked to horseradish peroxidase. The immunoreactive bands were visualized using the ECL plus western blotting chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). The densitometric analysis of the bands were performed with Image J (NIH, Bethesda, MD). A positive control was run throughout all gels for each antibody to standardize for variations in exposures and staining intensities. Negative controls were performed omitting the primary antibody. Band intensities were determined by densitometry and corrected by the respective intensities for GAPDH, upon reprobing.

Global transcriptional profiles (signature) (28–30)—For the multiple mRNA profile, RNA was isolated from aliquots of penile tissue from the control and BPA-treated rats and then maintained in RNA later, using RNeasy Plus Micro kit (Qiagen) with quality determined by the Agilent 2100 Bioanalyzer. Assays were performed in a duplicate set of penile tissue RNAs by the UCLA DNA microarray core, applying the Affymetrix Rat Gene array for 29,215 sequences. Only genes that were up- or down-regulated by at least 2-fold were considered unless specifically detailed. For the multiple miR profile, RNA was isolated from other tissue aliquots using the *mir*Vana™ miRNA isolation kit (Ambion), and analysis was carried out by LC Sciences (Houston, TX) for all miR transcripts listed in the Sanger miRBase Release 18.0.

Statistical analysis

Values are expressed as the mean ± SEM. The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by single factor ANOVA, followed by *post hoc* comparisons with the Tukey test. Differences among groups were considered statistically significant at $P < 0.05$.

RESULTS

Exposure of 10 weeks old Fischer 344 male rats for 4.5 months to BPA, given in the drinking water at calculated intakes of approximately 1 and 0.1 mg/kg/day of BPA, did not affect body weight (Fig. 1A). There was a statistically significant 58% reduction in the level of serum testosterone (Fig 1B) with the higher dose (1.0 mg/kg/day), and a 30%, but non-significant decrease at the lower dose (0.1 mg/kg/day). There was a much more pronounced (88% and 66%) decrease in the serum estradiol levels (Fig 1C) in the higher and lower BPA doses, respectively. The serum BPA concentration, reflecting the levels remaining after a 3 day washout period, was significantly increased in the rats exposed to 1 and 0.1 mg/kg/day, by 10.8-and 2.1-fold, respectively, with only a slight increase in the levels of free BPA (Fig 1D).

The alteration in serum testosterone and estradiol levels by BPA in these adult animals (7 months old at completion) was accompanied by a moderate CVOD. By cavernosometry, there was a non dose-dependent 82% and, paradoxically, 245% significantly higher drop rates for the higher and lower BPA doses, respectively (Fig 2 A). EFS of the cavernosal nerve gave a significant, but non dose-dependent, decrease of 12% and 13%, respectively, for the maximal intracavernosal pressure (MIP)/mean arterial pressure (MAP), thus implying a mild form of ED (Fig 2 B).

Histochemical staining of penile shaft tissue sections using Masson trichrome demonstrate a significant 48% and 34% reduction in the SM cell (SMC)/collagen ratio in the corpora cavernosa of the rats exposed to the 1 and 0.1 mg/kg/day doses of BPA, respectively (Fig 3 A). It was determined that this reduction in the SMC/collagen ratio was primarily due to a loss of smooth muscle cells, as confirmed by the significant 60% and 26% reduction, respectively, of the corporal area occupied by calponin, a marker of smooth muscle cells that is not expressed in myofibroblasts (Fig 3 B).

These findings are in agreement with the significant roughly 2-fold increase in corporal apoptosis (TUNEL assay) observed in the tissue sections for both BPA dosages (Fig 4 A), presumably affecting the smooth muscle cells. The immunohistochemical staining of α-SMA, a marker of both smooth muscle cells and myofibroblasts, was significantly decreased by 39% and 25%, respectively, in the corporal tissue of the high and low dose BPA-treated specimens (B). The α-SMA/calponin ratio was increased from 1.81 in the control rats to 2.80 in the 1 mg/kg/day BPA-treated rats, a 1.5-fold increase, indicating that in a setting of smooth muscle loss, a substantial part of the α-SMA + cells in the BPA treated corpora cavernosa are indeed myofibroblasts. The lower BPA dose treated corporal tissue showed an α-SMA /Calponin ratio of 1.83, suggesting that myofibroblast induction is unlikely to have occurred in this case.

