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Effect of chronic oestrogen administration on androgen receptor expression in reproductive organs and pituitary of adult male rat

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Summary

Following chronic (15 or 30 days) treatment with oestradiol 3-benzoate (75 μg rat^{-1} day^{-1} in 100 μl of olive oil) to adult rats, androgen receptor (AR) expression was analysed simultaneously in testis, epididymis, seminal vesicle, prostate and pituitary utilising three independent tools i.e. immunohistochemistry, Western blotting and RT-PCR. All the five organs showed higher AR transcriptional activity gradually increasing from 15 to 30 days of oestrogen treatment. However, the AR protein expression either through immunostaining or Western blotting demonstrated a significant decline in all the reproductive organs. In the pituitary, on the other hand, the decline coincided with a distinct breakdown of the AR protein into two bands with increasing duration of treatment. Serum and intra-testicular testosterone levels were found significantly lowered. Spermatogenesis was adversely affected with concurrent decrease in weights of testis and accessory sex organs. Decrease in testis weight was consistent with the reduction in the number of maturing germ cells per tubule. Despite the decrease in weight, accessory sex organs like epididymis, seminal vesicle and prostate were completely devoid of any apoptotic cells which were characterised only in testis and pituitary. Seminiferous epithelium demonstrated a marked increase in the number of germ cells undergoing apoptosis. However, the rate of cell apoptosis was much higher in the pituitary than in the testis at the end of 30 days treatment. It is therefore concluded that degradation of AR protein expression after oestrogen treatment is probably directly linked to an increase in cell apoptosis both in testis and pituitary.

Introduction

Chronic oestrogen exposure in foetal or neonatal life has been implicated for the apparent increase in male reproductive disorders like cryptorchidism, hypospodiasis, low sperm counts and testicular cancer in men (Sharpe, 2003). These alterations are considered as symptoms of a syndrome known as the testicular dysgenesis syndrome (Skakkebaek *et al.*, 2001; Skakkebaek & Jorgensen, 2005) reportedly altered in areas close to industry and exposed to environmental and industrial chemicals possessing oestrogenic potency (Mackenzie *et al.*, 2005). It is perceived that these abnormalities first arise during foetal development (Delbes *et al.*, 2006). Male rats treated neonatally with diethylstilboestrol (DES) revealed suppression of androgen action and gross abnormalities in reproductive tract (McKinnell *et al.*, 2001). Furthermore, studies on the sons of women who were exposed to DES during pregnancy show a similar range of abnormalities (Stillman, 1982; Toppari *et al.*, 1996).

On the other hand, oestrogen is also known to have adverse effects on spermatogenesis, which are reversible following withdrawal of treatment (Toyama *et al.*, 2001). Similar findings are also reported in adult male rats in which oestradiol 3-benzoate (EB) affects not only spermatogenesis but also induces apoptosis in the prevailing

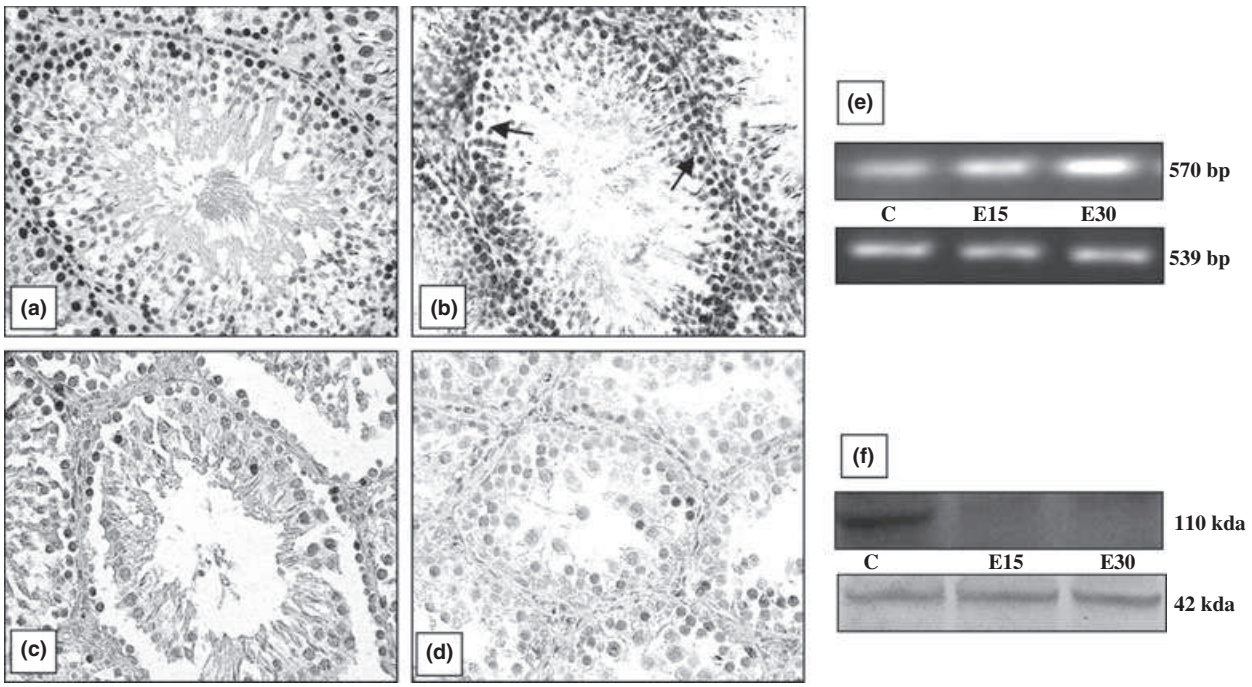


Fig. 1 Androgen receptor (AR) immunolocalisation in representative testis sections of rat; (a) negative control, (b) age matched vehicle treated control showing positive immunostaining in the germ and Sertoli cell nuclei (➡) compared with decrease in staining intensity after (c) 15 days and (d) 30 days of chronic EB treatment, (e) semiquantitative RT-PCR showing AR mRNA expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: internal control, β-actin), (f) Western blot showing AR protein expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: β-actin); 400× (the figure is representative of three identical experiments).

population among the dwindling number of germ cells (Chaki *et al.*, 2006). While foetal and neonatal exposure with oestrogen induces irreversible abnormal development of reproductive organs, the effect in adult animals is completely reversible with the stoppage of treatment. This may be explained by the fact that neonatal treatment with DES can cause life long suppression of plasma testosterone levels (Atanassova *et al.*, 1999) while post-pubertal DES administration clearly results in reduced androgen production during the course of treatment (Abeny & Keel, 1986).

