Sperm DNA: organization, protection and vulnerability –
from basic science to clinical application

The Acridine Orange test including flow cytometry (SCSA) and chromatin staining methods

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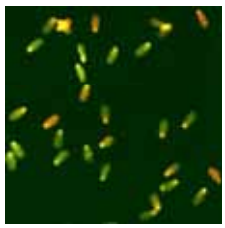
The rethorical view!

- Intact DNA looks green
- Damaged DNA looks red
- Red is bad!

- Increased Red/Red+Green is said to be due to fragmented sperm DNA



Physiology:
DNA in the nuclei of living cells becomes single-stranded (ss) during
replication, transcription and repair.

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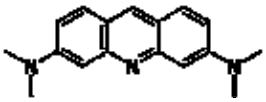
Disruption of the native structure may also result from modifications of the bases or sugar backbone induced by chemicals or light.
In living cells such changes are reversed by specialized enzymatic systems responsible for preventing loss of genetic information

Denaturation (= dsDNA into ssDNA) can be induced by
elevated temperature
alkali
acid
solvents
some drugs

and is used in lab protocols e.g FISH and SCSA

Acridine organe



Acridine orange is prepared from coal tar and creosote oil.

Acridine Orange and somatic cells

- nucleic acid selective fluorescent cationic*(+)* dye useful for cell cycle determination *(i.e the shift between 2n an 4n cells).*
- It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions respectively.
- Green: When bound to DNA, an excitation maximum at 502 nm *(eg 488 nm*) and an emission maximum at 525 nm (green). *(530+-30nm)*
- Red:With RNA, the excitation maximum shifts to 460 nm (blue) *488 nm*) and the emission maximum shifts to 650 nm (red) *(i.e. > 630 nm).*
- Orange: Acridine orange will also enter acidic compartments such as lysosomes and become protonated and sequestered.
- In these low pH conditions, the dye will emit orange light when exited by blue light.

AO bound to ssDNA by electrostatic forces

+ got stacked on each other

•AO binds to dsDNA by interchelation

Procedure flow cytometry with AO

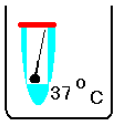
200 uL frozen sample

Freeze

When ?

-20C, -196 -80

Thaw - one at a time



Why?

Dilute to 1-2 milj/mL

Why?

Standardizations

- 5000 events = spermatozoa to be measure in duplicates, 200 spermatozoa per second if not, dilute the sample.

Dilution

- TNE **(**Tris-NaCl-EDTA) buffer
- (0,01M Tris-HCl(Sigma),
- 0,15 M NaCl(Sigma),
- 1mM EDTA **(**etylene diamine tetraacetic acid) (Sigma), pH 7,4)

Add " acid"

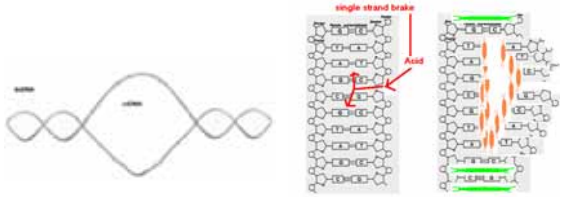
uL acid detergent tion to 200 uL TNE ed sample

Why?

To produce single stranded DNA

In somatic cells: Acid denatures DNA.

Plus the idea that this will be enhanced at strand break points.



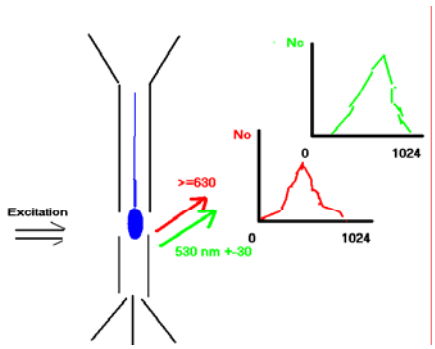
NB the denaturation increases with time

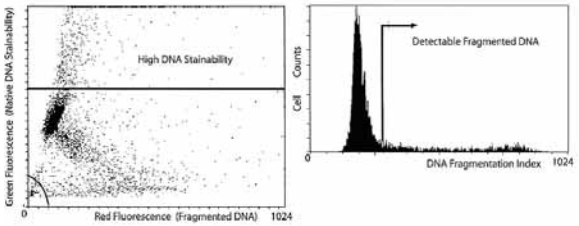
Production of single stranded DNA is stopped and AO given!

