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Loss and recovery of androgen receptor protein expression in the adult rat testis following androgen withdrawal by ethane dimethanesulfonate

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Abstract: Androgens are especially important for the maintenance of spermatogenesis in adulthood and the experimental withdrawal of testosterone (T) production by ethane dimenthanesulfonate (EDS) is a valuable tool for studying androgen-dependent events of spermatogenesis. The aim of the present study was to investigate the specific changes in immunoexpression of androgen receptor (AR) in the testis in relation to degeneration and regeneration of Leydig cell (LC) population and seminiferous epithelium. Immunohistochemistry for AR and 3β-hydroxysteroid dehydrogenase (3β-HSD) as well as TUNEL assay for apoptosis were performed on testicular sections of control and EDS-treated rats. Serum LH and T levels were measured by RIA. Our results revealed a total loss of AR immunoexpression from the nuclei of Sertoli (SCs), LCs and peritubular cells during the first week after EDS administration and that coincided with severe drop in T levels. Two weeks after EDS administration, the AR expression was recovered in these cells but normal stage-specificity in SCs was replaced by uniform intensity of AR immunostaining at all the stages of the spermatogenic cycle. The stage-specific pattern of androgen expression in SCs with a maximum at stages VII-VIII appeared 5 weeks after treatment. LC immunoreactivity for 3β-HSD at different time points after EDS administration correlated with values of T concentration. The maximal germ cell apoptosis on day 7 was followed by total loss of elongated spermatids 2 weeks after EDS treatment. Regeneration of seminiferous epithelium 3 weeks after EDS administration and onwards occurred in tandem with the development of new LC population indicated by the appearance of 3β-HSD-positive cells and gradual increase in T production. The specific changes in AR after EDS including their loss and recovery in Sertoli cells paralleled with degenerative and regenerative events in Leydig and germ cell populations, confirming close functional relationship between Sertoli, Leydig and germ cells. (www.cm-uj.krakow.pl/FHC)

Key words: Androgen receptor - Sertoli cells - Leydig cells - Spermatogenesis - Testis - EDS

Introduction

Androgens are especially important in male sexual differentiation in fetal life, pubertal sexual maturation and the maintenance of spermatogenesis in adulthood. The effects of androgens are mediated through the androgen receptor (AR), a 110 kDa nuclear receptor that binds testosterone (T) with high affinity and regulates gene expression in the target tissues [12] . Several pieces of evidence indicated the preferential action of androgens at stages VII-VIII of the spermatogenic cycle of the rat [15, 22] and this coincided with the maximal expression of AR protein in Sertoli cells (SCs) [8]. As immunoexprssion of AR in peritubular (PTCs) and Leydig cells (LCs) does not vary according to the stages of the cycle, it is presumed that androgen support for spermatogenesis is primarily mediated through the Sertoli cells [23]. This conclusion is supported by recent demonstration that Sertoli cell selective ablation of AR (SCARKO mice) results in arrest of spermatogenesis during meiosis [10].

Experimental manipulation of T levels during development [17] and adulthood [8, 25, 27] results in a loss of AR immunoexpression in the Sertoli cells, suggesting the specific role of T-ligand for the proper functioning of its own receptor. Treatment of adult rats with ethane dimethanesulfonate (EDS) selectively and temporarily eliminates LCs in the testis resulting in rapid loss of T

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production and hence is a valuable tool for investigating androgen-dependent events of spermatogenesis [25]. AR protein expression was investigated within the first 6 days after EDS treatment, when dramatic loss of AR expression in SCs was found, whereas germ cell compartment remained still quantitatively normal. Recovery of the normal stage-specific pattern of AR expression in the SCs was achieved by immediate therapy with high dose T within the first 3 days post EDS [25].

AR protein expression has not been examined in a long-term period after EDS administration when new population of LCs develops resulting in gradual restoration of their steroidogenic function. In this respect, the aim of the present study was to investigate the specific changes in immunoexpression of AR in the testis in relation to degeneration and regeneration of LC population and spermatogenesis and to extend our understanding of the mechanisms via which androgens act in the testis.