The molecular mechanism whereby oestrogen mediates such divergent effects is not completely understood and there are conflicting reports on the way it is mediated in different reproductive organs. In the prostate, neonatal exposure to oestrogen induced lobe-specific alterations in AR receptor expression (Prins, 1992). The intracellular mechanism of AR down-regulation is described to be mediated at the post-translational level characterised by AR degradation through proteosome pathway with no difference in the mRNA levels in response to oestrogen exposure (Woodham *et al.*, 2003). By contrast, adult rats fed with dietary phytooestrogens for 3 days demonstrated an increased AR mRNA expression in the initial segment of the epididymis (Glover & Assinder, 2006). This study was therefore initiated in adult rats exposed to EB at a higher pharmacological dose to determine the AR expression both at the mRNA and protein levels in different reproductive organs (testis, epididymis, prostate and seminal vesicle) and simultaneously in the pituitary through immunostaining, Western blotting and RT-PCR.

Materials and methods

Animals, treatment and sample preparation

Adult male rats, Holtzman strain, weighing 200–220 g were maintained under controlled temperature (25 ± 2 °C) and constant photoperiodic (12 h light : 12 h dark) conditions. Food and water were provided *ad libitum*. Twenty-four animals were divided into four groups of six animals each receiving either olive oil or oestrogen (EB in olive oil) as described below.

Group I: Vehicle control 15 days (olive oil 100 μl rat^{-1} day^{-1})

Group II: Vehicle control 30 days (olive oil 100 μl rat^{-1} day^{-1})

Group III: Oestradiol 3-benzoate 15 days (EB, 75 μg rat^{-1} day^{-1} in 100 μl of olive oil)

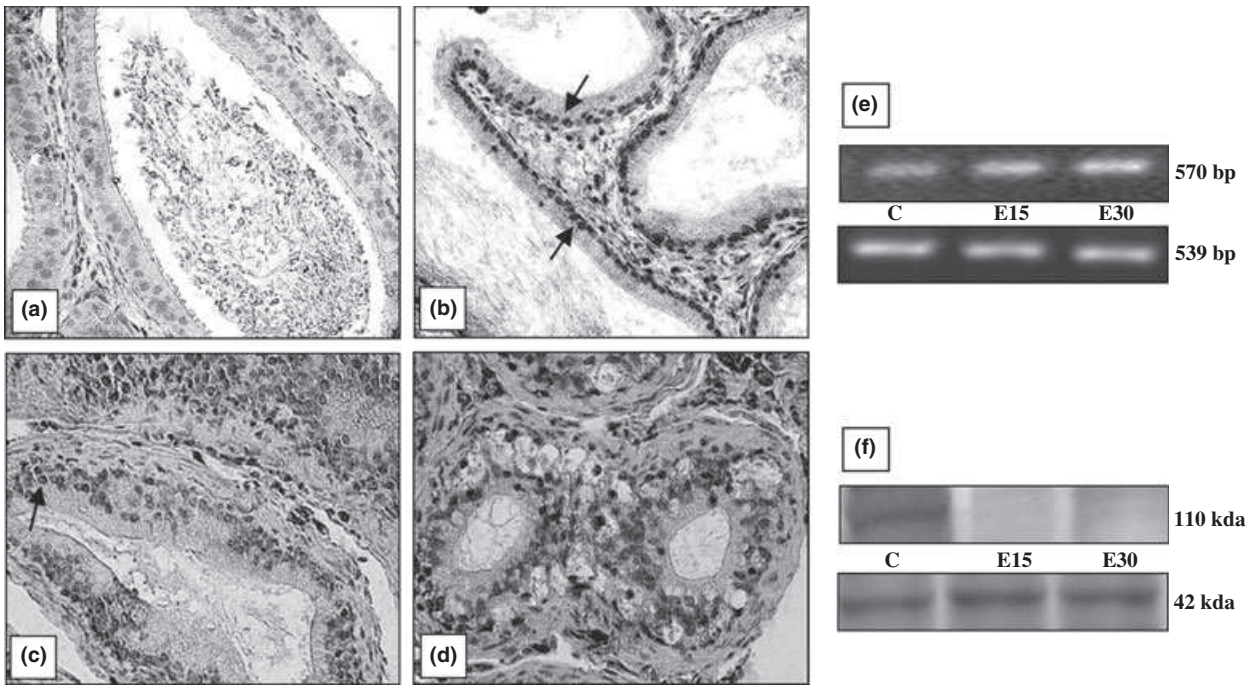


Fig. 2 Androgen receptor (AR) immunolocalisation in representative epididymis sections of rat; (a) negative control, (b) age matched vehicle treated control showing positive staining in the epithelial and stromal cell nuclei (➡) compared with decrease in staining intensity after (c) 15 days and (d) 30 days of chronic EB treatment, (e) semiquantitative RT-PCR showing AR mRNA expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: internal control, β-actin), (f) Western blot showing AR protein expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: β-actin); 400× (the figure is representative of three identical experiments).

Group IV: Oestradiol 3-benzoate 30 days (EB, 75 μg rat^{-1} day^{-1} in 100 μl of olive oil)

Oestradiol 3-benzoate (Sigma, St Louis, MO, USA) was dissolved first in isopropanol, resuspended in olive oil and administered intramuscularly. At the end of the treatment, the animals were bled and killed under aether anaesthesia with strict adherence to Standard Institutional Guidelines for Animal Care. Appropriate clearance from Animal Ethics Committee of the Institute was earlier obtained. Serum was separated and stored at −20 °C till assayed for testosterone. Testis, epididymis, prostate, seminal vesicle and pituitary gland were dissected, weighed and divided in two equal parts. One part was immediately fixed in buffered formalin while the rest was frozen in liquid nitrogen and stored at −70 °C till utilised for RT-PCR, Western blotting and intra-testicular testosterone.

Immunohistochemistry (IHC)

The protocol was strictly followed as per manufacturer's (Lab Vision Corporation, Feremont, CA, USA) instructions provided in the kit. Briefly, the paraffin-embedded sections were deparaffinised in xylene and rehydrated in alcohol series, immersed in dH_2O and washed two times in PBS pH 7.4. The sections were heated in sodium citrate buffer (10 mmol l^{-1}, pH 6.0) for 10 min in a microwave oven for antigen retrieval, washed in PBS and incubated with the blocking solution (provided in the kit) for 5 min at RT to block nonspecific binding sites. Sections were incubated with AR primary antibody (Lab Vision Corporation) (dilution 1 : 100 in PBS) overnight at 4 °C, washed four times in PBS and incubated with biotinylated second antibody ready-to-use followed by streptavidin alkaline phosphatase solution for 20 min each. The colour was developed using fast red as substrate followed by light counterstaining with aqueous haematoxylin.

