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Original Articles

Differentiation of Adult Leydig Cells in Relation to Spermatogenesis in Experimental Conditions of Androgen Deprivation: Scientific Contribution by Prof. Michail Davidoff to Development of Our Knowledge on Adult Leydig Cell Population

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The present paper provides an overview of our long collaborative studies with Prof. Michael Davidoff on the EDS (Ethane Dimethanesulfonate) experimental model for androgen deprivation and to evaluate our contribution for new knowledge about protein expression of cellular markers for differentiation of adult Leydig cells in relation to spermatogenesis. Degenerative and regenerative events in spermatogenesis and time specific changes in androgen receptor expression in Sertoli cells occur in tandem with development of new adult Leydig cell population after EDS indicating close functional relationship between Sertoli cells, Leydig cells and germ cells. By comparison of long term EDS model with short term EDS plus testosterone model we provided new understanding about regulation of androgen receptor by its own ligand testosterone.

Key words: Leydig cells, Sertoli cells, spermatogenesis, androgen receptor, testis

Introduction

Our studies on the development of adult Leydig cell population in relation to spermatogenesis started in the late nineties of the past century under the supervision by Prof. Michail Davidoff. Together with studies on neuroendocrine nature of adult Leydig

cells carried out in the Institute of Experimental Morphology and Anthropology, Prof. Davidoff prompted our studies on consecutive stages of differentiation of adult Leydig cells. He started application of long term experimental model in rat for treatment with ethane dimethanesulfonate (EDS) which is a toxin that selectively and temporally eliminates adult Leydig cells within the first 48 hours [9]. This is followed by severe androgen deficiency due to drop in testosterone levels below 0.1 ng/ml. Thus, the EDS long term model is unique tool to study development of the regenerating adult Leydig cell population that occurred within 7 weeks. The EDS experimental model is a useful tool to investigate androgen dependent events in spermatogenesis, as well. We compare our findings with data generated by a short term EDS experimental model with testosterone administration provided by Prof. Richard Sharpe from the Centre for Reproductive Health, Edinburgh, UK [13].

The aim of the present paper was to provide an overview of our long collaborative studies with Prof. Davidoff on the EDS experimental model and to evaluate our contribution for new knowledge about cellular markers for stages of differentiation of regeneration adult Leydig cell population in relation to spermatogenesis. By comparison of long term EDS model with short term EDS plus testosterone we provided new knowledge about the regulation of androgen receptor (AR) by its own ligand testosterone.

Materials and Methods

Leydig cell ablation followed by testosterone withdrawal in adult rats was induced by single intraperitoneal injection of 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 [8]. The testes were removed at 7, 14, 21, 35 and 49 days after treatment followed by fixation in Bouin's fluid for 24 hours. Short term EDS treatment with testosterone administration was used to assess the effect of acute testosterone manipulation. Thus, 6 days after EDS-induced testosterone withdrawal a single subcutaneous injection of testosterone ester (25 mg) was applied and testicular samples were taken 4 h later [13]. ABC-HRP immunohistochemistry was performed on 5 μm thick paraffin cross sections for visualization of different testicular cellular markers. For Leydig cells they were 3β-hydroxysteroid dehydrogenase (HSD) [1, 4]; 11β-HSD [10]; Insulin-like 3 peptide (INSL3) and its receptor LGR8 [12], receptors for T3 thyroid hormone (c-erbAα and c-erbAβ [7]). For Sertoli cells – AR [1] and cyclin D2 [13]; for germ cells – testicular angiotensin converting enzyme (tACE) [2]. Apoptotic germ cells were visualized by TUNEL method [5]. Ultrastructural studies were performed by routine TEM [3]. Plasma samples were stored at -20°C until used for hormonal analysis of testosterone and LH by RIA [5]. Statistical analysis was performed by unpaired Student T-test.

