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Chronic High Dose Intraperitoneal Bisphenol A (BPA) Induces Substantial Histological and Gene Expression Alterations in Rat Penile Tissue Without Impairing Erectile Function

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Abstract

Introduction—Bisphenol A (BPA), released from plastics and dental sealants, is a suspected endocrine disruptor and reproductive toxicant. In occupationally exposed workers, BPA has been associated with erectile dysfunction (ED).

Aims—To determine whether long-term exposure to high doses of BPA in the rat affects serum levels of testosterone (T) and estradiol (E2), and induces corporal histopathology and resultant ED.

Methods—Young rats were injected intraperitoneal (IP) injection daily with BPA at 25 mg/kg/day or vehicle (n = 8/group). Erectile function was measured at 3 months by cavernosometry and electrical field stimulation (EFS). BPA was assayed in serum, urine, and penile tissue, and serum T and E2 were determined. Quantitative Masson trichrome, terminal deoxynucleotidyl transferase dUTP nick end labeling, Oil Red O, immunohistochemistry for calponin, α -smooth muscle actin, and Oct 4 were applied to penile tissue sections. Protein markers were assessed by Western blots and 2-D minigels, and RNA by DNA microarrays.

Main Outcome Measures—Erectile function, histological, and biochemical markers in corporal tissue.

Results—In the BPA-treated rats, total and free BPA levels were increased in the serum, urine, and penile tissue while serum T and E2 levels were reduced. In addition, the corpora cavernosa demonstrated a reduction in smooth muscle (SM) content, SM/collagen ratio, together with an increase in myofibroblasts, fat deposits, and apoptosis, but no significant change in collagen content or stem cells (nuclear/perinuclear Oct 4). In the penile shaft, BPA induced a downregulation of Nanog (stem cells), neuronal nitric oxide synthase (nitrergic terminals), and vascular endothelial growth factor (angiogenesis), with genes related to SM tone and cytoskeleton upregulated 5- to 50-fold, accompanied by changes in the multiple protein profile. However, both cavernosometry and EFS were unaltered by BPA.

Conclusions—While rats treated chronically with a high IP dose of BPA developed hypogonadism and a corporal histo- and molecular-pathology usually associated with ED, no changes were detected in erectile function as measured by EFS and cavernosometry. Further studies using alternate routes of BPA administration with various doses and length of exposure are needed to expand these findings. Kovanecz I, Gelfand R, Masouminia M, Gharib S, Segura D, Vernet D, Rajfer J, Li DK, Liao CY, Kannan K, and Gonzalez-Cadavid NF. Chronic high dose intraperitoneal bisphenol A (BPA) induces substantial histological and gene expression alterations in rat penile tissue without impairing erectile function.

Keywords

Erectile Dysfunction; Fibrosis; Corporal Veno-Occlusive Dysfunction; Penis

Introduction

Bisphenol A (BPA) is a monomer chemical that is used in the manufacturing of polycarbonate plastics, epoxy resins, dental sealants, and other plastics. As such, it is found in the lining of both food and beverage cans. Since it is released, particularly upon moderate heating into the water supply and the ecosystem, it poses not only an environmental risk but an occupational risk for workers, who may suffer exposure by inhalation or oral/dermal contamination [1–7].

Exposure to BPA in experimental animals affects multiple organs (testis, ovaries, prostate, etc), and it is suspected of being a reproductive risk and endocrine disrupting chemical because of its well-documented “selective estrogen receptor modulator” properties [8–13]. In fact, BPA accounts for the majority of estrogenic activity that leaches from landfills into the ecosystem [10], although it can have either estrogenic or anti-estrogenic effects. In addition, it has weak anti-androgenic activity through direct binding to the androgen receptor at higher doses than required for the estrogen receptor, and indirectly by reducing testosterone levels via its effects on the testis [9]. These main actions are compounded by alteration in the expression of hormone receptors, and by effects on various enzymes and metabolic pathways [13]. However, it is unlikely that, at least in postnatal exposure to low doses of BPA, these relatively weak sex hormone-related effects could induce erectile dysfunction (ED) and/or an underlying histopathology [14]. BPA binds with higher affinity to the estrogen-related receptor γ than to the estrogen receptor α , and its chemical structure closely resembles bisphenol A diglycidyl ether, an antagonist of the peroxisome-proliferator-activated receptor gamma [15–17].