Materials and methods

Animals and treatments. Adult Wistar rats bred in our animal house were maintained under standard conditions. Food and water were provided *ad libitum*. Testosterone withdrawal in adult rats was induced by single intraperitoneal injection of ethanedimethane sulphonate (EDS) at a dose of 75 mg/kg body weight dissolved in dimethylsulfoxide and water (1:3, v/v). EDS is not commercially available and was synthesized in our laboratory from ethylene glycol and methane sulfonylchloride as described by Jackson and Jackson [13]. Control rats were treated with the vehicle alone. Animals were sacrificed at 1, 3, 7 ,14, 21 and 35 days after EDS. Plasma samples were stored at -20˚C until used for hormonal analysis of testosterone and LH by RIA. Testes were fixed in Bouin's solution, dehydrated and embedded in paraffin.

Imminohistochemistry. Immunohistochemistry was performed on dewaxed sections (5 μm) with antigen retrieval (5 min in 0.01 M citrate buffer, pH 6.0) for visualization of AR or without antigen retrieval for 3β-HSD. This was followed by endogenous peroxidase blocking in 3% (v/v) H_2O_2 in methanol for 30 min at room temperature (RT). For all washes between antibody or reagent incubations, 0.05 M TBS, pH 7.4, 2×5 min at RT was used. Tissue sections were blocked in TBS containing normal swine serum (NSS; 1:4) and 5% BSA before an overnight incubation at 4˚C with primary antibodies diluted in NSS. For detection of AR and Leydig cells, rabbit polyclonal antibodies, clone N20, sc-816, Santa Cruz Biotechnology, diluted 1:200 and antibody against 3β-HSD, diluted 1:1000, kindly provided as a gift form Prof. I. Mason (Edinburgh University), were applied, respectively. A swine anti-rabbit biotinylated secondary antibody (E0353, DAKO), diluted 1:500 in NSS was applied for 30 min at RT. Bound antibodies were visualized by incubating the sections with ABC complex/HRP reagent (K0355, DAKO) for 30 min followed by color development with 3,3'-diaminobenzidine tetrahydrochloride chromogene substrate (K3468, Liquid DAB+ kit, DAKO), monitored microscopically. Sections were counterstained with hematoxylin, dehydrated and coverslipped using Pertex mounting medium (CellPath, Hemel Hempstead, UK)

The specificity of immunostaining was checked for each antibody using previously established procedures. For AR, this involved preabsorption of the primary antibody with peptide immunogen (Santa Cruz N-20 peptide sc-816P) and for 3β-HSD it involved substitution of the primary antibody by normal swine (blocking) serum. To allow comparative evaluation of the immunostaining, tissue sections from control and treated animals were processed in parallel on at least three occasions to ensure reproducibility of results; on each occasion tissue sections from four to six animals in each experimental group were immunostained and the intensity of immunostaining was scored using an arbitrary scale ranging from negative (-) through weakly positive (+) to intensely positive (+++).The scores were used to compile table of results.

***In situ* assay of apoptosis and quantification of apoptotic cells.** Paraffin cross sections (5 μm) of seminiferous tubules were mounted on slides coated with 2% 3-aminopropyltriethoxysaline (Sigma), deparaffinized and rehydrated. Apoptotic cells were detected *in situ* by using terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-11-dUTP nick end labelling (TUNEL) method that resulted in a high degree of specificity and low background staining [23]. For assessment of apoptosis, the percentage of seminiferous tubules with apoptotic germ cells was determined by scoring 100 randomly selected tubules per section on four sections from different animals at each time point after EDS administration [29]. Tubules with even a single apoptotic cell were classified as containing apoptotic cells. The number of apoptotic cells per tubule was assessed on four sections. The apoptotic index was calculated by multiplying the percentage of tubules containing apoptotic germ cells by the number of apoptotic germ cells per tubule at each time point after EDS. The data obtained were statistically analyzed by Student's t-test.

Hormone measurements. Serum levels of T were measured using an enzyme-linked immunosorbent assay adapted from an earlier RIA method of Sharpe and Bartlett [21] as described previously [1]. Serum T levels were measured after hexane-ether extraction of the samples, using [^{3}H] testosterone (Amersham International, UK) as tracer. The concentrations of LH were determined by a RIA method as described by Fraser and Sandow [11] using kits supplied by the NIDDK (Bethesda, MD).

