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Androgen-dependent morphology of prostates and seminal vesicles in the Hershberger Assay: Evaluation of immunohistochemical and morphometric parameters*

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Summary. The aim of this study was to evaluate androgen-like effects using immunohistochemical and morphometric methods. Therefore, orchiectomized Wistar rats ($n \geq 13$) were treated s.c. with 1 mg/kg bw/day testosterone propionate (TP) for 7 days and compared to orchiectomized rats without TP substitution (OX) and to an untreated intact control group. Sections obtained from prostates and seminal vesicles were stained with polyclonal and monoclonal antibodies against the androgen receptor (AR) and assessed densitometrically (intensity of the immunoreaction) and morphometrically (epithelial height, luminal area). TP caused an enhancement of staining intensity and an increase in organ weights, epithelial height and luminal area. The use of proliferation markers (PCNA, MIB-5) showed also a highly significant increase of immunoreactive cells in TP-substituted orchiectomized rats compared with the OX group. Based on the present data, the densitometric analysis of AR-immunoreactivity as well as the assessment of proliferation markers, epithelial height and luminal area proved to be sensitive parameters for the evaluation of androgen effects on prostates and seminal vesicles. In further studies these parameters will be used to test several industrial xenooestrogens as well as phytooestrogens on their possible androgenic capacity.

Key words: Androgenic reactions – Androgen receptor – Prostate – Seminal vesicle – Densitometry – Morphometry – Proliferation markers

Introduction

The rodent Hershberger Assay using castrated peripubertal male rats measures the androgenic or anti-androgenic effects of test chemicals on several accessory glands/tissues (e. g. ventral and dorso-lateral prostate and seminal vesicles with coagulating glands) by means of organ weight measurements (Hershberger et al. 1953). Generally, accessory sex glands and tissues are dependent upon androgen stimulation to gain and maintain weight during or after puberty. If endogenous testicular sources of androgens are removed, exogenous sources of androgens are necessary to increase or maintain the weights of these tissues (Ashby and Lefevre 2000; Kim et al. 2002; Breckwoldt et al. 1991). All anabolic/(anti-) androgenic compounds (drugs) in clinical use have been screened by means of this *in vivo* bioassay (Kühnel 1970; Kühnel 1974). This assay is one of the assays in the proposed

* Dedicated to Professor Dr. med. Bernhard Tillmann on the occasion of his 65th birthday.

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Tier I screening battery by EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee). Tier I sites are those that have greater potential to require long-term or emergency cleanup work under the Federal Superfund program. These are sites which have a release of a hazardous substance, pollutant or contaminant that has caused, or is likely to cause exposure to humans or contamination of a sensitive environment. They typically involve contamination of drinking water, surface water, air or soils which have either caused or are likely to cause exposure to nearby populations, or have contaminated, or are likely to contaminate sensitive environments such as for example wetlands, national parks and habitats of endangered species (EDSTAC, EPA 1998).

However, the measurements of body and organ weight deliver only a limited amount of information, especially if this assay will be used to carry out a more subtle assessment of the androgenic properties of an unknown substance. Additional parameters such as morphometric and qualitative data are therefore required to determine the androgenicity of a given substance, in particular if the effects are of low degree.

The present study was undertaken to analyze these additional parameters and to assess their sensitivity for the evaluation of androgen-like effects using immunohistochemical and morphometric techniques.

Material and methods

Animals and housing. Wistar rats (male HdrBrHan from Harlan Winkelmann, Borchen, Germany), weighing about 150 g (age of 2 weeks) were randomly separated into different groups. 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 ± 3 °C, a relative humidity of 30–70% and artificial 12 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).

Treatment of animals. Animals were partitioned into three groups ($n \geq 13$ in each group). Two groups were orchiectomized. Seven days after orchiectomy one group was treated subcutaneously with testosterone propionate (TP) in arachis oil for seven days (kindly provided by Schering AG) and compared to vehicle treated, orchiectomized rats without TP substitution (OX) and to a vehicle treated intact control group (intact). After the treatment animals were sacrificed by decapitation. Seminal vesicles and prostates were harvested surgically, weighed and immediately fixed in 4% neutral buffered paraformaldehyde.