The histological damage caused by BPA on differentiated cells, mostly in the corpora cavernosa, was accompanied by a substantial reduction in stem cells, as indicated by the decrease in the number of cells positive for the key stem cell marker Oct 4 (Fig 5 A). Immunofluorescence estimation showed a significant decrease of 92% and 86% of the Oct 4a isoform which is the true stem cell transcription factor that is located in the nuclei (31) for the 1 and 0.1 mg/kg/day BPA doses, respectively, There was a 90% and 82% reduction, respectively, of the perinuclear Oct 4 (putatively the same nuclear Oct 4a isoform) and a smaller decrease in corporal cytoplasmic Oct 4b, which is not stem cell related (Fig 5 B).

The corporal SMC loss and potential fibrosis induced by BPA are compounded by substantial fat deposition, as indicated by a significant increase of 3.8- and 3.5-fold, respectively in response to the 1 and 0.1 mg/kg/day doses, compared to the control (Fig 6 A). The histopathological alterations suggestive of a BPA-induced corporal lipofibrosis are accompanied by a decrease in α-SMA in the penile tissue homogenate determined by quantitative western blot (Fig 6 B), similar to the one in the immunohistochemical determination restricted to the corpora cavernosa, but not in calponin which may reflect the presence of the corpus spongiosum tissue smooth muscle in the homogenate. No changes occurred in the content of nitrergic nerve terminals as assessed by nNOS and NF-70 expression (not shown).

The changes in protein expression induced by BPA in the corpora cavernosa are paralleled by changes in the expression of a few genes within the global transcriptional signature revealed by DNA microarrays. Out of 29,216 rat sequences, the means of independent determinations on RNA from pools of 3 penile shafts, showed for the 1 mg/kg/day BPA dose that 16 genes were up-regulated by >2.0, and, 104 genes if this ratio threshold is lowered to 1.5. There was only 1 gene down-regulated if the ratio threshold for downregulation is set at <0.5, and 21 genes if the ratio threshold is <0.66. The selected up-regulated genes (Table 1) include those involved in inflammatory pathways, which were increased 1.5-2.4-fold, and which were robustly expressed in the specimens of the control group (no BPA). The down-regulated genes, such as keratins and cadherin, were mainly related to epithelial mesenchymal transition (EMT) (32,33). Only a handful of the down-regulated genes had their mRNA levels affected by the lower BPA dose, and in a few cases the changes were opposite to the ones seen with the higher BPA dose, but in general the lower dose exerted on the genes upregulated by the higher dose the same trend of effects.

The microRNA (miR) global expression profile showed changes induced by BPA exposure at the higher 1 mg/kg/day dose, where out of 595 sequences, 31 were down-regulated to <0.55, but only 3 were up-regulated by >1.5 (Table 2). As in the case of the mRNAs, the most down-regulated miRs, such as miR-200, -203, and -205, were related to EMT, and in many cases the same genes were also related to fibrosis and inflammation. In contrast to the mRNA profile, the lower dose BPA caused some substantial down-regulation of miR-82, -203a, -347, -377-3p, or -328a The lower dose BPA also caused upregulation of other miRs such as miR-451-5p.

DISCUSSION

This is the first demonstration of ED caused by BPA in an animal model. In this experimental design, ED, specifically CVOD, was induced in 10 week old rats after 4.5 months of the continuous oral ingestion of BPA in the drinking water. In these animals that developed ED, it was estimated that the serum levels of BPA were lower than the serum BPA levels calculated to have been present in those men who developed ED solely from an occupational exposure to BPA (10–12). As such, the current work suggests that BPA exposure could be the first specific chemical environmental risk identified for ED. Taking together with the observations from our previous study, which showed that despite using higher doses of BPA than this current study, the intraperitoneal administration of BPA for a shorter period (3 months) and in younger (1.5 month old) rats failed to uncover any ED, this suggests that the combination of the mode of administration, length of exposure, and age, may determine the overall impact on erectile function. However, both of our studies in the rat showed a reduction in sex steroid levels, a corpora cavernosal histopathology characterized by a reduction in the smooth muscle and stem cell contents together with an increase in corporal myofibroblasts, apoptosis, and fat deposition, combined with alterations in global transcriptional gene expression in RNA obtained from the penile shaft tissues. To our knowledge, the current report is the first one on global miR expression in penile tissue in any condition, or for that matter on the alteration of the miR profile in any organ or tissue by exposure to BPA in an animal model, as well as the effects of BPA on the transcriptional mRNA profile in penile tissue.