There are very few studies of BPA reported in humans, but in exposed workers the finding of higher BPA level in the urine has been associated with an increased risk of both ED as assessed by self-questionnaires, and also with semen deterioration [14,18–20]. A similar association of BPA exposure with disease was recently reported in polycystic ovary syndrome [21]. The human study of ED in men exposed to BPA did not discriminate as to whether the sexual dysfunction is (i) peripheral, by inducing corporal lipofibrosis, loss of corporal relaxation, and/or damaging the cavernosal nerves; (ii) central, impairing the hypothalamic control of erection; or (iii) systemic, by reducing testosterone production that would affect both (i) and (ii).

With the exception of a study in the rabbit, where a high dose BPA given intraperitoneally had a detrimental effect on penile corporal histology and the resultant in vitro relaxation of the corporal smooth muscle (SM) [22], there is no other experimental report related to the human data on ED. In the current work we investigated whether prolonged exposure of young rats to intraperitoneal BPA, at one half of the “lowest observed adverse effect level” (LOAEL) [8] (i) affects body weight, (ii) leads to BPA accumulation in tissues involved in the peripheral mechanism of penile erection, (iii) reduces the levels of serum testosterone and estradiol, (iv) induces loss of SM cells and lipofibrosis in the corpora cavernosa and changes in the expression of genes related to corporal compliance, and (v) causes ED, specifically corporal veno-occlusive dysfunction (CVOD). This dose was chosen to be high enough to potentially affect the histology of the corpora cavernosa and resultant penile

erection in a proof of concept while still being safe in terms of the current LOAEL definition.

Materials and Methods

Animal Procedures

The 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 Institutional Animal Care and Use Committee at LABioMed. All 1.5-month old male Fischer 344 rats were injected daily with intraperitoneal BPA at 25 mg/kg/day (1/2 of the LOAEL) or corn oil vehicle (n = 8/group). Treatments were interrupted 3 days before completion (washout) at 3 months, and erectile function was measured as follows:

Dynamic Infusion Cavernosometry (DIC)—DIC was performed as previously described [23,24]. Briefly, basal intracavernosal pressure (ICP) was recorded, and 0.1 mL papaverine (20 mg/mL) was administered through a cannula into the corpora cavernosa. The ICP 5 minutes after the injection was recorded as “papaverine response.” Saline was then infused through another cannula, increasing infusion rate by 0.05 mL/minute every 10 seconds, until the ICP reached 100 mm Hg (“infusion rate”), then the infusion rate was adjusted to maintain a steady ICP level just above 100 mm Hg (“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 (EFS) of the Cavernosal Nerve—EFS was performed preceding cavernosometry as previously described [25,26]. Briefly, under anesthesia, the cavernosal nerve was exposed and hooked by a bipolar platinum electrode. Systemic arterial and ICP measurements were obtained by simultaneous intrafemoral artery and cavernosal catheterization, respectively. EFS was applied at 5 V (to increase sensitivity to detect small changes in the response) and a frequency of 15 Hz for 60 seconds, separated by 1-minute intervals, with a Lab-Trax-4/24T data acquisition device with built-in stimulator (WPI, Inc. Sarasota, FL, USA). Arterial and intracavernosal blood 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 was calculated to normalize for variations in blood pressure.

BPA and Hormonal Assays

BPA was assayed in blood, urine, and fresh penile tissue by high performance liquid chromatography– tandem mass spectrometry (HPLC–ESI–MS/MS) methods [27]. Testosterone and estradiol were assayed in serum by applying validated HPLC– MS/MS methods [28].

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 μ m). Adjacent tissue sections were used for the following [23–26]: (i) Masson trichrome staining for collagen (blue) and smooth muscle cells (SMC) (red); (ii) apoptotic index by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction with the Apoptag kit (Millipore, Billerica, MA, USA); (iii) immunodetection with monoclonal antibodies against: α -smooth muscle actin (ASMA) as a SM and myofibroblast marker (Sigma kit, Sigma Diagnostics, St Louis, MO, USA); (iv) calponin as marker for SM only mouse monoclonal, 1:100 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA); and (v) Oct 4 as stem cell marker mouse monoclonal, 1:100 (Santa Cruz Biotechnology Inc.). For immunodetection, sections were then incubated with biotinylated anti-mouse immunoglobulin G (IgG), followed by ABC complex (Vector labs, Temecula, CA, USA) and AEC chromogen peroxidase substrate (Sigma Diagnostics). 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 optimal cutting temperature compound (OCT) and used for obtaining frozen tissue sections that were subjected to Oil Red O staining for detecting fat droplets [29].

