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The xenoestrogen bisphenol A in the Hershberger assay: androgen receptor regulation and morphometrical reactions indicate no major effects

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Abstract

We evaluated androgen-like effects of bisphenol A (BPA) using orchiectomized Wistar rats. Animals were treated p.o. either with vehicle or with 3, 50, 200, 500 mg/kg bw/day BPA ($n = 13$) for 7 days. One group was treated s.c. with 1 mg/kg bw/day testosterone propionate (TP). Flutamide (FL) (3 mg/kg bw/day, p.o.) was used to antagonize androgen effects of the suprapharmacological dose (500 mg/kg bw/day) of BPA. Androgen-like effects of BPA on prostates and seminal vesicles were assessed by the Hershberger assay, densitometric analysis of androgen receptor (AR) immunoreactivity, cell proliferation-index and a morphometric analysis. Absolute weights of prostates and seminal vesicles were not increased by BPA, whereas the relative weights were increased at higher doses of BPA, most likely due to a decrease in body weight. Staining intensity for AR immunoreactivity was increased at low but not at higher doses of BPA in comparison to the orchiectomized rats. BPA at all doses tested did not cause an increase of the cell proliferation-index. Epithelial height and glandular luminal area were increased by low doses of BPA, whereas higher doses caused a decrease of these parameters. The data provide evidence that BPA does not exert major androgenic effects.

Keywords: Bisphenol A; Prostate; Seminal vesicle; Immunohistochemistry; Morphometry; Densitometry; Androgen receptor regulation; PCNA; MIB-5; Proliferation markers

1. Introduction

BPA is a chemical monomer used primarily to make epoxy-resins, polycarbonate (PC) plastic products and the flame retardant tetrabromobisphenol A [1]. A number of so-called xenobiotics, including pesticides (*p*,*p*′-DDT), plasticizers (BPA) and a variety of other industrial chemicals (polychlorinated biphenyls) contain a phenolic ring that mimics the A-ring of estradiol and have been reported to have hormonal or antihormonal activity [2,3]. Although the level of exposure to these xenobiotics may be, if any, very low, they may exert their potential toxicity or endocrine disturbance in human beings and wildlife.

Recent in vitro studies demonstrate, in fact, that xenobiotics can bind with estrogen receptors and activate them, resulting in gene expression [4–6]. BPA slightly induced MCF-7 cell proliferation at a level of 0.1 μM and maximum proliferation at 10 μM [6]. Also in in vivo studies, BPA showed estrogenic activity. Plasma free testosterone levels were dramatically decreased following 8 weeks of BPA treatment [7]. It was claimed by vom Saal that, the exposure of pregnant mice to extremely low concentrations of certain xenobiotics, for instance, results in offspring with lower sperm production, increased prostate size or alters maternal behaviour, postnatal growth rate and reproductive function in female mice [8–11]. Other work groups, in contrast, found an uterotrophic response (increase in uterine wet weight) at doses up to 100 mg/kg BPA for 3 days [12–14].

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Also Gupta [15] claimed an enhancement of the anogenital distance and the prostate size of fetuses, when pregnant CD-1 mice were treated with BPA in the microgram range per kg bw/day. BPA induces inappropriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells (LNCaP). Takao reported a significant decrease in plasma free testosterone levels at 50 μg BPA/ml in drinking water (14 mg/kg/day). No significant effect (although a trend in the same direction) was observed neither after 4 weeks of exposure nor after 4 and 8 weeks exposure to 5 μg of BPA/ml drinking water (0.14 mg/kg/day) [7].

In addition, Kim et al. did not detect any androgenic or anti-androgenic activities of BPA in Hershberger assay [16]: the BPA doses used were 10–1000 mg/kg/day.

The present study was carried out to clarify the androgenic potential of BPA in a broad dose range from "ultralow", "pharmacological" to "suprapharmacological" in rats using the standard Hershberger assay with additionally androgen-sensitive parameters.

Additional parameters such as morphometric and qualitative data are therefore required to determine the androgenicity of a given substance, in particular if the expected effects are of lower degree.

2. Materials and methods

2.1. Animals and housing

Wistar rats (male HdrBrHan from Harlan Winkelmann, Borchen, Germany), weighing about 150 g (age of 2 weeks) were separated into different groups by randomized procedure. They received tap water and ssniff R 10, laboratory standard rat diet (in pellet form) ad libitum (ssniff Spezialdiäten GmbH, Soest, Germany). Groups of 2–4 animals were kept in Makrolon cages type IV with ssniff bedding (3/4 Faser) at $22 \pm 3\,^{\circ}C$, a relative humidity of 30–70% and artificial 24 h light. After acclimatization animals were orchiectomized under Ketanest/Rompun—anesthesia (Ketanest 10 mg/kg bw from Parke-Davis, Berlin, Germany and Rompun 2 mg/kg bw from Bayer AG, Leverkusen, Germany).