The histological and molecular alterations observed in this study may underlie the development of CVOD but at the same time they also seem to suggest that by themselves these changes in the cavernosal tissue are not sufficient to cause ED, and other factors are needed to elicit ED in the rat. The 60% reduction in corporal smooth muscle associated with CVOD in the animals receiving the 1 mg/kg/day dose of BPA agrees with the most severe smooth muscle loss observed in impotent men with CVOD (34). However, the 26% decrease in smooth muscle in the rats exposed to a low dose of BPA did not lead to CVOD, despite in men this is associated with CVOD and in the rabbit leads to a reduction in cavernosal expandability as a surrogate of CVOD (34,35). Our data suggest that the effects of BPA on the corporal smooth muscle are not mediated by neuropraxia, a main factor in the corporal fibrosis occurring after cavernosal nerve damage (20,21), since we did not observe significant decrease in corporal tissue nNOS or NF70, i.e., in nitrergic nerves.

In turn, the reduction of stem cells shown by the decreased expression of Oct 4 suggests that endogenous stem cell reduction may negatively impact the potential repair of corporal tissue damaged by BPA (36,37). In addition, we are emphasizing for the first time in penile pathophysiology, and specifically in ED, the potential contribution to these processes of alterations in the multiple miR profile, and their relevance to BPA effects that have only been reported in a few studies in cell cultures or other tissues (18,19). We believe that the use of the global miR transcriptional signatures in penile tissue and potentially in serum for investigating the pathophysiology of erectile dysfunction as well as its relationship with vascular disease where there is already substantial literature, may open up new investigative opportunities. Altogether, our results suggest that the ED reported in men after occupational

exposure to BPA could be, as would be expected, due to a peripheral effect of this agent on the penile corpora cavernosa, although a central effect at the brain level cannot be discarded (38,39).

One consideration regarding this study is the question of how representative the BPA exposure conditions were, when compared with occupational exposure in men, or even in terms of environmental risks to the whole population. BPA exposed male workers in epoxy resin and BPA-manufacturing factories developed general sexual dysfunction including ED, shown by an almost 4-fold increased risk of erectile difficulty and a 7-fold increased risk of ejaculation difficulty, among other sexual dysfunctions, as self- reported in two well validated questionnaire tools (40,41). In that study, BPA levels were not estimated in serum but only in urine, and expressed as 467 μg/gCr (creatinine) in the occupationally exposed workers (10–12), versus an equivalent value of 11 μg/gCr (creatinine) in the unexposed workers. We have estimated that these urine values in the workers are equivalent to 0.61 versus 0.01 μg/ml, respectively, where the latter controls were comparable to those in the general U.S. population (5–7),

Considering that in our previous study the values of total BPA in urine in the BPA treated vs. control rats (4.35 vs. 0.02 μg/ml) corresponded to 1.09 vs. <0.001 μg/ml in serum, the ratio of the urine/serum values was close to 4. Since no determinations in urine were carried out in the current paper, this factor may then be applied to translate the current serum values of 0.034 μg/ml for the higher BPA dose, and 0.001 μg/ml for the lower dose, into urine values of approximately 0.136 and 0.004 μg/ml, respectively. These values would be 4 and 25 fold lower than the urine levels in men environmentally exposed to BPA in the above mentioned human study (10–12). The lower dose of BPA used in our rat study would only be double the average BPA exposure level in the general US population, i.e. within potential environmental ranges. This is obviously an oversimplification, considering the inaccuracy of this ratio and the differences in renal clearance between rats and men, but they provide an approximate comparison. However, this speculation requires direct validation and should only be taken as an approximation, particularly considering that our determinations were done after a 3 days washout period.