Quantitative image analysis (QIA) was performed by computerized densitometry using the ImageProPlus 5.1.1 program (Media Cybernetics, Silver Spring, MD, USA), coupled to an Olympus BHS microscope equipped with a Spot RT color digital camera [23–26]. For Masson, ASMA, Calponin, and Oil Red O staining, 40 $\times$ magnification pictures were taken comprising the whole cross section of the penile shaft. For TUNEL, 12 fields at 200 $\times$ 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 three matched sections per animal and eight animals per group were analyzed.

Sections of paraffin-embedded testicular tissue were used for hematoxylin/eosin and TUNEL staining for qualitative assessment without subsequent QIA.

Determinations in Fresh Tissue

Western Blots [23–26]—Penile tissue homogenates (about 50 mg fresh tissue stored at -80°C until use) were obtained using Bullet Blender Storm 24 (Next Advance, Inc, Averill Park, NY, USA) using one scoop of SSP14B (1.4 mm) beads and four SSB32 beads (3.2 mm) in lysis boiling buffer consisting in 1% sodium dodecyl sulfate (SDS), 1.0 mM sodium orthovanadate, 10 mM Tris pH 7.4, and protease inhibitors (3 μ M leupeptin, 1 μ M pepstatin A, 1 mM phenyl methyl sulfonyl fluoride), cutting the tissue into small pieces, adding the beads and the lysis buffer, placing in the blender, running 5 minutes at speed 8 three times and centrifuged at 16,000 g for 5 minutes. For Western blot analysis supernatant proteins (20–30 μ g) were subjected to Tris-HCL polyacrylamide gel electrophoresis using 7–15% Mini Protean TGX precast gels (Bio-Rad, Hercules, CA, USA) in Tris/glycine/SDS running buffer. Proteins were transferred overnight at 4°C to polyvinylidene difluoride membranes (Bio-Rad) in transfer buffer (Tris/glycine/methanol), and the next day, the nonspecific binding was blocked by immersing the membranes into 5% non-fat dried milk, 0.1% (v/v) Tween 20 in Tris-buffered saline (TBS) for 1 hour at room temperature. After several

washes with washing buffer (TBS Tween 0.1%), the membranes were incubated with the primary antibodies in incubating buffer (5% nonfat milk in TBS-0.1% Tween) for 1 hour at room temperature.

Antibodies were as follows: (i) calponin 1, mouse monoclonal (Santa Cruz Biotechnology, Inc.), 1:500; (ii) neuronal nitric oxide synthase (nNOS), rabbit monoclonal (Abcam, Cambridge MA, USA), 1:750; (iii) platelet/endothelial cell adhesion molecule 1, (CD-31), mouse monoclonal (Abcam), 1:1,000; (iv) α -smooth muscle actin (ASMA), mouse monoclonal (Sigma), 1:1,000; (v) neuro-filament 70 (NF70) mouse monoclonal (Millipore), 1:500; (vi) vasoendothelial growth factor (VEGF) mouse monoclonal (Santa Cruz Biotechnology), 1:500; (vii) Oct 4 rabbit polyclonal (BioVision, Milpitas, CA, USA), 1:500; (viii) Nanog rabbit polyclonal (Millipore), 1:500; and (ix) mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal (Millipore), 1:1,500, as a reference housekeeping protein.

The membranes after several washes with washing buffer were incubated for 1 hour at room temperature by a secondary antibody linked to horseradish peroxidase. After several washes, the immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent (Thermo Fisher Scientific, Pittsburgh, PA, USA) detection system. The densitometric analysis of the bands were performed with Image J (NIH, Bethesda, MD, USA). 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.

Collagen Content—Collagen was estimated by the picrosirius red procedure [30], using aliquots of the tissue homogenates prepared for Western blotting, mixing it with Sirius Red saturated in picric acid incubated for 30 minutes, and centrifuged at 15,000 g for 5 minutes to pellet the collagen. This pellet was rinsed once with 0.1 M HCl to remove excess dye, centrifuged again, and the bonded dye was extracted in 0.5 M NaOH, clarified, and measured spectrophotometrically at 550 nm. The standard curve is Type I Collagen, acid soluble, (Sigma Chemical Corp) from 0–100 μ g. Values are expressed as μ g of collagen per mg of tissue.

Multiple Protein Expression [31]—Multiple protein expression was obtained using penile shaft specimens from three rats in each group that were subjected to 2D gel electrophoresis (first dimension: immobilized pH gradient strips; second dimension: 24 cm gels), identifying differential spots by merging the fluorescent tag images and applying a data acquisition system. No trypsin digestion/nano electrospray liquid chromatography tandem mass spectrometry (Nano-ESI-LC-MS-MS) analysis was performed.

