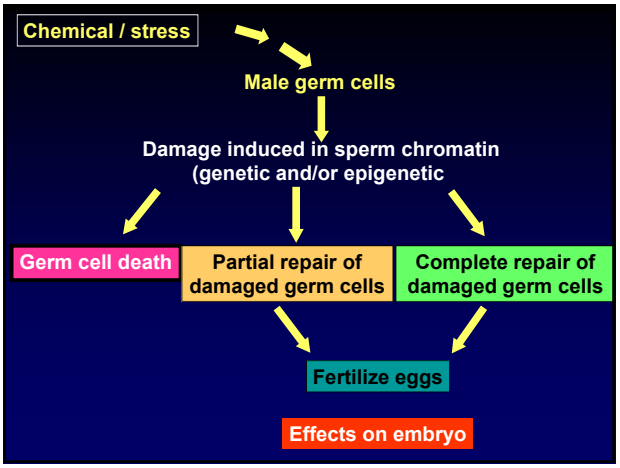
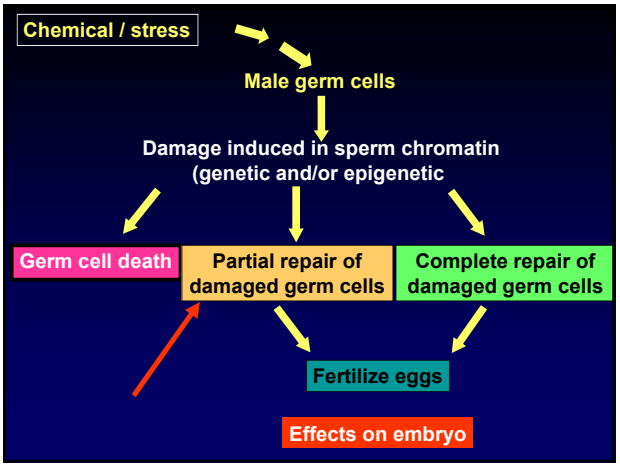
Use of sperm DNA tests to evaluate pre- and post-chemotherapy sperm quality

Bernard Robaire

Sperm DNA: organisation, protection and vulnerability: from basic science to clinical application
ESHRE Campus symposium
Stockholm, Sweden
21-22 May 2009

Departments of Pharmacology and Therapeutics and of Obstetrics and Gynecology
McGill University





Animal models
- Cyclophosphamide
  (commonly used anticancer drug)
- Bleomycin / Etoposide / Cisplatin
  (Testis cancer)

Human studies
- Testis Cancer
- Hodgkin Lymphoma
- Infertility patients

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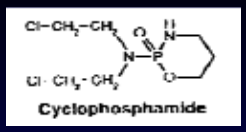
Acknowledgements
Cyclophosphamide Studies

Barbara Hales

Tara Barton
Alexis Codrington
Jianping Qiu

March of Dimes



Anticancer Agent

(Non)-Hodgkin Lymphoma
Lymphocytic Leukemia
Breast
Ovarian
Lung

Immunosuppressive Agent

Lupus Erythematosus
Wegener Granulomatosis
Graft-versus-Host Disease

Sperm Quality: Chromatin Biomarkers

- Sperm decondensation
  - in vitro, in vivo
- Breaks and cross-links and integrity of chromatin
  - Alkaline elution
  - Comet Assay
  - SCSA / acridine orange assay
  - TUNEL assay
  - Chromosomal aberrations (FISH)
- Chromatin template function
  - DNA replication
  - RNA transcription – germ cell
  - qPCR
- Chromatin structure
  - Protamine / histones
  - Disulfide links
  - Nuclear matrix
- Chromatin epigenome
  - DNA methylation / histone acetylation
  - protamine – histone ratio
  - piRNAs, microRNAs

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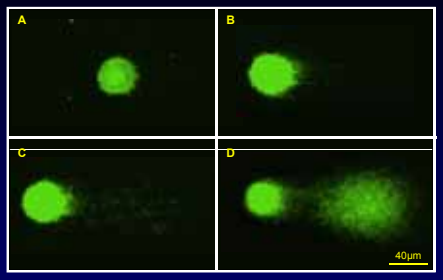
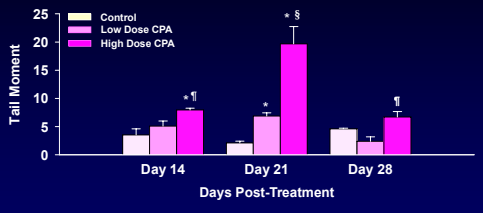
COMET ASSAY



Tail Extent Moment = $\frac{\text{Tail Length x \% Tail DNA}}{100}$

Comet Assay -Tail Extent Moment

Sub-Chronic Exposure

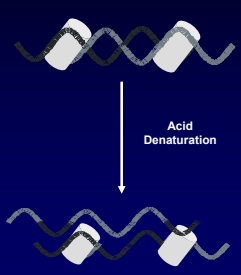


* Significantly different from time-matched controls
§ Significantly different from time-matched low-dose CPA
¶ Significantly different from dose-matched day 21 CPA

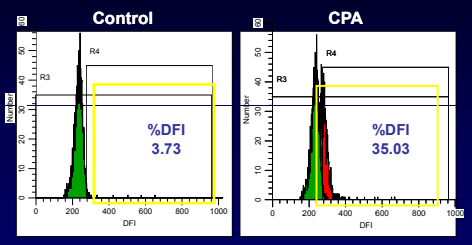
Germ Cells Targeted

14 Days = Elongated Spermatids
21 Days = Elongating Spermatids
28 Days = Round Spermatids

DNA DENATURATION
ACRIDINE ORANGE - SCSA®

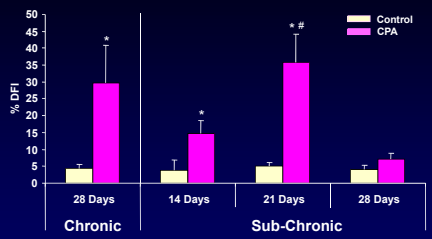


DFI: DNA Fragmentation Index
DFI= red fluorescence / total fluorescence



%DFI: % Abnormal cells

Acridine Orange – SCSA® Assay - %DFI



* Significantly different from time-matched controls
\# Significantly different from sub-chronic day CPA 14 and 28

Germ Cells Targeted

14 Days = Elongated Spermatids
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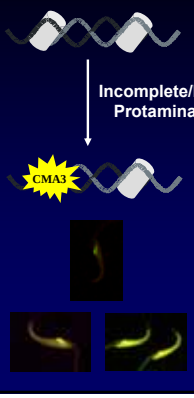
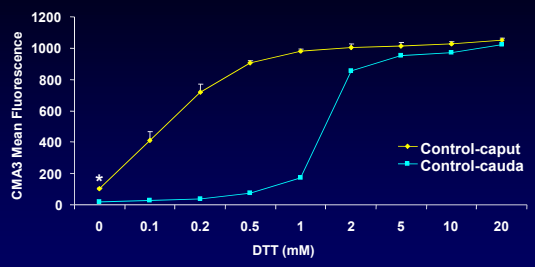
28 Days = Round Spermatids