2.2. Treatment of animals

Seven days after orchiectomy animals were partitioned into eight groups ($n = 13$ in each group). They were treated p.o. with 3, 50, 200 and 500 mg BPA/kg/day dissolved in propylene glycol for 7 days. BPA was purchased from Fa. Bayer (PtNr. 97.001/Prod.Nr. 04111095, CasNr. 80-05, Leverkusen, Germany). Another group of orchiectomized animals was treated s.c. with testosterone propionate (TP) 1 mg/kg bw in arachis oil. Flutamide (FL) 3 mg/kg bw p.o. in combination with 500 mg BPA was used to antagonize possible androgen effects of the "suprapharmacological" dose of BPA. These groups were compared to vehicle (propylene glycol) treated, orchiectomized rats without any other substitution (OX) and to a vehicle treated intact control group (Intact). Propylene glycol was purchased from Merck, Darmstadt, Germany. TP and FL were kindly provided by Schering AG, Berlin, Germany. After the treatment animals were sacrificed by decapitation and seminal vesicles and prostates were harvested surgically, weighed and immediately fixed in 4% neutral buffered paraformaldehyde for 24 h.

2.3. Immunohistochemistry

After fixation the specimens (prostate, seminal vesicle) were dehydrated in ascending series of alcohol, embedded in paraffin and cut in sections of 5 μm thickness (10 sections per specimen). For the immunohistochemical visualization of androgen receptors and proliferation markers the following primary antibodies were applied using the standard procedure protocols provided by the manufacturer: anti-androgen-receptor (1:100, sc-815, rabbit polyclonal, Santa Cruz Biotechnology, CA, USA), anti-androgen-receptor (1:100, 554224, mouse monoclonal, BD PharMingen, Germany), anti-PCNA (1:200, PC-10, mouse monoclonal, Novocastra, New Castle, United Kingdom) and anti-MIB-5 (1:100, M 7248 mouse monoclonal, DakoCytomation, Denmark).

2.4. Densitometry and morphometry

Intensity of immunohistochemical staining was determined densitometrically, while epithelial height and luminal area of the glandular ducts were measured morphometrically (KS 100, KS RUN, Zeiss-Vision, Jena, Germany). Microscopy was performed with an Axiophot light microscope (Zeiss, Jena, Germany) equipped with a high resolution scanner camera (Axiocam, Zeiss, Germany).

All images had a uniform size of 1300 × 1030 pixel. Since the images were generated by using a 20× objective and 1.0 optovar the final resolution of the edge lengths of one pixel in the resulting image is 0.32 μm. This resolution was large enough for deciding which profile of glandular ductus in the field of vision was suitable for densitometric measurements. Gray values were transformed pixel by pixel into optical densities [17].

Five measurements were performed within each section. Five sections were examined per animal resulting in 25 measurements for each animal. The mean values were calculated and compared between the different groups.

For morphometric measurements the software package KS 100 3.0 (Zeiss-Vision, Jena, Germany) was used. The epithelial height was determined by using a 40× objective and 1.0 optovar. For the determination of the luminal area a 10× objective and 1.0 optovar was used. The quantitative assessment of proliferating cells was performed using a 40× objective and 1.0 optovar. One thousand cells per section were counted excluding those which due to the section did not show a nucleus to avoid overestimation of the total cell number. A two-sided t-test at a significance level of $p < 0.05$

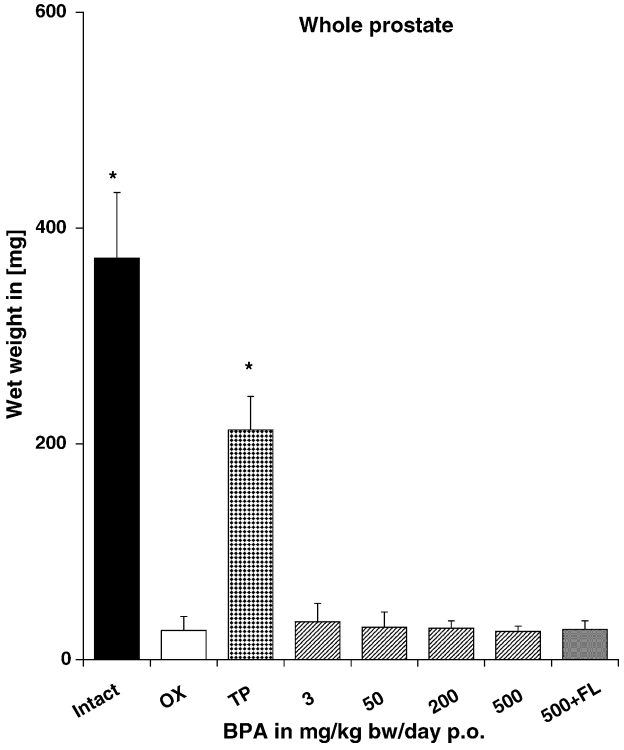

Fig. 1. Comparison of absolute wet weights of the whole prostate in (mg) between the Intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

