Sperm in genetic peril
Oxidative stress and sperm DNA damage

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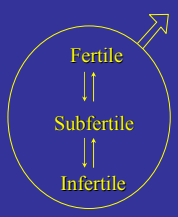
Sub/infertility: a multifactorial disease

Genetic defects
- CAVD
- Robertsonian translocations
- Y-chromosome deletions

→ CAVE ICSI

Life-style
- Nutrition → obesity
- **Tabacco**
- Alcohol
- Drugs
- Clothing
- Hot-baths
- (stress)



Environment & Profession
- Hormone disruptors
  xeno-oestrogens
  anti-androgens
- Toxic substances
  (lead, CS_2, ...)

Genital diseases
- **varicocele**
- **genital infections**
- anti-sperm-antibodies
- acquired damage
- iatrogenic

sperm DNA damage

Sperm from infertile men

- impaired rates of fertilization in vivo / in vitro
- impaired embryo development
- high rates of pregnancy loss
- high rates of offspring morbidity
  - Dominant genetic disease
  - Infertility
  - Cancer (childhood)

Baker & Aitkin, Reprod Biol Endocrinol, 3, 67, 2005

sperm DNA damage: different mechanisms

Abnormal chromatin packaging

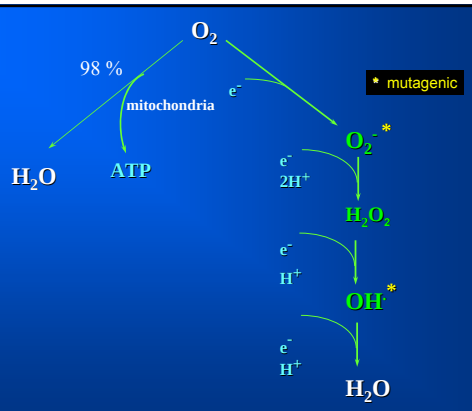
Manicardi et al., 1995, Sakkas et al., 1999

Apoptosis

Sakkas et al., 1999

Oxidative stress mechanism

Sharma & Agarwal, 1996; Aitkin & Krausz, 2001; Agarwal & Said, 2005; Lewis & Aitkin, 2005

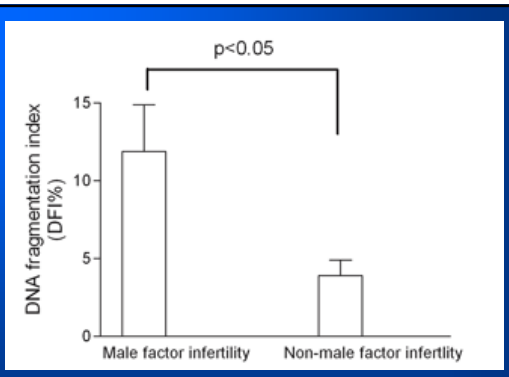


Free O_2^- radicals

- Cataract
- Collagen diseases
- Cancer
- Age
- AIDS
- ARDS
- ... Male sub(in)fertility

Evidence for oxidative stress and ⇓ sperm quality

- Sperm motility & sperm-oocyte fusion negatively correlated with lipoperoxidative potential
  - Jones et al., 1978,1979, Aitken et al., 1993; Gomez et al., 1998
- High levels of oxidative DNA damage in spermatozoa of infertile men compared to fertile men
  - Irvine et al., 2000; Henkel et al., 2003; 2005