DNA Microarray Transcriptional Profile (Signature) [32,33]—RNA was analyzed using duplicate penile tissue aliquots preserved in RNA later, obtained from the control and BPA-treated rats, and analyzed using the Affymetrix Rat Gene array for 29,215 genes. Only genes that were up- or downregulated by >2 fold were considered unless specifically detailed.

Statistical Analysis

Values were expressed as mean $\pm$ standard error of the mean. Comparisons between the treated and control groups were made by unpaired two tail *t*-test using GraphPad Prism v5.01 statistical program (GraphPad Software Inc., San Diego, CA, USA). *P* < 0.05 was considered statistically significant.

Results

Six- to seven-week-old Fischer 344 male rats, an NIH-selected model for reproductive studies, were exposed for 3 months to daily BPA given intraperitoneally at 25 mg/kg/day. This selected “proof of concept” dose is the highest within an ongoing multiple dose Food and Drug Administration-NIH study to ascertain under good laboratory practice conditions the impact of BPA on various rat tissues and dysfunctions. The way of administration was chosen based on the need to dissolve BPA in oil at the selected dose and to facilitate the daily long-term treatment, and considering that intraperitoneal administration has been widely used for BPA studies [10,11]. Weekly body weight gain was slowed down and final body weight was significantly reduced by 19% at completion, as determined following a 3-day washout period (Figure 1A). This was accompanied by an 82% reduction in the level of serum testosterone, and a 40% decrease in serum estradiol (Figure 1B). In the testis, this was associated with only mild spermatid damage and increased apoptosis on paraffin-embedded sections of the testis (Figure 1C). There was also epithelial thickening and partial atrophy of the seminal vesicles (not shown).

Compared with negligible values in the controls, the total BPA levels in the serum and urine were substantially increased in the rats exposed to BPA, at 1.09 and 4.34 $\mu\text{g/mL}$, respectively, even after the 3 days washout (Figure 2 top). The free unconjugated BPA in the exposed rats was respectively only 6.1% and 13.1% of the total BPA. The total BPA levels in penile shaft tissue resembled those in the serum (0.85 $\mu\text{g/g}$), but the proportion of free BPA was higher (17.8%) than in the serum (Figure 2, bottom).

Surprisingly, by cavernosometry (*n* = 8/group), when compared with controls, these BPA-treated rats did not demonstrate CVOD, as measured by the normal ICP induced by papaverine (88 ± 2.6 vs. 68 ± 2.0 mm Hg), and by the normal drop rate upon saline infusion (21 ± 1.6 vs. 24.0 ± 2.0). EFS of the cavernosal nerve performed in two additional animals/group gave a MIP/MAP ratio slightly higher than in controls (1.02 vs. 0.80), so that the erectile response to EFS was also normal. Therefore, no other additional animals were subjected to EFS.

However, a histochemical determination on penile shaft tissue sections with Masson trichrome showed a significant 33% reduction of the SM/collagen ratio in the corpora cavernosa of the rats exposed to BPA (Figure 3 left). These data imply that this change is due to a loss of SMC, which was confirmed by the significant 51% reduction in the corporal area occupied by calponin, as detected by immunohistochemistry, a SMC marker which is not expressed in myofibroblasts (Figure 3 right). In fact, no significant changes were found in collagen content in the penile shaft homogenate as estimated by picrosirius red (146 ± 24

in BPA vs. 156 ± 26 $\mu\text{g}/\text{mg}$ tissue in control), but it was increased in reference to the SM content.

There was a significant increase in the immunohistochemical staining of ASMA, a marker of both SMC and myofibroblasts (Figure 4 left). The ASMA/calponin ratio was increased from 0.87 in the control rats to 2.31 in the BPA-treated rats, a 2.7-fold increase, indicating that a substantial part of the ASMA cells in the BPA corpora cavernosa are indeed myofibroblasts. The corporal SMC loss induced by BPA is compounded by substantial fat deposition, as indicated by the 2.7-fold increase (as compared with the control) in the area occupied by fat droplets, as estimated by Oil Red O staining in frozen sections (Figure 4 right).

