Lebanese International Fertility Summit
2 – 3 October 2015
Hilton Beirut Habtoor Grand
Oxydative stress, DNA fragmentation & male infertility

Stéphane Droupy MD, PhD
Professor of Urology
CHU de Nîmes - Université Montpellier 1
Rationale

• Delayed conception affects 15% of couples

• Male factor subfertility accounts for up to 50% of these cases, and 50% are idiopathics.

• 30% to 80% of male factor subfertility cases are believed to be due to the damaging effects of oxidative stress.

• How to identify those men with infertility due to OS?

• Is it possible to prevent or to reverse OS damage?

• How antioxydant supplements can improve natural fertility or ART?

Sperm DNA and fertility
Sperm DNA

- Integrity of paternal genome → viable pregnancy
- Sperm DNA Fragmentation is incompatible with normal embryo development.

- **Causes of sperm DNA damage**
  - Protamine deficiency
  - Lipid peroxidation
  - Failure to repair DNA strand breaks

- Spermatozoa have few repair mechanisms,

- Major role of epididymis in post testicular DNA damage

Lewis et al Reproductive Biomedicine Online 2013
Semen analysis

- **Minimum standard** (WHO, 2010)
  - seminal plasma by volume, appearance and liquefaction
  - spermatozoa, concentration, motility and morphology

- **The strict Tygerberg criteria for morphology** (WHO)
  - spermatozoa capable of penetrating cervical mucus and binding to the zona pellucida

- No link between normal head morphology and the genetic quality of a spermatozoon

- Sperm DNA testing add further information
Semen analysis / DNA damage

• Progressive motility is the only semen parameter that correlates with sperm DNA damage.
  - a real-time functional test of sperm vitality.

• Fertilization rates dependent upon both sperm progressive motility and DNA fragmentation,
  - DNA Frag: OR: 24.18 (95% CI 5.21–154.51)
  - Progressive motility OR: 4.81 (95% CI 1.89–12.65)

Gharagozloo and Aitken Human Reprod 2011
DNA damage and fertility

- Fertile men with normal semen parameters = high levels of DNA integrity,

- Infertile men, with abnormal semen parameters, = decreased DNA integrity.

- A significant number of infertile men with normal semen parameters have abnormal DNA integrity despite

- DNA damage (fragmentation - decondensation) may explain idiopathic infertility
Analysis of sperm DNA integrity
Analysis of sperm chromatin integrity

- **The Comet assay**
  - single-cell gel electrophoretic assay that quantifies broken strands of DNA in individual spermatozoon
  - Suitable for oligozoospermic and testicular samples

- **Clinical Thresholds:**
  - Male infertility (25%), OR: 120 (95% CI 13–2700)
    - 95% of fertile donor <25%
    - 98% infertile men >25%
  - Success with IVF (25–50%)
  - Need for ICSI (>50%)

Analysis of sperm chromatin integrity

- Sperm chromatin structure assay (SCSA)
- Measure of DFI and the presence of immature sperm nuclei with abnormal proteins and/or altered protamine/histone ratios
- Threshold for natural and intrauterine insemination conception: 30% DFI
- In OATS: 10%

Analysis of sperm chromatin integrity

- The sperm chromatin dispersion (SCD) (Halo test)
- Cutoff value of 27% in couple when women has a reduced ovarian reserve to predict live birth.

**Fig. 1** Nucleoids from human sperm cells obtained procedure: Nucleoids with big halo of DNA dispersed sized (b), small (c) and without halo and degenerated.

Analysis of sperm chromatin integrity

- **Terminal deoxynucleotidyl transferase (TdT)** mediated deoxyuridine triphosphate (dUTP)-fluorescein **Nick-End Labelling assay**

- **Prediction of male infertility:** 20%
  - No fertile donor above 20%
  - 65% of infertile men > 20%

Correlation was found between the cytometric assays and SCSA, as has been previously reported (Chohan et al., 2010; Garcia-Peiró et al., 2011). This is given that the two assays are thought to be measuring different aspects of chromatin integrity. The presence of many single-stranded DNA breaks could lead to double-stranded DNA damage. This is interesting as SCD and SCSA might be detecting some aspects related to chromatin fragmentation, and Comet and TUNEL assays have been shown to detect DNA damage. These data suggest that differences between their values. These data suggest that differences between their values. These data suggest that differences between their values.