Our current results in the rat treated with 1.0 and 0.1 mg/kg/day of BPA extend to a comparatively 75 and 750 fold lower dose of BPA via this oral exposure when compared to the observations in the rabbit penis following intraperitoneal BPA administration (13). These investigators showed the in vitro impairment of pharmacologically induced relaxation in rabbit corporal strips with the inference that this may lead to ED. However, the exact mechanism of how this impairment in corporal relaxation occurs remains unknown. It should be noted that BPA is assumed to account for the majority of estrogenic activity that leeches from landfills into the ecosystem (15,42,43), and therefore has the potential to affect the human population through its known mediation of the estrogen-related receptor γ, which has greater affinity for BPA than the estrogen receptor α (43,44). Whether these estrogenic effects of BPA play any role remains to be studied.

Experimental clarification is also needed as to whether the faint anti-androgenic activity of BPA by direct binding to the androgen receptor (at higher doses than required for activation

of the estrogen receptor), or its indirect effects by reducing testosterone levels through testicular damage (45–48), may contribute to the corporal histopathology. These main actions are compounded by alteration of the expression of hormone receptors, as well as effects on various enzymes and metabolic pathways. However, it is unlikely that, at least in postnatal exposure to low doses of BPA, these relatively weak effects could induce ED and/or an underlying histopathology (9).

The moderate impact of the 1 mg/kg/day dose of BPA on the global transcriptional expression of mRNAs in the penile shaft suggests that there is an incipient process of inflammation and EMT, but is not indicative of frank corporal fibrosis, and in any case the 10-fold lower dose was inactive in this respect. Much more informative are the transcriptional changes in global miR expression, suggesting that the miR changes by the 0.1 mg/kg/day dose were an earlier process that did not yet impact considerably on the expression of their respective gene targets. However, longer exposures to BPA at this low dose may eventually lead to the miR changes to be reflected on the mRNAs expression.

Of those miRs that were down-regulated in the penile shaft, miR-203, an estrogen-upregulated inhibitor of smooth muscle replication and inducer of SMC de-differentiation that is related to inflammation but not to fibrosis, was reduced by 87% at the 1 mg/kg/day dose (49,50). Remarkably, EMT, a process that plays a pivotal role in wound healing, tissue regeneration and organ inflammation and fibrosis, as well as cancer (32,33,51), is affected by miR-203 as well as the considerably downregulated miRs -200 and -205, and by the lesser downregulated miRs-347, -377, and -494 (52–54). The miR-200a, -200b, and -200c, as well as miR-429 and miR-141, belong to the same MIR family, and EMT is affected by at least the first four of these miRs EMT is a direct contributor to the kidney myofibroblast population in renal fibrosis, specifically in diabetic nephropathy (32).

In turn, miRs -29, -200, -203, -210, -328, -494, and -1224 are involved in the regulation of inflammation-related genes (55,56), and miRs -29 and -200 are particularly related to fibrosis (57,58). None of the miRs whose expression we found to be affected by BPA in the penile shaft tissue have been reported to be related to BPA effects in other systems. The same, with the above cited exception of miR-203, applies to estrogen-mediated effects. miR-328 is drastically down-regulated in the pulmonary artery after a hypoxic assault (59), whereas miR-494 reduces CFTR abundance and function in the airway epithelium in cystic fibrosis (60). The other miRs whose levels we found to be affected by BPA in the penile shaft are new additions on the database and not yet well studied (mainly those > miR-400) or their respective publications are not directly pertinent to potential BPA effects on the corpora cavernosa. Taken together, these observations suggest that miRs-29, -200 (various), -203, -205, and -494 appear to be the more interesting for future studies on the impact of BPA on penile erection.