The histological damage caused by BPA on differentiated cells, mostly in the corpora cavernosa, did not significantly affect the number of the key stem cell marker Oct 4 + cells (Figure 5). Immunofluorescent microscopy showed the total disappearance of the very few cells positive for the true stem cell transcription factor isoform that is located in the nuclei [34], but not of the perinuclear Oct 4, which is presumably similar to the nuclear isoform. There was no change in the cytoplasmic Oct 4, which is not considered as stem cell related.

The histopathological alterations suggestive of BPA-induced corporal lipofibrosis was accompanied by a 35% increase in apoptosis as estimated by the TUNEL assay (Figure 6 left), and by a 52% reduction in VEGF expression in the penile shaft tissue (containing in addition to the SM, tunica albuginea and corpus spongiosum tissues), as estimated by Western blot (Figure 6 right), and a 42% reduction in nNOS present in nitrergic nerve terminals. There were no changes in the content of nerve terminals as indicated by NF70, or of endothelium as indicated by CD31. There is also a 70% BPA-induced decrease in the level of Nanog, a key stem cell marker [35], but no change in Oct 4a expression, representative of the very few cells with nuclear or perinuclear Oct 4 shown on Figure 5. Other alterations are qualitatively evident in protein expression within the penile shaft, including a number of up- and downregulated spots in 2D-gel electrophoresis. The changes are indicated by the merge of alternatively tagged extracts with fluorescent red and green dyes attached to the control and BPA-exposed specimens (Figure 7).

The changes in protein expression induced by BPA in both the corpora cavernosa (histoimmunohistochemistry) and in the penile shaft (Western blot, 2D-gel electrophoresis) are paralleled by an even more pronounced series of changes observed in DNA expression analysis of the penile shaft RNA, carried out using DNA microarrays. Out of 29,216 rat sequences, the mean values of independent determinations from 2 separate pools of 2–3 penile shafts each, showed 236 genes upregulated by BPA greater than 2-fold and 67 downregulated by more than twofold, where only genes expressed at greater than a normalized threshold value of 50 were included.

The upregulated genes include mRNAs for some genes (among others) related to cytoskeleton remodeling, calcium flow, myofibroblast pheno-typic switch, and SM tone [36,37], which were increased 2- to 50-fold over the level of expression measured in the untreated specimens (50–600 in a conventional scale). The downregulated genes include

mRNAs for some genes (among others) related to anti-fibrotic/anti-inflammatory, and pro-fibrotic/pro-inflammatory pathways [38], as well as some that may act both ways and are strongly expressed, and that were decreased two- to fourfold from a very high level of expression in the untreated specimens (700–11,800 on the arbitrary scale in which GAPDH is expressed at a level of 9,000–11,000) (Table 1). Considering that the DNA microarray assays were performed in duplicate using pool of individual rat RNAs, no individual reverse transcription polymerase chain reactions (RT-PCR) were carried out because the considerable detected transcriptional changes exerted by BPA made this confirmation unnecessary.

Discussion

The current results are the first demonstration that prolonged exposure to BPA given to young rats intraperitoneally at comparatively high, but safe levels, intended for a proof of concept, causes substantial changes that can affect the reproductive system. Besides a marked decrease in body weight, it also leads to a considerable uptake of BPA in the penile shaft tissue, low serum testosterone levels presumably due to testicular damage, and a corpora cavernosal histopathology characterized by a loss of SM, accumulation of myofibroblasts and fat depots, changes in protein expression consistent with neural and angiogenic damage and stem cell decrease (as judged by Nanog, but not Oct 4, expression), and alterations in the transcriptional signature of the penile shaft for genes related to cytoskeleton remodeling, myofibroblast switch, and fibrotic and inflammatory pathways. Despite the hypogonadism and the corporal histo- and molecular-pathology, no alterations in the peripheral erectile response could be detected by either cavernosometry or EFS of the cavernosal nerve. Therefore, the current work provides some indirect experimental assumptions for the underlying histopathology of the ED observed in men subjected to occupational exposure to BPA, but does not show the expected direct impact on penile erection.

This begs the question as to the possible reasons for the discrepancies between (i) the corporal histological/biochemical changes and the resilience of erectile function; and (ii) the experimental and human studies in terms of dosage, mode and length of exposure, age, and general validity of the rat model of ED. The observation that penile erection was not impacted despite the very low levels of serum testosterone induced by BPA is discrepant since in our own previous studies [39,40], castration of adult rats reduced the erectile response to EFS by 50%. However, the 0.63 mg/dL of serum testosterone detected in the BPA-treated rats is still much higher than the negligible levels present after castration, and the hormonal threshold for the serum testosterone affecting erectile function may not have been breached. In addition, the blood specimens were taken only at completion of the study, and since the actual time course of testosterone depletion is unknown, the impact on erectile function could have been relatively recent and of short duration. However, the question remains open on whether this hypogonadism is responsible for changes observed in the corpora cavernosa, namely the reduction of corporal SM and nNOS, and the induction of fat infiltration, processes that in other contexts have been shown to be caused by the decrease of serum testosterone.