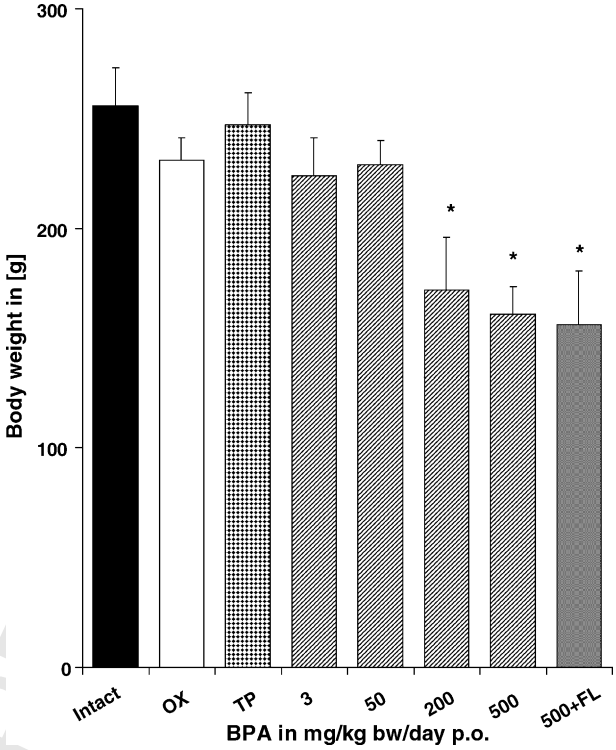

Fig. 2. Comparison of body weights in (g) between the Intact group, orchiectomized group (OX) and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

was applied for statistical comparison. Data were depicted as mean ± standard deviation (S.D.).

3. Results

3.1. Hershberger assay

In contrast to TP, BPA induced no effect on absolute weights of prostate (Fig. 1) and seminal vesicle (not shown). BPA at high doses of 200 and 500 mg/kg bw caused a decrease in body weights (Fig. 2) and a significant increase in relative weights of prostate and seminal vesicle (not shown). A simultaneous administration of FL had no further effect in BPA treated animals. Animals treated with 200, 500 and 500 + FL BPA showed severe signs of gastro-intestinal toxicity.

3.2. Densitometric analysis

The staining intensity after incubation with the polyclonal antibody (1:100, sc-815, rabbit polyclonal, Santa Cruz Biotechnology, CA, USA) directed against AR was 75 ± 21 (Intact), 43 ± 12 (OX), 61 ± 24 (TP), 65 ± 27 (BPA 3), 57 ± 19 (BPA 50), 48 ± 18 (BPA 200), 39 ± 9 (BPA 500) and 33 ± 10 (BPA 500*FL) for the prostates and 42 ± 17 (Intact), 11 ± 10 (OX), 19 ± 11 (TP), 8 ± 5 (BPA 3), 11 ± 15 (BPA 50), 7 ± 3 (BPA 200), 3 ± 1 (BPA 500) and 15 ± 17 (BPA 500 + FL) for seminal vesicles, respectively. In the prostate tissue the intensity of staining was significantly higher in both the Intact and TP group (*t*-test, $p < 0.05$, $n = 13$) compared to the OX group. BPA at lower doses (3 and 50 mg/kg bw) increased AR immunoreactivity and staining intensity of prostate tissue, but reduced them at higher doses (200 and 500 mg/kg bw). In seminal vesicles, the intensity of AR staining was reduced by orchiectomy in comparison with the Intact control. The treatment of orchiectomized animals with BPA showed no dose-dependent effects (not shown).

Using the anti-androgen-receptor monoclonal antibody (1:100, 554224, mouse monoclonal, Novocastra, New castle, United Kingdom) the staining intensity of AR in the prostates revealed the following optical density values: 104 ± 20 (Intact), 56 ± 17 (OX), 77 ± 22 (TP), 99 ± 7 (BPA 3), 103 ± 23 (BPA 50), 78 ± 16 (BPA 200), 49 ± 23 (BPA 500) and 36 ± 6 (BPA 500 + FL). Statistical analysis confirmed that the intensity of staining was significantly higher in both the intact and TP group (*t*-test, $p < 0.05$, $n = 13$) compared to the OX group (Figs. 3 and 4A–H). Thus, the data obtained for both antibodies used provide evidence that orchiectomy results in a reduced staining intensity of AR, whereas substitution with TP enhances the immunoreactive signal of AR. The intensity of staining was significantly increased in prostate after treatment with lower doses of BPA (3 and 50 mg/kg bw). At 500 mg/kg bw staining intensity was similar to the castrated control, but the combina-

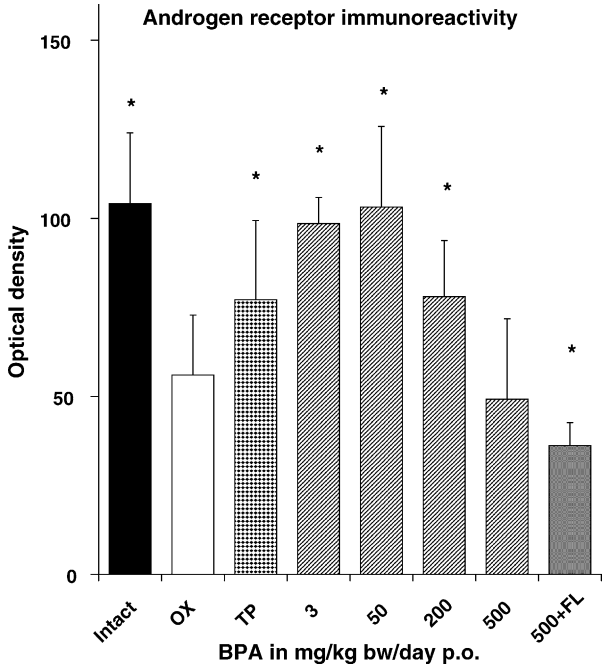

Fig. 3. Densitometric values of the Intact group, orchiectomized group (OX), TP group and the BPA-treated groups (3, 50, 200, 500 and 500 + FL) after immunohistochemical staining (monoclonal antibody) of the androgen receptor in the prostate. Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

tion of BPA (500 mg/kg bw) with FL significantly reduced the staining intensity (Figs. 3 and 4A–H). Absolute organ weights (prostate, seminal vesicle) at 200 and 500 mg/kg/day (40/100 fold the NOAEL (no-observed-adverse-effect-level)) were not significantly altered (Fig. 1). The NOAEL is the greatest concentration or amount of a substance e.g. BPA, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure.