Western blot analysis

Frozen tissues (testis, epididymis, prostate gland, seminal vesicles and pituitary gland) in liquid nitrogen were grinded in ice-cold RIPA (radioimmuno-precipitation assay) buffer pH 7.5 containing 100 μl of protease inhibitor cocktail (Roche, Basel, Switzerland). Following centrifugation at 15000 g for 20 min at 4 °C, the supernatant was collected and stored at −20 °C. Aliquots of supernatant were assayed for protein content (Bradford, 1976) and electrophoresed through 10% SDS–PAGE and transferred to polyvinyl difluoride membrane (0.45 μm). Membranes

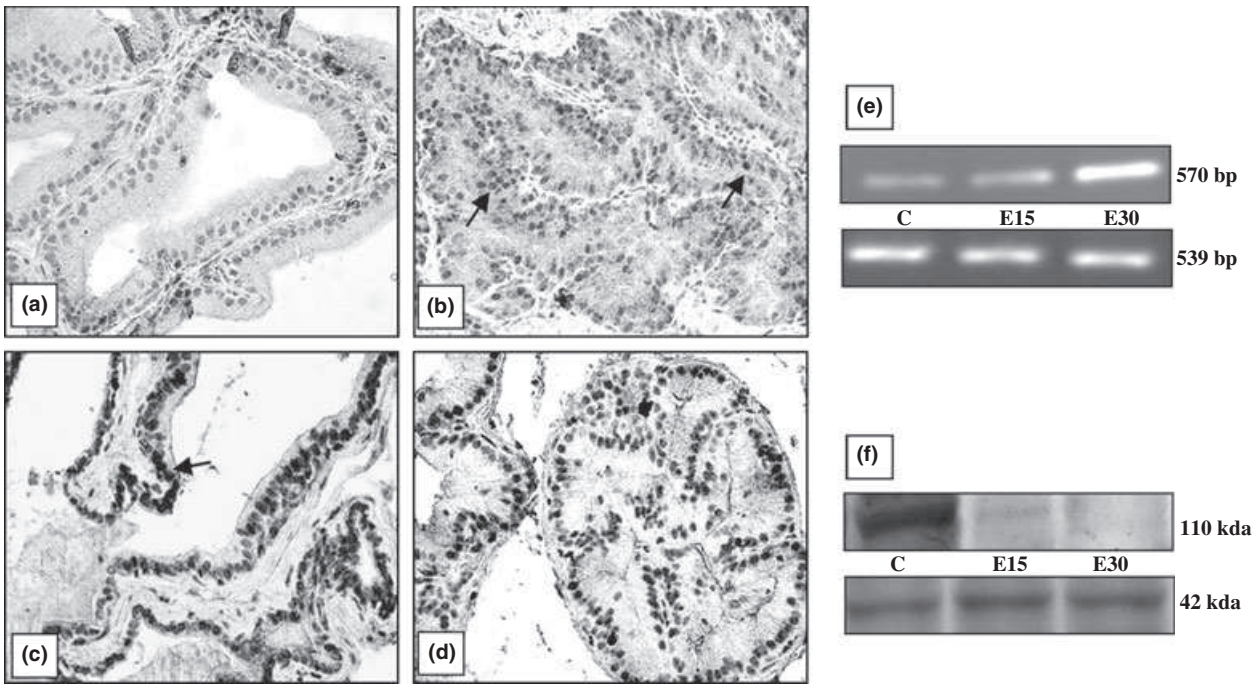


Fig. 3 Androgen receptor (AR) immunolocalisation in representative prostate sections of rat; (a) negative control, (b) age matched vehicle treated control showing positive staining in the epithelial and stromal cell nuclei (➞) compared with decrease in staining intensity after (c) 15 days and (d) 30 days of chronic EB treatment, (e) semiquantitative RT-PCR showing AR mRNA expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: internal control, β-actin), (f) Western blot showing AR protein expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: β-actin); 400× (the figure is representative of three identical experiments).

were blocked with 3% gelatin provided in the immunoblot assay kit (Bio-Rad Laboratories, Philadelphia, PA, USA). Membranes were washed and incubated with AR primary antibody (1 : 2000, Lab Vision Corporation) for overnight at 4 °C. Equal loading of samples with β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was carried out. The membranes were finally treated with goat anti-rabbit second antibody conjugated with alkaline phosphatase supplied in the immunoblot kit and the colour was developed as per the manufacturer's (Bio-Rad Laboratories) instructions.

Semiquantitative RT-PCR

Total RNA was isolated from testes, epididymis, prostate gland, seminal vesicle and pituitary using acid guanidinium thiocyanate–phenol–chloroform extraction as described (Chomczynski & Sacchi, 1987). Complementary DNA was made using 2 μg of total RNA in the presence of Avian myeloblastosis virus reverse transcriptase from kit (Promega Corporation, Madison, WI, USA). The primer sets used as a template to obtain PCR products of AR were 5′-TGC TGC CTT GTT ATC TAG TCT CA-3′ and 5′-ACC ATA TGG GAC TTG ATT AGC AG-3′ and for β-actin were 5′-CTG TGC CCA TCT ATG AGG GTT AC -3′ and 5′-AAT CCA CAC AGA GTA CTT GCG CT -3′. For semiquantitative analysis, β-actin was used as an internal control and co-amplified with AR mRNA by using β-actin primer and AR primer, 50 pmol each. All PCR reactions were performed for 25 cycles with an annealing temperature of 60 °C in 2.5 mM $MgCl_2$.

The band intensities of AR and β-actin as obtained for each organ were quantified using the densitometric software; Lab Works Image Acquisition and analysis Software version 4.0 (UVP, CA, USA) and the differences in ratios (value of AR/β-actin) between groups were compared.

