International Symposium

Alternative in vitro methods to characterize the role of Endocrine Active Substances (EASs) in human hormone-targeted tissues

Rome, December 17, 2012

Human sperm (epi)genetic biomarkers to assess the impact of Endocrine Active Substances on male reproductive function

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Endocrine disruption is one of the most relevant and contentious topics in environmental science and reproductive toxicology

50 years ago...
Endocrine disruption is one of the most relevant and contentious topics in environmental science and reproductive toxicology.

20 years ago...
Semen quality has deteriorated in many countries during the last 60 years.

Carlsen E, Giwercman A, Keiding N, Skakkebaek NE.
Evidence for decreasing quality of semen during past 50 years.
BMJ 305: 609-613, 1992

Each man in this room yields half his grandfather yielded!
Secular trends in male reproductive disorders: should we be concerned?

- Increased incidence of testicular cancer worldwide
- Increased incidence of cryptorchidism and hypospadias
- Growing demand for ART in affluent countries
The “oestrogen hypothesis”


Abstract

The incidence of disorders of development of the male reproductive tract has more than doubled in the past 30-50 years while sperm counts have declined by about half. Similar abnormalities occur in the sons of women exposed to diethylstilbestrol (DES) during pregnancy and can be induced in animals by brief exposure to exogenous oestrogen/DES during pregnancy. We argue that the increasing incidence of reproductive abnormalities in the human male may be related to increased oestrogen exposure in utero, and identify mechanisms by which this exposure could occur.

DES competes with oestradiol for ER binding
The “environmental xenoestrogen hypothesis”


Endocrine Disruptors
Environmental Estrogens
Endocrine Modulators
Eco-Estrogens
Environmental Hormones
Xeno-Estrogens
Hormone-related Toxicants
Hormone Interferers
Endocrine-Active Substances

Endocrine Disruptors Website
http://ec.europa.eu/environment/endocrine/index_en.htm
New biomarkers of semen quality

Semen quality is conventionally assessed by visual scoring of sperm number and properties according to WHO guidelines

Need for new markers that may better:

- discriminate infertile from fertile men
- predict pregnancy outcome (also after ART)
- predict risk of adverse reproductive events
- give information about sperm genomic integrity

Sperm DNA damage as a new candidate biomarker

Biomonitoring-epidemiology
Clinics
In vivo toxicology
In vitro toxicology
DNA DAMAGE

Pathologies
- Varicocele
- Thalassemia major
- Diabetes type 1
- Spinal cord injury
- Chlamydia T.
- Mycoplasma

Cancer & Mutagenic Chemo/Radio Therapy
- Ribavirin (hepatitis C)
- SSRI (depression)

Genetic background

Drugs
- Smoking
- Coffee

Life style
- Lead
- Styrene
- Acrylonitrile
- PAHs
- Organophosphoric pesticides
- Insecticides (carbaryl, fenvalerate)

Work
- POPs
- Phthalates
- Air pollution
- Radiofrequency electromagnetic radiation
- Heat

Environmental xenobiotics

Age

Infertility
- Pregnancy loss
- Developmental defects
- Infant mortality
- Infertility
- Genetic diseases in the offspring

Normal embryogenesis and birth

DNA REPAIR

(in the oocyte)

Defective

Normal
Sperm DNA Integrity Assay Methods

**SCSA**
(Sperm Chromatin Structure Assay) *Evenson et al., 1980*

**TUNEL Assay**
(in situ Terminal deoxynucleotidyl transferase driven UTP Nick End Labeling)
*Gorczyca et al., 1993*

**Comet Assay**
(single cell gel electrophoresis)
Alkaline (pH>13) *Singh et al., 1989*
Neutral (pH=8) *Singh & Stephens, 1998*

**SCD (Sperm Chromatin Dispersion) HaloTest**
*Fernandez et al., 2003*
FACTORS TO BE CONSIDERED IN SPERM DNA DAMAGE PRODUCTION AND DETECTION