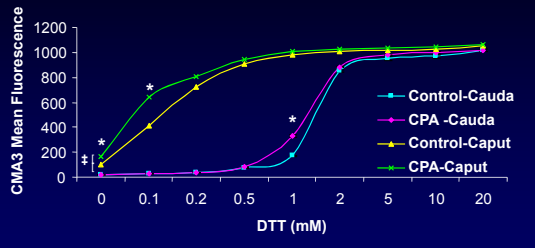
Protamination - Chromomycin A3



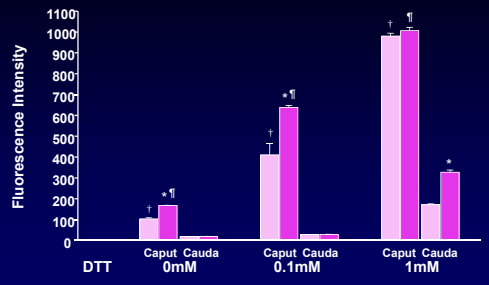
CHRONIC CPA EXPOSURE



CHRONIC CPA EXPOSURE

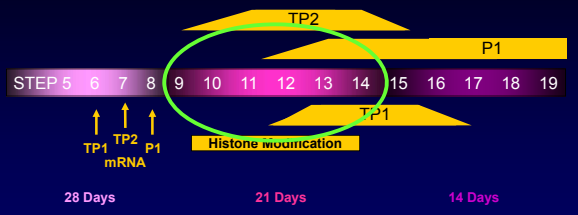


CHROMOMYCIN A3 - CHRONIC EXPOSURE



* Significantly different from matched controls
† Significantly different from control cauda sperm
¶ Significantly different from CPA cauda sperm

Chromatin Remodeling



Histone-protamine exchange is a key component of chromatin remodeling

NUCLEAR MATRIX EXTRACTION

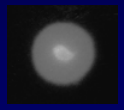
Separate Sperm Head and Tail by Sonication and Sucrose Density Gradient Centrifugation

Remove Peri-nuclear Material w/ 1% SDS

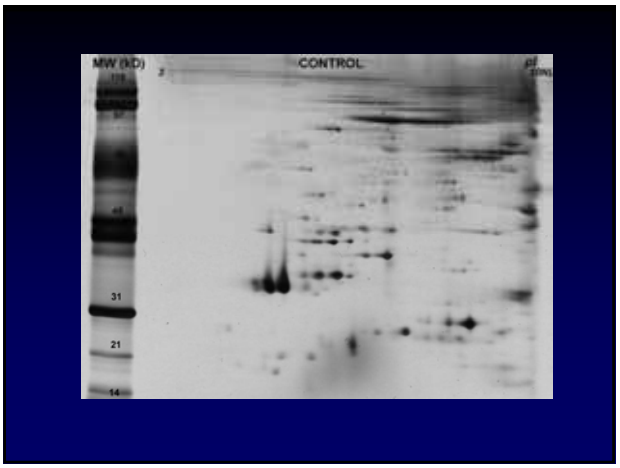
DTT and Low Salt (0.25M $(NH4)_2SO_4$)