Irrespective of the mechanism, it is likely that at least part of the erectile difficulty occurring in men after occupational exposure to BPA (10,11) could be due to the local long-term direct toxic effect of BPA on the corpora cavernosa and possibly an effect on the pelvic ganglia, which integrates the peripheral machinery of penile erection. The ejaculation complaints vocalized by these patients may result from effects on the testis, prostate or

seminal vesicles. These assumptions do not exclude potential central effects on sexual arousal and the control of erection occurring in the brain. Additional studies are needed to elucidate these issues and to fully assess the true environmental effects of BPA on erectile function using even lower doses of BPA and longer periods of exposure, and/or determining whether BPA may exacerbate the impact of the predominant risk factors of ED, such as aging, diabetes, or cavernosal nerve damage (61).

ACKNOWLEDGEMENTS

This work was supported by grant NIH-NIEHS R21ES019465 and partially by grant NIH-NIEHS 1U01ES020887, to NGC

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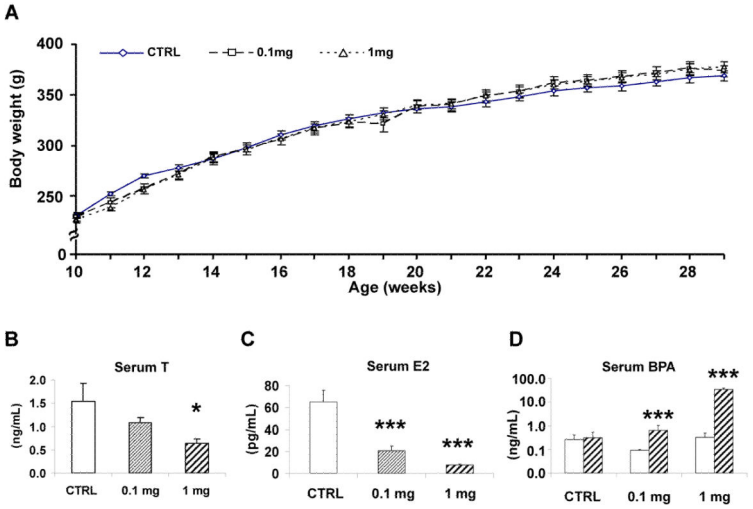

Figure 1. BPA given orally (1 and 0.1 mg/kg/day) to 2.5 month old Fisher 344 rats for 4.5 months did not affect body weights but reduced the serum levels of testosterone and estradiol and increased total and free BPA levels

A: time course of body weight; B: serum testosterone, C: estradiol, and D: BPA levels at completion. CTR: control; n=8/group. *p <0.05; ***p<0.001.

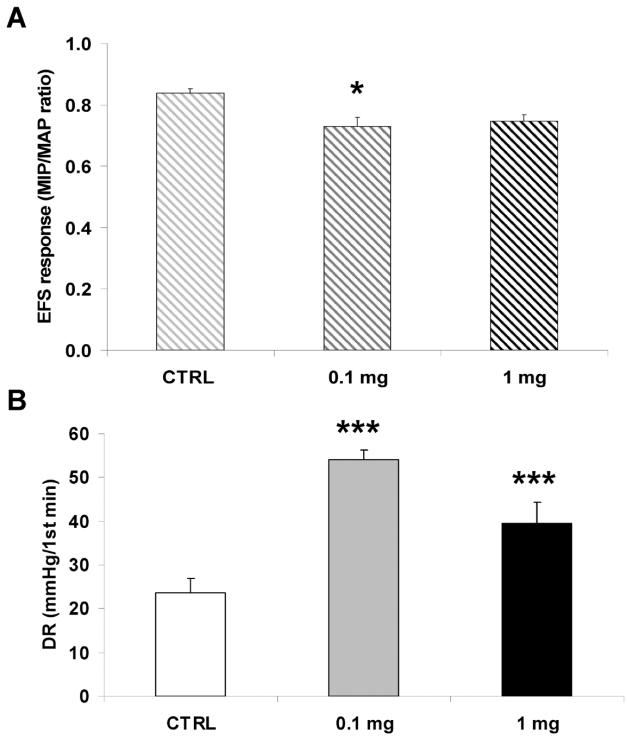

Figure 2. BPA exposure caused a slight reduction in the erectile response to EFS of the cavernosal nerve and moderate CVOD

A: EFS of the cavernosal nerve was applied at 5 Volts. B: the drop rates determined by dynamic infusion cavernosometry. n=8/group. *p <0.05; ***p<0.001.