3.3. Cell proliferation

The assessment of cell proliferation markers yielded the following data in rat prostates: The percentage of immunoreactive epithelial cells for MIB-5 was 85 ± 9% in the Intact group, 9 ± 1% in the OX group and 90 ± 2% in the TP group. BPA treated groups displayed: 10 ± 1% (BPA 3), 8 ± 1% (BPA 50), 2% (BPA 200), 1% (BPA 500) and 1% (BPA 500 + FL). The percentage of immunoreactive epithelial cells for MIB-5 in the OX group was significantly (*t*-test, $p < 0.05$, $n = 13$) reduced compared to the intact and TP group (Figs. 4I–P and 5). Similar results were obtained for the relative amount of cells immunoreactive for PCNA: 90 ± 9% in the Intact group, 10 ± 2% in the OX group and 88 ± 9% in the TP group, 2% (BPA 3), 4% (BPA 50), 2% (BPA 200), 5 ± 1% (BPA 500) and 6 ± 1% (BPA 500 + FL). The percentage of epithelial cells immunoreactive for PCNA in the OX group was significantly (*t*-test, $p < 0.05$, $n = 13$) reduced compared to the Intact and TP group (Figs. 4Q–X and 5). Whereas orchiectomy caused a considerable decrease of cell proliferation, administration of TP could reverse this effect and induced a cell proliferation index similar to the Intact group. The assessment of both proliferation markers revealed that BPA showed at all doses tested no stimulation of proliferating activity in prostate.

3.4. Morphometry

The mean epithelial height of prostate glands measured 17 ± 2 μm in both the Intact and TP group, whereas the mean epithelial height was significantly (*t*-test, $p < 0.05$, $n = 13$) decreased to 11 ± 1 μm in the OX group. BPA treated groups displayed following data: 14 ± 2 μm (BPA 3), 14 ± 2 μm (BPA 50), 10 ± 1 μm (BPA 200), 9 ± 1 μm (BPA 500) and 8 ± 1 μm (BPA 500 + FL). Similar significant (*t*-test, $p < 0.05$, $n = 13$) data were obtained for the epithelial height of seminal vesicle glands: 18 ± 3 μm (Intact), 9 ± 2 μm (OX) and 15 ± 2 μm (TP), 11 ± 1 μm (BPA 3), 11 ± 1 μm (BPA 50), 8 ± 1 μm (BPA 200), 8 ± 1 μm (BPA 500) and 8 ± 1 μm (BPA 500 + FL), respectively (Fig. 6).

The mean luminal area of prostate glands was 131 000 ± 40 000 μm^2 in the Intact group, 7000 ± 5000 μm^2 in the OX group and 87 000 ± 30 000 μm^2 in the TP group. BPA treated groups showed following data: 23 000 ± 16 000 μm^2 (BPA 3), 25 000 ± 12 000 μm^2 (BPA 50), 15 000 ± 10 000 μm^2 (BPA 200), 4000 ± 500 μm^2 (BPA 500) and 4000 ± 3000 μm^2 (BPA 500 + FL). The luminal area in the OX group was significantly (*t*-test, $p < 0.05$, $n = 13$) reduced compared to the Intact and TP group. In seminal vesicles the mean luminal area measured 113 000 ± 80 000 μm^2 (Intact), 7000 ± 5000 μm^2 (OX) and 151 000 ± 86 000 μm^2 (TP), 35 000 ± 43 000 μm^2 (BPA 3), 27 000 ± 20 000 μm^2 (BPA 50), 8000 ± 3000 μm^2 (BPA 200), 4000 ± 1000 μm^2 (BPA 500) and 3000 ± 2000 μm^2 (BPA 500 + FL), respectively. Similarly as observed for the prostates the luminal area in the OX group was significantly (*t*-test, $p < 0.05$, $n = 13$) reduced compared to the Intact and TP group (Fig. 7).

These morphologic observations clearly reveal that orchiectomy causes a substantial reduction of both epithelial height and luminal area of the prostate gland and seminal vesicles. If TP is substituted both parameters return to values similar to those found in the Intact group. Lower doses of BPA caused an increase in epithelial height and luminal area of prostate and seminal vesicle, while high doses reduced the epithelial height of prostate significantly in comparison to the orchiectomized group.