✓ Chromatin reorganization

✓ DNA repair capability loss

In vitro exposure of ex vivo sperm to EASs can be investigated to test the hypothesis of direct effects of these compounds on terminally differentiated mature gametes and as pre-screening approach to their toxicological assessment.
<table>
<thead>
<tr>
<th>Sperm species</th>
<th>Compounds tested</th>
<th>Assay</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>bisphenol A</td>
<td>Comet, TUNEL</td>
<td>- Bennetts et al., Mutat Res 2008</td>
</tr>
<tr>
<td>Human</td>
<td>chlorpyrifos</td>
<td>SCSA</td>
<td>+ Salazar- Arredondo et al., Reprod Toxicol 2008</td>
</tr>
<tr>
<td>Human</td>
<td>chlorpyrifos-oxon</td>
<td>SCSA</td>
<td>+ Salazar- Arredondo et al., Reprod Toxicol 2008</td>
</tr>
<tr>
<td>Human</td>
<td>daidzein</td>
<td>Comet</td>
<td>+ Anderson et al., Teratog Carcinog Mutagen 1997; Anderson et al., Mutat Res 2003</td>
</tr>
<tr>
<td>Human</td>
<td>diazinon</td>
<td>SCSA</td>
<td>+ Salazar- Arredondo et al., Reprod Toxicol 2008</td>
</tr>
<tr>
<td>Human</td>
<td>diazoxon</td>
<td>SCSA</td>
<td>+ Salazar- Arredondo et al., Reprod Toxicol 2008</td>
</tr>
<tr>
<td>Human</td>
<td>dibromochloropropane</td>
<td>Comet</td>
<td>+ Anderson et al., Teratog Carcinog Mutagen 1997</td>
</tr>
<tr>
<td>Human</td>
<td>1,2,3,4-diepoxybutane</td>
<td>Comet</td>
<td>+ Anderson et al., Teratog Carcinog Mutagen 1997; Anderson et al., Mutat Res 2003</td>
</tr>
<tr>
<td>Human</td>
<td>Diethylstilbestrol</td>
<td>Comet, TUNEL</td>
<td>- Bennetts et al., Mutat Res 2008</td>
</tr>
<tr>
<td>Human</td>
<td>β-estradiol</td>
<td>Comet</td>
<td>+ Anderson et al., Teratog Carcinog Mutagen 1997; Anderson et al., Mutat Res 2003</td>
</tr>
<tr>
<td>Human</td>
<td>ethylene glycol monoethyl ether</td>
<td>Comet</td>
<td>+ Anderson et al., Teratog Carcinog Mutagen 1997</td>
</tr>
<tr>
<td>Human</td>
<td>genistein</td>
<td>Comet</td>
<td>+ Anderson et al., Teratog Carcinog Mutagen 1997; Anderson et al., Mutat Res 2003</td>
</tr>
<tr>
<td>Human</td>
<td>2-hydroxyestradiol</td>
<td>TUNEL</td>
<td>+ Bennetts et al., Mutat Res 2008</td>
</tr>
<tr>
<td>Human</td>
<td>4-hydroxyestradiol</td>
<td>TUNEL</td>
<td>- Bennetts et al., Mutat Res 2008</td>
</tr>
<tr>
<td>Human</td>
<td>methyl-parathion</td>
<td>SCSA</td>
<td>+ Salazar- Arredondo et al., Reprod Toxicol 2008</td>
</tr>
<tr>
<td>Human</td>
<td>methyl-paraoxon</td>
<td>SCSA</td>
<td>+ Salazar- Arredondo et al., Reprod Toxicol 2008</td>
</tr>
<tr>
<td>Human</td>
<td>myricetin</td>
<td>Comet</td>
<td>+ Anderson et al., Teratog Carcinog Mutagen 1997; Anderson et al., Mutat Res 1998</td>
</tr>
<tr>
<td>Human</td>
<td>Nonylphenyl</td>
<td>Comet</td>
<td>+ Anderson et al., Teratog Carcinog Mutagen 1997; Anderson et al., Mutat Res 2003</td>
</tr>
<tr>
<td>Human</td>
<td>PCB77</td>
<td>Comet</td>
<td>+ Baumgartner et al., Mutat Res 2011</td>
</tr>
<tr>
<td>Human</td>
<td>TCDD</td>
<td>Comet</td>
<td>+ Baumgartner et al., Mutat Res 2011</td>
</tr>
</tbody>
</table>

**References:**
- Bennetts et al., Mutat Res 2008
- Salazar- Arredondo et al., Reprod Toxicol 2008
- Baumgartner et al., Mutat Res 2011
Limits of application of these tests reside in the simulation of a direct contact between the sperm cell and the reprotoxins only, with no information on interactions with previous stages of spermatogenesis having consequences for sperm genetic integrity.
In vitro production of functional sperm in cultured neonatal mouse testes

Takuya Sato¹, Kumiko Katagiri¹, Ayako Gohbara¹, Kimiko Inoue², Narumi Ogonuki², Atsuo Ogura², Yoshinobu Kubota¹ & Takehiko Ogawa¹,³