Results and Discussion

Our studies started with development of regenerating new Leydig cell population after EDS treatment. Leydig cells were visualised by specific cytoplasm marker 3β-HSD that is a key enzyme in androgen biosynthesis indicative for acquirement of steroidogenic competence of Leydig cells (**Fig. 1A**). Complete loss of Leydig cells

was evident on day 7 after EDS injection (**Fig. 1B**). On the 14th day after EDS, first steroidogenic Leydig cells, that are newly-formed adult Leydig cells, appeared in testicular interstitium between seminiferous tubules. Later, on day 21st and day 35th numerous Leydig cells were seen (**Fig. 1C**) [1].

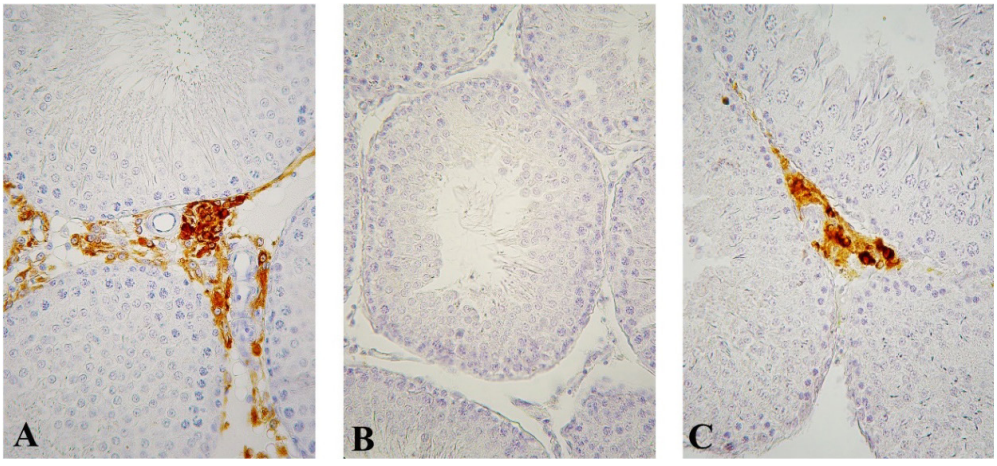

Fig. 1. Immunoexpression of 3β-HSD in control and EDS-treated adult rat testis. **A** – Control; **B** – 7th day after EDS; **C** – 21st day after EDS. ×400

Electron microscopy observation on day 7 after EDS revealed presence of spindle-shaped cells with elongated nucleus and little cytoplasm that were situated in the interstitium. These cells were supposed as presumptive progenitor cells for Leydig cells that were negative for 3β-HSD. Three weeks after EDS treatment the ultrastructure of Leydig cells revealed oval shape with round nucleus and numerous lipid inclusions that were characteristic for the next two steps for Leydig cell differentiation – newly-formed and immature adult Leydig cells. These cells corresponded to 3β-HSD positive cells [4]. Five weeks post EDS mature adult Leydig cells appeared as evident by their morphological characteristics – round nucleus with prominent nucleolus, abundant smooth endoplasmic reticulum and mitochondria with tubular crists [3].

Together with acquirement with steroidogenic competence newly-formed Leydig cells start expression of neuronal markers as glial fibrillary acidic protein (GFAP), tyrosine kinase A, glial cell line–derived neurotrophic factor (GDNF), neuron-specific enolase, neural cell adhesion molecule [7].

In later studies we followed the expression of cell markers specific for differentiating Leydig cells – 11β-HSD [10], Insulin like 3 peptide and its receptor LGR8 [12], T3 thyroid hormone receptors c-erbAα and c-erbAβ [11].

11β-HSD is enzyme that catalyzes the reversible conversion of physiologically active corticosterone to the biologically inert 11β-dehydrocorticosterone and thus protects the Leydig cells against the suppressive effect of glucocorticoids. The developmental pathway of adult Leydig cell population is accompanied with increase in the 11β-HDS activity. Therefore, 11β-HDS can be used as a marker for their functional maturity. First immune-positive for 11β-HSD cells appeared on day 14 after EDS that supposed to be progenitor cells transforming into newly-formed Leydig cells. Progressive increase in the 11β-HSD2 reaction intensity and the number of positive

cells occurred later than day 21st post EDS with maximum on day 35 [10]. Our data suggested that the changes in 11β-HSD2 expression can be used for evaluation of adult Leydig cell differentiation in rat testis.