At 30 s exactly add solution pH 6,0 with AO

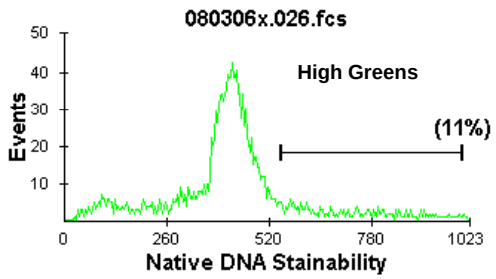
AO 6 mg/L in pH 6,0:

0,1 M citric acid (Sigma),
0,2 M Na2PO4 (Sigma),
1 mM EDTA(Sigma),
0,15 M NaCl, pH 6,0

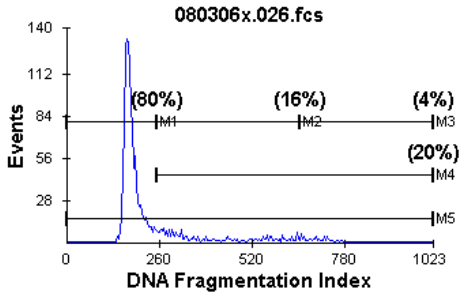




Collection of green fluorescens

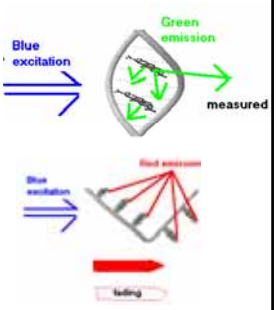


The proportion of red light of all light emitted. (red/ red+green)



AO itself can induce strand-breaks and cause single stranded DNA

- AO emits light (= energy) that can
- 1) induce strand-breaks
- 2) Cause DNA-denatura-tion and AO is then stacked on emitting red
- Consequence:
- Green light decrease
- Red light increase, but fades more rapidly than green



Tytus Bernas et al 2005; Photochemistry and Photobiology 81(4): 960-969

Consequences for measurements?

- Bound to ds DNA green G+
- Bound to ss DNA red R+
- The amount (intensity) of red a question of stacking R+
- **+ light**
- Decreases ds DNA G-
- And increases ssDNA R+
- Red fades >green R-

One could speculate that the more DNA that is avalaible

- Acid pretreatment (creates ssDNA with time) (especially where strand-breaks are present)
- Exposure time
- AO + light-induced strand-breaks and ssDNA
- will, in spite of AO-red fading with time, create

more red and less green

But availability shifts.

Thus AO is a major test of sperm chromatin availability i.e packageing?

Increased temperature denaturates DNA. Condensed spermatozoa resist temp better.

UV-light absorption at 285 nM

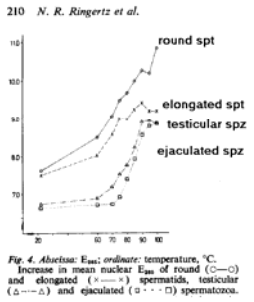


Fig. 4. Abscissa: E_{285}; *ordinate:* temperature, °C. Increase in mean nuclear E_{285} of round (○—○) and elongated (×—×) spermatids, testicular (△---△) and ejaculated (□ · · · □) spermatozoa.

Nils Ringertz, Barton Gledhill Zebigniew Darynkiewicz Expt Cell Res 1970

The sperm nucleus closes!

- These changes in the DNP express themselves as
- a decrease in stainability by the Feulgen reaction
- a lowered capacity to bind basic dyes
- a decrease in the ability of the complex to bind H*-Actinomycin D.
- Inactivation of the genome + condensation of the chromatin

Nils Ringertz, Barton Gledhill Zebigniew Darynkiewicz Expt Cell Res 1970

Experiments: One

- How is the AO-assay influenced by sperm storage in seminal plasma
- From 30 min post ejaculation until 24 hours post-ejaculation.
- (NB Time interval 0 to 30 minutes is not covered here)

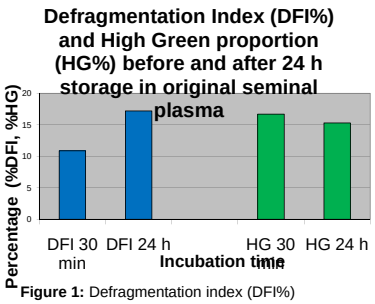
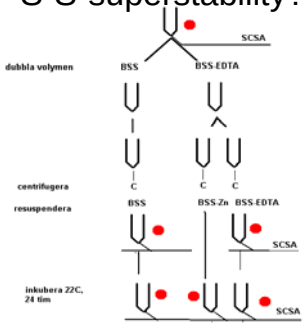
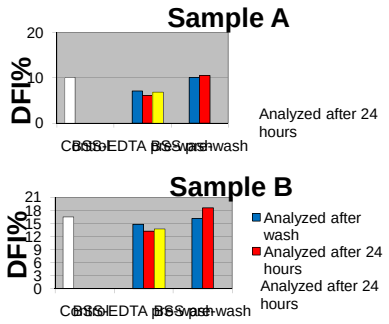


Figure 1: Defragmentation index (DFI%) increased signficantly during 24 hour storage in original seminal plasma (N=13, *P*<0.01). No significant difference was observed in the proportion High Green (HG%).

Experiments: Two
Can DFI% diminshed by induced S-S-superstability?





CONCLUSIONS

- Storage of spermatozoa in semen induces changes in the structural stability of the chromatin.
- In this investigation relatively more red emission from Acridine Orange could be detected in sperm heads after storage in seminal plasma, indicating increased access to DNA or increased acid denaturation of DNA.