Results

As previously shown, EDS induced apoptosis of the adult LC population in the testis resulting in total loss of LCs within the first 24 hours [2]. The absence and regeneration of LCs and their steroidogenic activity after EDS administration was proved by immunostaining for 3β-HSD which is the key enzyme in testicular steroidogenesis and a specific marker for visualization and quantification of LCs [1]. In controls, intense immunostaining was observed in LC cytoplasm (Fig. 1A). A total loss of LCs on day 3 and 7 after EDS was confirmed by lack of 3β-HSD-immunopositive cells in the testis (Fig.1B). First 3β-HSD-positive cells could be seen in the testicular interstitium two weeks after EDS administration and the intensity of 3β-HSD immunostaining was comparable to the control (Fig. 1C, Table 1). After day 21, the number of 3β-HSD-immunoreactive cells markedly increased (see Fig. 1. in Davidoff *et al.* [9]). LC immunoreactivity for 3β-HSD correlated with values of serum T levels measured by RIA. A severe drop in T concentration below 0.1 ng/ml on day 7 after EDS treatment was followed by gradual increase in T levels after two and three weeks (Fig. 2). LH remained elevated until the 3rd week after EDS administration.

In control rats, AR was expressed in the nuclei of LCs, PTCs and SCs but not in germ cells. Stage-specific immunoexpression of AR was evident in SC nuclei with

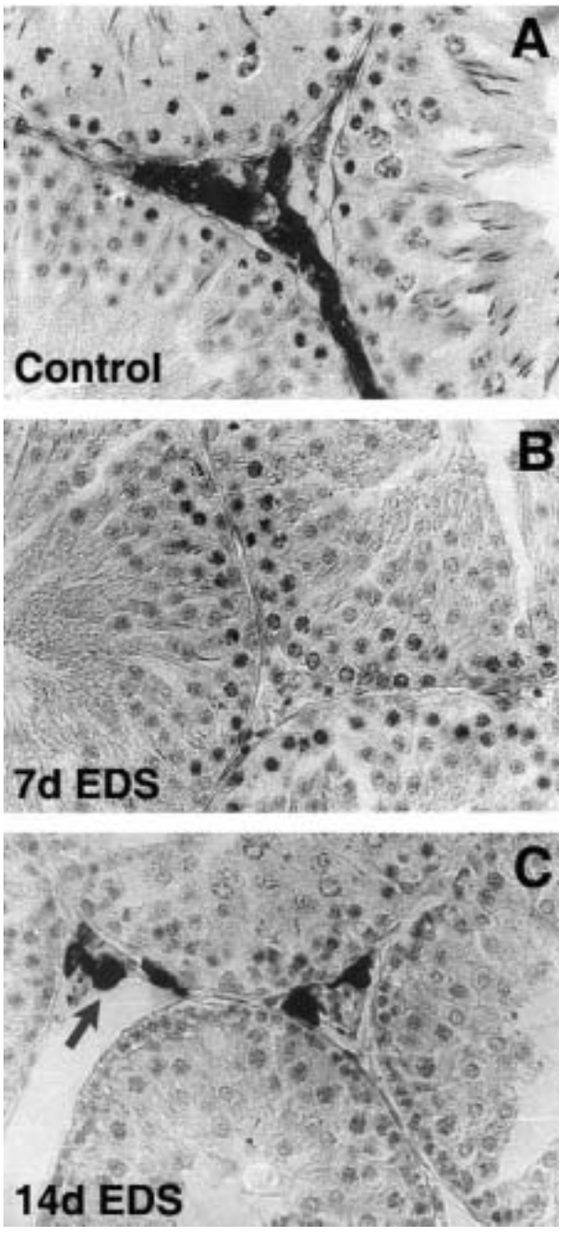

Fig. 1. Immunoexpression of 3β-HSD in LCs of rat testis after EDS treatment. × 400. **A.** Control testis; **B.** Testis from rat 7 days after EDS administration. Note the absence of 3β-HSD immunostaining in the interstitium; **C.** Testis from rat 14 days after EDS. Note the appearance of new LCs (arrow) revealed by 3β-HSD immunoreactivity with intensity comparable to the controls.

the most intense expression at stages VI-VIII of the spermatogenic cycle and the lowest expression at stages IX-XIV (Fig. 3A, Table 1), as reported previously [8, 25, 27]. Seven days after EDS administration there was a total loss of AR immunoexpression from SCs, LCs and PTCs, as indicated by lack of AR immunoreactivity in the testis (Fig. 3B). Two weeks after treatment, AR immunoexpression was recovered in these cell types but