INSL3 localized predominantly in the gonadal tissues. During fetal life INSL3 is produced by fetal Leydig cell population (different from adult Leydig cells) being critical for testicular descent before birth. In postnatal life regulation of INSL3 gene is independent of that for steroidogenic enzymes. INSL3 is a useful marker of Leydig cell differentiation status. INSL3 is a specific ligand for LGR8 receptor. Pattern of INSL3 and LGR8 receptor protein expression were similar with that of 11β-HSD [12]. It was obvious that INSL3/LGR8 ligand-receptor system, in auto-paracrine fashion, has influence on adult type Leydig cell differentiation.

The pattern of protein expression of T3 thyroid hormone receptors (c-erbAα and c-erbAβ) was similar to that of other Leydig cell differentiation markers mentioned above (11β-HDS, INSL3/LGR8). Data were in support for regulatory role of thyroid hormones in the differentiation of adult Leydig cell population in postnatal life [11].

Together with our studies on the development of regenerating Leydig cell population after EDS treatment, we focused our interest on spermatogenesis in condition of testosterone deprivation, as well. It is well known that germ cell development is strongly dependent on proper androgen levels [14]. Spermatogenesis is a complex process involving many consecutive steps of germ cell differentiation that occurs in stage specific manner called stages of seminiferous epithelium. In rat there are 14 stages that differ by association in germ cell types and subtypes – spermatogonia, spermatocytes and spermatids. Different stages have specific requirement of testosterone and FSH levels. Middle stages (VII–VIII) require maximal testosterone levels and they are considered as androgen-dependent stages.

To identify specific changes in seminiferous epithelium in condition of testosterone insufficiency we applied immunostaining for tACE – specific marker for elongating spermatids. Testicular ACE is expressed in a stage specific manner. The enzyme is localized in the cytoplasm of elongating spermatids from step 8 to step 19 of spermiogenesis. Weak immunoreactivity was seen in elongating spermatids step 9 (stage IX of spermatogenic cycle) followed by progressive increase in elongating

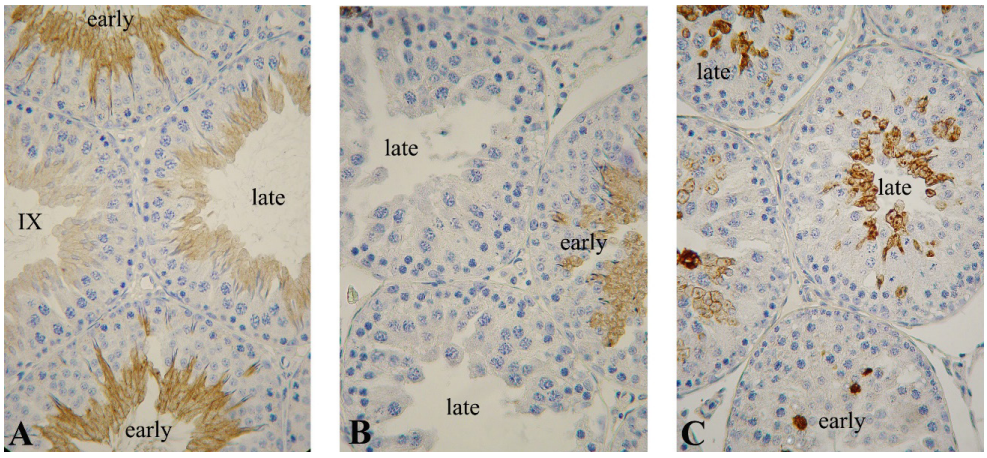

Fig. 2. Immunoexpression of testicular ACE in control and EDS-treated adult rat testis. **A** – Control; **B** – 7th day after EDS; **C** – 21st day after EDS. ×400