Based on current knowledge, it is not possible to propose whether the putative decrease in stem cell content indicated by the downregulation of Nanog in the penile shaft may be related to low testosterone levels, as opposed to a direct effect of BPA on stem cells that has been documented in various systems [41,42]. Whatever the cause, this putative endogenous stem cell reduction may affect the potential repair of corporal tissue damage by BPA [43]. However, the reason why Oct 4a expression was not significantly reduced needs to be clarified.

In turn, the changes in corpora cavernosa homeostasis are difficult to reconcile with the lack of effects on the erectile response. It may be speculated that the young age of the rats, the absence of an excessive corporal collagen deposition at this stage, and perhaps an insufficient period of exposure to affect the erectile response were responsible for the erectile persistence in the face of the changes induced by BPA. Our own parallel work [44] involving longer periods of exposure and older rats, under an oral administration of even lower doses, instead led to a moderate CVOD, suggesting that this may be the case. Alternatively, an unknown compensatory functional mechanism may operate in the BPA-exposed rats, similar to the one we observed in a previous study where rats exposed to chronic smoking maintained a normal erectile response to EFS despite the induction of a considerable alteration of corporal histo-chemical and biochemical markers [45], which in other rat models of risk factors of ED have been shown to lead to an impaired response to EFS [25,26,38,39].

The single previous article describing BPA effects on the penile erectile mechanism and histology in an animal model [22] reported that exposure of 3-month-old rabbits to an average intraperitoneal dose of BPA roughly triple the one that we applied here, but for only 12 days, caused at 4 and 8 weeks (i) a considerable reduction of the in vitro relaxation of phenylephrine-precontracted penile corpora cavernosa strips that was induced by acetylcholine, L-arginine, and sodium nitro-prusside; (ii) a similar effect on the contractile responses to noradrenaline; (iii) a histochemically detected (Masson trichrome) thickening of the tunica albuginea and overproduction of collagen in both the corpora and the tunica (fibrosis); and (iv) a subtunical deposition of fat and decrease of subtunical space. This study did not measure ED in vivo, was based on a short exposure to a comparatively high “toxic” dose and did not measure BPA levels. However, the histopathological changes in the rabbit penis resemble what we have observed in the rat penis albeit with our much lower dose. The impairment of in vitro rabbit corporal relaxation induced by the higher dose is in agreement with our prediction that the BPA-elicited changes may eventually become detrimental to penile erection in vivo.

The main issue is therefore how the alterations we have observed in the current work translate to the human studies on occupational exposure [14,18,19]. The recently published report by Li et al. [18], demonstrated that BPA-exposed male workers in epoxy resin and BPA-manufacturing factories developed general sexual dysfunction and ED. This population (184 subjects) exposed to BPA in their workplace, compared with workers from control factories (404 subjects), had almost fourfold increased risk of erectile difficulty and sevenfold increased risk of ejaculation difficulty, among other sexual dysfunction problems.

There was a 40- to 50-fold increase in BPA excretion, over urine BPA levels in controls which were comparable to those in the general U.S. population [5–7,18–20].

The male sexual dysfunction outcomes in this human study were based exclusively on self-reporting to two questionnaire tools [46,47] that do not allow conclusions in terms of (i) where the primary effects would occur: central sexual stimulation and control of erectile function in the brain vs. the peripheral mechanism of penile corporal relaxation to induce the erectile response [48–50]; (ii) what is the risk threshold level of cumulative BPA exposure; and (iii) what are the cellular and molecular mechanisms for these effects? However, it may be assumed that at least part of the erectile difficulties were due to a local long-term toxic effect of BPA on the corpora cavernosa and/or pelvic ganglion affecting the peripheral machinery of penile erection. The ejaculatory problems may result from the effect on the testis, prostate, or seminal vesicles [51]. These assumptions do not exclude potential central effects in the brain on sexual arousal and the control of erection.