In situ end labelling (ISEL)

In situ Apoptosis Detection Kit (R & D Systems Inc., Minneapolis, MN, USA) was used to identify apoptotic cells by detecting DNA fragmentation through a combination of enzymology and immunohistochemistry techniques. Paraffin embedded sections of testis, epididymis, prostate gland, seminal vesicle and pituitary gland were deparaffinised in xylene rehydrated in alcohol, immersed in dH_2O and washed in PBS. To make the DNA accessible to the labelling enzyme, the cell membranes were permeabilised with proteinase K. Endogenous peroxidase activity was quenched using hydrogen peroxide and biotinylated

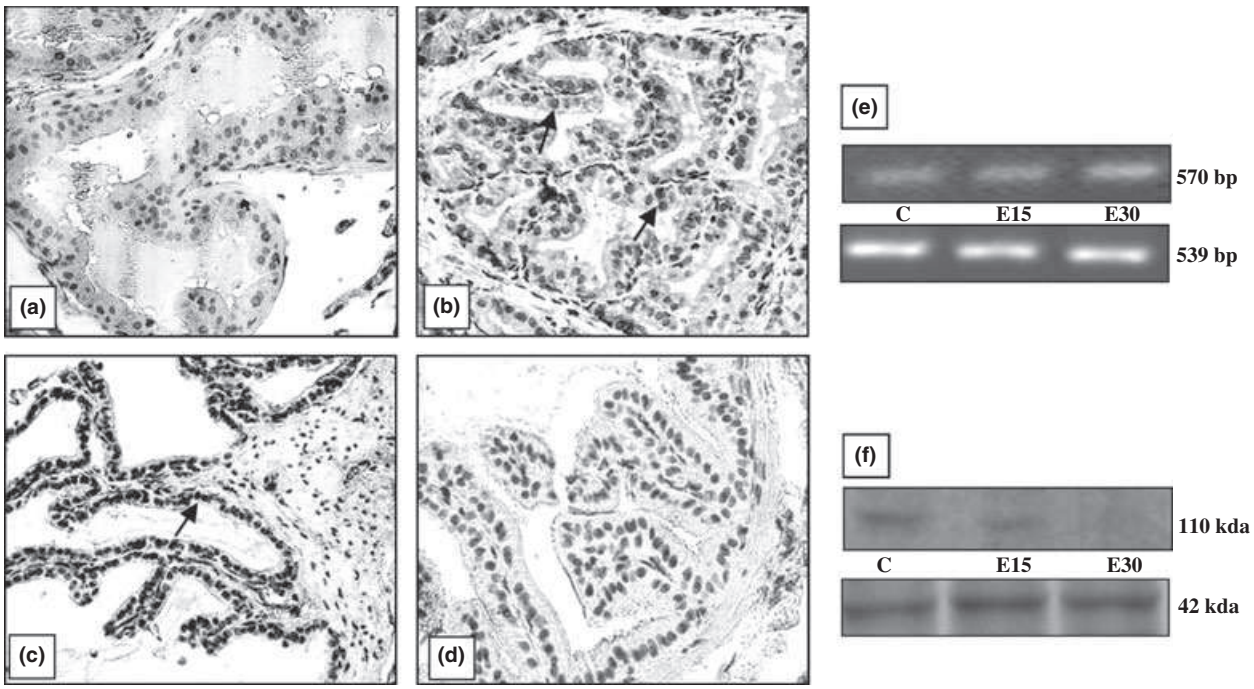


Fig. 4 Androgen receptor (AR) immunolocalisation in representative seminal vesicle sections of rat; (a) negative control, (b) age matched vehicle treated control showing positive staining in the epithelial and stromal cell nuclei (➡) compared with decrease in staining intensity after (c) 15 days and (d) 30 days of chronic EB treatment, (e) semiquantitative RT-PCR showing AR mRNA expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: internal control, β-actin), (f) Western blot showing AR protein expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: β-actin); 400× (the figure is representative of three identical experiments).

nucleotides were incorporated into the 3′-OH ends of the DNA fragments by terminal deoxynucleotidyl transferase. The biotinylated nucleotides were detected by using streptavidin-horseradish peroxidase conjugate followed by the substrate, diaminobenzidine (DAB). The enzyme reaction generates an insoluble coloured precipitate where DNA fragmentation has occurred. DAB-stained tissues sections were examined using a Nikon microscope image analyser and photographed. ISEL positive cells were counted out of every 100 cells examined serially in each section.

Quantitation of spermatogenesis

Quantitation of the spermatogenesis in the testes of control and treated rats ($n = 6$) was carried out as described (Russell & Clermont, 1977). Briefly, 20 tubules in each testicular section (one section each from five different regions of testis) were randomly picked up and included in the analysis. Numbers of different types of germ cells present in a tubule were recorded through microscopic examination. An ocular calibrated micrometre fitted to the eyepiece was used to measure diameters of seminiferous tubules and germ cell nuclei. All the nuclear count (crude counts) of the germ cells were corrected for differences in nuclear diameter by the formula of Abercrombie (1946), i.e. true count = (crude count × section thickness)/(section thickness + nuclear diameter of germ cells) and tubular shrinkage by the Sertoli cell correction factor (Clermont & Morgantaler, 1955).

Radio immunoassay of testosterone

Radio immunoassay of testosterone was performed following methods of Abraham (1974) with modifications. The antibodies for the purpose were raised in the laboratory. Aether extraction of serum samples was carried out prior to setting up the radioimmunoassay. The inter- and intra-assay variations were 4.3% and 2.3% respectively.

Statistical analysis

Quantitative data are expressed as mean ± SD. Statistically significant differences between groups were determined by student's t-test. P-value <0.05 was considered statistically significant.

Results

The AR protein expression found using immunostaining, Western blotting and the semiquantitative RT-PCR in