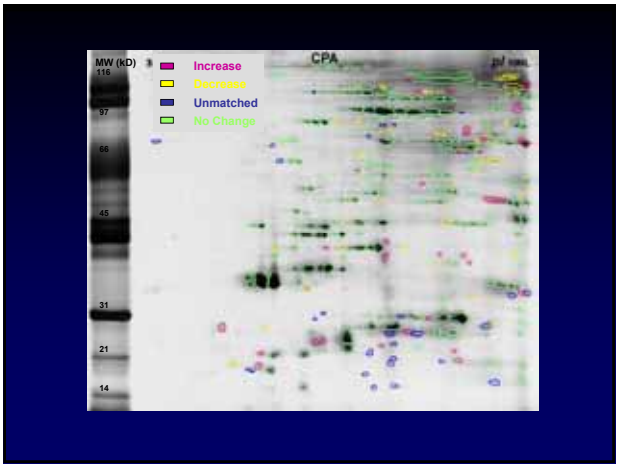
DNase Digestion of Chromatin

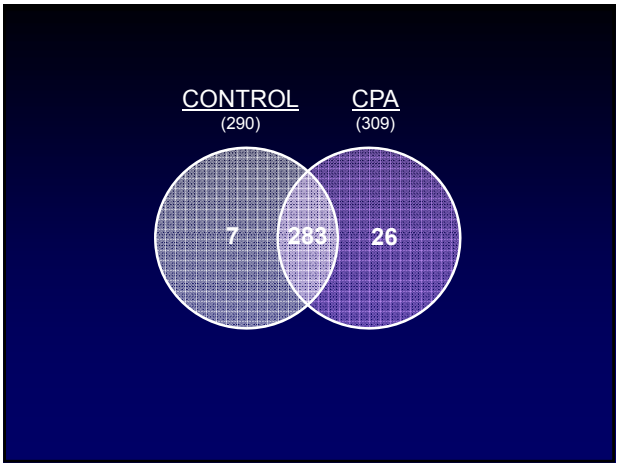
2D Gel Electrophoresis

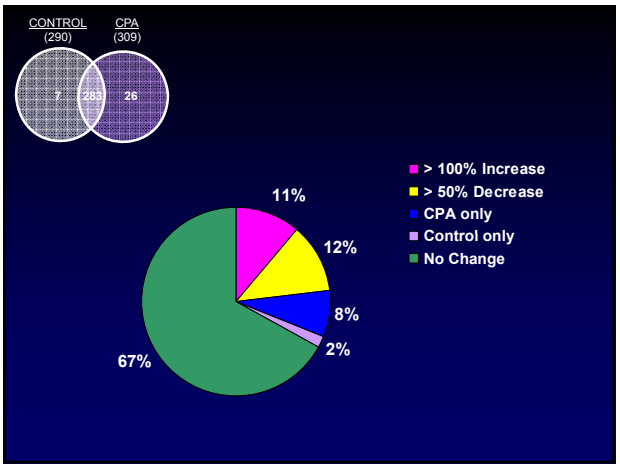


Sperm DNA-nuclear matrix halo structure
Biology of Reprod. 60:702-706, 2000


MW (kD)
CONTROL
31
21
14

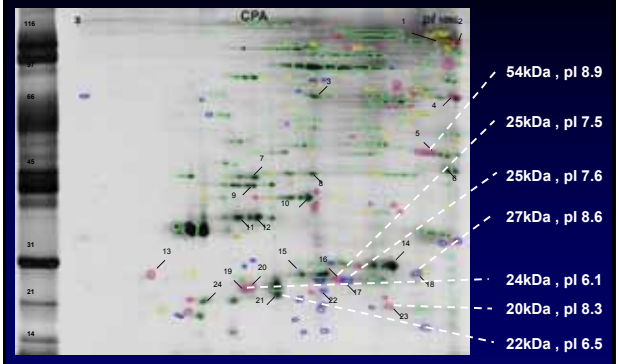

MW (kD)
CPA
Increase
Decrease
Unmatched
No Change
116
97
66
45
31
21
14


CONTROL
(290)
CPA
(309)
7
283
26

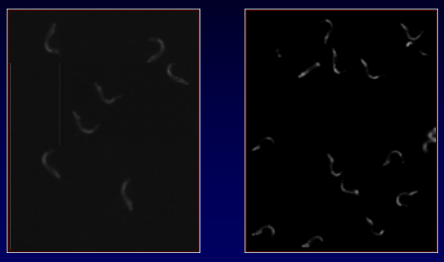


Nuclear Matrix Proteins Identified	Function
DnaJ/Hsp40, subfamily B, member 6	Co-Chaperone
DnaK-type molecular chaperone hst70	Co-Chpaerone
F-actin capping protein beta subunit	Cytoskeleton
Keratin K5	Cytoskeleton
Organic anion transporter/Testis specific transporter	Nucleocytoplasmic Transport
Phosphoprotein phosphatase 1 gamma catalytic chain	Signal Transduction
cAMP-specific 3,5-cyclic phosphodiesterase 4C	Signal Transduction
Chromodomain protein Y-chromosome-like	Transcriptional Co-repressor / Histone Acetyltransferase
Glutathione-S-transferase, mu 5	Antioxidant Defense
LPP LIM domain preferred translocation partner	Nucleocytoplasmic Transport
Outer dense fiber protein	Cytoskeleton
Phosphatidylethanolamine binding protein	Lipid Binding
Phospholipid hydroperoxide glutathione peroxidase	Antioxidant Defense
Poly(rC) binding protein 1	(Post-) Transcriptional Control
Proteosome subunit beta type 4 precursor	Metabolism
Similar to Ran-interacting protein MOG1	Nucleocytoplasmic Transport
Triosephosphate isomerase 1	Glycolysis
Hypothetical protein DKFZp434H2215	?
Hypothetical protein MGC 26988	?
Unknown protein for MGC 95189	?

Glutathione Peroxidase 4



Nuclear Matrix Extracts: GPX4



Control CPA

Male mediated developmental toxicity: chemotherapy, sperm chromatin, psychosocial and progeny outcome

- Project 1: Impact of Combination Chemotherapy on Reproductive Health and Gamete Genetic Integrity in Humans
- Project 3: Impact of Chemotherapeutic Regimens on Male Reproduction and Progeny Outcome in the Rat Model
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The Team

Project 1:
Clinical Andrology

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McGill University
Bernard Robaire
Raghu Rajan
Cristian O'Flaherty
Farida Vaisheva

University of Montreal
Valerie Desilets

Health Canada
Tye Arbuckle

Project 2:
Psychosocial Study

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McGill University

University of Montreal
Marie Achille

Tom Baker Cancer Center,
University of Calgary
Barry Bultz

Project 3:
Animal Models

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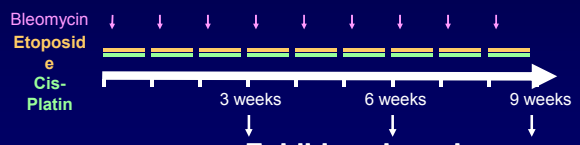
Barbara Hales
Louis Hermo
Jacquetta Trasler
Makoto Nagano
Geraldine Delbes
Donovan Chan
Farida Vaisheva
Ludovic Marcon
Adrienne Bieber

Grant # HGG-62294

Testis Cancer Chemotherpy: Animal Model

- **Testicular can cer chemotherapy cocktail (BEP):**
  - **Bleomycin:** causes cleavage of DNA strands
  - **Etoposide:** topoisomerase II inhibitor
  - **Cis-Platin:** alkylating agent causing DNA cross-links

- **Doses:** conversion from surface to mg/kg
- **0X, 1/3X, 2/3X and 1X** - Bleomycin: 1.5mg/kg/day
  - Etoposide: 15mg/kg/day
  -Cis-Platin: 3mg/kg/day



Epididymal cauda sperm

Animal Models

SD and BN rats

Regimens that mimic those used for
- **Non-Hodgkin's lymphoma (CHOP)**
- **Testis cancer (BEP)**

Assess consequences of treatment
- **Male reproductive system**
  - **(weights, hormones, histology)**
- **Sperm chromatin quality**
- **Progeny outcome**
- **Reversibility of effects/Germ stem cells**
- **Gene expression profiling during spermatogenesis**
- **Epigenetic effects - DNA methylation**
- **Stem cell transplantation**

Chronic treatment with BEP results in:

- Effects on body, testis, and epididymis weights
- Abnormal testis histology
- Decreased spermatid head count
- Significant effects on sperm motility, morphology, and quality
- No effects on litter size, sex ratio, pre- or post-implantation loss
- Decreased post-natal survival

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- in vitro, in vivo

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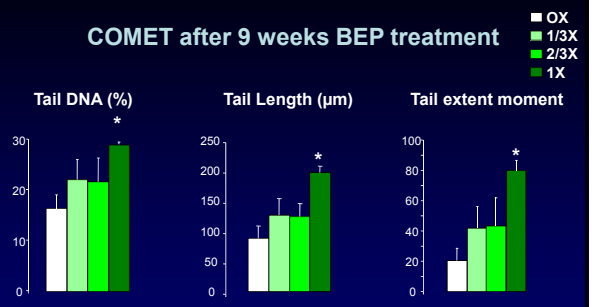
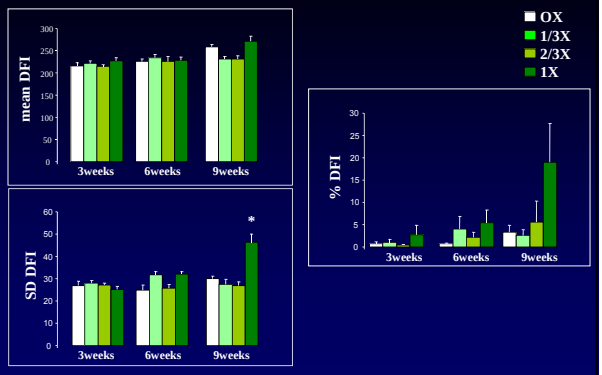
COMET Assay

COMET after 9 weeks BEP treatment



Increased number of DNA strand breaks

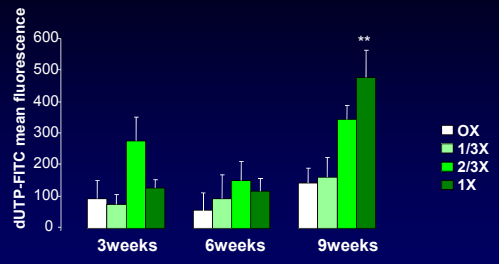
Acridine Orange Assay– Results



Increased susceptibility to denaturation =alteration of chromatin structure

TUNEL Assay – Results

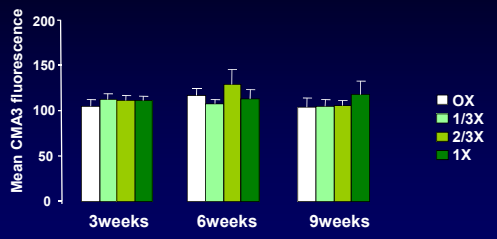
dUTP-FITC fluorescence after BEP treatment