The study by Li et al. [18] indicates that the urinary excretion of BPA reaches 467 $\mu\text{g/gCr}$ (creatinine) in 75 percent of the occupationally exposed workers vs. an equivalent value of 11 in the unexposed workers. Considering an average 1.3 g/L of creatinine in the urine [52] and 1.6 L of urine/day, this would translate into approximately 972 $\mu\text{g/day}$ (exposed) vs. 23 $\mu\text{g/day}$ (unexposed), or 0.61 vs. 0.01 $\mu\text{g/mL}$ urine, respectively. In our results, the level in urine in the BPA-exposed rats was 4.2 $\mu\text{g/mL}$ or only 6.9-fold higher. This suggests that, leaving aside species differences, the intraperitoneal route is much less effective than the oral or inhalation routes, since the estimated intake of BPA in the human would be 18.5 $\mu\text{g/kg/day}$ in the exposed workers, as compared to the much higher 25 mg/kg/day in the rat, or about 3.5 mg/kg/day after accounting for differences in surface between the species [53].

In the case of the male reproductive system, the testis appears to be a developmental and adult target at low BPA doses (i.e., less than the LOAEL dose), mainly due to induced deficits in testosterone production and spermatogenesis [9,10,54]. Additional targets are the epididymis, prostate, and seminal vesicles (involved in ejaculation). The doses in these studies ranged from 0.005 to 20 mg/kg/day (uterus, testis) [10,11]. Other forms of administration, including oral (chow, oil), subcutaneous injection, and osmotic minipump ranged from 0.01 to 20 mg/kg/day , and some surpassed the LOAEL, using up to 200 mg/kg/day (ovary, prostate, testis). Therefore, although the dose chosen here is much higher than the one used in the human studies, since it was delivered intraperitoneally it is likely within the dosages normally employed experimentally for studying effects on reproduction in terms of actual BPA levels.

In summary, our data in rats chronically exposed to one-half of the LOAEL dose of BPA, leading to an urinary excretion calculated as sevenfold higher than in the occupationally exposed workers, show that this exposure is detrimental to the corpora cavernosa through its induction of a corporal histopathology normally associated with ED. In addition, there is an associated hypogonadism that is also a key factor in ED [55], even if the ED itself could not be detected due speculatively to a functional compensatory mechanism. Taken together, our current laboratory results indirectly support the human findings through the induced histopathology and alterations in gene expression, and are complemented at the functional

level by our parallel study where a mild ED could be detected with oral administration of lower doses and longer exposures associated with significant histological and biochemical alterations [44]. This suggests that the different kinetics and metabolism of the intraperitoneal and oral administration may affect the extent of the functional impact.

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Figure 1.

BPA given intraperitoneally (25 mg/kg/day) to 1.5-months-old Fisher 344 rats for 3 months reduced body weights as well as serum levels of testosterone and estradiol, and induced apoptosis in testicular tubules. (A) Time course of body weight increase; (B) serum hormone levels at completion; $n = 8/\text{group}$. * $P < 0.05$; ** $P < 0.01$. (C) Representative paraffin-embedded tissue sections of the testis that were subjected separately to hematoxylin/eosin staining (HE) and apoptotic cell detection (TUNEL) for a qualitative assessment. BPA = bisphenol A; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling

Figure 2.

BPA-exposed rats had substantial increases in total and free BPA levels in the serum, urine, and penile shaft tissue. HPLC determinations were done as indicated; CTR: control; n = 8/group. ** $P < 0.01$; *** $P < 0.001$. BPA = bisphenol A; HPLC = high performance liquid chromatography

Figure 3.

BPA exposure induced a reduction in the smooth muscle content in the penile corpora cavernosa. Bottom: quantitative image analysis (QIA) values; $n = 8/\text{group}$. Left: SM/collagen ratio determined by Masson trichrome staining on paraffin-embedded penile shaft tissue sections followed by QIA of the corporal region; Right: SM content estimated by immunohistochemistry/QIA for calponin in the corpora. Top: representative photomicrographs (200 $\times$); Bottom: QIA values. $n = 8/\text{group}$; $*P < 0.05$; $***P < 0.001$. BPA = bisphenol A; SM = smooth muscle

Figure 4.

BPA exposure induced an increase in myofibroblasts and fat deposition in the penile corpora cavernosa. Bottom left: the SM cells and myofibroblasts content were estimated by immunohistochemistry/QIA for ASMA in the corpora and the myofibroblast content was calculated as indicated in the text by the ASMA/calponin ratio. Top left: Representative photomicrographs (200 $\times$). Bottom Right: Frozen tissue sections were stained with Oil-Red O and subjected to QIA in the corpora. QIA. $n = 8/\text{group}$; $*P < 0.05$; $**P < 0.01$. Top: Representative photomicrographs (40 $\times$). Top: representative photomicrographs (200 $\times$). ASMA = α -smooth muscle actin; BPA = bisphenol A; QIA = quantitative image analysis; SM = smooth muscle

Figure 5.