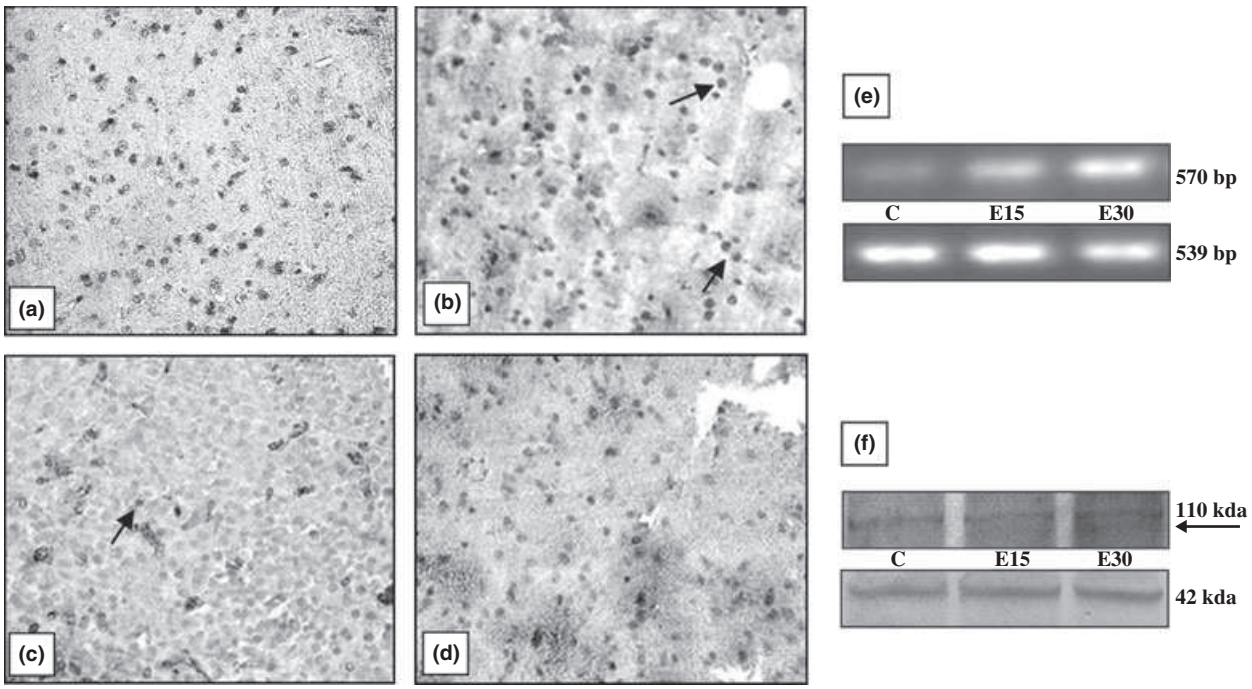


Fig. 5 Androgen receptor (AR) immunolocalisation in representative pituitary sections of rat; (a) negative control, (b) age matched vehicle treated control showing positive staining of the nuclei (➡) compared with decrease in staining intensity after (c) 15 days and (d) 30 days of chronic EB treatment, (e) semiquantitative RT-PCR showing AR mRNA expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: internal control, β-actin), (f) Western blot showing AR protein expression in control versus 15 and 30 days of EB exposure (upper lane: AR, lower lane: β-actin). Note the degradation of the AR protein (⟶); 400× (the figure is representative of three identical experiments).

different organs is shown in Figs 1–5. All the organs (testis, epididymis, seminal vesicle, prostate and pituitary), showed a marked decline in the intensity of immunostaining at the end of 30 days of EB treatment compared with vehicle treated controls. In testis, conspicuous staining was noted in the cells close to the basement membrane which included Sertoli cells, gonial cells and spermatocytes (Fig. 1b–d). Positive staining was also observed in peritubular myoid cells and Leydig cells but was absent in sections incubated with nonimmune serum (Fig. 1a). AR immunostaining in the epididymis was intensely observed both in epithelial as well in stromal cells. A pronounced reduction in the intensity of expression was visible after 30 days compared with 15 days of EB treatment (Fig. 2b–d). Immunoexpression in the vehicle treated adult prostate was characterised by positive staining in all the luminal epithelial, stromal and periacinar smooth muscle cells which gradually declined with increasing duration of oestrogen treatment (Fig. 3b–d). The trend was similar in the seminal vesicle with intense AR staining in epithelial and stromal cells of control rats, which faded gradually after oestrogenisation (Fig. 4b–d). In the pituitary, it was difficult to differentiate the acidophils and basophils on the basis of staining. However, the intensity of immunostaining followed a similar trend, gradually decreasing with the increase in the duration of EB treatment (Fig. 5b–d). No immunostaining was seen in tissue sections (negative control) incubated with nonimmune serum (Figs 2–5a).

Identical to immunostaining, immunoblots of all the reproductive organs (testis, epididymis, seminal vesicle and prostate) demonstrated a marked attenuation in AR protein expression following oestrogen treatment. However, AR protein in these organs failed to be probed in the immunoblots at the end of 30 days of EB treatment (Figs 1–4f). Only the pituitary presented a different picture in which AR protein was immuno-precipitated into two different specific entities or bands (Fig. 5f). By contrast, all the five organs demonstrated a consecutive rise in AR mRNA levels with respect to the increase in the duration of oestrogen treatment (Figs 1–5e). This was clearly resolved from the data obtained from the densitometric analysis of band intensities of AR transcripts in between groups and in different organs (Table 1). A summary observation of the rise and fall of AR expression following oestrogen treatment in different organs is presented in Table 2.

The spermatogenesis was severely affected both quantitatively and qualitatively. There was a significant decrease in seminiferous tubule diameter and the number of

Table 1 Densitometry showing band intensities of AR and β-actin in different organs

Organs	Control	15 days	30 days
Testis	1.035 ± 0.127	1.302 ± 0.159*	1.377 ± 0.180*
Epididymis	0.906 ± 0.052	1.006 ± 0.068	1.063 ± 0.092*
Prostate	0.853 ± 0.066	0.908 ± 0.080*	1.225 ± 0.100**
Seminal vesicle	0.721 ± 0.040	0.737 ± 0.040	0.918 ± 0.106*
Pituitary	0.633 ± 0.026	0.870 ± 0.011***	0.982 ± 0.065**

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control.

Table 2 Summary of AR expression in different organs after EB treatment

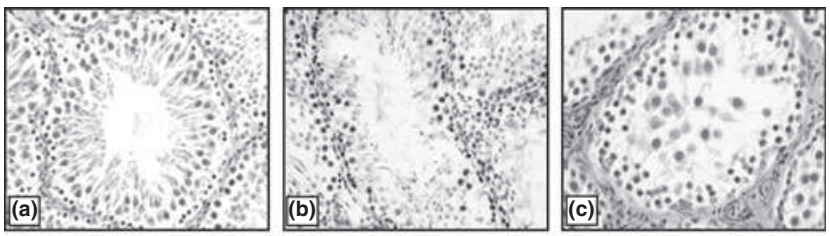
Organs	Immunostaining	Western blotting	RT-PCR
Testis	↓	↓	↑
Epididymis	↓	↓	↑
Prostate gland	↓	↓	↑
Seminal vesicles	↓	↓	↑
Pituitary gland	↓	↓	↑

prevailing germ cells per tubule (Fig. 6). While the number of elongated spermatids declined drastically by 15 days, most of the tubules were devoid of elongated spermatids and spermatozoa after 30 days of EB treatment (Fig. 6). Loss of germ cells in the seminiferous tubules was consistent with the rise in number of apoptotic germ cells per tubule (Fig. 7). Germ cell apoptosis was not found specific to any particular germ cell type. There was decrease in weights of testis and epididymis with increasing duration of oestrogen treatment (Table 3). The reduction in the weight of accessory reproductive organs like prostate and seminal vesicle was significant ($P < 0.001$) after 15 days of EB treatment. However, no substantial decline was observed subsequently after 30 days treatment (Table 3). Cell apoptosis was also not found prevalent (data not shown) in all these organs. On the other hand, a significant rise in the number of apoptotic cells in the pituitary was observed at the end of 30 days of EB treatment (Fig. 8). Intra-testicular and serum testosterone levels declined significantly as a result of oestrogen treatment (Fig. 9).