Increased number of DNA strand breaks

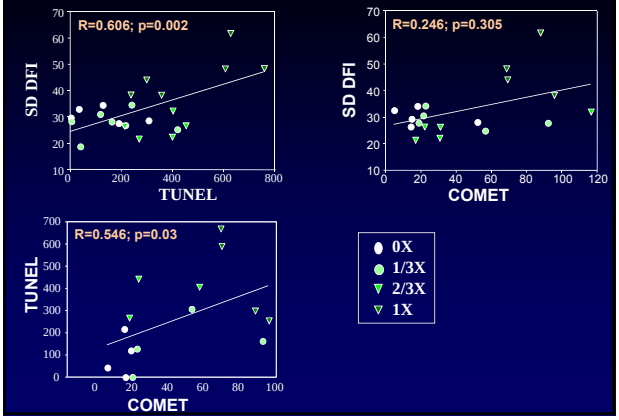
CMA3 assay

CMA3 fluorescence after BEP treatment



No change in protamine content

Correlation study (9 week data)



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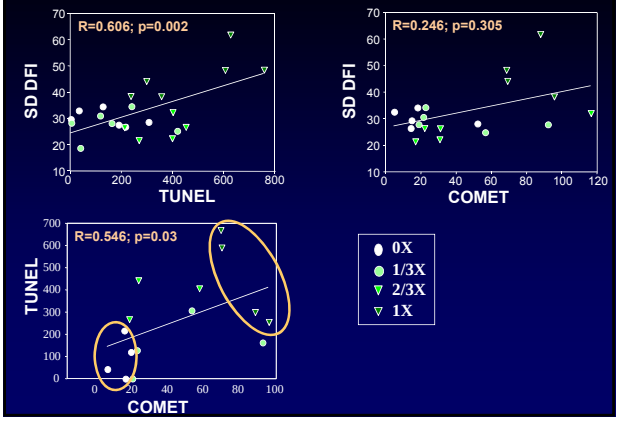
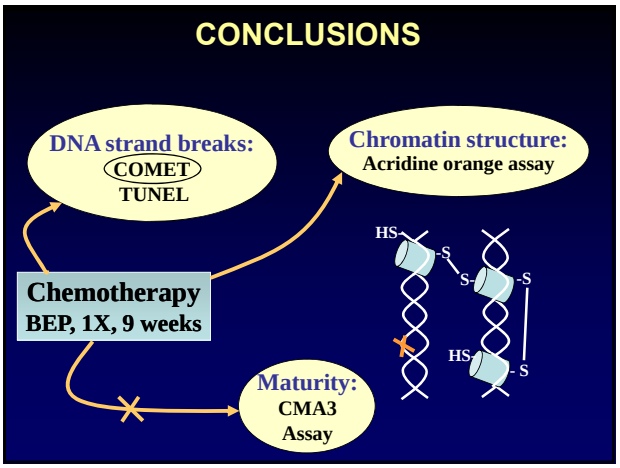


CONCLUSIONS



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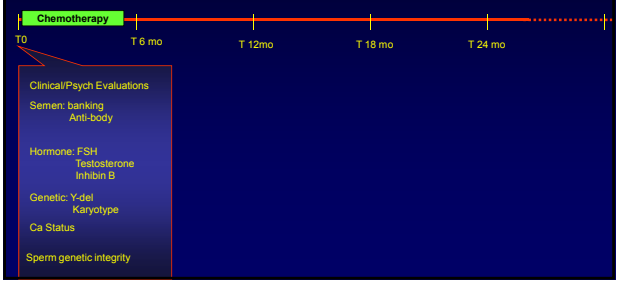
Chemo patients (N=30 for each cancer group)



Time Line

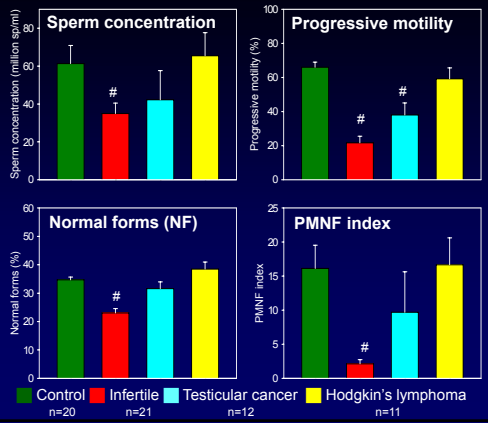
Study Gps

- Testis Ca — Chemo
- HL — Chemo
- NHL — Chemo

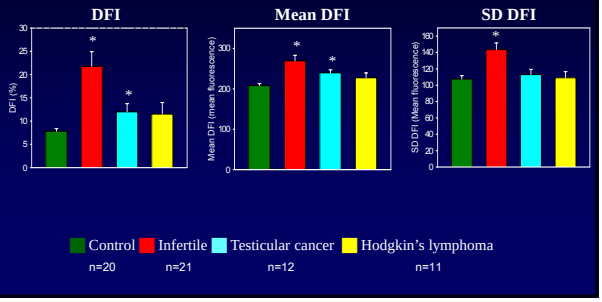
Control Gps

- Infertile Control
- Normal Control

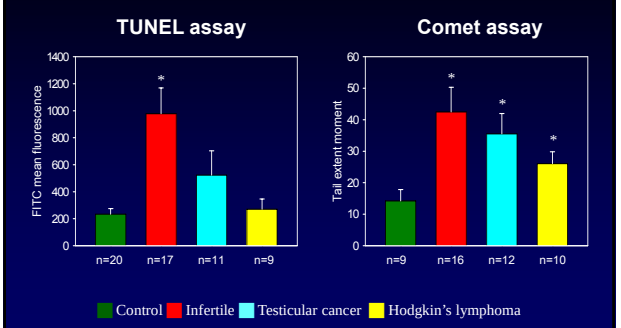
Semen Parameters



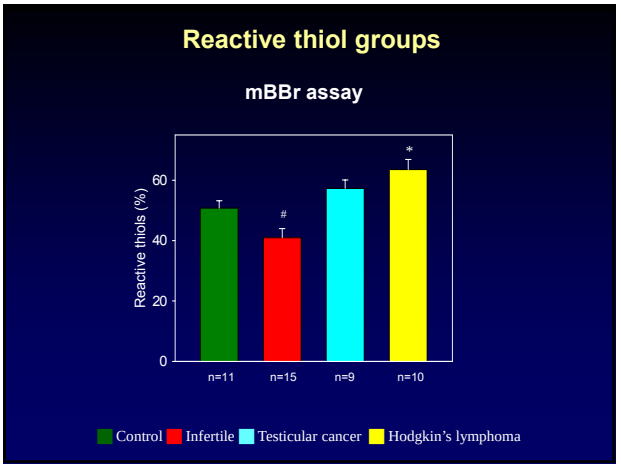
Acridine Orange Assay
Sperm chromatin structure assay® (SCSA®)