© 2013 American Society of Andrology and European Academy of Andrology

Ribas Maynou et al Andrology 2013
Sperm nuclear decondensation index (SDI)

• Failures in condensation induce delays in the first cell cycle with further detrimental consequences at some point for the developing embryo.

• High decondensation:
  - ‘no fertilization’ syndrome in ICSI
  - early developmental arrests
  - Miscarriages

• The SDI can be measured by microscopy on 200 cells using aniline blue.

• No pregnancy if SDI > 30%

Hamidi et al, Zygote 2014
Oxydative stress and fertility
Oxydative stress

• **Oxidative stress:**
  - reactive oxygen species (ROS) > the semen’s natural antioxidant defences
  - cellular damage to the sperm

• **Spermatozoa are vulnerable to oxidative stress**
  - high polyunsaturated fatty acid content Mb: **lipid peroxidation**
  - Deficiencies in intracellular anti-oxidant enzyme protection
  - Limited capacity for DNA repair

• Reproductive tract, epididymal and seminal plasmas, contains enzymatic and non-enzymatic antioxidant molecules to protect spermatozoa.
  - First line defence against ROS: **SOD, GPx, Catalase**
  - Second line: ROS Scavengers: **Vit C, E**

---

### Table 2.

<table>
<thead>
<tr>
<th>Enzymatic Antioxidants</th>
<th>Non-Enzymatic Antioxidants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione peroxi</td>
<td>Catalase</td>
</tr>
<tr>
<td>Glutathione reduct</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td></td>
</tr>
<tr>
<td>Proteins like Albumin, Tran Haptoglobin, Ceruloplasin Glutathione (GSH)</td>
<td></td>
</tr>
</tbody>
</table>

---

**Figure 1.**

A schematic representation of the types of antioxidants to minimize free radicals in seminal plasma. There is a strong positive correlation between immature spermatozoa and ROS production, which is negatively correlated with sperm quality and elevated ROS. The retention of excess reactive oxygen species (ROS) even at concentrations below the World Health Organization cutoff value for leukocytospermia (concentration > 1x10⁶ peroxidase positive leukocytes/mL semen) is an indicator of excessive ROS even at co-factors that prevent them from initiating a chain reaction. When transition metals become loosely bound to ROS, they can initiate a redox cycle, which prevents them from initiating a chain reaction. The definitions of stress, radical, compound, or the free radical-induced damage: enzymatic and non-enzymatic antioxidants are the line of defense against an oxidative insult. Peroxynitrite (ONOO⁻), nitric oxide (NO), and nitric dioxide (NO₂⁻) are key players in the physiological processes such as capacitation, hyperactivation, and sperm-egg interaction. Peroxidase positive leukocytes in semen have been observed to be the main source of ROS. Spermatozoa carry cytoplasmic droplets that are the link between poor sperm quality and elevated ROS. The seminal plasma contains two different classes of antioxidants to minimize free radicals, particularly OH radicals, which have been observed to be the main source of ROS. The seminal plasma contains two different classes of antioxidants to minimize free radicals, particularly OH radicals, which have been observed to be the main source of ROS.
Reactive Oxygen Species

- **Principal sources of endogenous ROS:**
  - Leukocytes (infection, inflammation) $\text{O}_2^-$ $\xrightarrow{\text{SOD}} \text{H}_2\text{O}_2$
  - Abnormal spermatozoa: Mitochondrial ROS and impairment of chromatin compaction (prolamination)

- **Environmental factors that increase levels of ROS**
  - high temperatures, electromagnetic radiation,
  - pesticides and pollution (estrogen-like xenobiotics)
  - lifestyle factors of advanced age, alcohol consumption, smoking, stress, obesity and poor diet.

