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

Methods to assess the status of the sperm DNA:

The Comet and TUNEL Assays

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Learning Objectives

- Background and nature of Comet and Tunel assays
  - What is measured?
  - Differences in results due to method variants
  - Differences due to different DNA organization
- Notes on the Halosperm® assay
- Alternative interpretation of assay results

Origin of the Comet assay

- Undamaged DNA is believed to remain in organized structure with nuclear proteins while damaged DNA cannot maintain the high degree of organization
- Single-cell gel electrophoresis – originally after exposure to chemical substance
  - Detection of genotoxic potential of chemical compounds
  - Human biomontoring – effects on entire human organisms
  - Developed for various somatic cell types and tissues
  - Requires relatively few cells, rapidly performed, relatively eas

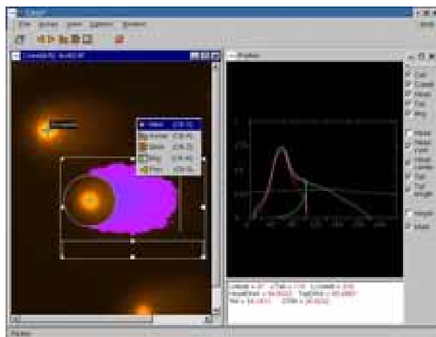
Origin of the Comet assay

- Principle:
  - Cells embedded in an agarose matrix
  - Cell lysis – salt, detergent, pH
  - Electrophoresis, pH
  - DNA staining
  - Microscopy
  - Image analysis

Origin of the Comet assay

- Alkaline Comet – high pH
  - Single- and double strand DNA breaks
  - Alkali labile sites
  - Incomplete repair sites
  - Crosslinks
- Neutral Comet – pH around 7
  - single- and double strand breaks
- Standardization
  - International recommendations, somatic cells:
    - International Workshop on Genotoxicity Testing (IWGR)
  - Studies on germ cells:
    - only limited data from studies done under controlled conditions and GLP

Calculation of results CASP – Comet Assay Software Project
www.casplab.com



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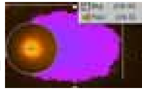


- **HeadArea** Area of the comet head
- **TailArea** Area of the comet tail
- **HeadDNA** Sum of intensities of pixels in the head
- **TailDNA** Sum of intensities of pixels in the tail
- **HeadDNA%** Percent of intensity of pixels in the comet head
- **TailDNA%** Percent of intensiyt of pixels in the comet tail
- **HeadRadius** Radius of the comet head
- **TailLength** Length of the comet tail
- **CometLength** Length of the entire comet from head area to end of tail
- **HeadMeanX** Center of gravity of intensity in the head (x coordinate)
- **TailMeanX** Center of gravity of intensity in the tail (x coordinate)
- **TailMoment** TailDNA% x TailLength
- **OliveTailMoment** TailDNA% x (TailMeanX-HeadMeanX)

Alterations of the Comet assay for sperm investigations

- Additional steps to allow access to the sperm DNA
  - Many different approaches, various background levels
  - In general much higher "background" levels in spermatozoa
- Sperm Chromatin Decondensation
  - Proteinase K after lysis, various temperatures, duration 3-18 h
  - RNase and Proteinase K
  - DMSO
  - DTT – zinc-binding and disulfide-bridge cleaving agent
    - Combined with Lithium diiodosalicylate (LIS)
      - Shortest time for decondensation
      - Reduced baseline DNA breaks

Alterations of the Comet assay for sperm investigations

- No standard developed for sperm Comet electrophoresis
  - pH
  - Time
  - Voltage and amperage
- Alkaline conditions increase DNA migration (background) in somatic cells
  - NOT due to pre-existing DNA breaks
  - Due to alkali-sensitive sites
  - In published sperm studies 5-97% of DNA migrates!

Alterations of the Comet assay for sperm investigations

- Sperm Comet assay often run under neutral conditions
  - Supposedly lower background
  - Alkaline conditions appear to result in higher variability between studies
  - NOT specific for DNA double strand breaks
  - Mainly single-strand breaks
  - Lower sensitivity

Studies of genotoxicity in vitro exposure

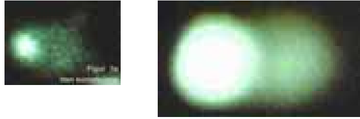
- DNA of ejaculated sperm is resistant to X-ray or H_2O_2 exposure
  - Difficult to induce DNA breaks with external agents WHEN THE NUCLEAR ORGANIZATION IS INTACT!
- Exposure to chemical compounds
  - Agents able to bind to and interact with DNA
    - Presence during decondensation?
- Still valid to investigate potential DNA damaging compounds *in vitro*