spermatids step 12-14 at late stages (XII-XIV) and in elongating spermatids step 15-18 at early stages form the next cycle (I-VI). Maximal intensity was reached in mature spermatozoa step 19 (stage VIII) (**Fig. 2A)**. We found on 7th day after EDS that late stages (IX-XIV) were depleted from elongated spermatids (step 9-14) whereas early stages (I-VI) remained intact (**Fig. 2B**). On day 14th post EDS elongating spermatids were completely lost from the seminiferous tubules. On day 21st, first sign of recovery of spermatogenesis was seen with the appearance of elongated spermatids in late stages (step 9-14), but early stages were still depleted from elongated spermatids (step 15-19) (**Fig. 2C**). On day 35 spermatogenesis is fully recovered [2]. Therefore our data indicated that depletion and recovery of elongating spermatids after EDS treatment occurred in stage-specific pattern. It can be concluded that degenerative and regenerative events in germ cell (spermatid) population occur in tandem with development of new adult Leydig cell population after EDS treatment.

Having in mind that apoptosis is a hormonally-induced process, we enumerated apoptotic cell visualized by TUNNEL method. Data were presented as number of apoptotic cell per tubule; percent of seminiferous tubules containing apoptotic cells and apoptotic index after multiplying of first two parameters. There was a similar trend in the three apoptotic parameters. Maximal germ cell apoptosis was established on day 7 when testosterone levels dropped to lowest value. Apoptotic cells decreased in number in the course of recovery of Leydig cell population as evident by gradual increase in testosterone levels but still under control range (**Table 1**). LH levels were elevated as a result of lack of testosterone feedback control on pituitary gland. Our results indicated that quantitative pattern of germ cell death after testosterone deprivation revealed in advance the kinetic of germ cell depletion and regeneration in a long period after EDS [5].

Table 1. Summary of EDS–induced changes in germ cell apoptosis, testosterone levels and LH plasma levels in adult rats. Data represent mean ± SD (n=4; *** p< 0.001, ** p< 0.01, * p< 0.05, ns – non significant, in comparison with control value)

Day after treatment	Number of apoptotic cells per ST	% of ST with apoptotic cells	Apoptotic Index	Testosterone levels (ng/ml)	LH levels (ng/ml)
Control	1.61 ± 0.18	6.81 ± 2.03	11.00 ± 3.57	2.14 ± 0.39	0.86 ± 0.18
7	3.55 ± 0.07 ***	40.80 ± 8.91 ***	144.14 ± 21.40 ***	< 0.1	3.66 ± 1.76 **
14	1.58 ± 0.14 ns	18.43 ± 1.59 ***	29.27 ± 4.90 ***	0.51 ± 0.25 ***	3.97 ± 1.18 ***
21	1.84 ± 0.15 ns	15.61 ± 6.02 *	29.20 ± 13.32 *	1.27 ± 0.3 **	not measured
35	1.34 ± 0.26 ns	18.22 ± 1.12 ***	24.46 ± 5.88 **	not measured	not measured

ST – seminiferous tubule

Androgen support for spermatogenesis is mediated by AR that is expressed in the nuclei of Leydig cells, peritubular cells and Sertoli cells but not in germ cells indicating that androgen support for developing germ cells is dependent on Sertoli cells. [6] There

is a state-specific pattern of expression of AR within the spermatogenic cycle. Low levels are seen at late stages (IX-XI) followed by increase in early stages (I-VI) and maximal levels are reached at VII-VIII stages (androgen dependent when spermatozoa are released into the tubular lumen) (**Fig. 3A**).