- The relation between seminal fructose and large increase in DFI% is likely to depend on increased oxidative environment due to presence of seminal vesicular fluid.
- During storage there was no change in the proportion sperm with high green emission (%HG), indicating an unaffected access to double stranded DNA.

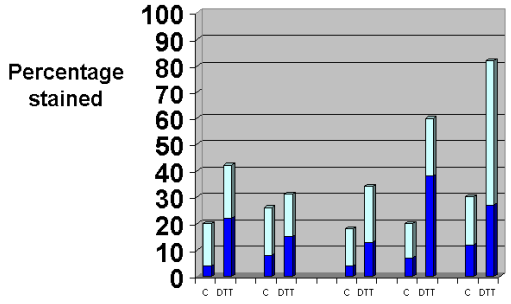
- Experimental zinc deprivation of the sperm chromatin may decrease the access to DNA.

  This might be due to that –SH groups, freed by zinc extraction, got committed into disulfide bridges which are known to increase the stability of the human sperm chromatin. Further experiments are required to verify these findings.

Aniline Blue

- Idea: Blue coloured spermatozoa means lysine-containing histones left and indicates deficient incorporation of protamines?
- Validated by Terquem & Dadoune (1982): Aniline Blue binds solely to Lysine as did lysine –specific aPTA (alcoholic phosphotungsic acid)
- Experiment: Assay run on spermatozoa with and without pre-expsure to DTT.

Aniline Blue staining of ejaculated spermatozoa exposed to buffer and buffer with DTT 5 min

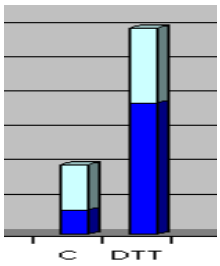


Conclusions…..

DTT increase the amount of histones in ejaculated spermatozoa? No

Chromatin stability influence the detectability of histones by aniline blue. Yes

To detect spermatozoa with increased amounts of histones ejaculated spermatozoa need be pretreated……..
5min=60 min



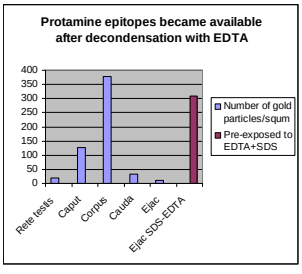
Other examples on the availability to the sperm chromatin

- The availability to epitopes of boar protamine decreased during epididymal passage and was re-found after exposure to SDS-EDTA.

Table 1. Number of colloidal gold particles/area of nuclear sections (in μm^2) of boar spermatozoa, collected at different areas of post-testicular transit

	Epididymis			Ejaculate	
Rete testis ($n=62$)	Caput ($n=50$)	Corpus ($n=63$)	Cauda ($n=76$)	No treatment ($n=79$)	Treatment with SDS/EDTA ($n=74$)
19·0 ± 27·2[a]	126·5 ± 29·9[b]	376·9 ± 9·4[c]	34·3 ± 15·6[a]	9·5 ± 2·9[a]	307·7 ± 23·5[c]

Values are mean ± s.e.m. for the no. of sections indicated (n) from 6 boars.
Values with different superscripts differ significantly ($P<0·05$).



Rodriguez-Martinez H, Courtens JL, Kvist U, Plöen L. J Reprod Fertil. 1990 Jul;89(2):591-5. Immunocytochemical localization of nuclear protamine in boar spermatozoa during epididymal transit.

Final remarks 1

- Many methods has been designed to characterize the ” integrity of the sperm DNA”.
- The original protocols developed and validated on somatic cells and not on spermatozoa.

Final remarks 2

- None of the protocols take in consideration Neither that the availability to the sperm chromatin undergoes severe changes with the respect to its degree and type of stabilization (disulfide-bridge dependent, zinc-dependent).
- Nor that these changes are influenced by the ejaculatory sequence and the time of exposure to seminal plasma.

Final remarks 3

- **A positive finding** means that the probe reached the chromatin and gave a signal back to the investigator.
- Increased red light from AO stained means that at least some single stranded DNA was detected.
- It could mean that
- DNA was (1) available and (2) single stranded and
- DNA was (1) available and (2) double stranded and (3) denaturated by the acid treatment and by AO+light and
- DNA was (1) available and (2) double stranded and (3) had strand-breaks that (4) was unraveled by the acid.
- **In conclusion: A positive finding means some DNA was available**, and that is no good sign for a sperm.

Final remarks 4

- A negative finding means that the probe used by the investigator did not reach the chromatin in question.
- This could be a healthy sign – there was no ss DNA
- Or there were strandb-breaks but the normal stabilization did not allow the assay to reveal them.
- Or there was increased ssDNA but due to a secondary superstabilization by excess disulfide-bridges the probe did not reach them.

Final remarks 5

- In conclusion, to me
- A positive signal (pos TUNEL, AO-red, Increased DFI%, Toludine+,Sperm swelling in SDS, positive COMET) tells that the sperm chromatin is available and susceptible to damage.
- A negative signal can be true or false due to superstabilization. A zinc-deficient chromatin is available and can be harmed. It is likely to undergo excess superstabilisation by S-S,which, in turn decrease its availability and give a false negative signal.

- Forward to physiology