BPA exposure induced a decrease in the number of stem cells as evidenced by nuclear and perinuclear Oct 4 expression in the penile shaft. (A) representative pictures (200×) of the merge of Texas red immuno-detected Oct 4 + nuclei and DAPI stained nuclei. (B) QIA. BPA = bisphenol A; DAPI = 4',6-diamidino-2-phenylindole; QIA = quantitative image analysis

Figure 6.

BPA exposure induced an increase in corporal apoptosis and a reduction of angiogenesis, nitrergic nerves, and stem cells in the penile shaft without affecting the nerve terminals or the endothelium content. (A) Apoptosis was evaluated by the TUNEL reaction in adjacent sections to those in Figures 3–5 followed by QIA and the apoptotic index was calculated. Top: representative photomicrographs (200×); Bottom: QIA values. $n = 8/\text{group}$; $*P < 0.05$; $***P < 0.001$. (B) Frozen homogenates of aliquots of penile shaft tissue extracts were subjected to immunoblotting by Western blot followed by densitometry of the selected bands. Top: Photomicrographs of the blots. Bottom: QIA. $n = 4/\text{group}$; $*P < 0.05$. The bar graphs correspond to an $n = 8$ in two separate gels per antigen, and the representative pictures are only for one half of the specimens. Variable loads are corrected by ratios to housekeeping GAPDH gene. BPA = bisphenol A; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; QIA = quantitative image analysis; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling

Figure 7.

BPA exposure induced up- and downregulation of certain penile shaft proteins as detected by 2D-gel electrophoresis. Merge of BPA (BPA9) and control (NB5) protein extracts labeled with alternative stains. Arrows indicate proteins considerably up- or downregulated by BPA. BPA = bisphenol A; NB = non BPA treated

BPA exposure induced in the penile shaft the upregulation of certain genes related to cytoskeleton remodeling and myofibroblast/fibrosis pathways, and the downregulation of certain genes related to fibrosis and inflammation pathways. Mean of two different penile shaft comparisons in pools of two to three penises each. Among functionally relevant genes only both changes >2 fold were averaged. BPA/C: ratio of gene expression in BPA vs. control RNA; CEV lowest threshold: 50. BPA

Table 1

Upregulated genes			Downregulated genes		
Gene	BPA/C	CEV	Gene	BPA/C	CEV
Cytoskeleton remodeling					
Myosin light polypep 1	49.10	65	Anti-fibrotic/anti-inflammatory		
Parvalbumin	38.00	34	iNOS (NOS 2)	0.43	1,562
Actinin α3	32.40	187	Urokinase	0.46	2,069
Titin	22.80	43	Metalloprotein I	0.37	10,744
Myosin light chain phosph	20.60	64	Suppressor of cytokine signaling-3	0.42	2,598
Nebulin	16.80	80	Adrenomedullin	0.40	973
Troponin C2 fast	13.80	115	MMP9	0.25	693
Myosin bind prot C, fast	12.30	59	MMP8	0.20	1,599
Myosin light polypep 3	5.00	57	Pro-fibrotic/pro-inflammatory		
Myosin light chain kinase	4.45	53	Interleukin 1B (IL1B)	0.50	1,476
Protein phosphat inhib 1	3.48	182	ICAM 1	0.50	1,058
Tropomyosin 2	2.89	601	Serpine 1 (PAI 1)	0.34	3,029
ATPase Na/K	2.24	559	Interleukin 6 (IL6)	0.28	1,388
Calcium flow			Interleukin 1 receptor 2 (IL1R2)	2.24	559
Calsequestrin	30.60	49	Unknown or both ways		
Ca chan volt depend γ1	10.10	60	α-2 microglobulin PGCL 4	0.35	1,783
Myofibroblast/fibrosis/oxidative stress					
α1SKM actin	21.3	415	Chemokine ligand 2	0.40	11,795
Myopalladin	8.16	58			
Actc1	3.21	257			
Endothelin 1	1.71	444			

BPA = bisphenol A; CEV = control expression value; ICAM = intercellular adhesion molecule 1; NOS = nitric oxide synthase; PAI = plasminogen activator inhibitor; PGCL = poly(glycolide-co-caprolactone)