Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 21
Sofia ● 2015

Morphology

Experimental Approaches for Identification of Biomarkers for Male Infertility

N. Atanassova

Dept. Experimental Morphology, Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

The incidence of disorders of human male reproductive health has increased more than double in the past 30 years while sperm counts have declined by about half. Similar abnormalities occur in sons of women treated with estrogenic hormones during pregnancy and they can be experimentally induced in animals by brief exposure to exogenous estrogens during perinatal life [6]. Hormones (mainly estrogens) determine subsequent risk of cancers of the male reproductive organs, e.g. testicular and prostate cancers. Endocrine disrupting chemicals that are widely spread in the environment act as weak hormones being estrogen or androgen receptor agonists or antagonists. Hence, they cannot be ignored as a potential involvement in human reproductive disease [7].

A complex system of morphological, quantitative and functional criteria was developed for identification and evaluation of androgen and estrogen action and the balance between both of hormones in the male reproductive system applying experimental approach involving single or combined treatments with different doses potent (DES) or weak estrogens (Bisphenol-A, Octylphenol, phytoestrogen-Genistein), GnRH-antagonist and anti-androgen Flutamide [2]. The role of each somatic cell types of the testis (Sertoli cells, Leydig cells, peritubular cells, testicular arteriole smooth muscle cells) in androgen signaling was established via comparative and detailed studies on genetic model total and selective targeted disruption of androgen receptor (AR) in these cells [1, 2, 5, 8, 9, 10]. Another experimental model, complementary to the latter, is androgen withdrawal after selective ablation of Leydig cells by ethane dimethanesulfonate (EDS) [2, 3].

Another risk factor for male infertility is diabetes mellitus (DM), but the underlying mechanisms involved are poorly understood. Recently, experimental model was developed for induction of hyperglycaemia in neonatal (on day 1), peripubertal/developing (on day 10) and adult (on day 60) rats by treatment with streptozotocin [4].

Schematic demonstration of treatment regimens was shown in **Table 1** together with semiquantitative presentation of plasma levels of steroid hormones and gonadotrophins.

Table 1. Schematic demonstration of treatment regimens and semiquantitative presentation of plasma levels of steroid hormones (testosterone and estradiol) and gonadotrophins (FSH and LH). DES – diethylstilboestrol; GnRHa – GnRH-antagonist; EDS – ethane dimethanesulfonate; ARKO – AR knockout; pnd – postnatal day; N – normal; nm – non measured

	Treatments	**Doses**	**Regimen**	**Androgen**	**Estrogen**	**FSH**	**LH**
1	Control	20 µl corn oil	2-12 pnd	N	N	N	N
2	DES-10	10 µg	2-12 pnd	↓↓↓↓↓	↑↑↑↑↑↑	↓↓↓↓	↓↓↓↓
3	DES-1/0.1	1 µg/ 0.1µg	2-12 pnd	↓-N	↑↑↑↑/ ↑↑	N/ ↑	↓/ N
4	Bisphenol-A	0.5 mg	2-12 pnd	↑	↑	↑	N
5	Octylphenol	150 mg/kg	2-12 pnd	↑	↑	↑	nm
6	Genistein	4 mg/kg	2-18 pnd	nm	↑	N	nm
7	GnRHa	10 mg/kg	2, 5 pnd	↓↓↓↓↓		↓↓↓↓	↓↓↓↓
8	Flutamide	50 mg/kg	2-12 pnd	AR↓↓↓		N	N
9	Testosterone	200 µg	2-12 pnd	↑↑		↓↓↓↓	nm
10	DES+T	as 2 and 9	as 2 and 9	↑↑	↑↑↑↑↑↑	↓↓↓	↑↑↑
11	DES-0.1+GnRHA	as 3 and 7	as 3 and 7	↓↓↓↓↓	↑↑	↓↓↓↓	↓↓↓
12	DES-10+GnRHA	as 2 and 7	as 2 and 7	↓↓↓↓↓	↑↑↑↑↑↑	↓↓↓↓	↓↓↓↓
13	DES+Flutamide	as 3 and 8	as 3 and 8	AP↓↓↓	↑↑	N	N
14	EDS	75 mg/kg	60 pnd	↓↓↓↓↓↓	↓↓↓↓↓↓	↑↑	↑↑↑
15	streptozotocin	65 mg/kg	1/10 pnd	↓/N	nm	nm	nm
16	AP -/- ARKO	total		↓/N	nm	↑↑	↑↑
17	AP -/- SCARKO	Selective in Sertoli cells		N	nm	N	N
18	AP -/- PTMARKO	Selective in Peritubular cells		N	nm	↑↑	↑↑↑
19	AP -/- LCARKO	Selective in Leydig cells		N	N	N	N
20	AP -/- SMARKO	Selective in testicular arteriole smooth muscle cells		N	nm	N	↑↑