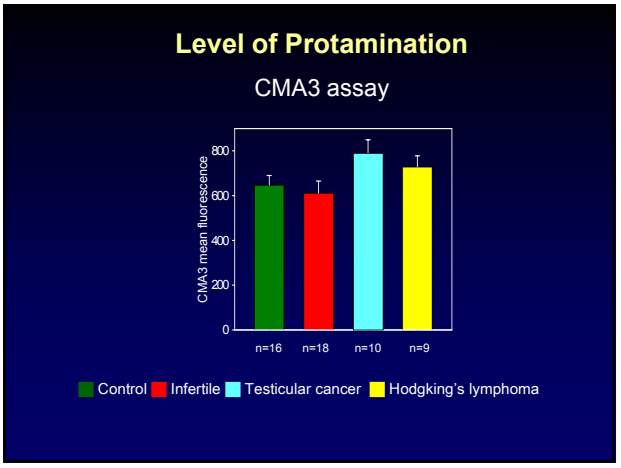
Sperm DNA strand breaks



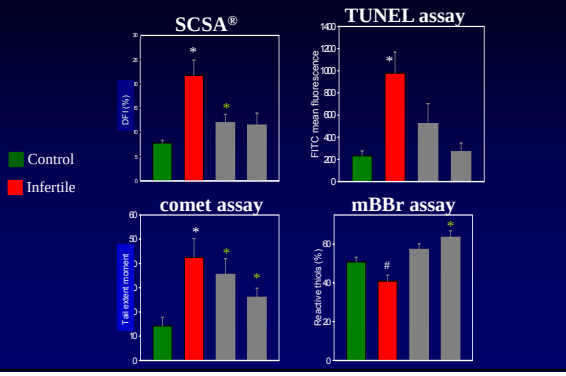
Reactive thiol groups



Level of Protamination



Reliability of assays to predict sperm chromatin quality based on the fertile and infertile populations



Assay	Criterion	Sensitivity		Specificity	
		%	95% IC	%	95% IC
DFI (SCSA®)	> 10.3 %	76	52.8-91.7	90	68.3-98.5
TUNEL	> 389 F.U.	92	63.9-98.7	94	71.2-99
comet	>18.38 mm	81	54.3-95.7	88.9	51.7-98.2
mBBr	< 32.8 %	42	15.3-72.2	100	66.2-100

Sensitivity is the proportion of true positives that are correctly detected by the assay.

Specificity is the proportion of true negatives that are correctly detected by the assay.

Testicular cancer patient sperm quality: PRIOR to treatment

	Semen analysis (PMNF index)	
	Normal	Abnormal
DFI (SCSA)	40	43
TUNEL	40	67
Comet	100	29
mBBr	0	100

Red: patients with low WHO sperm parameteres (%).

Green: patients with high WHO sperm parameteres (%).

Hodgkin's lymphoma patient sperm quality: PRIOR to treatment

	Semen analysis (PMNF index)	
	Normal	Abnormal
DFI	30	100
TUNEL	22	n.d.
comet	80	n.d.
mBBr	0	100

Red: patients with low sperm chromatin quality (%).

Green: patients with high sperm chromatin quality (%).

n.d.: not determined

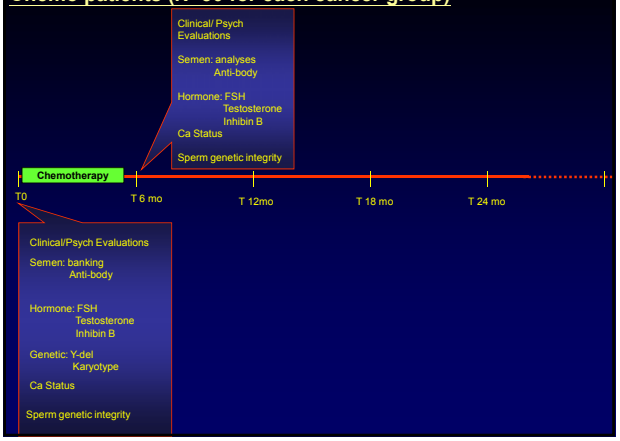
Correlations among sperm chromatin assays and semen parameters

	Sperm concentration ($x10^6$ sp/ml)	Total Sp ($x10^6$ sp)	Motility (%)	Progressive motility (%)	Normal forms (%)	PMNF Index
DFI (n=64)	--	--	-0.64	-0.60	-0.33	-0.34
Mean-DFI (n=64)	--	--	-0.57	-0.51	-0.28	-0.27
SD-DFI (n=64)	-0.34	--	-0.52	-0.51	-0.36	-0.41
Free SH (n=64)	--	--	0.35	0.40	0.49	--
Log FITC fluoresc. (n=48)	--	--	-0.34	-0.33	--	--

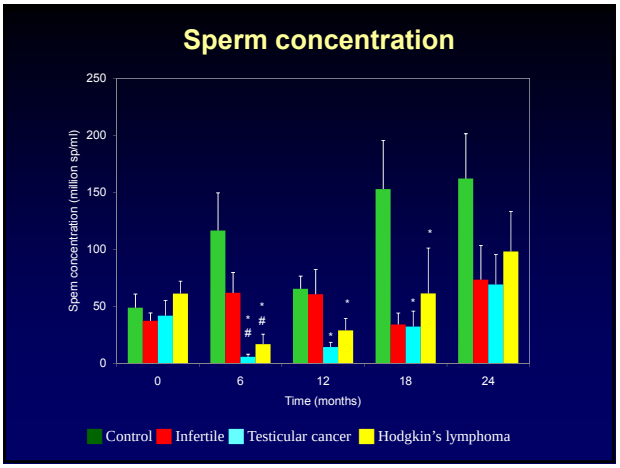
CONCLUSIONS

* Spermatozoa from cancer or infertile patients have lower sperm chromatin quality than in the control group.
* SCSA®, TUNEL and comet assays similarly predict sperm chromatin quality in infertile patients.
* In cancer patients, sperm chromatin anomalies can be identified best using the comet assay.
* Routine semen parameters fail to predict sperm chromatin quality.

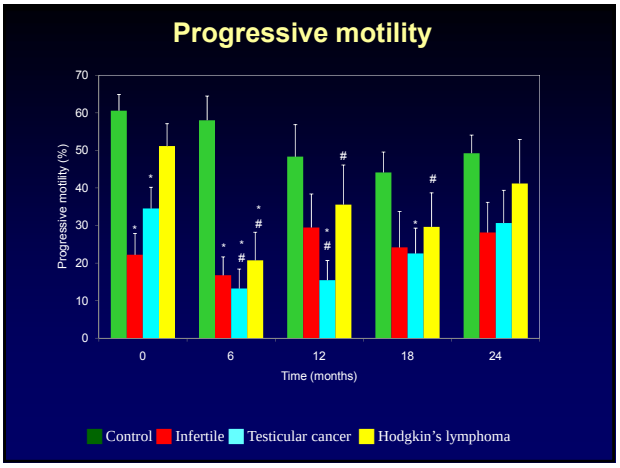
Chemo patients (N=30 for each cancer group)