- **Genetic disposition:**
  - Glutathione S transferase polymorphism might be an important source of variation of susceptibility of spz to OS in patients with idiopathic infertility
Measurment of OS

- Measurement of ROS:
  - Fluorescence on spermatozoa / DCFH2-DA
  - Analysis of seminal plasma oxydative parameters
    - MDA (Malondialdehyde)
    - Protein Carbonyl group
    - Nitrotyrosine
    - Total thiol group
    - FRAP: ferric reducing antioxidan power

Aktan et al Fertil Steril 2013
Agarwal et al 2008

Figure 2. Flow diagram showing the various methods to measure seminal stress.
The value of sperm DNA adduct analysis

• DNA adduct formation in human spermatozoa has direct, negative impacts on fertility and is a good predictor of treatment outcome.

• 3 factors associated with the formation of oxidative DNA adducts (ageing, smoking and infertility) are also with DNA damage in human spermatozoa in the germ line.
Clinical data

Male infertility
Pregnancy
Offspring
fertile men (6). Besides hormonal abnormalities, Y-chromosome deletions, and abnormal karyotypes (2, 3), the loss of DNA integrity intensive oxidations appears to be a serious hurdle in such treatment. It has been suggested that fertilization through ICSI could be an approach to overcome these barriers. However, whether the sperm of idiopathic infertile men is under extensive oxidative stress, although the semen characteristics were found to be normal according to WHO standards.
Oxydative stress imbalance and infertility

• Increased production of ROS and/or modification in the levels of antioxidant defences are implicated in the occurrence of many sperm defects. These include reduction of sperm motility, spermatozoa-oocyte fusion, and acrosine activity

• Spermatozoa of oligozoospermic patients have been confirmed as a very important source of ROS

• ROS-induced motility decrease is associated with a growth of lipid peroxidation measured as malondialdehyde (MDA) and DNA modifications

• OS could be a cause for hyperviscosity of seminal plasma
Histone retention and sperm DNA damage measured by Comet and TUNEL assays were associated with fertilization rate (P = 0.012). The predictive power was significantly higher using the alkaline Comet assay when compared with TUNEL and FCCE as was a significant decrease in pregnancy rates in patients when age of damaged sperm was above the threshold value of 82% for Comet assay and 10% threshold value measured by TUNEL.

between nuclear proteins and ART outcomes. We noted a strong relationship between sperm DNA damage and ART outcomes (fertilization rate, embryo quality, blastocyst quality, implantation rate and pregnancy rate). The predictive power was significantly higher using the alkaline Comet assay when compared with TUNEL and FCCE as was a significant decrease in pregnancy rates in patients when age of damaged sperm was above the threshold value of 82% for Comet assay and 10% threshold value measured by TUNEL.

### Limitations, Reasons for Caution

- A potential drawback of this study is that it is cross-sectional. Generally in such studies more than one variable that could cause the effect. Analysing sperm is one part of the equation; there are also a number of female factors the potential to influence ART outcomes. Therefore, given the large and well-established role of female factors in infertility, normal sperm integrity and protamination do not necessarily ensure clinical pregnancy in ART. Thus, female factors can reduce the prognostic value of DNA tests. Further, our use of native semen instead of prepared sperm may have iatrogenically increased the DNA damage.

### Wider Implications of the Findings

Alteration in sperm nuclear protein affects sperm DNA integrity. Further, with this dataset, TUNEL and Comet assays appeared more predictive of ART success than FCCE.
Metaanalysis

• 16 cohort studies (3,106 couples)

• High-level sperm DNA fragmentation has a detrimental effect on outcome of IVF/ICSI:
  ▪ Decreased pregnancy rate
  ▪ Increased miscarriage rate.
  ▪ type of procedure (IVF vs. ICSI)
    ▪ Lower pregnancy rates in IVF but not in ICSI cycles,
    ▪ higher miscarriage rates in both IVF and ICSI cycles.