In vivo exposure – human biomonotoring

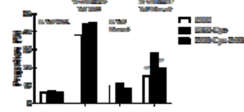
- Few results from studies on humans – much variation
- Age, tobacco smoking, environmental exposure
- Confounding factors
  - Abstinence time (storage before ejaculation)
  - Post-ejaculatory storage conditions

Chromatin stabilization and Comet assay

– Exposure of sperm to Cysteine results in huge increase in DNA loss



– If Cysteine is washed away before Comet assay, Tail Moment is not increased



– *Tu et al, 2009, Poster presentation at American Society of Andrology, Philiadelphia, PA, USA, April 4-7.*

Origin of the TUNEL assay

– **T**erminal deoxynucleotidyl transferase d**U**TP **n**ick **e**nd **l**abeling

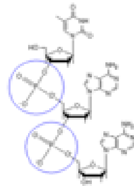
– Assessment of programmed cell death – apoptosis

– Non random DNA breaks by endogenous endonucleases creating short, regular DNA sections
  - – Fast renewing tissues – gut epithelium

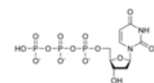
Origin of the TUNEL assay

– Markers of "open" DNA endings – "nicks"
  - – Single strand breaks of phosphodiester bonds

– Proteins stripped off cell nuclei with proteinase K, 15 min, room temperature
  - – Inactivation of endogenous peroxidase with H_2O_2
  - – Addition of Terminal deoxynucleotidyl transferase (TdT) that labels DNA endings with dUTP



phosphodiester bonds
source: Wikipedia



dUTP - Chemical structure of Deoxyuridine triphosphate; source: Wikipedia

Adjustment of the TUNEL assay for human spermatozoa

- Cell lysis and nuclear decondensation
  - NaCl, EDTA, Tris, detergent
  - RNase
  - Proteinkinase K
- Fixation with paraformaldehyde
- Permeabilization with detergent

- Assessment by fluorescence microscopy or flow cytometry

TUNEL assay for human spermatozoa

- Expose to nucleotides (dUTP) and TdT enzymes
  - Positive control: DNase I treatment
  - Negative control: absence of TdT enzymes

- More stain bound to DNA endings:
  - Positive controls (DNase I)
  - Exposure to H_2O_2

TUNEL assay and chromatin stability

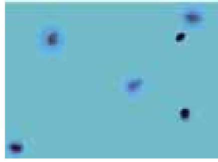
- Using In Situ Cell Detection Kit (Fluorescein)
  - Positive controls only stained 15-53% of sperm
  - High percentage TUNEL-positive spermatozoa related to
    - Low seminal zinc concentration
    - Long abstinence time
    - Long time between ejaculation and start of TUNEL preparation

% TUNEL (N=9), coefficient of correlation (r_c)

	r_c	r_c^2	P=
Zinc conc. (mM)	-0.73	0.53	0.026
Abstinence time (days)	0.80	0.63	0.01
Analysis delay (min)	0.95	0.90	0.0001
% TUNEL (Pos. control)	0.31	0.09	ns (0.42)
Sperm conc. (10^6/mL)	-0.17	0.03	ns (0.67)

From Björk et al, 2009, Poster presentation at the American Society of Andrology, Philadelphia, PA, USA, April 4-7.

Some notes on the Halosperm® assay



Principle

- Gel embedded spermatozoa
- Denaturation with 0.08 N HCl
- Deproteinization with detergent, DTT and washed with EDTA
- Dehydrated with increasing ethanol concentration

Interpretation

- Spermatozoa **with** DNA-halo: low DNA fragmentation
- Spermatozoa **without** DNA-halo: fragmented DNA outside the core of the nucleus washed away

Fernandez et al (2003) The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl, 24, 59-66.

Clinical usefulness – basic aspects

- A test that correlates well with for instance ART outcome is not necessarily clinically useful
- A clinically useful test must distinguish between at least two groups of patients
  - Overlap between groups is a bad ”sign” for the test
- The frequency of the ”disorder” in the investigated population is also an essential factor
  - A rare state of health can be concealed by numerous false positive cases

Clinical usefulness – basic aspects

- Good Laboratory Practice (GLP) – *diagnosis* and *prognosis* for infertile couples
  - validation and evaluation of methods using statistical measures like Odds Ratio and Relative Risk, not correlations
  - ”What is the probability that this patient has a certain disorder?”
- Reproductive Toxicology
  - Evaluation of effects of chemical compounds
    - Comparison of groups with different exposure
    - Comparison of spermatozoa with different exposures
  - Difference between controls and exposed individuals or cells
    - ”Where is the baseline level?”

Summary

- Results from Comet, Tunel and Halosperm® assays
  - Depends on access to the DNA
    - Level and type of sperm chromatin stabilization likely to play a crucial role for the outcome
  - Use in reproductive medicine is different from reproductive toxicology
    - Absolute level of DNA damage cf. Relative effect of exposure

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