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 receptor and proliferation markers the following primary antibodies were applied: anti-androgen-receptor (1 : 100, sc-815, rabbit polyclonal, Santa Cruz Biotechnology, California, 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).

After deparaffinization in xylene and descending series of alcohol the sections were treated in 0.3% hydrogen peroxide in distilled water to quench endogenous peroxidase activity, rinsed 2 times in 0.01 M phosphate buffered saline (PBS) containing 0.1% Triton X-100 (PBS-TX), incubated for 10 min in 10% normal goat serum (NGS) in PBS-TX to block nonspecific antigen binding sites, incubated with the above mentioned primary antibodies diluted in PBS containing 1% NGS overnight, rinsed in PBS, incubated with biotinylated secondary antibodies (1 : 200, Dako, Denmark) for 1 h, rinsed in PBS and incubated with StreptAvidin-Biotin (SAB)-Complex (Biocare Medical, USA) conjugated to horseradish peroxidase for 1 h. Peroxidase activity was revealed with the chromogen 3-amino-9-ethyl-carbazole (AEC, Zymed, USA) for 2 minutes. Sections stained with proliferation markers were counterstained with Mayer`s hematoxylin for 10 minutes. Omission of primary antibodies resulted in the absence of a specific labelling.

Morphometry and densitometry. 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 have 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 adequate for deciding which profile of glandular ductus in the field of vision is suitable for densitometric measurements. By using densitometry, gray values were transformed pixel by pixel into optical densities (Oberholzer 1996), followed by calculation of the sum, mean and standard deviation. Densitometry was performed in gray level images with a dynamic range of 256 intensities. Each pixel had a value between 0 and 255, where 0 is darkest (black) and 255 is lightest (white). We used the software package KS 400 for image analysis.

The glandular duct to be measured was centered into the optical field at the resolution mentioned above. After focusing and adaption of light intensity (3200 K) the section was removed and a white image (w) was generated iteratively. The adaption of light intensity was carried out by the following procedure. Firstly the illumination was adjusted, so that the objects of interest could be clearly identified. Thereafter, an image without any histologic object was grabed and the mean pixel intensity was calculated. If the mean pixel intensity differed much from the pixel intensity obtained from preceding measurements, the light intensity was adapted until the mean pixel intensity adapted similar values to those in the previous measurements. The mean gray value of the white image was determined for each section in order to obtain the same illumination condition within each specimen examined. After white image calibration the glandular duct of interest was grabed and its immunoreactive epithelium was surrounded interactively. Within this defined epithelial area the mean optical density (OD) and the variance of OD were determined. 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 were used. The quantitative assessment of proliferating cells was performed using a 40× objective and 1.0 optovar. We have counted 1000 of those cells per section exhibiting nuclei.

A two-sided t-test at a significance level of $p < 0.05$ was applied for statistical comparison. Data were depicted as means ± standard deviation (SD).

Results

Hershberger Assay. Measurement of the body weight yielded no significant differences between the intact group ($n \geq 13$), OX group ($n \geq 13$) and TP group ($n \geq 13$). However, examination of wet weights (absolute and relative) of prostates and seminal vesicles showed that the orchiectomy in the OX group induced a significant (t-test, $p \leq 0.05$, $n \geq 13$) decrease in organ wet weights in comparison to the intact group, which was nearly reversed by an administration of a pharmacological dose of TP in the TP group (Fig. 1).

Densitometric analysis. Using the monoclonal antibody the staining intensity of AR in the prostates revealed the following optical density values: 104 ± 20 (intact), 56 ± 17 (OX), and 77 ± 22 (TP). Statistical analysis confirmed that the intensity of staining was significantly higher in both the intact and TP group (t-test, $p \leq 0.05$, $n \geq 13$) compared to the OX group (Figs. 2, 7 A–C).

The staining intensity after incubation with the polyclonal antibody against AR was 75 ± 21 (intact), 43 ± 12 (OX) and 61 ± 24 (TP) for the prostates and 42 ± 17 (intact), 11 ± 10 (OX) and 19 ± 11 (TP) 11 ± 10 for seminal vesicles, respectively. In the prostate tissues the intensity of staining obtained by the polyclonal antibody was significantly higher in both the intact and TP group (t-test, $p \leq 0.05$, $n \geq 13$) compared to the OX group.