Spermatogenesis is one of the most complex and longest processes of sequential cell proliferation and differentiation in the body, taking more than a month from spermatogonial stem cells, through meiosis, to sperm formation¹,². The whole process, therefore, has never been reproduced in vitro in mammals³–⁵, nor in any other species with a very few exceptions in some particular types of fish⁶,⁷. Here we show that neonatal mouse testes which contain only gonocytes or primitive spermatogonia as germ cells can produce spermatids and sperm in vitro with serum-free culture media. Spermatogenesis was maintained over 2 months in tissue fragments positioned at the gas–liquid interphase. The obtained spermatids and sperm resulted in healthy and reproductively competent offspring through microinsemination. In addition, neonatal testis tissues were cryopreserved and, after thawing, showed complete spermatogenesis in vitro. Our organ culture method could be applicable through further refinements to a variety of mammalian species, which will serve as a platform for future clinical application as well as mechanistic understanding of spermatogenesis.

Sato et al., Nat Commun. 2011 Sep 13;2:472
To implement the 3Rs principle of reduction, refinement and replacement of animals for reproductive toxicity assessment, human biomonitoring studies offer a complementary approach to in vitro tests.

**Pros**

- Potential effects on our species
- Potential effects of “real life” (chronic, complex mixture, low-level) exposures can be evaluated

**Cons**

- Slow and expensive
- Limited in the ability to draw causal inferences
## DDT & sperm DNA damage

<table>
<thead>
<tr>
<th>Assay</th>
<th>Exposed</th>
<th>DDE plasma conc (ng/ml lipid)</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMET neut</td>
<td>212 infertile US men 36.1±5.2 yrs</td>
<td>254 (72.5-7776)</td>
<td>N.S.</td>
<td>Hauser et al., 2003</td>
</tr>
<tr>
<td>SCSA</td>
<td>175 Swedish fishermen</td>
<td>334 (80-887)</td>
<td>N.S.</td>
<td>Rignell-Hydbom et al., 2005</td>
</tr>
<tr>
<td>TUNEL</td>
<td>652 fertile men (200 Inuits, 166 Sweden, 134 Poland, 152 Ukraine) 33.7 (18-67.5) yrs</td>
<td>790 (6-13000)</td>
<td>DFI N.S.</td>
<td>Stronati et al., 2006</td>
</tr>
<tr>
<td>SCSA</td>
<td>707 fertile men (193 Inuits, 178 Sweden, 141 Poland, 195 Ukraine) 33.7 (18-67.5) yrs</td>
<td>790 (6-13000)</td>
<td>DFI N.S.</td>
<td>Spanò et al., 2005</td>
</tr>
<tr>
<td>SCSA</td>
<td>202 South Africans from Limpopo Province (malaria)</td>
<td>215,500 ± 210,600 (10-997,000)</td>
<td>DFI ↑ (r = 0.12)</td>
<td>De Jager et al., 2009</td>
</tr>
</tbody>
</table>

TOTAL 1296
## Bisphenol A/Parabens/PFAs & sperm DNA damage

<table>
<thead>
<tr>
<th>Assay</th>
<th>Exposed</th>
<th>BPA urinary conc (ng/ml) mean (min-max)</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMET (neut)</td>
<td>190 infertile US men 36.4±4.1 yrs</td>
<td>1.4 (ND-36.4)</td>
<td>↑</td>
<td>Meeker et al., 2010a</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>COMET (neut)</td>
<td>132 infertile US men 36.7±5.4 yrs</td>
<td>1.4 (ND-36.4)</td>
<td>↑</td>
<td>Meeker et al., 2010b</td>
</tr>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Parabens urinary conc (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMET (neut)</td>
<td>132 infertile US men 36.7±5.4 yrs</td>
<td>MP 28.6 (5.1-1080) PP 3.7 (0.4-294) BP NC (ND-64.5)</td>
<td>↑</td>
<td>Meeker et al., 2010b</td>
</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>PFOS, PFOA, PFNA, and PFHxS blood conc (ng/ml)</td>
<td></td>
<td>Specht et al., 2012</td>
</tr>
<tr>
<td>SCSA TUNEL</td>
<td>604 fertile European men (199 Inuits) 36.7±5.4 yrs</td>
<td>PFOS (Greenland) 51.9 (12-161) PFOS (Poland) 18.6 (8-40) PFOS (Ukraine) 8.1 (3-30)</td>
<td>↑</td>
<td>Specht et al., 2012</td>
</tr>
</tbody>
</table>

**Notes:**
- ↑ indicates an increase.
- ND: Not detected.
- SCSA: Single cell gel electrophoresis assay.
- TUNEL: Terminal deoxyribonucleotidyl transferase dUTP nick end labeling assay.
Male gonads and progenitors of male germ cells start forming in utero! Prenatal life might be a highly sensitive developmental stage, especially regarding hormonally mediated effects and epigenetic signatures!
The masculinization programming window
Male development is totally hormone-dependent
Fetal basis of adult onset diseases

Key developmental events in human reproductive tissue, gland and organ development during the critical windows.