4. Discussion

4.1. Methodologic approaches

4.1.1. Hershberger assay

Although the Hershberger assay is a valid quantitative method for evaluating androgenic or anti-androgenic proper-

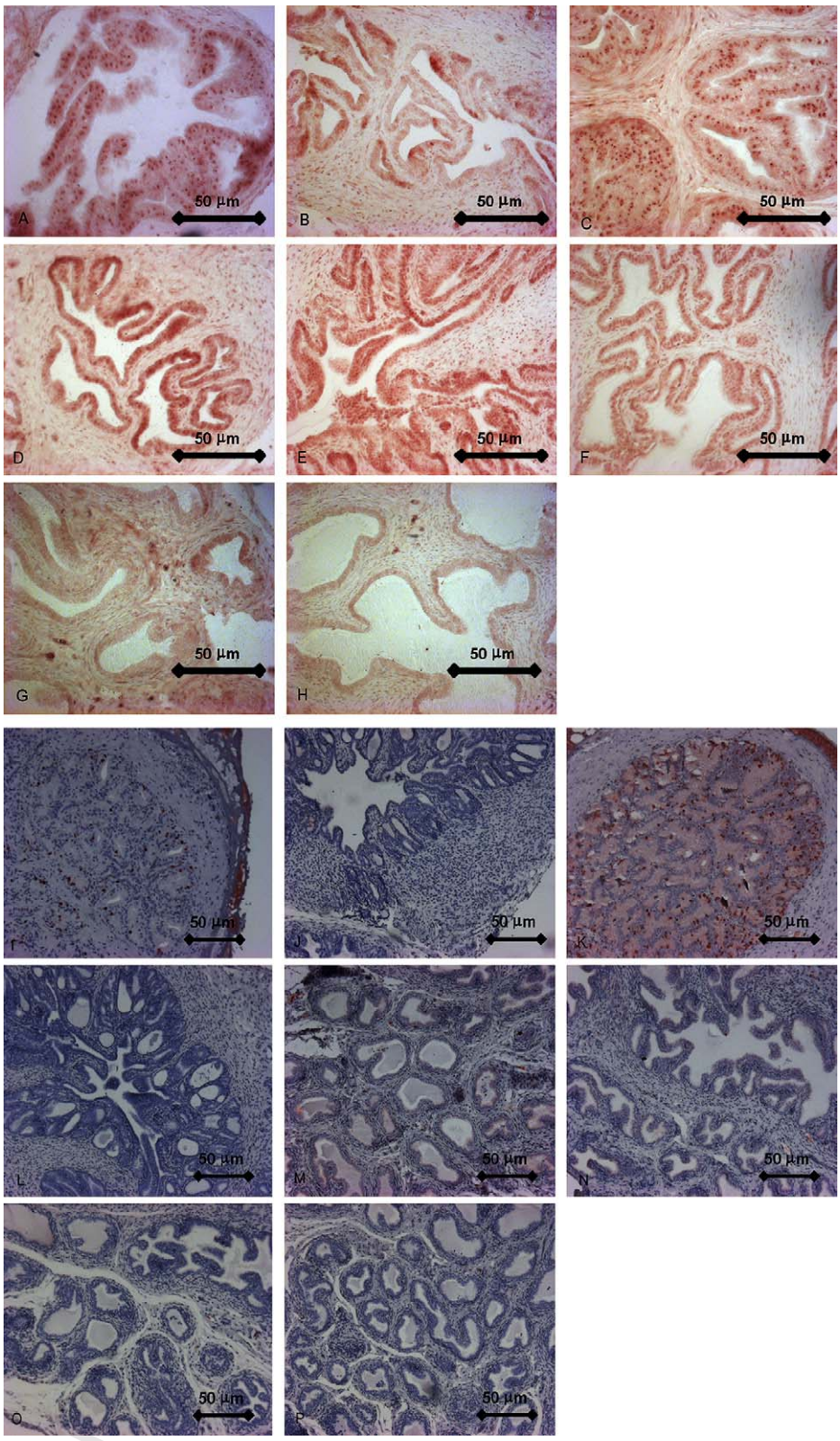

Fig. 4. All panels show photographs of the prostate. Zones are described according to McNeal [35]. A–H: Immunohistochemical staining of androgen receptor (monoclonal antibody) showing the transition zone of the Intact group (A), OX group (B) and TP group (C) in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Original magnification 20×. I–P: Immunohistochemical staining of MIB-5 showing the peripheral zone of the Intact group (I), OX group (J) and TP group (K) in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Original magnification 10×. Q–X: Immunohistochemical staining of PCNA showing the transition zone of the intact group (Q), OX group (R) and TP group (S) in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Original magnification 10×.