Table 1. Semiquantitative evaluation of the effect of EDS treatment on immunoexpression of 3β-HSD in LC cytoplasm and AR in SC nuclei

Experimental groups	AR immunoexpression in SCs			3β-HSD immuno-expression in LCs
	Stages I-VI	Stages VII-VIII	Stages IX-XIV	
Control	+	+++	+	+++
EDS 6d	-	-/+*	-	-
EDS + testosterone 6d	+	+++	+	-
EDS 7d	-	-	-	-
EDS 14d	+++	+++	+++	+++
EDS 21d	+++	+++	+++	+++
EDS 35d	+	+++	+	+++

*-/+ indicates that a few cells are immunopositive, whereas most are negative

there was no clear evidence for stage-specific expression of AR in SCs (Table 1). Instead, uniform intensity of Sertoli cell AR immunostaining in the tubules from all the stages was observed (Fig. 3C). Three weeks after EDS treatment, the uniform pattern of AR immunoexpression in SCs was still present and strong immunostaining was even found in SCs from Sertoli cell only (SCO) tubules (Fig 3D). The normal stage-specific pattern of AR immunoexpression was recovered 5 weeks after EDS administration.

As recently reported [25], administration of 25 mg testosterone ester at the time of EDS injection and 3 days later prevented the loss of AR from the testis and maintained the normal stage-specific pattern in SC nuclei on day 6 after EDS (Table 1).

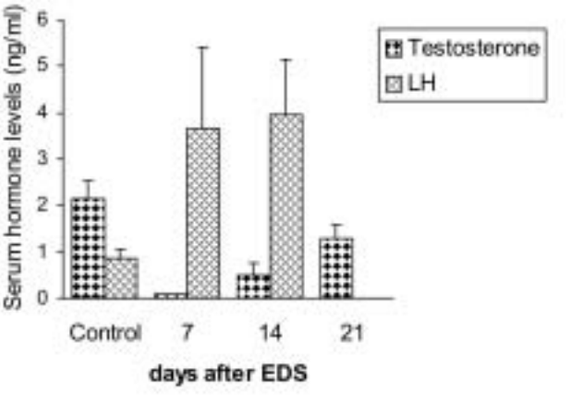

Fig. 2. Levels of testosterone and LH at different time points after EDS treatment. Data represent means±SD (n=5 for each experimental group). All the differences in values between treated and control animals were significant ($p<0.01$).