The data provide evidence that orchiectomy results in a reduced staining intensity of AR, whereas substitution with TP enhances the immunoreactive signal of AR.

Morphometric analysis. 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, $p \leq 0.05$, $n \geq 13$) decreased to 11 ± 1 µm in the OX group. Similar significant (t-test, $p \leq 0.05$, $n \geq 13$) data were obtained for the epithelial height of seminal vesicle glands: 18 ± 3 µm (intact), 9 ± 2 µm (OX) and 15 ± 2 µm (TP), respectively (Fig. 3).

The mean luminal area of prostate glands was 131.206 ± 40.000 μm^2 in the intact group, 6.559 ± 5 000 μm^2 in the OX group and 86.892 ± 30.000 μm^2 in the TP group. The luminal area in the OX group was significantly (t-test, $p \leq 0.05$, $n \geq 13$) reduced compared to the intact und TP groups. In seminal vesicles the mean luminal area measured 113.188 ± 80.000 μm^2 (intact), 7.476 ± 5.000 μm^2 (OX) and 150.769 ± 86.000 μm^2 (TP), respectively. Similarly as observed for the prostates, the luminal area in the OX group was significantly (t-test, $p \leq 0.05$, $n \geq 13$) reduced compared to the intact and TP group (Fig. 4).

These morphologic observations clearly reveal that orchiectomy causes a substantial reduction of both epithelial height and luminal area of prostates and seminal vesicles. If TP is substituted both parameters return to values similar to those found in the intact group.

Cell proliferation. The assessment of cell proliferation markers yielded the following data in the 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. The percentage of

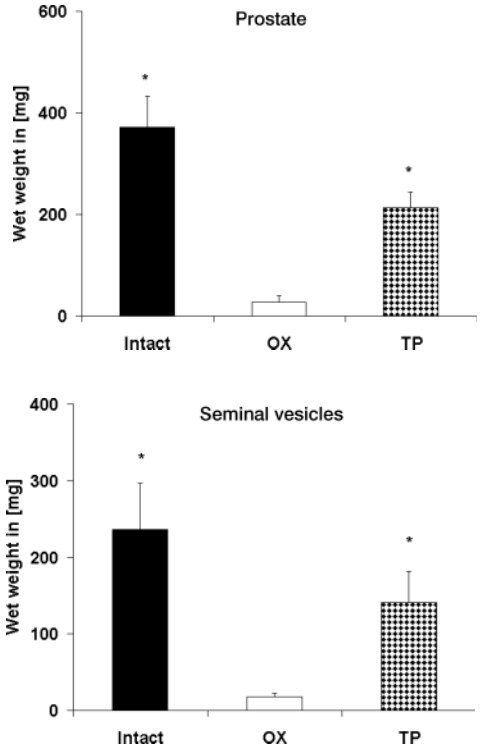

Fig. 1. Comparison of absolute wet weights of prostate and seminal vesicles between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

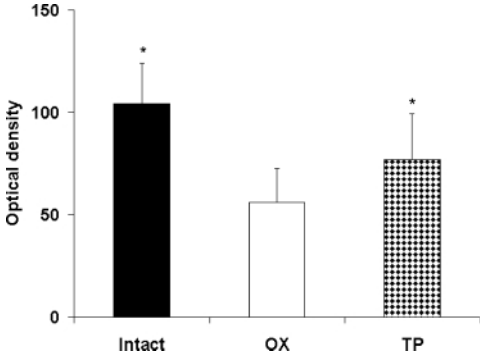

Fig. 2. Comparison of densitometric values between the intact group, OX group and TP group after immunohistochemical staining of androgen receptor in the prostate using a monoclonal antibody. Asterisks indicate statistically significant differences.