Critical window
Developmental period

15 years

Prenatal/perinatal exposure and reduced sperm count in the adult

**Tobacco smoke**


*Jensen et al.* Association of in utero exposure to maternal smoking with reduced semen quality and testis size in adulthood: a cross-sectional study of 1,770 young men from the general population in five European countries. Am J Epidemiol 159: 49-58, 2004

*Jensen et al.* Lower sperm counts following prenatal tobacco exposure. Hum Reprod 20: 2559-2566, 2005


**Dioxin**


**PCBs**

Epigenetics refers to mitotically/meiotically heritable resettable changes in gene expression not involving DNA sequence changes.

Epigenetic changes encompass an array of molecular modifications involving DNA and chromatin (DNA methylation, histone modifications, nc RNAs).
In utero exposure to EASs has been shown to promote transgenerational effects mediated by epigenetic mechanisms. Critical window: genome-wide demethylation in PGC.
### Reproductive and developmental effects of environmental chemicals on DNA methylation: in vivo effects from in utero exposures

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Species</th>
<th>Tissue/ce II</th>
<th>Genes</th>
<th>DNA methylation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinclozolin Methoxychlor (in utero)</td>
<td>Rat</td>
<td>Testis (F0-F4); Epididymal sperm (F2-F3)</td>
<td>Global and gene specific (e.g., lysophospholipase – LPLase, cytokine-inducible SH2 protein) (PCR methylation-sensitive restriction enzyme digestion analysis)</td>
<td>↓↑</td>
<td>Anway et al., 2005</td>
</tr>
<tr>
<td>Vinclozolin (in utero E8-14)</td>
<td>Fisher Sprague-Dawley Rat</td>
<td>Epididymal sperm (F1-F3)</td>
<td>Global (PCR methylation-sensitive restriction enzyme digestion analysis; alternate bisulphite DNA sequence analysis)</td>
<td>25 candidate DNA sequences altered</td>
<td>Chang et al., 2006</td>
</tr>
<tr>
<td>Vinclozolin (in utero E10-18)</td>
<td>FBV/N Mice</td>
<td>Sperm Tail Liver Muscle</td>
<td>H19, Gtl2 (paternal imprinting) Peg1, Snrpn, Peg3 (maternal imprinting) (pyrosequencing)</td>
<td>↓↑ disappear gradually (in somatic Peg3 in F2-3)</td>
<td>Stouder &amp; Paoloni-Giacobino, 2010</td>
</tr>
<tr>
<td>Vinclozolin (in utero E8-14)</td>
<td>Harlan Sprague-Dawley Rat</td>
<td>Sperm (F3)</td>
<td>Genome-wide (Array MeDIP-Chip Analysis) Mass spectroscopy Pyrosequencing</td>
<td>Changes in 16 promoters</td>
<td>Guerrero-Bosagna et al., 2010</td>
</tr>
<tr>
<td>Methoxychlor (adult, x1w)</td>
<td>FBV/N Mice</td>
<td>Sperm Liver Muscle</td>
<td>H19, Gtl2 (paternal imprinting) Peg1, Snrpn, Peg3 (maternal imprinting) (pyrosequencing)</td>
<td>In sperm only, in all but H19</td>
<td>Stouder &amp; Paoloni-Giacobino, 2011</td>
</tr>
<tr>
<td>Methoxychlor (in utero E10-18)</td>
<td>FBV/N Mice</td>
<td>Sperm Tail Liver Muscle</td>
<td>H19, Gtl2 (paternal imprinting) Peg1, Snrpn, Peg3 (maternal imprinting) (pyrosequencing)</td>
<td>↓↑ disappear gradually (only in sperm)</td>
<td>Stouder &amp; Paoloni-Giacobino, 2011</td>
</tr>
<tr>
<td>Vinclozolin Flutamide Methoxychlor (in utero)</td>
<td>Rat</td>
<td>Testis (F0-F1)</td>
<td>lysophospholipase – LPLase (&amp; spermatogenesis/fertility up to F3)</td>
<td>≈</td>
<td>Inawaka et al., 2009 Schneider et al., 2008</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>Agouti Viable yellow Mouse</td>
<td>Embryo</td>
<td>Agouti (Avy) intracisternal A particle CDK5 activator-binding protein (CabplAP)</td>
<td>↓↓</td>
<td>Dolinoy et al., 2007</td>
</tr>
<tr>
<td>PFOS (prenatal ED 2-21)</td>
<td>Sprague-Dawley (SD) rat</td>
<td>Liver</td>
<td>global DNA methylation (Methylamp, Epigentek) methylation of LiNE-1 regulatory region (COBRA) tumor suppressor gene glutathione S-transferase pi (GSTP) and p16 promoter methylation level (PCR cloning)</td>
<td>↓↓↓=</td>
<td>Wan et al., 2010</td>
</tr>
<tr>
<td>TCDD (in utero E14)</td>
<td>Jcl:ICR Mouse</td>
<td>Embryo</td>
<td>H19, Igf2 (bisulfite genomic sequencing)</td>
<td>↑</td>
<td>Wu et al., 2004</td>
</tr>
</tbody>
</table>
### Effects of environmental chemicals (including EASs) on DNA methylation: humans