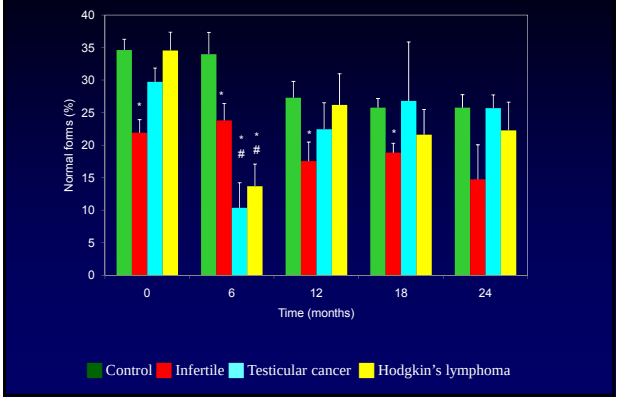
Sperm concentration



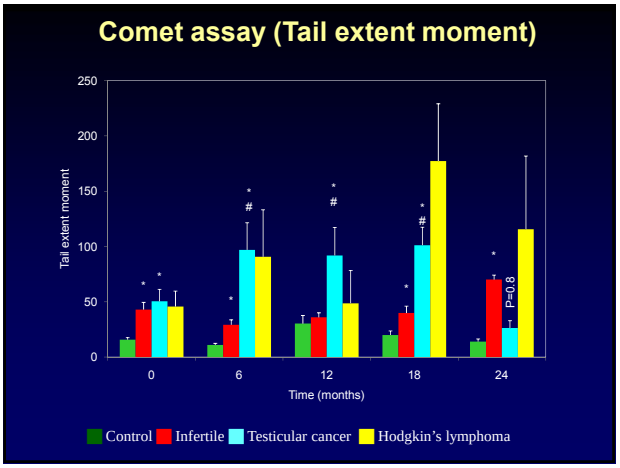
Progressive motility



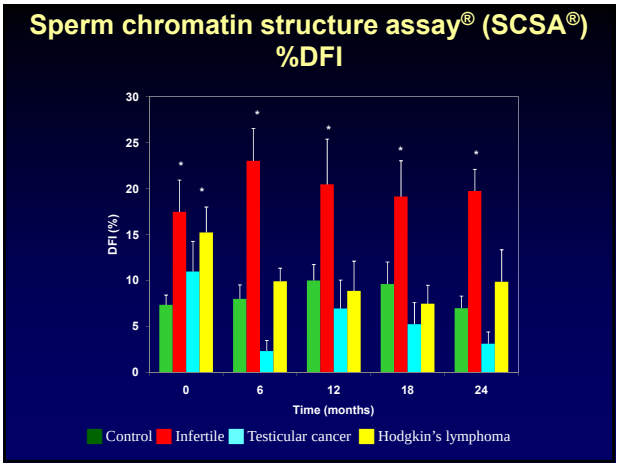
Normal forms



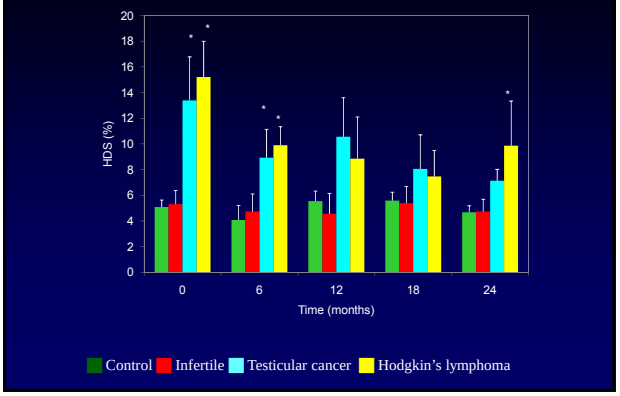
Comet assay (Tail extent moment)



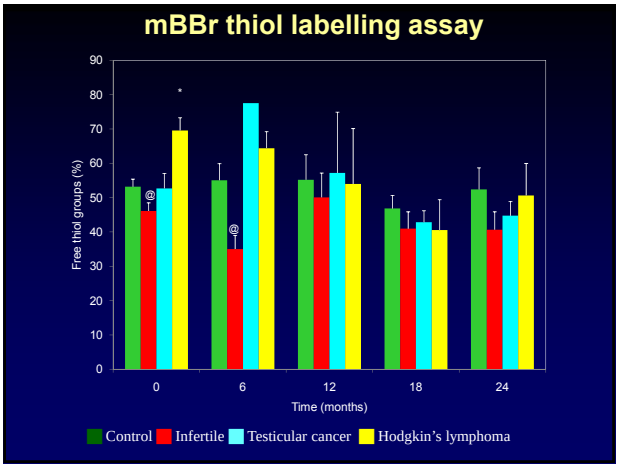
Sperm chromatin structure assay® (SCSA®) %DFI



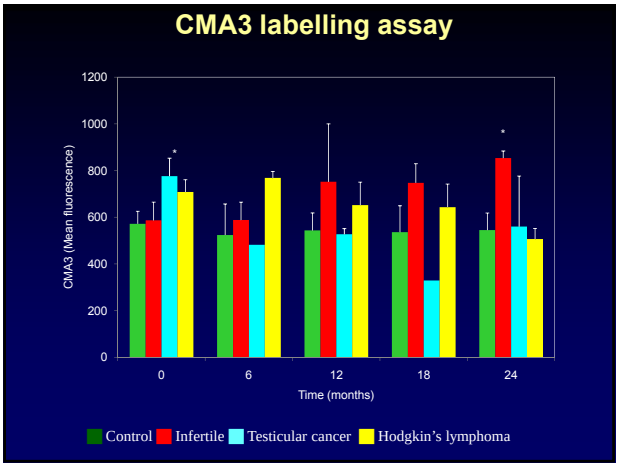
Sperm chromatin structure assay® (SCSA®) %HDS



mBBr thiol labelling assay



CMA3 labelling assay



SUMMARY

- Pre-chemotherapy, both cancer groups had poorer semen quality compared to controls.
- Among TC and HL patients, 67% and 60%, respectively, had < $5x10^6$ sperm/ml at 6 months post-chemotherapy.
- At 24 months, 60% and 57% of TC and HL, respectively, had normal sperm concentrations.

SUMMARY

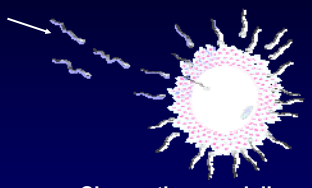
- Pre-chemotherapy, sperm DNA damage was higher in the cancer group than controls.
- This damage was increased further at 6 months and remained high by 24-month post-treatment.
- Pre-chemotherapy, cancer patients have low sperm DNA compaction.
- Levels of free thiols and of protamination in cancer patients are similar to controls at 18 months. In contrast, HDS remains high up to 24 months after treatment.

CONCLUSIONS

- Sperm generated post-chemotherapy maintain a significant degree of chromatin damage. Thus, survivors of TC and HL are at risk of having abnormal reproductive outcome.
- Proper counseling to these patients on reproductive risks and fertility preservation prior to chemotherapy is recommended.