• Assays detecting sperm DNA damage should be recommended to those suffering from recurrent failure to achieve pregnancy in ART.
Implications for the offspring

- DNA damage / **Paternal age**
  - Increase in neurological conditions:
    - epilepsy, spontaneous schizophrenia, autism and bipolar disease.
  - increased risk of cancer in the offspring
  - Appearance of dominant genetic mutations including achondroplasia and Apert’s syndrome

- **Paternal smoking** = oxidative stress = oxidative DNA damage in the spermatozoa
  - increased levels of cancer in the offspring

- alcohol consumption, exposure to radio-frequency electromagnetic radiation, chemotherapy, diabetes, heat, testicular torsion, oestrogenic steroids, anti-retroviral drugs, anti-epileptics, phthalate esters, heavy metals, acrylamide, arsenic, pesticides, herbicides, paracetamol, hypobaric hypoxia, cryostorage and indeed idiopathic infertility,
Antioxidants for male subfertility
Antioxidants for male subfertility (Review)
Schowell et al The Cochrane Library 2014, Issue 12

• **Live birth:**
  - Antioxidants may have increased live birth rates
    - (OR 4.21, 95% CI 2.08 to 8.51, P < 0.0001)
  - 4 RCTs, 277 men, (low quality evidence)
  - Result based on only 44 live births from a total of 277 couples in 4 small studies.

• **Clinical pregnancy:**
  - Antioxidants may have increased clinical pregnancy rates (OR 3.43, 95% CI 1.92 to 6.11, P < 0.0001)
  - 7 small RCTs, 522 men, (low quality evidence).
Type of antioxidant

- **Increased clinical pregnancy and Live Birth:**
  - vitamin **E** compared to placebo (OR 6.71, 95% CI 1.98 to 22.69, P = 0.002, 2 RCTs, 117 men, I² = 0%); for clinical pregnancy
  - Zinc compared to placebo or no treatment (OR 4.43, 95% CI 1.39 to 14.14, P = 0.01, 2 RCTs, 153 men, I² = 0%).
  - Combined: **Zn, VitC and E, lycopene, Garlic oil, Selenium:** versus placebo (OR 3.42, 95% CI 1.15 to 10.13, P = 0.03, 1 trial, 60 men).

- **Decreased sperm DNA fragmentation**
  - **Vit C and D** (MD -13.80, 95% CI -17.50 to -10.10, P < 0.00001, 1 RCT, 64 men),
  - **docosahexaenoic acid** MD -14.12, 95% CI -23.23 to -5.01, P < 0.002, 1 RCT, 36 men).

Schowell et al *The Cochrane Library* 2014, Issue 12
Twenty-seven of the 48 included trials were small in size (randomising between 20 and 60 men), and the estimates of the intervention effect tend to be more beneficial in smaller studies. Smaller studies also may not be as rigorous as the larger studies in their methodology (Higgins 2011).

The trial by Cavallini (Cavallini 2004) had unexplained differences in randomisation and analysis numbers and this may have introduced some reporting bias.

There may have been some publication bias in this review as, although we performed a comprehensive and wide ranging search including both full text and conference abstracts, we did not find any unpublished trials.

**Effects of interventions**

See: **Summary of findings for the main comparison**

Antioxidants versus placebo or no treatment for male subfertility

1. Live birth; type of antioxidant

See Analysis 1.1

Only four trials reported on live birth, three of these had methodological inadequacies as they did not describe their methods of randomisation or allocation concealment. The meta-analysis of these trials showed that antioxidants were associated with a increased live birth rate compared with placebo or no treatment (OR 4.21, 95% CI 2.08 to 8.51, P < 0.0001, 4 RCTs, 277 men, I² = 0%, low quality evidence) (Figure 4). This meant that within this population of subfertile men with an expected live birth rate of 5%, use of an antioxidant increased this rate to between 10% and 31% (Summary of findings for the main comparison).
Synthesis

• 19/20 studies: significant reduction of OS or DNA damage after oral antioxidant treatment.