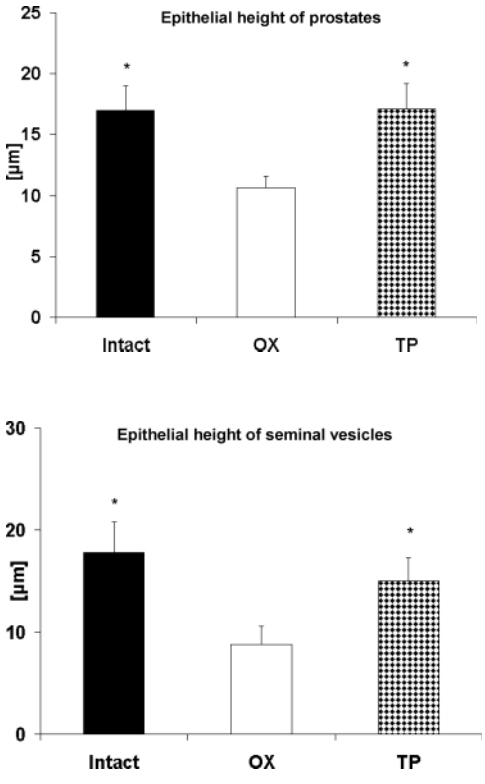

Fig. 3. Comparison of epithelial height of prostate and seminal vesicles between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

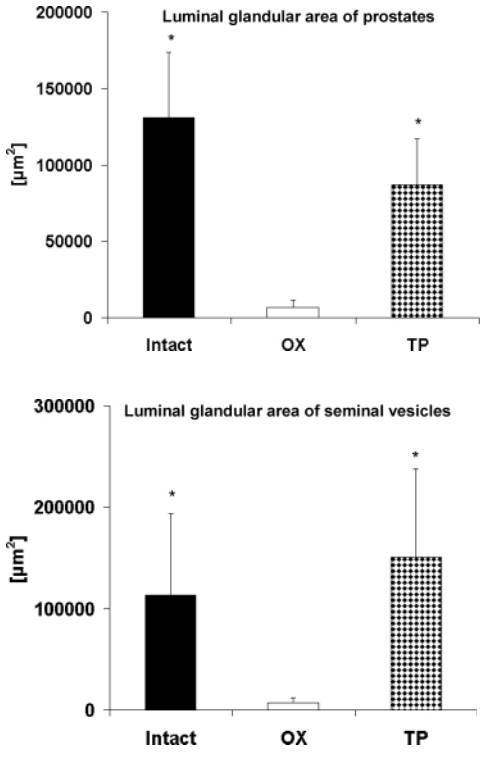

Fig. 4. Comparison of luminal glandular area of prostate and seminal vesicles between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

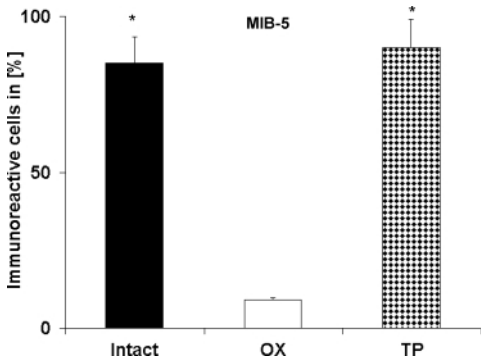

Fig. 5. Quantitative comparison of MIB-5-immunoreactive epithelial prostatic cells between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

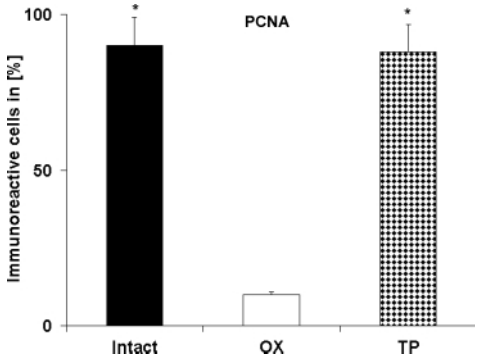

Fig. 6. Quantitative comparison of PCNA-immunoreactive epithelial prostatic cells between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

immunoreactive epithelial cells for MIB-5 in the OX group was significantly (t-test, $p \leq 0.05$, $n \geq 13$) reduced compared to the intact und TP group (Figs. 5, 7 D–F). Similar results were obtained for the relative amount of cells immunoreactive for PCNA: $90 \pm 9\%$ in the intact group, $10 \pm 2\%$ in the OX group and $88 \pm 9\%$ in the TP group. The percentage of epithelial cells immunoreactive for PCNA in the OX group was significantly (t-test, $p \leq 0.05$, $n \geq 13$) reduced compared to the intact und TP group (Figs. 6, 7 G–I).