Discussion

The present findings demonstrate that modulation of AR protein expression by oestrogen in all the five organs of adult rats (testis, epididymis, seminal vesicle, prostate and pituitary) was carried out through either translational or post-translational pathways as AR transcriptional activity was found up-regulated. Decrease in the weight of androgen-dependent organs (epididymis, seminal vesicle and prostate) was consistent with the decrease in serum levels of testosterone but independent of cell apoptosis found prevalent only in the testis and pituitary.

AR is a member of the steroid receptor super family with an apparent molecular mass of 110 kDa with 918 amino acids (Chang *et al.*, 1988; Lubahn *et al.*, 1988). Abnormalities in the AR signalling pathway have been linked to male infertility, Kennedy's disease and prostate cancer. Regulation of the AR activity can be achieved by several different ways: modulation of AR gene expression,



Seminiferous tubule diameter and maturing germ cells/tubule in control versus EB treatment rats.

Treatment	Seminiferous tubule diameter (μm)	Prelepto-/leptotene spermatocytes	Pachytene spermatocytes	Round spermatids
Vehicle control	288 ± 12	37 ± 2.9	35 ± 11	130 ± 30
EB 15 days	255 ± 10	37 ± 3.2	29 ± 10	*35 ± 6.7
EB 30 days	*168 ± 15	*29 ± 5	*19 ± 3	*15 ± 5

Fig. 6 Histological sections of rat testes stained with haematoxylin and eosin. Representative section from (a) control testis show normal spermatogenesis, reduction in germ cell number per tubule is evident both in (b) 15 days and (c) 30 days of EB treatment; 400×.

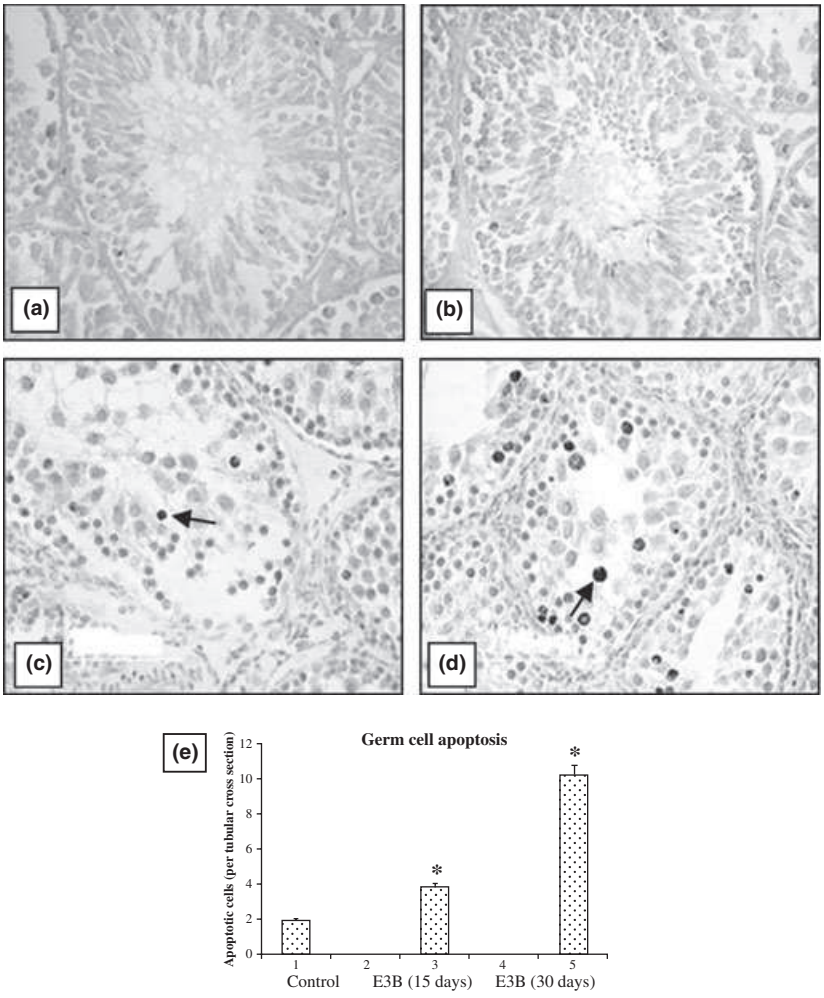


Fig. 7 *In situ* end labelling of DNA in testis sections. Representative sections from (a) negative control, (b) vehicle treated control, (c) 15 days and (d) 30 days of EB treatment. Note the rise in ISEL positive cells (➡) in testis sections from treated rats, also shown in (e) bar chart. *$P < 0.01$, **$P < 0.001$, 400×.

Table 3 Weight of reproductive organs from control and EB treated rats

Treatment	Testis (g)	Epididymis (g)	Prostate gland (g)	Seminal vesicle (g)
Vehicle control	1.4 ± 0.17	0.7 ± 0.11	0.416 ± 0.07	1.03 ± 0.235
EB (15 days)	0.85 ± 0.3*	0.339 ± 0.06*	0.141 ± 0.016*	0.3 ± 0.069*
EB (30 days)	0.24 ± 0.03*	0.232 ± 0.04*	0.139 ± 0.093*	0.306 ± 0.064*

*$P < 0.001$ compared with controls

androgen binding to AR, AR protein stability, AR nuclear translocation and AR transactivation (Lee & Chang, 2003). Both androgen and oestrogen regulate AR at the level of mRNA and protein. Neonatal exposure to oestrogens resulted in an immediate and sustained decrease in AR protein levels in the developing and adult rat ventral prostate that in turn led to abnormal growth, decreased secretary capacity and a dampened activational response to exogenous androgen replacement (Prins, 1997).