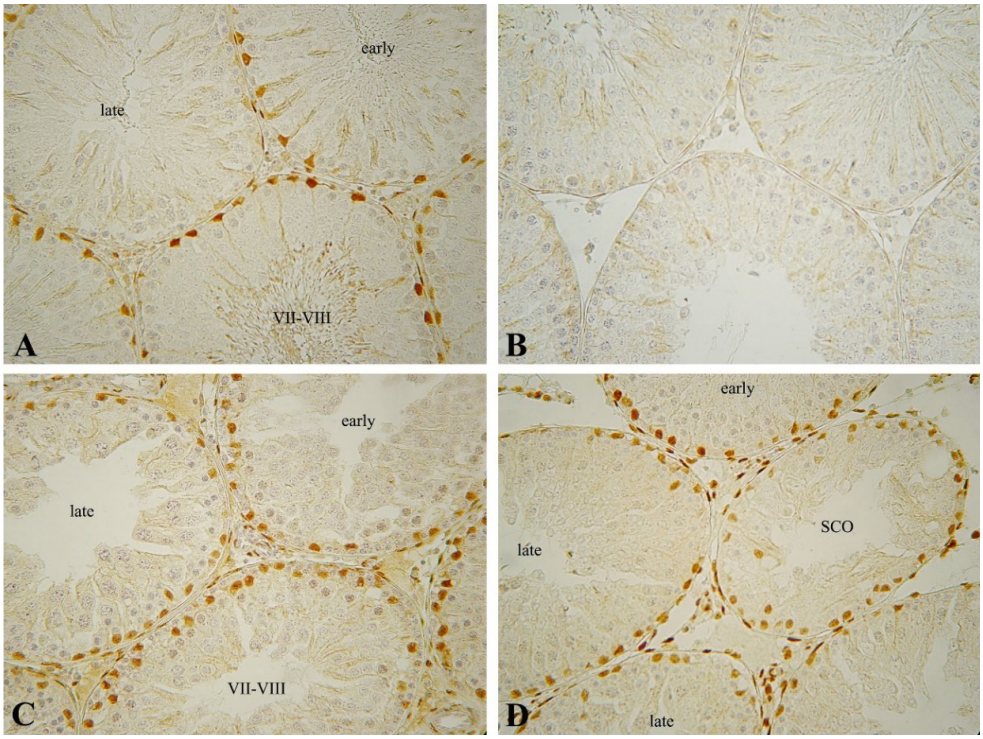

Fig. 3. Immunoexpression of Androgen receptor in control and EDS-treated adult rat testis. **A** – Control; **B** – 7th day after EDS; **C** – 14th day after EDS; **D** – 21st day after EDS. ×400

On day 7 after EDS we found complete loss in AR protein expression in Sertoli cells nuclei (**Fig. 3B**). Two and three weeks after EDS androgen receptor appeared with uniform strong intensity in all stages and stage-specific manner was not seen (**Fig. 3C**). Stage specificity was restored 5 weeks after EDS. Relating these findings to hormonal profile it seems that strong uniform AR expression could be considered as compensatory mechanism of unappropriate testosterone levels. There was no change in AR expression in Leydig cells. The specific changes in AR after EDS including its loss and recovery in Sertoli cells paralleled with degenerative and regenerative events in Leydig and germ cell populations confirming close functional relationship between Sertoli cells, Leydig cells and germ cells [1].

Short term EDS models with testosterone administration provided important data about regulatory mechanism of AR by its own ligand testosterone. Immune-staining for 3β-HSD was used to validate the model and lack of Leydig cells on day 6 after EDS and in testosterone administration conditions, as well (**Fig. 4B, C**).

On day 6 after EDS injection AR protein expression was completely lost in Sertoli cells nuclei (**Fig. 5B**). When testosterone was applied on the day of EDS injection

the spermatogenesis was maintained as well stage specific manner of AR protein expression (**Fig. 5C**). Moreovere, AR expression was restored when testosterone was applied for 4 days after 6 days of EDS condition. Our data suggested that maintenance of stage-specific AR expression in Sertoli cells after EDS requires acute administration of high amount of testosterone but not gradual increase in androgen production during long-term recovery of Leydig cell population after EDS.