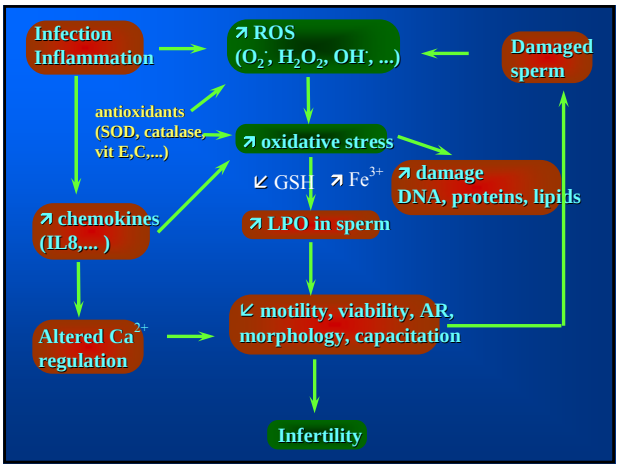


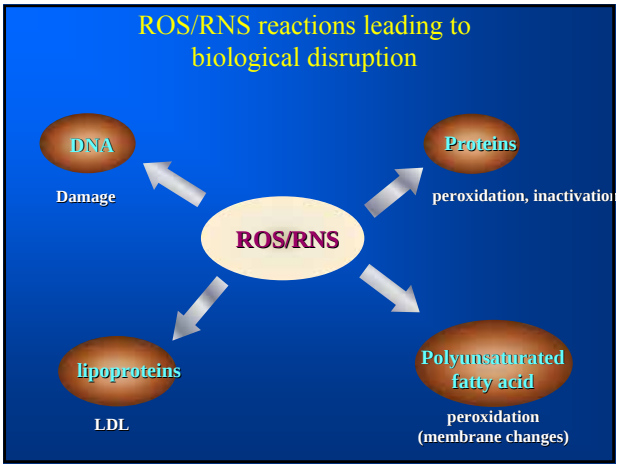
M Appasamy, RBM Online, 14,159, 2007

Sources of oxidative stress

- Spermatozoa // Polymorhonuclear leucocytes
  - Low levels of ROS required for nl sperm function
  - Inflammation (testis, epididymis, prostate ..) → high numbers of defective sperm & leucocytes → oxidative stress

Oxidative damage of mitochondrial DNA
⇓
reduced ATP production
⇓
nuclear DNA damage





Male subfertility : Sources of oxidative stress

Genital tract infections

Varicocele

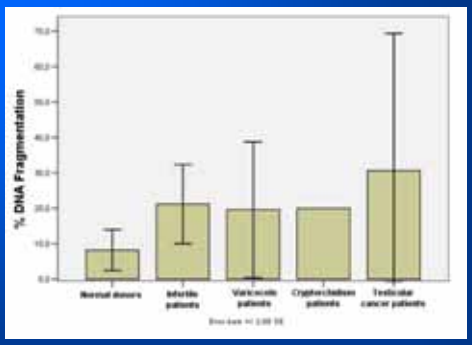
Undescended testis

Ageing

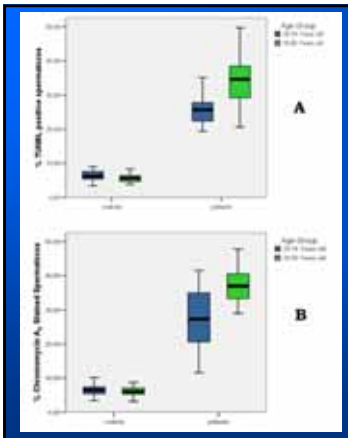
Cancer

Smoking

...



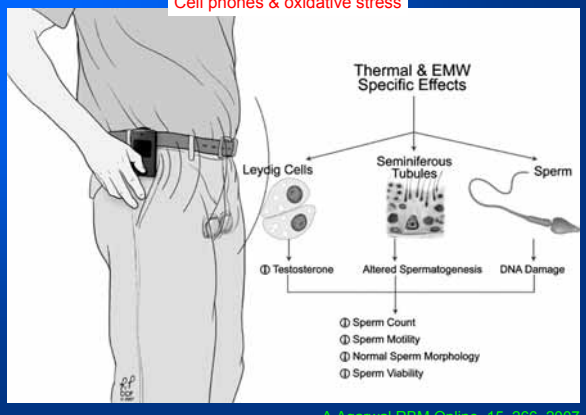
Angelopoulou et al., Reprod Biol Endocrinol, 5, 36, 2007



The effect of age on DNA fragmentation and chromatin packaging.
61 OAT patients were divided into two age subgroups
(20–34 yrs, n = 30; 35–50 yrs, n = 31).
49 healthy fertile controls were also divided according to their age
In the control group, the differences observed between the two age subgroups were not statistically significant.
The older patient subgroup demonstrated a significantly higher percentage of TUNEL positive ($P < 0.001$) and CMA3 stained ($P < 0.001$) spermatozoa compared to the younger patient subgroup.