• 100% of the studies (or 7/7) targeting asthenospermic patients showed a significant improvement in motility.

• No effect of antioxidants on sperm morphology

• Positive effects on concentration (3 studies).

• 6/10 studies improvement in fertilization or pregnancy rates,

• No perfect trials have been conducted to date

Gharagozloo and Aitken Human Reprod 2011
Type of antioxidant

- **Improve sperm motility**
  - Vit C 100mg/day: Yes
  - Carnitines: Yes
  - ZN + Vit C and E: Yes
  - Pentoxifylline: Yes
  - Selenium: debated
  - N-Acetylcysteine: debated
  - Zn alone: debated
  - Coenzymz Q10: debated
  - Folic acid: No
  - Magnesium: No
  - DHA: No
  - L acetyl carnitine + L carnitine > vitamin E + vitamin C

Schowell et al *The Cochrane Library* 2014, Issue 12
Antioxidants for male subfertility (Review)
Copyright © 2015 The Cochrane Collaboration. Published by John Wiley & Sons, Ltd.
Antioxydants in infertile men

- High doses of Vit C, Selenium, SOD & CoEQ10 decrease DFI but increase DSI.
Cocktails

- **Conceptio**: L-carnitine, 800mg de DHEA, zinc sélénium, vit E, B6 et du co Q10
- **Fertimax**: carnitine, vit C, vit E, zinc, sélénium, acide folique, co Q10
- **Bétaselen**: vit C, vit E, zinc, sélénium, béta carotène
- **Fertibiol**: zinc, vit B6, B9 B12, N acétyl cystéine, L-carnitine, caroténoïdes, vitamine E et de la DHEA (DFI>40%)
Cocktails

- **Condensyl**: NAC, vit E, zinc, vit B6, B9 et B12. (SDI>20%)

- **Oligobs Procréa**: vit B6, vit B, B12,C, E, zinc, mg, sé, chrome, inositol, taurine, d'oméga 3 DHEA

- **Procrélia**: zinc, vit B2 B6 B9 et 12, NAC (DFI>20%)

- **Proxeed**: L carnitine, carnitine, sé, co Q10, vitC, zinc, acide folique, vit B12
Conclusion

• Increased DNA fragmentation is associated with reduced fertility (Level B)

• The tests can be used as a predictor of fertility (Level C)
  ▪ cut-points have not been clearly established and validated.

• SCSA test with DNA fragmentation index (DFI) >30% would be associated with a lower pregnancy and delivery rate in IUI. (Level C)

• There is insufficient evidence (Level C) to recommend routine use of DNA integrity testing for patients undergoing IVF and IVF/ICSI or to predict pregnancy loss.

Guideline of the ASRM Fertil Steril 2013
Indications for DNA fragmentation Test

• Unexplained or persistent infertility
• Failure to conceive after 5-6 IUI despite good count and motility
• Low fertilization rates or poor embryo quality in IVF
• IR failure after IVF
• Recurrent miscarriage
• Prolonged stay in an environment exposed to reproductive toxins
• Abnormal semen analysis
• Advancing male age (>45y)
Conclusion

• Use of antioxidants may be effective in increasing a couple’s chances of having a live birth when compared to placebo and when compared to no treatment.

• Subfertile men with an expected live birth rate of 5% the use of antioxidant would increase this rate to between 10% and 31%

• Best Antioxidant combination: (adapted to SDI and DFI ?)
  - Carnitines + Zn + Pentoxifylline + Vit E
  - Avoiding: high doses of Selenium, CoEnz Q10 and Vit C

• Os and antioxidant in women ?: endometriosis, PCO

Schowell et al The Cochrane Library 2014, Issue 12