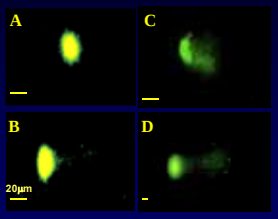
Assessing Consequences of Sperm Chromatin damage

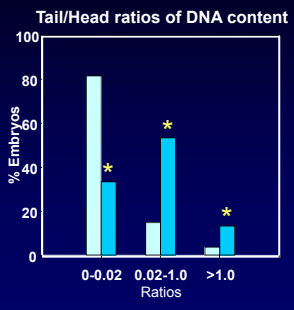
Toxicant



- Chromatin remodeling
- Pronuclear formation
- Zygotic genome activation
- Cell lineage determination

DNA damage in CPA-exposed sperm is transmitted to the conceptus

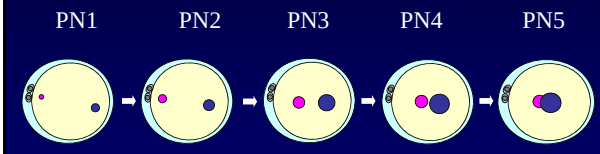




Pronuclear Stages in Zygotic Development

SALINE or CPA 6 mg/kg/day
4 WEEK DURATION

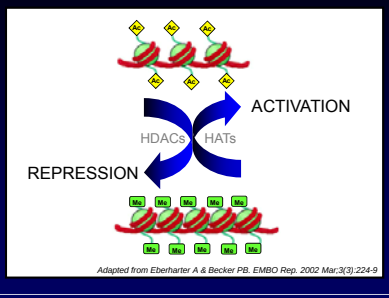
Naturally Cycling females
WEEK 5 OF TREATMENT



Maternal Pronucleus Paternal Pronucleus

Adapted from Adenot et al., Development 124, 4615-4625 (1997)

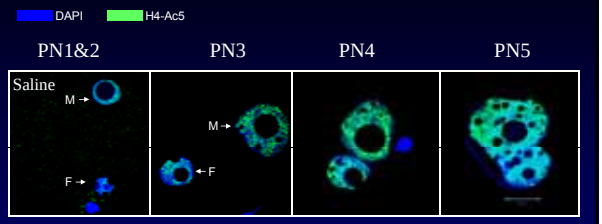
Histone Acetylation and Deacetylation Regulate Gene Transcription



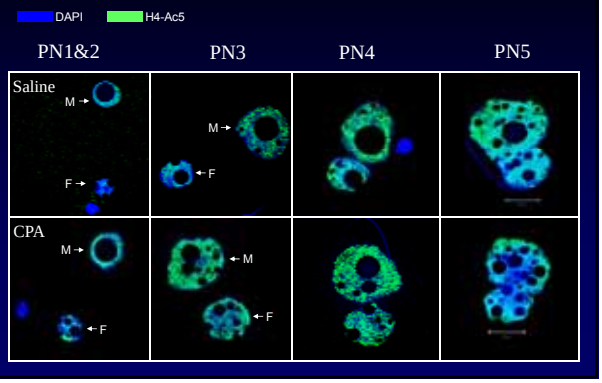
Adapted from Eberharter A & Becker PB. EMBO Rep. 2002 Mar;3(3):224-9

Core histone acetylation ⟶ correlated with activation of the zygotic genome in early embryonic development.

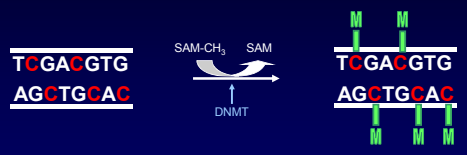
Abnormal Histone Acetylation Patterns In Rat Zygotes Fertilized by CPA Exposed Spermatozoa



Abnormal Histone Acetylation Patterns In Rat Zygotes Fertilized by CPA Exposed Spermatozoa



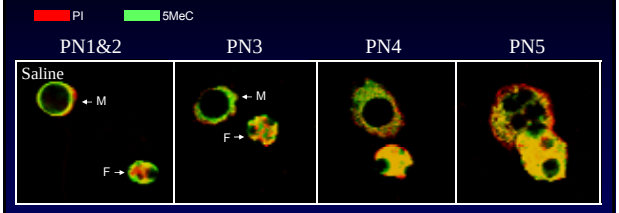
DNA Methylation: 5-position of cytosine



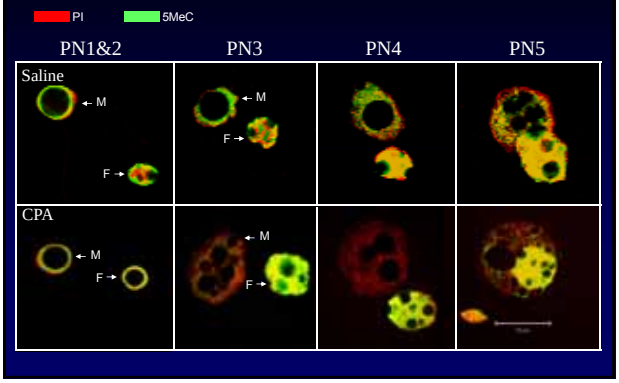
SAM = S-adenosylmethionine

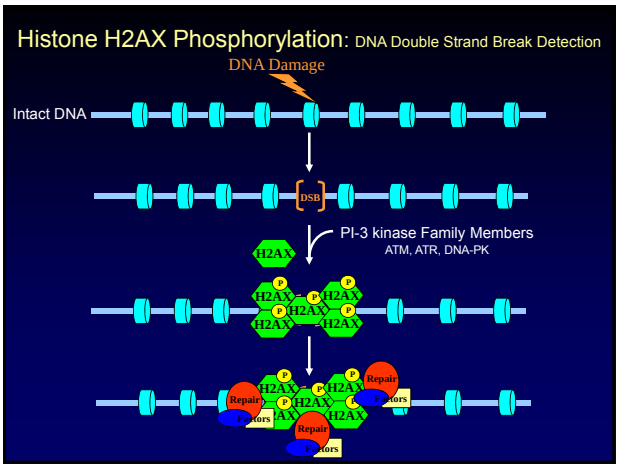
DNMT = DNA methyltransferases

Abnormal DNA Methylation Patterns In Rat Zygotes Fertilized by CPA Exposed Spermatozoa

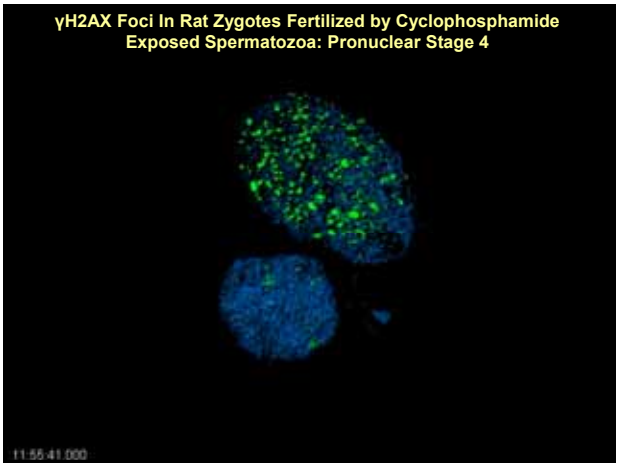


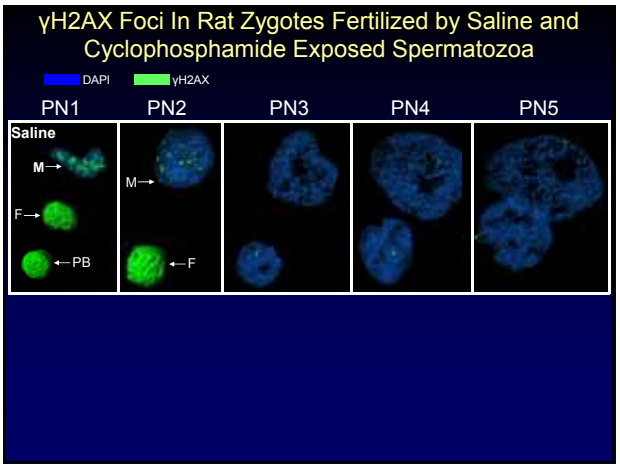
Abnormal DNA Methylation Patterns In Rat Zygotes Fertilized by CPA Exposed Spermatozoa