Thus, whereas orchiectomy caused a considerable decrease in cell proliferation, administration of TP could reverse this effect and induced a cell proliferation index similar to the intact group.

Discussion

Hershberger Assay. Although the Hershberger assay (Hershberger et al. 1953) is a valid quantitative method for evaluating androgenic or anti-androgenic properties of substances by measuring the organ weight of seminal vesicles and prostates, the findings obtained by this assay provide only limited information on the specifity of the

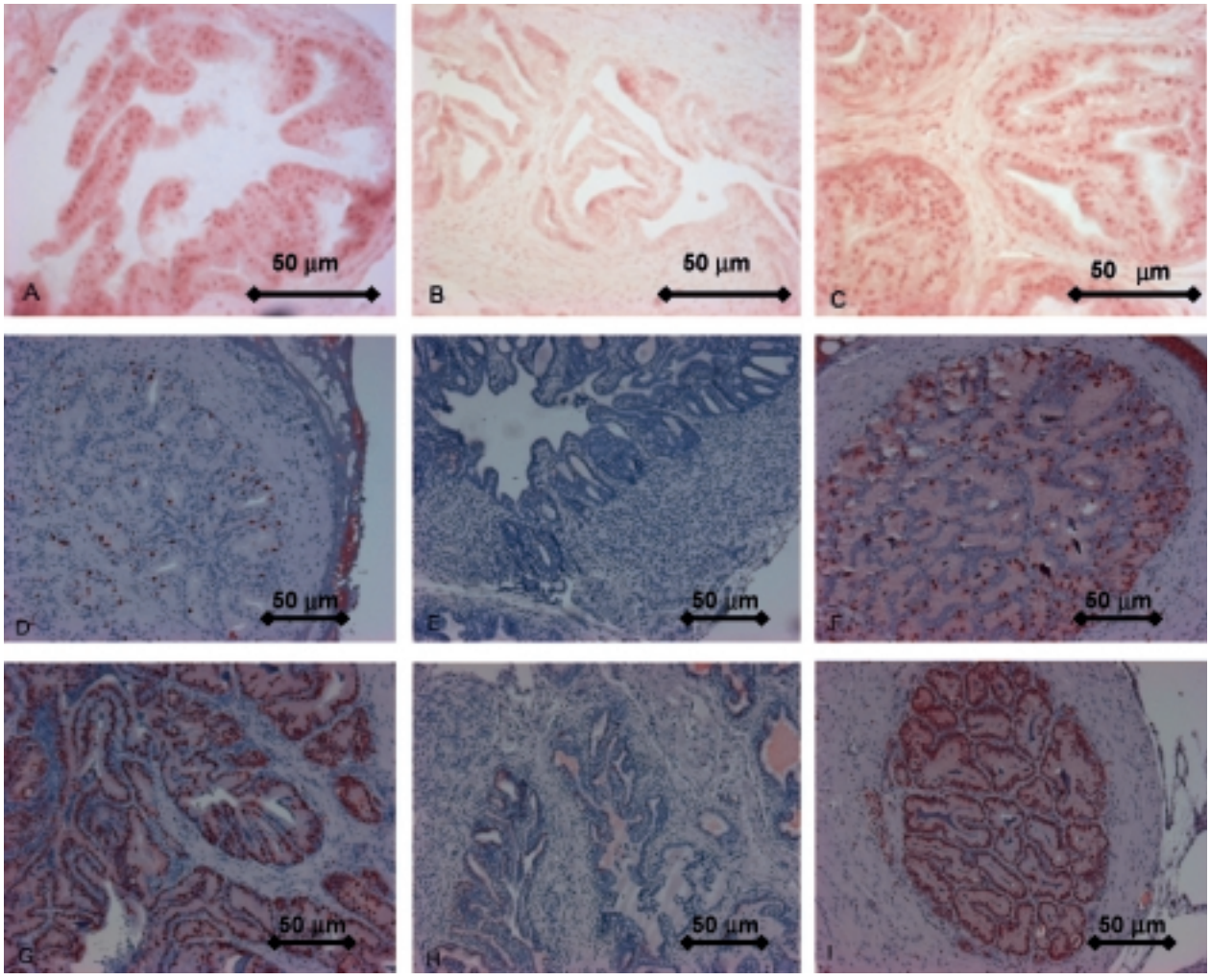

Fig. 7. All panels show photographs of the prostate. Zones are described according to McNeal (McNeal 1988).
A–C: Immunohistochemical staining of AR (monoclonal antibody) showing the transition zone of the intact group (A), OX group (B) and TP group (C). Original magnificaton 20×.
D–F: Immunohistochemical staining of MIB-5 showing the peripheral zone of the intact group (D), OX group (E) and TP group (F). Original magnification 10×.
G–I: Immunohistochemical staining of PCNA showing the transiton zone of he intact group (G), OX group (H) and TP group (I). Original magnificaton 10×.

observed effects. For example, the growth of seminal vesicles can be stimulated not only by androgens but also by oestrogenic substances, well known as a paradoxical effect of oestrogens (de Jongh 1935; de Jongh 1937; Freud 1933).

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 (Vollmer et al. 1994). Since the amount of nuclear AR present in the rat prostate has been demonstrated to be influenced by androgens (Moore et al. 1979; Blondeau et al. 1982), a densitometric analysis of AR-immunoreactive cells in prostates and seminal vesicles was performed by using immunohistochemical methods (Huggins and Hodges 2002; Rolf and Nieschlag 1998).

Densitometric analysis. It is known (Nabors et al. 1988; Mize et al. 1988) that the concentrations of biochemically active substances can be estimated from the optical density of the immunoreactive signal. 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 (De Boer et al. 2001).

Morphologic alterations. Morphologic or functional changes in the accessory reproductive organs have mainly

been used as hormone indicators. The main accessory glands of the male rat are the prostate and the seminal vesicle. The induction of tremendous size changes in these glands in castrated immature animals has been used for the assay of androgenic activity by Tschopp (Tschopp 1936).