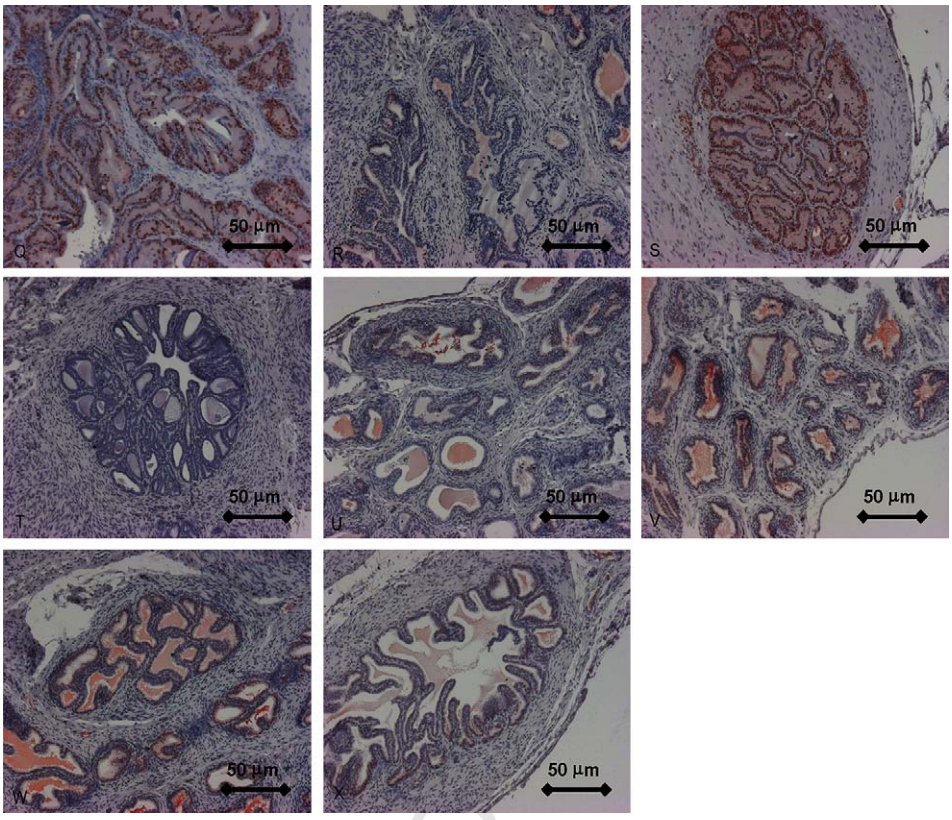

Fig. 4. (*Continued*).

ties of substances by measuring the organ weight of seminal vesicles and prostates, the findings obtained by this assay provide only limited information on the specificity of the observed effects when only the reactions of the organ weights are judged: For example, the growth of seminal vesicles can be stimulated not only by androgens but also by estrogenic substances, well known as a paradoxical effect of estrogens [18–20].

Morphologic and functional analysis of cellular parameters in male accessory organs may allow a more subtle and reliable assessment of the (anti-) androgenicity of substances; in previous studies we analyzed the regulation of tenascin expression [21]. Since the amount of nuclear AR present in the rat prostate has been demonstrated to be influenced by androgens [22,23], a densitometric analysis of AR-immunoreactive cells in prostates and seminal vesicles was performed by using immunohistochemical methods [24,25].

4.2. Densitometric analysis

It has been previously described that the concentrations of biochemically active substances can be estimated from the optical density of the immunoreactive signal [26,27]. As a result, we found a marked decrease in staining intensity of AR-positive cells after orchiectomy in comparison to the control group. This effect of orchiectomy was mostly reversed by an administration of a pharmacological dose of TP. The advantages of a computer-assisted densitometry are a faster scoring procedure of sections from large series and a higher reliability. However, the disadvantage of a semiquantitative approach is the possibility that relevant signals can easily be missed, so that comparative studies should be based on rather robust signals [28].

4.3. Influence of BPA

In this study lower doses of BPA (3 and 50 mg/kg bw/day) were found to cause an enhancement in staining intensity of AR in rat prostate. Morphometric data showed that lower doses of BPA cause an increase in epithelial height and luminal area of prostate and seminal vesicle, while high doses significantly reduce the epithelial height of prostate. These findings are similar to those observed in testosterone propionate substituted castrated rats. Gupta [15] proposed androgen-like effects of BPA in pregnant CD-1 mice at 0.05 mg/kg/day by observing an enhancement of the anogenital distance and the size of the prostate in fetuses. The androgen receptor (AR) binding affinity in prostate of fetuses was also increased significantly. In contrast, Kim et al. did not found any androgenic or anti-androgenic activities of BPA in Hershberger assay at 10–1000 mg/kg/day [16].

BPA at all doses tested exerted no significant effects on absolute weights of prostate and seminal vesicle. High doses of BPA (200 and 500 mg/kg bw/day) caused a significant