Angelopoulou et al., Reprod Biol Endocrinol, 5, 36, 2007

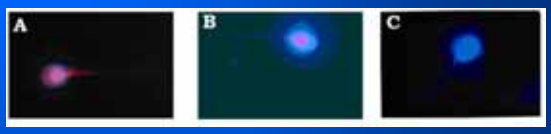
Cell phones & oxidative stress



A Agarwal,RBM Online, 15, 266, 2007

Detection of DNA damage: methods

Single-cell gel electrophoresis (Comet assay)	⇒ Evaluates DNA integrity (single & double-strand breaks)
TUNEL	⇒ Evaluates DNA fragmentation (single & double-strand breaks)
Acridine Orange Technique (AOT)	⇒ Distinguish between single & double-stranded
Sperm Chromatin Structure Assay (SCSA)	⇒ Acid DNA denaturation
In situ nick translation	⇒ Single-strand DNA breaks
Sperm Chromatin Dispersion Test (SCD)	⇒ Examines susceptibility of sperm DNA to acid denaturation
Acid Aniline blue	⇒ Stains lysine residues from persisting histones
Chromomycin A3 – CMA_3	⇒ Indirect visualization of nicked denatured DNA



TUNEL assay. TUNEL-positive nuclei (with double-strand nuclear DNA fragmentation) of spermatozoa as represented by the intense (A) and dull (B) Texas red fluorescence in the nuclear region. The healthy nuclei (without DNA fragmentation) are stained blue with DAPI (C) used as counterstain.

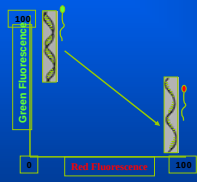
Angelopoulou et al., Reprod Biol Endocrinol, 5, 36, 2007

CMA3 staining. Two types of staining patterns were identified, bright and dull yellow fluorescence of the sperm nuclei (abnormal chromatin packaging) (A, B) and blue staining with DAPI in the healthy nucleus (normal chromatin packaging) seen in C.

Angelopoulou et al., Reprod Biol Endocrinol, 5, 36, 2007

Sperm Chromatin Structure Assay - SCSA

Native ds DNA: green fluorescence

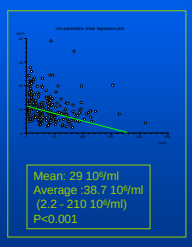
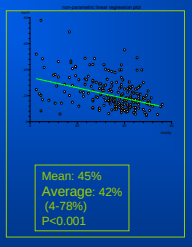
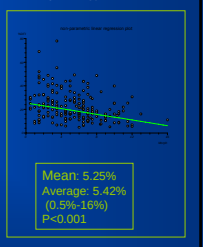


200 cells /minute
5000 cells analysed/sample

Denatured ss DNA: red fluorescence

Mouse, bull and human, Evenson et al., 1980

Negative correlation between % DFI and different sperm parameters

Concentration and DFI	Grade A and B motility and DFI	Morphology and DFI
		
Mean: 29 10^6/ml Average :38.7 10^6/ml (2.2 - 210 10^6/ml) P<0.001	Mean: 45% Average: 42% (4-78%) P<0.001	Mean: 5.25% Average: 5.42% (0.5%-16%) P<0.001

Sperm Chromatin Structure Assay - SCSA

- Larson et al (2000)
  - No ongoing IVF/ICSI pregnancies when DFI ≥27%.
- Larson-Cook et al (2003)
  - Correlation of IVF/ICSI outcome and SCSA parameters
- Viro et al (2004)
  - Correlation between SCSA parameters and fertilization, development to blastocyst stage and ongoing pregnancy rate

Impacts of sperm DNA damage

1. Impact on outcome of assisted reproduction
2. Impact on conventional semen parameters
3. Safety of using DNA damaged spermatozoa in ART
4. DNA damaged spermatozoa in cryopreservation