It was also explained that this AR down-regulation in the rat prostate as result of neonatal oestrogen exposure is not mediated at the transcriptional or translational level but rather through a post-translational mechanism at the level of AR protein degradation through ubiquitin-proteosome pathway (Woodham *et al.*, 2003). In the adult model as followed in this study, the AR protein levels continued to decline from the control levels to post-15 days of EB treatment, and by 30 days there was hardly any protein resolved in the immunoblots of different organs except the pituitary (Figs 1–5f). AS decline in AR protein levels was observed by post-15 days of EB treatment in all the organs, the involvement of transla-

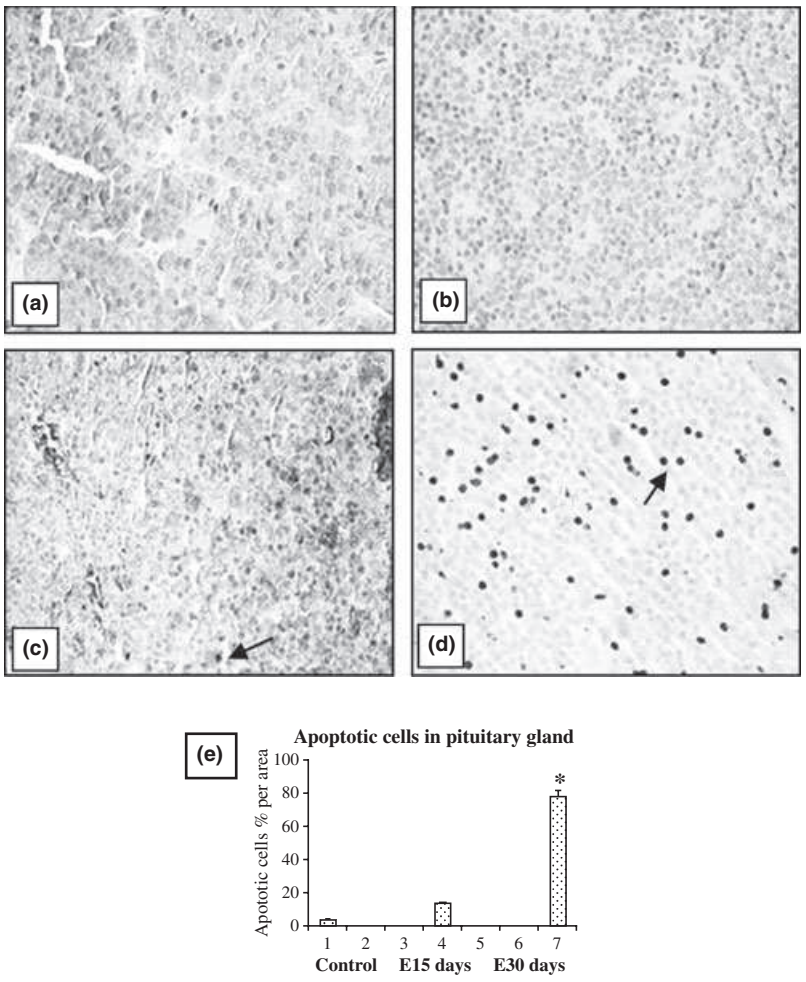


Fig. 8 *In situ* end labelling (ISEL) of DNA in pituitary sections. Representative sections from (a) negative control, (b) vehicle treated control, (c) 15 days and (d) 30 days of EB treatment. Note the rise in ISEL positive cells (➡) in pituitary sections from treated rats, also shown in (e) bar chart. $*P < 0.01$, $**P < 0.001$, 400×.

tional pathway in AR protein down-regulation cannot be ruled out. The complete disappearance of the AR protein probably supports the fact that it has undergone degradation following long-term EB (30 days) exposure, by ubiquitin-proteosome or some other pathway in adult rats needs further investigation. However, the process of AR degradation probably provides sufficient input for additional AR transcriptional activity and quantitatively production of more AR mRNAs as presently observed (Figs 1–5e).

That the AR protein is degraded at the post-translational level is further supported by the present findings in the pituitary gland. AR protein blot for the pituitary shows two specific bands instead of one, found closely allied to each other, possibly as a product of degradation (Fig. 5f). It is not clear at this stage whether AR protein degradation follows certain specific steps as seen in case of the pituitary or degrades without living any trace as shown in the immunoblots for reproductive organs. As the AR antibody recognises the two different specific epitopes, it is quite possible that these bands represent the initial stages of degradation of AR protein in the pituitary following both 15 and 30 days of EB treatment. Further investigation of AR protein levels in the adult pituitary following extended oestrogen exposure than the present one is needed to confirm the sequences of events leading to total AR degradation.

Complete androgen withdrawal that elevated mRNA levels was also associated with a decrease in AR protein levels in the adult prostate. This autologous nature of regulation of AR expression was reported earlier in rats utilising a castrate model (Prins & Woodham, 1995; Pelletier, 2002). In the other androgen insufficiency model induced by ethane dimethane sulphonate treatment, AR protein expression in testis and prostate was suppressed independently of the up-regulation in mRNA levels (Turner *et al.*, 2001; Tan *et al.*, 2005). The possible explanation for such a discordant trend in AR protein and mRNA in the same organ was described as the inability or lack of availability of AR messages to be translated

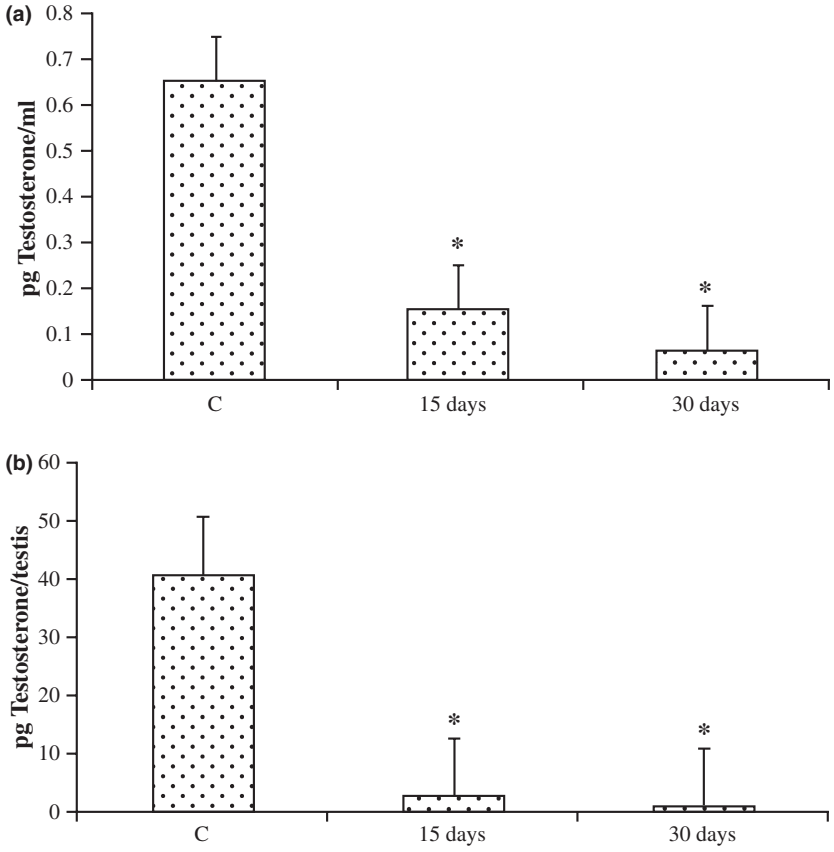


Fig. 9 (a) Serum and (b) intra-testicular testosterone levels show a gradual decline with the increase in the duration of oestradiol treatment (n = 6/group). *P < 0.001.

despite their cellular presence (Prins & Woodham, 1995). The other possibility is that under limited androgen availability as observed following chronic oestrogen treatment, androgens can regulate their own receptors both at transcription and translation levels completely independently of each other.