Detailed studies were performed on the testes and male reproductive tracts from rats subjected on treatment regiments shown in **Table 1**, as well as from AR-knockout mice. They involved stereological analysis, immunohistochemistry and measurement of plasma levels testosterone, FSH and LH [1, 2]. Based on data obtained quantitative and cellular markers was identified for experimentally induced male reproductive abnormalities that lead to infertility. They are summarized below:

I. Testicular biomarkers:

1. Quantitative macro-biomarkers of the testis:

a. Testis weight (mg) – indicative for total germ cell number;

b. Luminal percentage volume – indicative for Sertoli cells (SC) function to produce seminiferous tubule fluid.

2. Quantitative micro-biomarkers of spermatogenesis:

a. Absolute nuclear volume (ANV)/Number of Sertoli cells per testis indicative for SC function to produce Inhibin-B and is also used for monitoring of spermatogenesis;

b. Absolute nuclear volume/Number of Leydig cells (LC) per testis indicative for Testosterone production;

c. Germ cell apoptotic index = apoptotic cell /total germ cell number;

d. Absolute nuclear volume of germ cells population and their subtypes:

– Spermatogonia – type A and type In+B;

– Spermatocytes – early (leptotene+zygotene) and late meiotic (pachytene+diplotene);

– Spermatids – round (steps 1-7) and elongating (steps 8-19).

e. Cell ratios – Germ cell ANV/Sertoli cells ANV indicative for supporting function of Sertoli cells toward germ cells and hence for efficiency of spermatogenesis.

3. Cellular biomarkers:

a. Sertoli cell markers:

– nuclear: Androgen Receptor (AR), 27 kD Cyclin-dependent kinase inhibitor protein ($p27^{Kip1}$), Wilms' Tumor suppressor protein 1 (WT-1), GATA-4, Placentae and Embryos Oncofetal gene (Pem, marker for androgen regulation);

cytoplasmic: Anti Müllerian Hormone (AMH), Sulfated Glycoprotein-2 (SGP-2), Inhibin-α, – Retinoic Acid Receptor-α (RARα);

b. Leydig cell (LC) markers:

– nuclear: – Chicken Ovalbumin Upstream Promoter Transcription-Factor II (COUP TF-II) as marker for LC progenitor cells;

– cytoplasm: 3βHydroxysteroid Dehydrogenase (3βHSD) as marker for androgen production and steroidogenic capacity of LC; Insulin-like 3 factor (Insl-3) and 11βHSD (markers for LC differentiation), Estrogen Recepto-α (ERα).

c. Peritubular markers: α-Smooth Muscle Actin (αSMA), Desmin, Laminin, β-Tubulin isotype 3;

d. Germ cell markers: p63 (marker for meiotic spermatocytes); Testicular Angiotensin Converting Enzyme (tACE, marker for postmeiotic elongating spermatids step 8-19).

II. Biomarkers of male reproductive tract.

a. Quantitative measurements: area of rete testis and ductuli efferentes (indicative for accumulation of seminiferous tubule fluid); epithelial cell high of rete testis, ductuli efferentes, epididymis and ductus deferens; number of basal epithelial cell per μm length;

b. Epithelial cells: AR, ERα, ERβ, pan-cytokeratins;

c. Basal epithelial cells: p63 and Cytokeratin High Molecular Weight (CKHMW) – indicative for hyperplasia;

d. Stromal and periductal cells: αSMA, desmin.

III. Hormonal profiles.

a. Testosterone: indicative for LC steroidogenesis and androgen production;
b. FSH: indicative for adequate spermatogenesis;
c. LH: indicative for LC responsiveness to androgen;
d. Inhibin-B: indicative for Sertoli cell function and monitoring of spermatogenesis.

Acknowledgement: The author thanks to Professor Richard Sharpe for providing samples from experimental models for hormonal manipulations, to Professor Guido Verhoven, Dr. Karel DeGent and Professor Lee Smith for samples from AR knockout models and to Chris McKinnel for technical expertise in immunohistochemistry. I am also grateful to Professor Michail Davidoff, Professor Yvetta Koeva and Assoc. Professor Mariana Bakalska for studies on EDS experimental model as well as to Assoc. Professor Emilia Lakova for studies on streptozotocin induced DM model. Authors' work was supported in part by Grant DEER # 212844 funded by FP7-ENV-CP and by Grant # DO 02/113 funded by NF "Scientific Research" of Ministry of Education Youth and Science in Bulgaria.

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