Review: B Ozmen, RBM Online, 14, 384, 2007

(1) Impact on outcome of assisted reproduction

Two recent meta-analyses: contradictory results

Evenson & Wixon, 2006
SCSA-method
DFI closely related to IUI, IVF and ICSI clinical PR

↔

Li et al, 2006
SCSA-method
DFI not related to IVF and ICSI clinical PR
TUNEL method
Only IVF clinical PR related to DNA damage
No relation with IVF & ICSI fertilization rates

Studies according to method of diagnosis	*Pregnancy rate*	*Embryo quality and development*	*Early pregnancy loss*	*Live birth*
TUNEL				
Lopes *et al.*, 1998	–	–	–	–
Høst *et al.*, 2000	–	–	–	–
Tomlinson *et al.*, 2001	Decrease	No relation	–	–
Benchaib *et al.*, 2003	Decrease	Decrease	–	–
Henkel *et al.*, 2004	Decrease	Decrease, stops	–	–
Seli *et al.*, 2004	No relation	Decrease	–	–
Huang *et al.*, 2005	No relation	No relation	–	–
Benchaib *et al.*, 2006	Decrease	Decrease	Increase	Decrease
Borini *et al.*, 2006	Decrease	–	Increase	Decrease
COMET[a]				
Tomsu *et al.*, 2002	Decrease	Decrease	–	–
Morris *et al.*, 2002	–	Decrease	Increase	Increase
Nasr-Esfahani *et al.*, 2005[b]	–	Decrease	–	–
SCSA or SCD[c]				
Larson-Cook *et al.*, 2003	Decrease	No relation	–	–
Gandini *et al.*, 2004	No relation	Decrease	–	–
Virro *et al.*, 2004	Decrease	Decrease	–	Decrease
Bungum *et al.*, 2004	No relation	–	–	–
Payne, 2005	No relation	–	–	–
Zini *et al.*, 2005	No relation	Decrease	–	–
Check *et al.*, 2005	Decrease	Decrease	Increase	Decrease
Bungum *et al.*, 2007[d]	Decrease	–		

(2) Impact on conventional semen parameters

Most studies: DNA fragmentation rates higher in OAT patients
Gandini et al., 2000; Siddighi et al., 2004, Huang et al., 2005

No strong correlation between DFI and OAT
Larson-Cook et al., 2003

Drop of DFI (chromatine structure) after swim-up procedure
Gandini et al., 2004

Selection less powerful after gradient techniques
Sakkas et al., 2000; Tomlinson et al., 2001

(3) Safety of using DNA damaged spermatozoa in ART

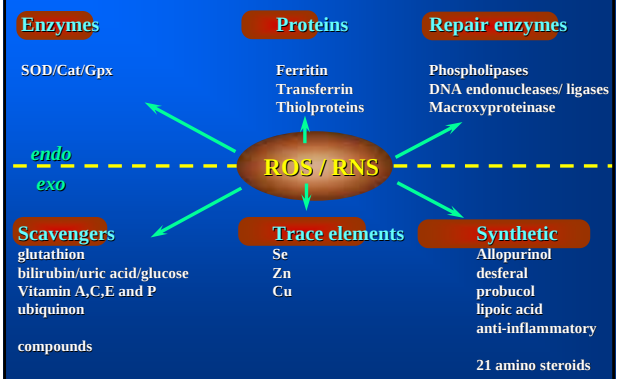
- **Sperm DNA damage** (Aitkin & Krausz, 2001)
  → promutagenic // mutations after fertilization
  → fixed in germline – responsible for infertility
  → childhood cancer – imprinting diseases
- **Follow-up studies of ICSI babies are reassuring**
  (Bonduelle et al., 1996, 1998, 2003; Hansen et al., 2002)
- **Significant decrease in PR & IR = defence mechanism**
  (Larson-Cook et al., 2003)
- **Selection of the best spermatozoon for ICSI**
  → High magnification optical system (Hazout et al., 2006)
  → Magnetic cell sorting (annexin V microbeads)
  (Said et al., 2005, 2006)