The ventral prostate is the most sensitive region among the accessory glands in rats. After castration at birth the accessory glands are most sensitive to exogenous androgen like testosterone at 40–60 days of age (Hooker 1937). In contrast, in the mouse the prostate is much less prominent and the seminal vesicles are comparatively insensitive (Voss and Loewe 1931; Deanesly and Parkes 1933).

Changes in the glandular epithelium under the influence of androgen administration take place before any substantial change in weight occurs. These changes were screened in a semi-quantitative approach by Moore and Gallagher (Moore and Gallagher 1930) and the quantitative assessment by Hansen (Hansen 1933) suggested that epithelial cell height in both prostate and seminal vesicles may serve as an androgen assay. A detailed dose-response curve of the effect of 14 daily injections of androsterone on the height of the vesicular epithelium in the castrated rat was given by Jacobsen (Jacobsen 1938).

Markers of cell proliferation. In order to further characterize androgenic effects two proliferation markers were used in this study. The proliferation associated antigen Ki-67 (MIB-5) and proliferating cell nuclear antigen (PCNA) have been used as proliferation and prognostic markers in a large number of studies, in particular in malignancies (Korkolopoulou et al. 1993; Kawai et al. 1994). PCNA is a highly conserved 36 kDa acidic nuclear protein that is expressed during cell replication and DNA repair (Shivji et al. 1992; Hall et al. 1990). PCNA interacts with DNA-polymerase delta and with RF-C protein to bind at DNA primer-template junctions. Immunostaining of S-phase nuclei will detect PCNA in sites of DNA synthesis. Ki-67 antigen is expressed during the G_1, S, G_2 and M phases of the cell cycle, but is not expressed during the G_0 (resting) phase. Because Ki-67 antigen has a short half-life, it can be used as a marker of actively proliferating cells (McCormick et al. 1993; Pinder et al. 1995).

Endothelial cell proliferation in male reproductive organs of adult rat is high and regulated by testicular factors. In the epididymis, the ventral and dorsolateral prostate lobes, and the seminal vesicles, endothelial cell proliferation decreased after testosterone withdrawal and increased following testosterone treatment (Franck Lissbrant et al. 2002).

In our study orchiectomy caused a dramatic decrease in the percentage of proliferating epithelial cells – an effect which could be almost completely reversed by the administration of testosterone.

Conclusion

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 currently being discussed as to their potential risk to the environment and humans.

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