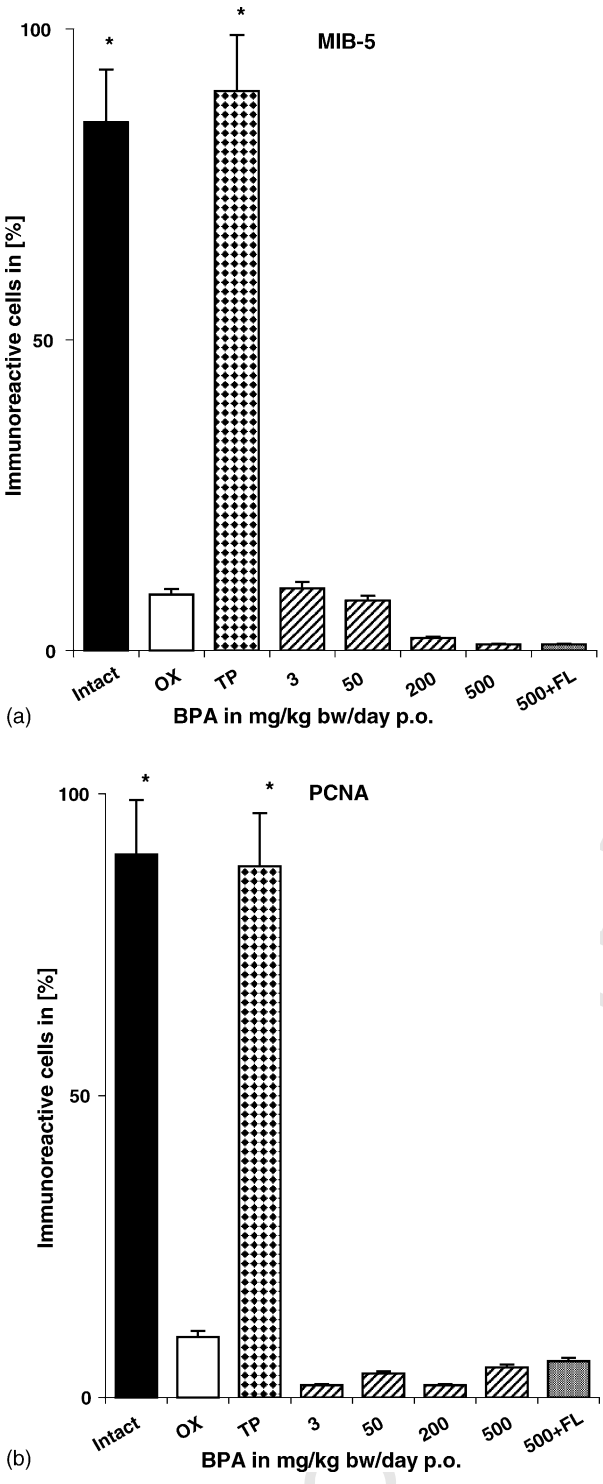

Fig. 5. Quantitative comparison of (a) MIB-5-immunoreactive and (b) PCNA-immunoreactive epithelial prostatic cells between the Intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

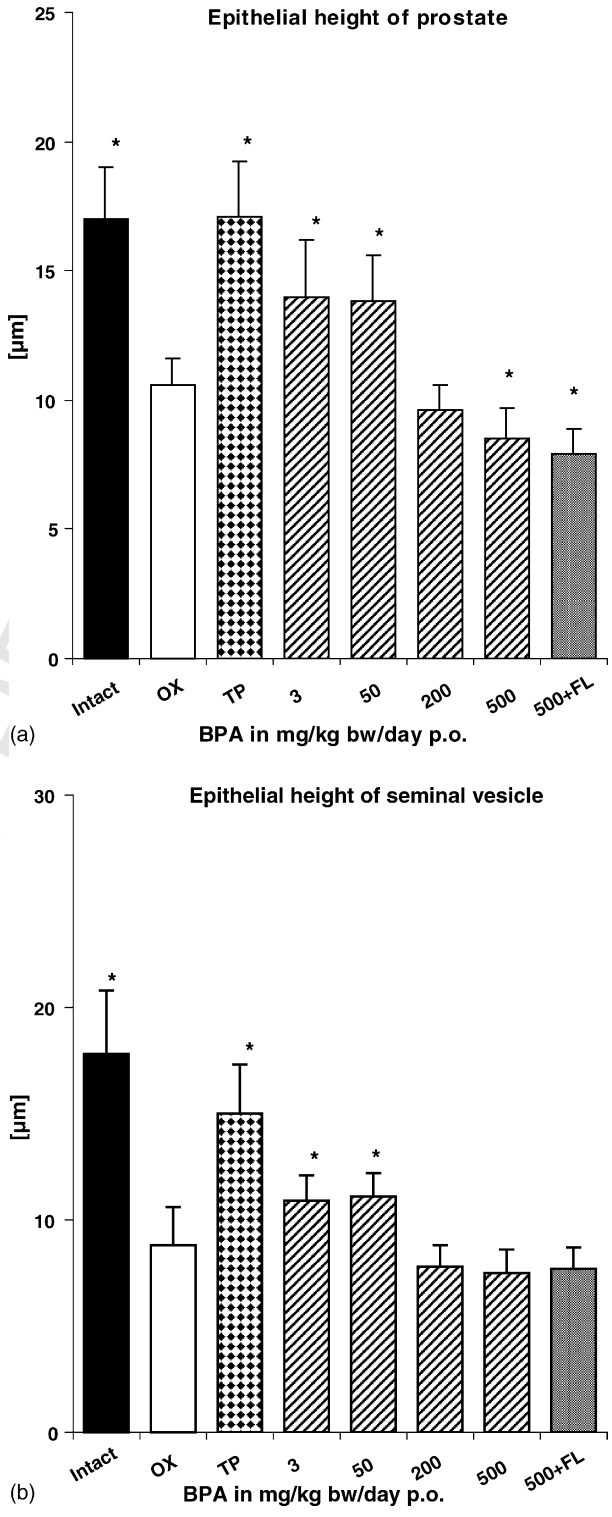

Fig. 6. Comparison of epithelial height of (a) prostate and (b) seminal vesicle between the Intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

increase in relative weights of prostates and seminal vesicles. These effects of BPA may be due rather to a toxicity-related significant decrease in body weights and well known general side effects, e.g. loss of appetite and diarrhea [29–31], than to an androgenic effect of this substance. An oral predictable no effect concentration ($PNEC_{oral}$) of 33 mg/kg food has been derived for the secondary poisoning assessment from a NOAEL of 50 mg/kg bw (based on a reduction in litter