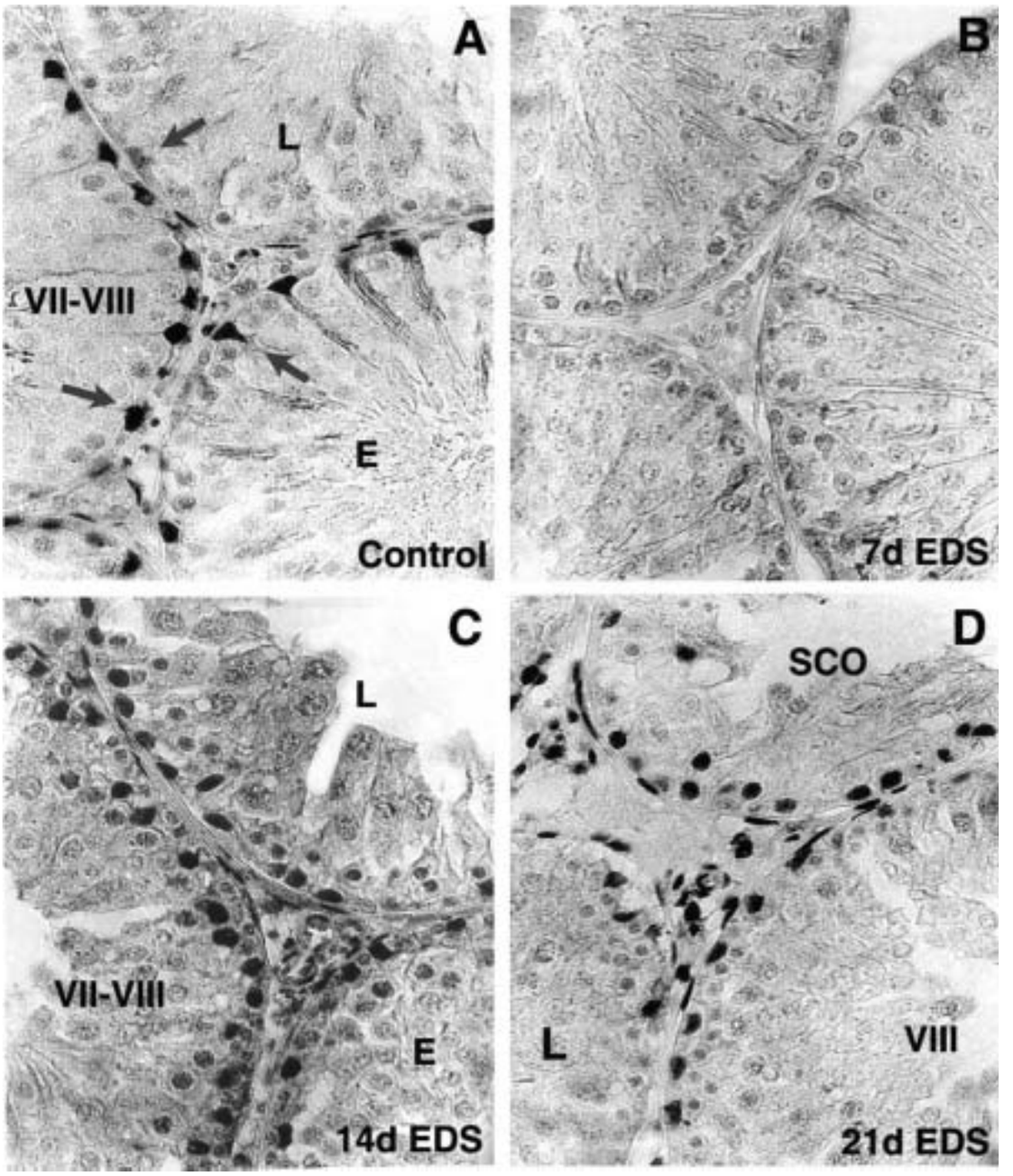

Fig. 3. Immunoexpression of AR in the rat testis after EDS treatment. × 400. **A.** Control testis. Note stage-specific staining in SC nuclei (arrows) indicated by strong intensity in early I-VI (E) and VII-VIII stages and weak immunostaining in late IX -XIV (L) stages; **B.** Testis from rat 7 days after EDS. Note the absence of AR immunoreactivity in the testis; **C.** Testis from rat 14 days after EDS. Note uniform intensity of AR immunostaining in SC nuclei in tubules from early (E), VII-VIII and late (L) stages; **D.** Testis form rat 21 days after EDS. Strong AR immunoexpression in SCs was found in Sertoli cell only (SCO) tubules.

First signs of seminiferous epithelium regression were manifested by a marked increase in germ cell apoptotic index on day 7 (144.14±21.40) in comparison with control rat testis (11.00±3.57). Afterwards the parameter decreased but remained still significantly higher, compared to control, on days 14 and 21 after EDS treatment (29.27±4.90 and 29.20±13.32, respectively). Germ cell apoptosis was followed by total loss of elongated spermatids from all the stages of the cycle by the 2nd week after EDS (see Fig. 3C). First signs of spermatogenesis recovery were manifested by the appearance of elongated spermatids at late stages 3 weeks after EDS.

Discussion

The biological action of androgens in the testis is mediated through the AR that functions as a transcriptional factor to regulate initiation and maintenance of spermatogenesis [12]. The stage-specific pattern of AR immunoexpression in the Sertoli cells reported by us and other authors [8, 25, 27] indicates that T exerts its supportive effect on spermatogenesis acting specifically at stages VII-VIII and that coincides with the maximal levels of overall protein secretion by seminiferous tubules [22].

In the rat, T withdrawal is known to disrupt spermatogenesis [5, 15] and we therefore tested the effect of T removal, using EDS, on the pattern of AR immunoexpression in the adult rat testis. The present study provides new data about recovery of AR immunoexpression in the testis in a long period after EDS treatment when new LC population is developed in tandem with degeneration and restoration of spermatogenesis. The lack of AR immunostaining in all testicular cell types, found during the first week, coincides with total loss of LCs proved by lack of 3β-HSD-immunopositive cells and the lowest plasma T concentrations. Comparative studies employing Western blotting and ligand-binding assay of testicular AR revealed very low level of immunodetectable AR protein on day 5 after EDS administration in contrast to normal binding ability of the receptor that may point to a structural modification of the AR [7]. It is possible that prolonged absence of androgens results in an unstable conformation of the AR which is more easily degraded within the cell [14, 27, 30].