Some divergent trends on the effects of oestrogen on AR gene transcription rate and mRNA quantity in the same organ or different parts of the same organ are reported. In the rat ventral prostate, nuclear run-on-assays showed no alteration in AR gene transcription following neonatal exposure of EB on days 1–5 compared with the controls. *In situ* hybridisation and quantitative RT-PCR revealed no difference in mRNA levels in the stromal or epithelial cells of the prostate in response to oestrogen exposure, which supports the contention that oestrogen-induced down-regulation of AR is mediated at the post-transcriptional level (Woodham *et al.*, 2003). In male rats fed with dietary phytooestrogens for 3 days, quantitative RT-PCR showed AR expression increasing in the initial but decreasing in the distal region of cauda epididymidis (Glover & Assinder, 2006). This is probably indicative of the fact that differential regulation of the steroid receptor (AR) does occur even in the same organ which could be associated with the specific differences in physiological function in between the regions. Compared with the controls, up-regulation of AR transcripts was consistently observed following EB treatment in all the organs in this study and the increase was proportional to the duration of treatment; more in 30 days than 15 days of treatment (Figs 1–5, Table 1).

Animals, in this study were exposed to high pharmacologic dose of oestrogen (75 μg day^{-1} rat^{-1}) with chronic interventions for longer periods (15 days and 30 days) and instead of a single reproductive organ, all the reproductive organs and the pituitary were simultaneously investigated for AR expression through immunostaining as a tool. In earlier studies, in rats treated with DES, AR immno-expression was virtually absent from the affected tissues like testis, epididymis and vas deferens (McKinnell *et al.*, 2001). Rat litters given EB on days 1, 3 and 5 and killed on day 90 demonstrated a significant reduction in AR immunoexpression in ventral and dorsal prostate. A near absence of prostate epithelial AR expression was reported due to impaired differentiation in these cells as a result of neonatal EB exposure (Prins, 1992). In our studies, not a single organ demonstrated a complete absence of AR expression on immunochemistry although a significant decline in the intensity of staining was resolved in all the organs from

treated rats compared with those of the controls. The suppression of immunoexpression of AR in the adult reproductive organs is similar to the effects seen in the reproductive organs (testis, epididymis and seminal vesicle) of pubertal rats (Williams *et al.*, 2001) and also in organs (vas deferens, prostate and seminal vesicle) of adult animals exposed to oestrogen (Bianco *et al.*, 2002) during the neonatal period (Atanassova *et al.*, 2005; Prins *et al.*, 2001). Androgen and oestrogen balance is believed to be critical for normal spermatogenesis to proceed and any impairment is always associated with sub-optimal androgen production and availability, which subsequently affects other reproductive tissues. Thus, the attenuation of AR immunoexpression in these tissues may be attributed partly to androgen insufficiency and partly to an anti-androgenic effect exerted by oestrogens.

Oestrogen administration to adult rats reportedly reduced reproductive organ weights, epididymal sperm numbers and fertility (Goyal *et al.*, 2001; Chaki *et al.*, 2006). Present findings are very much identical as chronic EB treatment for 30 days down-regulated serum and intra-testicular testosterone, reduced testis and accessory sex organ weights and adversely affected spermatogenesis (Fig. 6, Table 3). Spermatogenesis is a hormonally regulated process and testosterone availability is essential for the survival and maintenance of germ cells (Sinha-Hikim & Swerdloff, 1999; Woolveridge *et al.*, 1999). Although oestrogen is a survival factor (Pentikainen *et al.*, 2000), a higher level of the hormone is apoptogenic for germ cells *in vivo*. Neonatal oestrogen administration was associated with germ cell apoptosis in the testis as a result of suppression of serum testosterone levels (Atanassova *et al.*, 1999). In adult rats receiving oestrogen treatment, serum and intratesticular testosterone levels were severely depleted, leading to oxidative stress and germ cell apoptosis in the testis (Chaki *et al.*, 2006). Testosterone withdrawal was reportedly associated with the induction of apoptosis in the epididymis during the initial 5 days post-orchidectomy (Fan & Robaire, 1998). Similar findings on the induction of apoptosis in the prostate were described in organ cultures in Estrogen Receptor Knock Out (ERKO) mouse following high doses of oestradiol exposure which down-regulated prostate-specific AR protein expression (Taylor *et al.*, 2006). However, it is interesting to note that the decrease in the weight of reproductive organs in this study is associated with an increase in germ cell apoptosis only in the testis but not in the tissues that are entirely androgen-dependent (*viz.* epididymis, seminal vesicle or prostate). Unlike orchidectomy which drastically cuts down the source of androgen supply, oestrogen treatment in adults only reduces the same to sub-optimal levels (Fig. 7). In the present study, because of such sub-optimal but not complete withdrawal of androgen availability, cell apoptosis was not observed in accessory reproductive organs although there was down-regulation of AR protein expression. Like testis, pituitary on the other hand, demonstrated a significant increase in cell apoptosis following 30 days of EB treatment (Fig. 8).

Apoptosis in pituitary examined through light and electron microscopy has been reported in male rats in which hyperplasia of prolactin-secreting cells were induced by oestrogen implanted subcutaneously for 6 weeks (Drewett *et al.*, 1993). More recently in female rats, utilising TUNEL, it was confirmed that chronic oestrogenisation induces apoptosis in the anterior pituitary gland and 75% of these apoptotic cells were identified as lactotropes using immunofluorescence (Pisera *et al.*, 2004). The present findings in oestrogenised male rats confirmed the incidence of increased cell apoptosis in the pituitary and it is possible that the same cell types, lactotropes, are involved. However, its association with up-regulation of AR mRNA expression and protein degradation needs further investigation.

In conclusion, the above findings establish the fact that chronic oestrogen exposure to adult rats decreases AR immunolocalisation in testis, epididymis, seminal vesicle, prostate and pituitary which is associated with an increase in AR mRNA expression in these organs, probably initiating AR protein degradation at translational/post-translational level. It also induces cell apoptosis in the testis and pituitary but not in the accessory sex organs which needs to be investigated further in future studies. The mechanism of oestrogen action involves the suppression of androgen production and AR protein expression which may be common in the entire adult male reproductive tract.

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