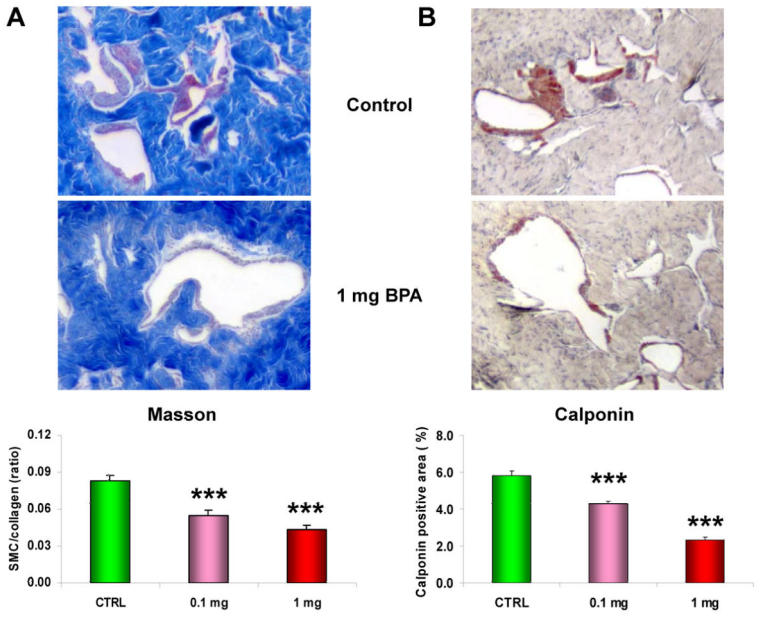

Figure 3. BPA exposure induced a reduction in the smooth muscle content in the penile corpora cavernosa

A: Smooth muscle/collagen ratio determined by Masson trichrome staining on paraffin embedded penile shaft tissue sections followed by quantitative image analysis (QIA) of the corporal region; B: smooth muscle content estimated by immunohistochemistry/QIA for calponin in the corpora. Top: representative photomicrographs (200X); Bottom: QIA values. n=8/group; ***p<0.001.

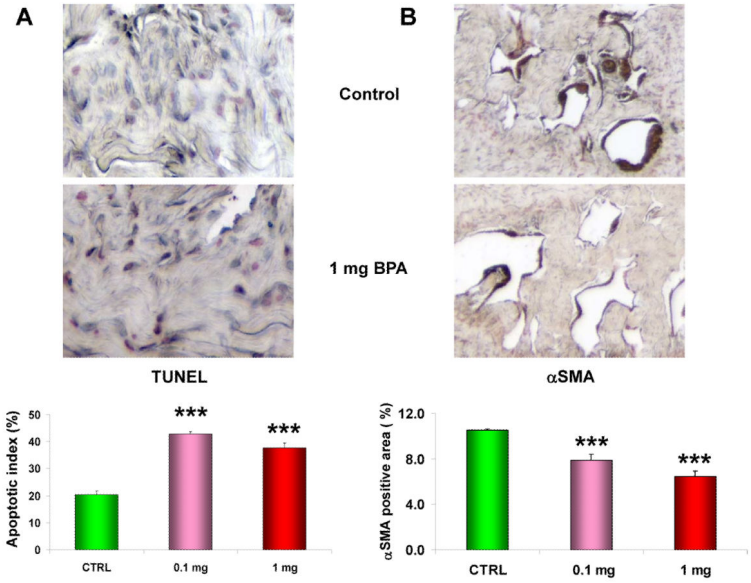

Figure 4. BPA exposure at 1 mg/kg/day induced an increase in apoptosis and of myofibroblasts in the penile corpora cavernosa