(4) DNA damaged spermatozoa in cryopreservation

- Sperm DNA damage can be induced by cryopreservation (animal studies)
- Probably no difference between standard slow-freezing/thawing and vitrification

Treatment & prevention of oxidative stress

Endogenic vs. exogenic ROS/RNS neutralisation



ROS neutralization & prevention mechanisms

- SOD superoxide dismutase
  $O_2^- + H_2O \longrightarrow H_2O_2$
  Cu, Zn dependent
- Catalase
  $2H_2O_2 \longrightarrow 2H_2O + O_2$
- Vitamin A,E,C — Redox chain
  Protection of PUFA's through competition with ROS
- Fe-chelation — Transferrin, Apotransferrin
  Prevention Fenton reaction
  $H_2O_2 + Fe^{2+} \longrightarrow OH^- + OH^- + Fe^{3+}$
  $H_2O_2 + Fe^{3+} \longrightarrow Fe^{2+} + HO_2^- + H^+$
  $\Rightarrow 2H_2O + O_2$
- Glutathion GSH — Redox chain
- Gpx glutathion peroxidase
  conversion oxidized into reduced GSH
  selenium dependent enzyme

Antioxidants

Side-effects

high dose vit A → embryotoxic teratogenic

high dose vit C → ⇑ abortion

⇑ renal calculi (oxalate) (offspring)

Treatment & prevention of oxidative stress

In-vitro studies (laboratory)

Human // non-randomized

Human // randomized

Reducing DNA-damage: preparation of semen

- Gradient centrifugation / swim-up / glass wool filtration → higher sperm DNA integrity
- Electrophoretic separation of sperm → higher sperm DNA integrity
  - High quality sperm = more electronegative
  - Separated from other electronegative cells due to small cross-sectional size

Kirchhoff & Schroter, 2001; Giuliani et al., 2004; Ainsworth et al., 2005

Table 1. Studies in animals and humans demonstrating the effects of antioxidant supplementation in IVF media.

Authors	***Antioxidant supplementation***	***Effects of antioxidants***
Animal studies		
Ali *et al.*, 2003	Cysteine, N-acetyl-l-cysteine, catalase and superoxide dismutase (SOD)	Addition of cysteine resulted in significant improvement in morula and blastocyst development rates. IVF – addition of antioxidants catalase, SOD and NAC (N-acetyl l-cysteine) significantly reduced morula and blastocyst stage development rates in bovine embryos.
Iwata *et al.*, 1998	SOD, catalase and mannitol	High concentration of glucose causes generation of reactive oxygen species (ROS). Low oxygen concentration significantly improved embryo development. Antioxidants SOD, catalase, and mannitol had no positive effects on embryo development.
Wang *et al.*, 2002	Vitamin C and vitamin E	Vitamin C was more effective than vitamin E in reversing ROS induced mouse embryotoxicity.
Human studies		
Tarin *et al.*, 2002	Ascorbate (62.5 µmol/l) supplementation in HTF (human tubal fluid) medium	No significant effects of ascorbate supplementation on fertilization.
Noda *et al.*, 1994	Low oxygen tension (5% O_2) and low illumination	Higher blastulation rates
Lighten *et al.*, 1998	Addition of human insulin-like growth factor-1 ligand	Enhanced embryo survival and blastocyst formation
Ali *et al.*, 2000	Antioxidants and chelators, i.e. catalase citric acid, desferroxamine, ethylenediaminetetraacetic acid, glutathione, pentoxyfylline and probucol	Better quality embryos generated

Treatment & prevention of oxidative stress

In-vitro studies (laboratory)

Human // non-randomized

Human // randomized

Reducing DNA-damage: use of testicular sperm

- Hypothesis:
  ROS can damage sperm for a long period in the epididymis → use of testicular sperm
  Henkel et al., 2003
- Contradictory results
  Greco et al., 2005; Nicopoullos et al;, 2004

Reducing DNA-damage: high-magnification ICSI

- Hypothesis:
  Intranuclear vacuoles, associated with alterations in chromatine packaging, are not visible with standard ICSI magnification