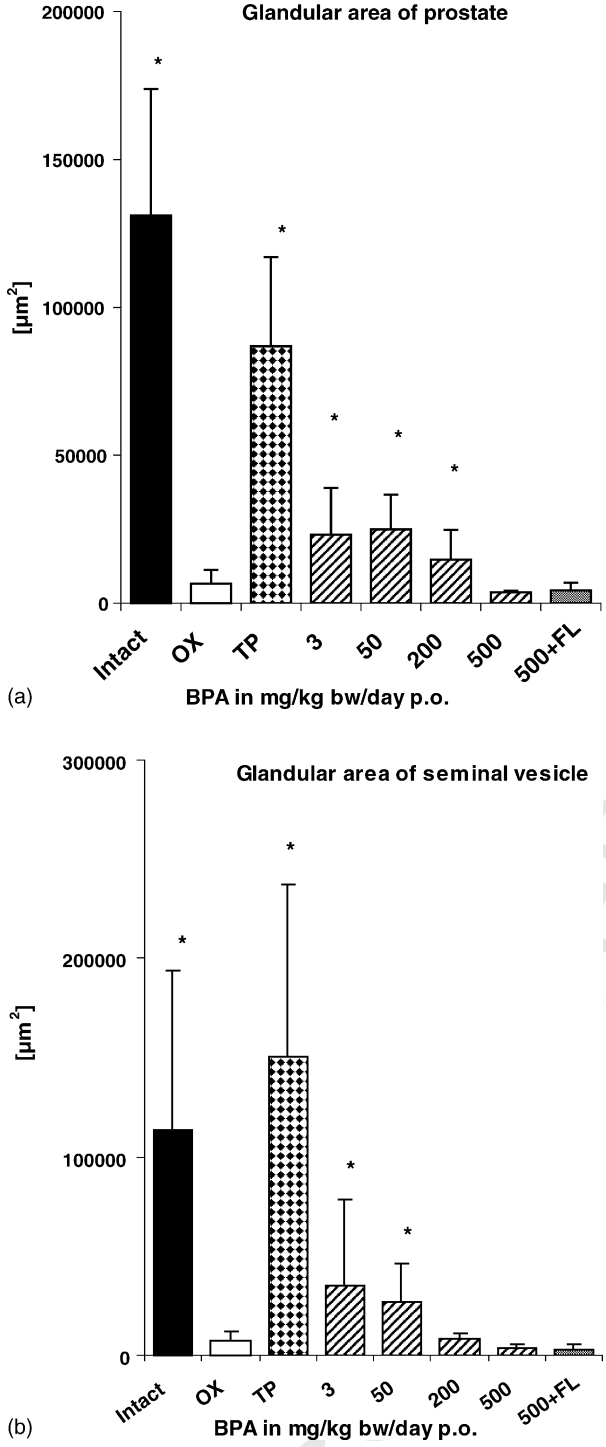

Fig. 7. Comparison of luminal glandular area of (a) prostate (μm^2) and (b) seminal vesicle (μm^2) between the Intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

size) from a three-generation multi-dose level feeding study in rats [31]. The PNEC represents the concentration below which an unacceptable risk should not occur. At 3 and 50 mg BPA/kg bw/day no effect on body weight, organ weight, cell proliferation in epithelial prostatic cells, but effects on androgen receptor immunoreactivity and epithelial height of prostate and seminal vesicles, glandular area of prostates and seminal vesicles was observed. Overall, in standard developmental studies in rodents, there is no convincing evidence that BPA is a developmental toxicant [32].

The estrogenic activity of BPA has been mostly observed in higher doses up to 100 mg/kg bw/day [12–14]: the mechanism concerning the androgen-like effect of the low-doses of BPA on the epithelial cells of the prostate is not clear at the present time. It may be difficult to correlate this BPA-effect with the known estrogenic property of this compound [12–14], since BPA had almost no effect on the seminal vesicle. Estrogens have been known to stimulate the development of the fibrous tissue and muscular walls of both prostate and seminal vesicle [20], without stimulating the epithelium and secretory activity [33]. Moreover, it seems very improbable, that the increase of the immunoreactive AR in the prostatic epithelial cells (not in the seminal vesicle) is up-regulated by estrogens. It is also unlikely that BPA, via an activation of adrenal androgen synthesis (the production of corticosteroid-binding globulin CBG and an activation of adrenal functions, leads to an increased steroidogenesis including androgen production), stimulates the prostate selectively, although estrogens have been considered to be one of the controllers of adrenal androgen secretion [34]. Further studies on the low-dose effects of BPA using an antiandrogen may be necessary to elucidate the selective effects on the prostate in the rat.

Based on the present data, the densitometric analysis of AR-immunoreactivity and the assessment of both cell morphology and cell proliferation proved to be independent and sensitive parameters for the evaluation of androgen effects on prostates and seminal vesicles. The combined application of these parameters may provide an additional tool to test the broad spectrum of endocrine active substances such as endocrine disruptors, which are actually discussed on their potential risk to the environment and humans.

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