A: Apoptosis was evaluated by the TUNEL reaction in adjacent sections to those in Fig. 3 followed by QIA and the apoptotic index were calculated; B: the smooth muscle cells and myofibroblasts content were estimated by immunohistochemistry/QIA in the corpora, and the myofibroblast content was calculated as indicated in the text. Top: representative photomicrographs (200X); Bottom: QIA values. n=8/group; ***p<0.001

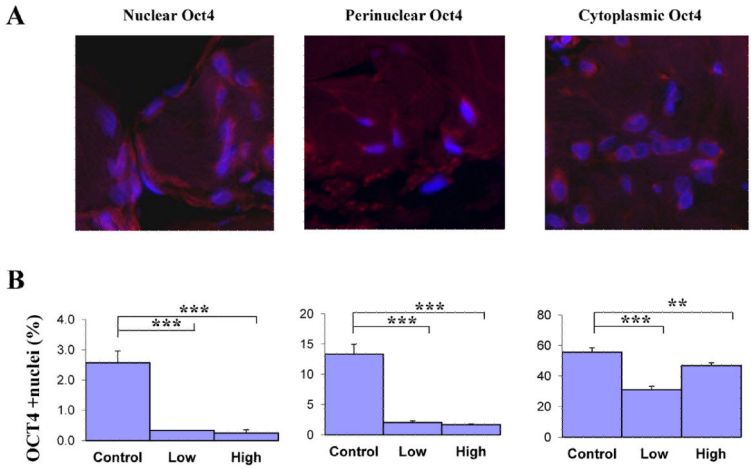

Figure 5. BPA exposure induced a decrease of stem cells as evidenced by nuclear and perinuclear Oct 4 expression in the penile shaft

A: representative pictures (200X) of the merge of Texas red immuno-detected Oct4+ nuclei and DAPI stained nuclei. B: QIA values. **p<0.01, ***p<0.001

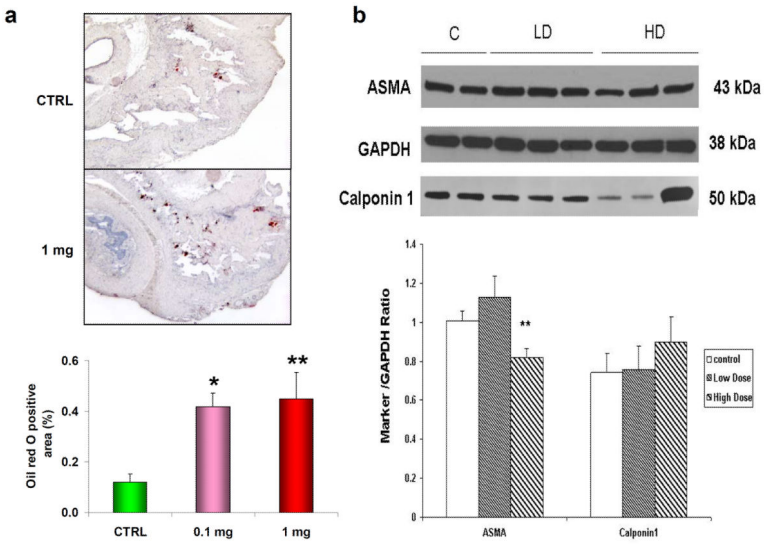

Figure 6. BPA exposure induced an increase in fat deposition in the penile corpora cavernosa and downregulation of α-SMA in the penile shaft

A: Frozen tissue sections were stained with Oil-Red O and subjected to QIA in the corpora. Top: Representative photomicrographs (40X). Bottom: QIA. n=8/group; **p <0.01; B: Aliquots of penile shaft tissue extracts were subjected to western blot followed by densitometry of the selected bands. Top: Photomicrographs of the blots. Bottom: QIA. n=4/group; *p<0.05, **p <0.01

Table 1

BPA exposure induced the upregulation of certain genes related to inflammatory pathways in the penile shaft.