The restoration of AR immunostaining in the testis found 2 weeks after EDS treatment occurred in tandem with the appearance of new generation of LCs as proved by the presence of 3β-HSD immunoreactivity observed in the present study and by detectable enzyme activity [4]. As 3β-HSD is a marker for LC differentiation in adult testis after EDS administration [26], the appearance of 3β-HSD-positive cells occurred at the time when progenitor LCs in the testis began to transform into immature LCs [3] and that in turn led to gradual restoration of T production. Immunoexpression of AR in SCs was restored 2 weeks after EDS but instead of stage-specific pattern, we observed uniform staining present at all the stages with the intensity identical to the maximal staining at stage VII-VIII in the controls. This uniformity of AR immunoexpression was maintained for a long period of 3 weeks and normal, stage-specific AR immunostaining appeared 5 weeks after EDS treatment when T levels returned to the normal range [5]. The lack of stage specificity of AR expression in SCs coincided with the lack of an appropriate level of T support, indicating the role of T-ligand in the proper functioning (immunodetectability) of its own receptor [8]. Therefore, maintenance of maximal intensity of AR imunoexpression at all the stages in EDS-treated rats could be interpreted as a possible compensatory mechanism of androgen signalling, needed for the regeneration of germ cell population occurring in the same period after EDS treatment. The great importance of adequate androgen signalling via AR in the SCs for spermatogenesis was recently demonstrated in transgenic mice with selective knockout of AR in the SCs [10]. Germ cells in turn can control production of several proteins by the SCs [6, 16]. Total loss of elongated spermatids [3] coincides with the time of absence of AR immunoexpression stage specificity in SCs. However, the studies involving methoxyacetic acid (MAA)-induced germ cell depletion do not indicate any changes in stage-dependent staining of AR [8]. This, of course does not eliminate the possibility that germ cells are involved in the local control of AR expression, as different mechanisms underlie germ cell depletion induced by MAA (directly destroyed pachytene spermatocytes) and EDS (hormonally induced germ cell apoptosis).

In agreement with other studies, immunodetectability of AR appeared to be controlled by the binding of T, since AR immunostaining was markedly reduced in EDS-treated rats and replacement of T at the time of EDS administration or for 4 hours 6 days after EDS restored the normal stage-specific pattern of AR staining in SCs [8, 27]. In addition, loss of AR in the male reproductive tract of castrated rats was restored within 15 min of androgen administration [19]. These results demonstrated that T probably decreases the rate of AR degradation, controlling the synthesis of its own receptor, as previously demonstrated in prostate and other tissues [14, 18]. Studies on EDS-treated rats supplemented with synthetic metabolisable or nonmetabolisable androgens showed that stage-dependent pattern of AR immunoexpression in SCs is not due to androgen metabolism [28]. Although the pattern of AR staining was very similar in EDS+T and control rats, the quantitative levels of staining were somewhat lower in the former animals [8]. This could be due to suppression of FSH levels by T replacement [5, 23], if FSH is important in maintaining normal AR expression. Recent studies using EDS experimental model demonstrated a possible non-cell cycle role of cyclin D2 in regulating stage-dependent expression of the AR in SCs [25].

Comparative analysis of our data on the recovery of AR in SCs in a long period after EDS administration (5 weeks) and data from EDS plus T supplemented rats (1 week duration; [27]) suggest that acute administration of high amount of T is sufficient to restore quickly (within 3-6 days) the stage-specific expression of AR in SCs, whereas gradual increase in T production during long-term recovery after EDS lasted 3 weeks. Different manner of the AR immunoexpression recovery we found in the SCs and LCs probably reflects the different mechanisms via which both cell types maintain their own levels of AR [20] . The specific changes in AR after EDS treatment including loss and recovery of AR in Sertoli cells paralleled with degenerative and regenerative events in Leydig and germ cell populations, confirm

a close functional relationship between Sertoli, Leydig and germ cells.

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