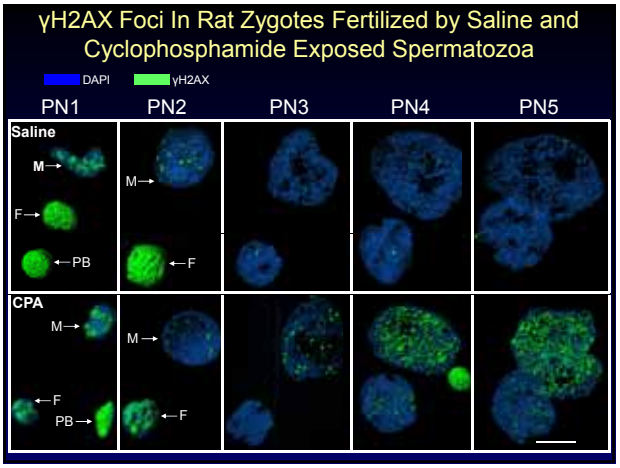


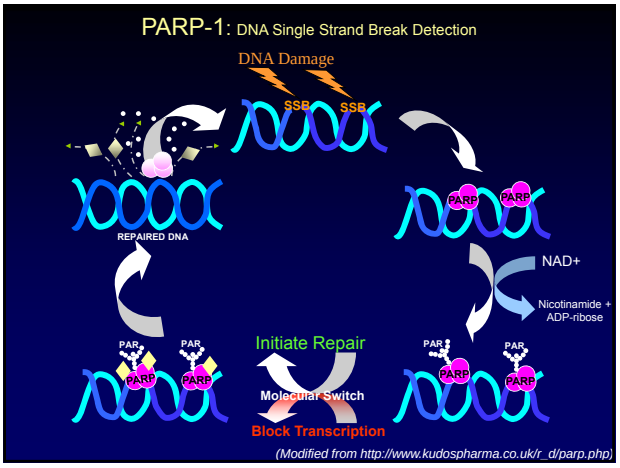

Histone H2AX Phosphorylation: DNA Double Strand Break Detection
DNA Damage
Intact DNA
DSB
PI-3 kinase Family Members
ATM, ATR, DNA-PK
H2AX
P
Repair
Factors


γH2AX Foci In Rat Zygotes Fertilized by Saline Exposed Spermatozoa: Pronuclear Stage 4

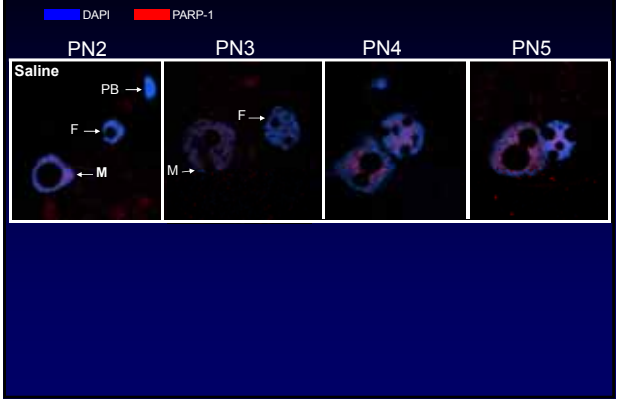

γH2AX Foci In Rat Zygotes Fertilized by Cyclophosphamide Exposed Spermatozoa: Pronuclear Stage 4


γH2AX Foci In Rat Zygotes Fertilized by Saline and Cyclophosphamide Exposed Spermatozoa
DAPI
γH2AX
PN1
PN2
PN3
PN4
PN5
Saline
M
F
PB
M
F

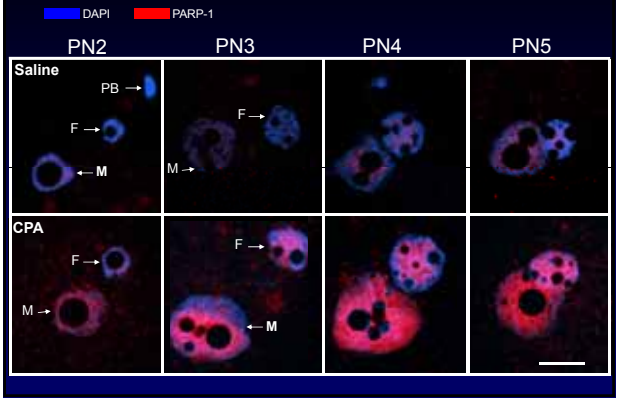

γH2AX Foci In Rat Zygotes Fertilized by Saline and Cyclophosphamide Exposed Spermatozoa
DAPI
γH2AX
PN1
PN2
PN3
PN4
PN5
Saline
M
F
PB
M
F
CPA
M
F
PB
M
F


PARP-1: DNA Single Strand Break Detection
DNA Damage
SSB
SSB
REPAIRED DNA
PARP
PARP
NAD+
Nicotinamide +
ADP-ribose
PAR
PAR
PARP
PARP
Initiate Repair
Molecular Switch
Block Transcription
(Modified from http://www.kudospharma.co.uk/r_d/parp.php)

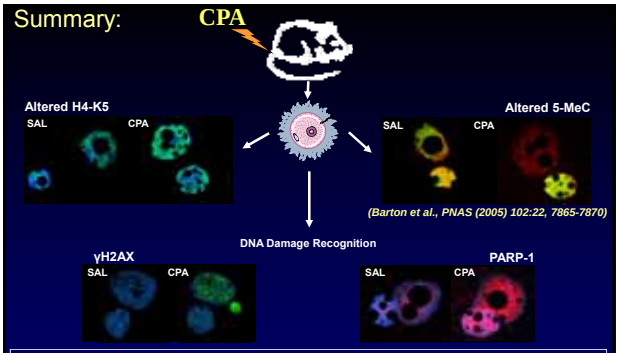
PARP-1 In Rat Zygotes Fertilized by Saline and Cyclophosphamide Exposed Spermatozoa



PARP-1 In Rat Zygotes Fertilized by Saline and Cyclophosphamide Exposed Spermatozoa



Summary:



- Pronuclear cross talk is implemental in the regulation of epigenetic reprogramming and DNA damage recognition in the zygote.
- The response of the early embryo to disturbances in spermatozoal genomic integrity plays an important role in determining developmental outcome.