<table>
<thead>
<tr>
<th>Noxia</th>
<th>Tissue/cell</th>
<th>Genes/sequences</th>
<th>Δ DNAm</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air pollution (PM&lt;2.5, black</td>
<td>PBL (718 elderly participants</td>
<td>Global (Alu, LINE-1) (bisulfite pyrosequencing)</td>
<td>LINE-1 ↓</td>
<td>Baccarelli et al., 2009</td>
</tr>
<tr>
<td>carbon) 7 d</td>
<td>in Boston)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air pollution (PM10) 3 d</td>
<td>PBL (63 Italian steel workers,</td>
<td>Global (Alu, LINE-1) iNOS (inducible nitric oxide synthase) (bisulfite pyrosequencing)</td>
<td>= ↓</td>
<td>Tarantini et al., 2009</td>
</tr>
<tr>
<td>Ambient air particles (PM1 &amp; 10) 3 d</td>
<td>Brescia)</td>
<td>4 tumor suppressor genes (APC, p16, p53, RASSF1A) (bisulfite pyrosequencing)</td>
<td>APC/p16 ↑ p53/RASSF1A↓</td>
<td>Hou et al., 2011</td>
</tr>
<tr>
<td>Benzene</td>
<td>PBL (156 Italian workers, Milan)</td>
<td>Global (Alu, LINE-1) P15 MAGE (bisulfite pyrosequencing)</td>
<td>↓ ↑ ↓</td>
<td>Bollati et al., 2007</td>
</tr>
<tr>
<td>Benzene</td>
<td>PBL (156 Italian workers, Milan)</td>
<td>Global (Alu, LINE1) (bisulfite pyrosequencing) high-resolution GC-MS</td>
<td>↓ ↓</td>
<td>Fustinoni et al., 2012</td>
</tr>
<tr>
<td>PAHs</td>
<td>PBL (92 Polish workers)</td>
<td>p53, HIC1 P16, IL-6 Global (Alu, LINE-1) (bisulfite pyrosequencing)</td>
<td>↓ ≈ ↑</td>
<td>Pavanello et al., 2009</td>
</tr>
<tr>
<td>POPs</td>
<td>PBL (131 Greeland Inuits)</td>
<td>Global (Alu, LINE-1) (bisulfite pyrosequencing)</td>
<td>Alu ↓</td>
<td>Rusiecki et al., 2008</td>
</tr>
<tr>
<td>POPs</td>
<td>PBL (86 South Koreans)</td>
<td>Global (Alu, LINE-1 bisulphite pyrosequencing)</td>
<td>Alu ↓</td>
<td>Kim et al., 2010</td>
</tr>
</tbody>
</table>
Conclusions

Mature germ cells may be used in vitro as indicators for the evaluation of reprotoxins.

However, limits of applicability of these tests reside in the simulation of a direct contact between the sperm cell and the reprotoxins only, with no information on interactions with previous stages of spermatogenesis having consequences for sperm genetic integrity.

Probable improvements from new in vitro mammalian spermatogenesis models.

Genetic and epigenetic biomarkers will be integrated to provide a global in vitro assessment of semen quality.
Conclusions

Epidemiology studies are still necessary

Quandaries:

1. how to disentangle the effects where EASs are responsible from those due to other environmental or lifestyle-associated exposures that can impinge on the reproductive system
2. how to disentangle effects in the adult from in utero exposures occurred almost 15 years before (fetal masculinisation and expression of fertilizing ability in late puberty)

need for mother and child cohorts

Updated comprehensive reviews conclude that current epidemiologic evidence does not support with sufficient certainty the view that EASs contribute to an increase in male reproductive disorders; neither does it provide sufficient grounds to reject this hypothesis.

Endocrine disruption continues to be one of the most relevant and controversial topics in environmental science and in reproductive toxicology.
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