Gene		BPA-L	BPA-H	CEV*	EMT relationship
Inflammation pathways					
CXCL1	Chemokine (C-X-C motif) ligand 1	1.83	2.43	0.95	−
IL6	Interleukin 6	1.58	2.08	1.22	+
IL1B	Interleukin 1B	1.09	1.99	0.95	+
CCL2	Chemokine (C-C motif) ligand 2	1.69	1.98	1.22	+
PLAU	Urokinase	1.36	1.66	0.95	+
COX2	Cyclooxigenase 2	1.38	1.58	1.22	+
PTGS2	Prostaglandin-endoperoxide synthase 2	1.30	1.58	0.95	+
CD248	Endosialin	0.99	1.57	1.22	−
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	1.26	1.52	0.95	−
CADM3	Cell adhesion molecule 3	1.10	1.49	1.22	−
NOS 2	Nitric oxide synthase 2	0.98	1.47	0.95	+
Other pathways					
ERRFI1	ERBB receptor feedback inhibitor 1	1.29	1.47	6574	+
E-NCAM	Embryonic form of neural cell adhesion molecule	0.94	0.63	819	+
KRT 1	Keratin 1	8.21	0.59	364	+
KRT 4	Keratin 4	0.94	0.64	4661	+
KRT 15	Keratin 15	0.90	0.69	6324	+
KRT 19	Keratin 19	1.06	0.70	1410	+
KRT 14	Keratin 14	2.24	0.71	533	+
CDH1	Cadherin 1	1.14	0.73	1059	+
KRT 8	Keratin 8	0.95	0.74	312	+
KRT 7	Keratin 7	1.22	0.75	507	+

The DNA microarray assays were performed in RNA extracted from pools of 3 penises. Among functionally relevant genes only those with changes >1.5 fold or < 0.66 were entered. BPA-H/C: ratio of gene expression in the high dose of BPA (1 mg/kg/day) versus control RNA; BPA-L/C: similar but with the low dose (0.1 mg/kg/day); CEV*: control expression value normalized by the ratio of GAPDH expression in this assay and a previous one (14). The relationship to inflammation and EMT is indicated by + through ++++, according to the number of citations in Pubmed retrieved by linking as key words the miR to the pathological process.

Table 2

BPA exposure mainly induced the down-regulation of a set of micro RNAs in various pathways in the penile shaft.

Micro RNA	BPA-L	BPA-H	CEV*	EMT	Fibrosis	Inflammation
Up-regulated						
568	1.13	1.74	446	−	−	−
451-5p	1.83	1.41	869	−	+	+
Down-regulated						
664-1-5p	0.71	0.55	2081	−	−	−
296-3p	1.07	0.53	2321	+	+++	++
377-3p	0.53	0.52	1556	+	+	−
1224	0.58	0.50	751	−	−	+
665	0.58	0.50	751	−	−	−
182	0.38	0.47	470	+	+	+
672-5p	0.38	0.47	464	−	−	−
483-5p	0.93	0.46	1333	+	−	+
1306-3p	1.48	0.44	683	−	−	−
210-3p	1.46	0.42	683	+	+	+
3584	0.69	0.39	1064	−	−	−
494-3p	0.68	0.36	1450	+	+	+
206-3p	1.48	0.36	1595	−	+	+
200c	0.92	0.35	1478	++++	++	++
328a	0.54	0.32	1818	−	−	+
347	0.34	0.31	448	+	−	−
200b	0.93	0.29	2174	++++	++	++
6216	0.35	0.28	994	−	−	−
6215	0.43	0.26	968	−	−	−
429	1.11	0.24	652	+	−	+
200a	1.38	0.19	963	++++	++	++
205	0.87	0.19	7027	+++	+	+
203a	0.21	0.13	2309	++	−	++

The detection of miRs was performed in RNA extracted from pools of 3 penises. Among functionally relevant genes only those with changes >1.4 or <0.55 fold after high dose exposure (and having the same direction of change at the low dose) were entered. BPA-H/C: ratio of gene expression in the high dose of BPA (1 mg/kg/day) versus control RNA; BPA-L/C: similar but with the low dose (0.1 mg/kg/day); CEV: control expression value. The relationship to inflammation, fibrosis and EMT is indicated by + through ++++, according to the number of citations in Pubmed retrieved by linking as key words the miR to the pathological process.