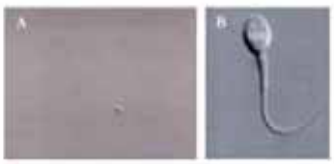
  → use of Nomarski differential interference contrast optics combined with a digitally enhanced secondary magnification system

- Better ICSI outcome in both high and low DNA damage

  Bartoov et al., 2002, 2003; Berkovitz et al., 2005; Hazout et al., 2006

Conventional ICSI
Magnification X 400

High-magnification ICSI
Magnification X 6600



Repeated ICSI-failure – 125 patients
Comparison: last conventional ICSI vs high-magnification ICSI
No difference in FR, cleavage & embryo morphology
Increased PR (40.8 vs 6.4 %), birth rate (17.6 vs 0 %)

Hazout A, RBM Online, 12, 19, 2006

In vivo studies

humans

Year	First Author	result(s)	study group
1992	Dawson	↗ sperm quality	heavy smokers
1993	Lenzi	↗ sperm quality	male factor infertility
1995	Kessopoulou	↗ fertility potential	high level of ROS in semen
1996	Geva	↗ fertility potential	normospermia IVF : poor fertilization

antioxidants ↔ sperm DNA fragm & decondensation

Menezo RBMOnline, 14, 418, 2007

- Study-population
  - >= 2 previous IVF-ICSI failures
  - DFI & sperm decondensation (SCMC) before and after 90 days of Tt
  - Tt: antioxidant vitamins + Selenium + zinc
- Results

  ⇒ decrease in DNA-fragmentation (- 19.2 %)

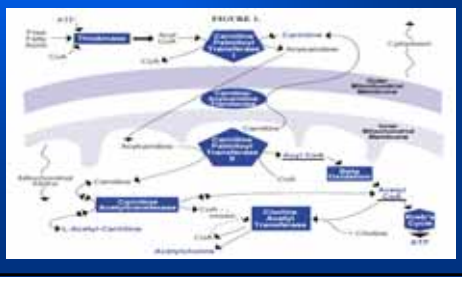
  ⇒ Increase in sperm decondensation (+ 22.8 %)
- Comment

Vit C: able to open the cystin net – may interfere with paternal gene activity

L-carnitine and semen quality

Agarwal & Said, RBM Online, 8, 376, 2004

Lack of good studies



Treatment & prevention of oxidative stress

In-vitro studies (laboratory)

Human // non-randomized

Human // randomized

Antioxidants and semen quality

Wong et al., F&S, 77, 491, 2002

- 108 fertile & 103 subfertile men
- Double-blind, randomized, placebo controlled
- 4 groups
  - Zinc sulphate 66 mg, folic acid 5 mg
  - Placebo + FA, placebo + Zn, Zn + FA, 2 placebos
- Results
  ⇒ Zinc and folate; associated with ⇑ total normal sperm count (74%) and ⇑ sperm morphology (4%)

Antioxidant and ICSI outcome

Greco et al., HR, 20, 2590, 2005

- 38 men: >= 15 % DFI
  1 failed ICSI attempt
- 1gr Vit C & 1gr Vit E for 2 months
- 29 (76 %): decrease in DFI ⇒ 2nd ICSI attempt
- Results
  No difference in FR, cleavage rate & embryo morphology
  Significant ⇑ clinical PR (48.2 vs 6.9 %) and IR (19.6 vs 2.2 %)

Antioxidant and IVF/ICSI outcome

Tremellen et al., Aust N Z J Obstet Gynaecol, 47, 216, 2007

- 60 men with severe OAT
- Randomized, double-blind placebo-controlled trial
- 3 months Menovit or placebo before IVF trial
- Results
  No difference in FR & embryo morphology
  Significant ⇑ viable* PR (38.5 vs 16 %)

  * viable = > 13 weeks

Conclusion

- Sperm DNA damage can cause male subfertility
- Oxidative stress is an important source of DNA damage
- Different methods of detection
- DNA damage: impact on outcome & safety of ART
- Future: new techniques for sperm selection and detection (ICSI)
- First optimistic results with oral antioxidants and ART outcome (IUI, IVF, NC)