A possible candidate involved in the regulation of stage-specific expression of AR was considered Cyclin D2 that play a role different from that in the cell cycle [13]. In control conditions Sertoli cells do not express

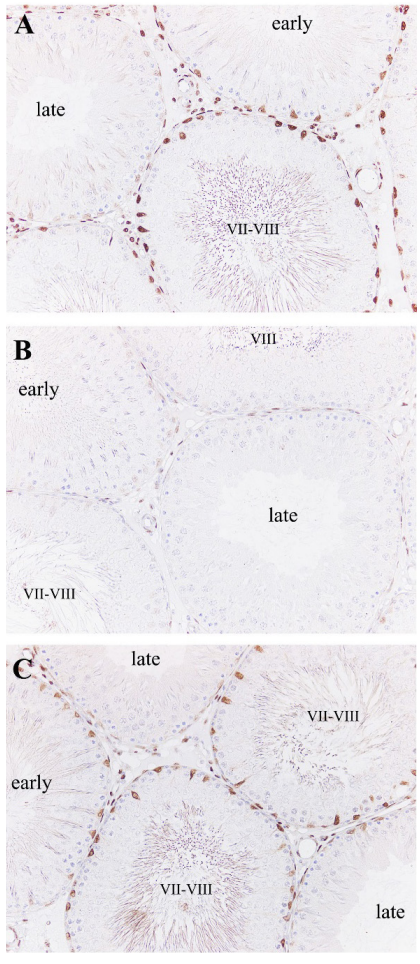

Fig. 4. Immunoexpression of 3β-HSD in adult rat testis: Control (**A**), 6th day after EDS-injection (**B**); 6th day after EDS with testosterone administration (**C**). ×400

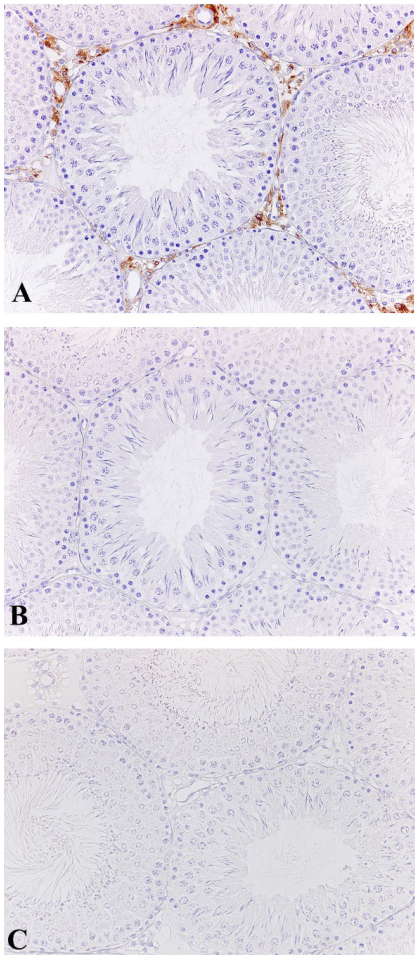

Fig. 5. Immunoexpression of Androgen receptor in adult rat testis: Control (**A**), 6th day after EDS-injection (**B**); 6th day after EDS with testosterone administration (**C**). ×400

Cyclin D2. After 6 days EDS Cyclin D2 protein expression appeared in Sertoli cell nuclei at late stages and testosterone administration recovered control pattern. It seems that Cyclin D2 is a negative regulator of AR expression in Sertoli cells.

Conclusion

The current overview of our data from collaborative studies with Prof. Davidoff on the EDS experimental model for androgen deprivation provided new knowledge about protein expression of cellular markers for differentiation of adult Leydig cells in relation to spermatogenesis. The morphology and protein expression of Leydig cell markers suggested that development of new adult Leydig cell population after EDS repeats the normal dynamics of differentiation of postnatal Leydig cells within a similar time range. Degenerative and regenerative events in spermatogenesis and time specific changes in androgen receptor expression in Sertoli cells occur in tandem with development of new adult Leydig cell population after EDS indicating close functional relationship between Sertoli cells, Leydig cells and germ cells. By comparison of long term EDS with short term EDS plus testosterone model we provided new understanding about regulation of androgen receptor by its own ligand testosterone.

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