Human Sperm Nuclear DNA Fragmentation Assays and Their Values in Assisted Conception

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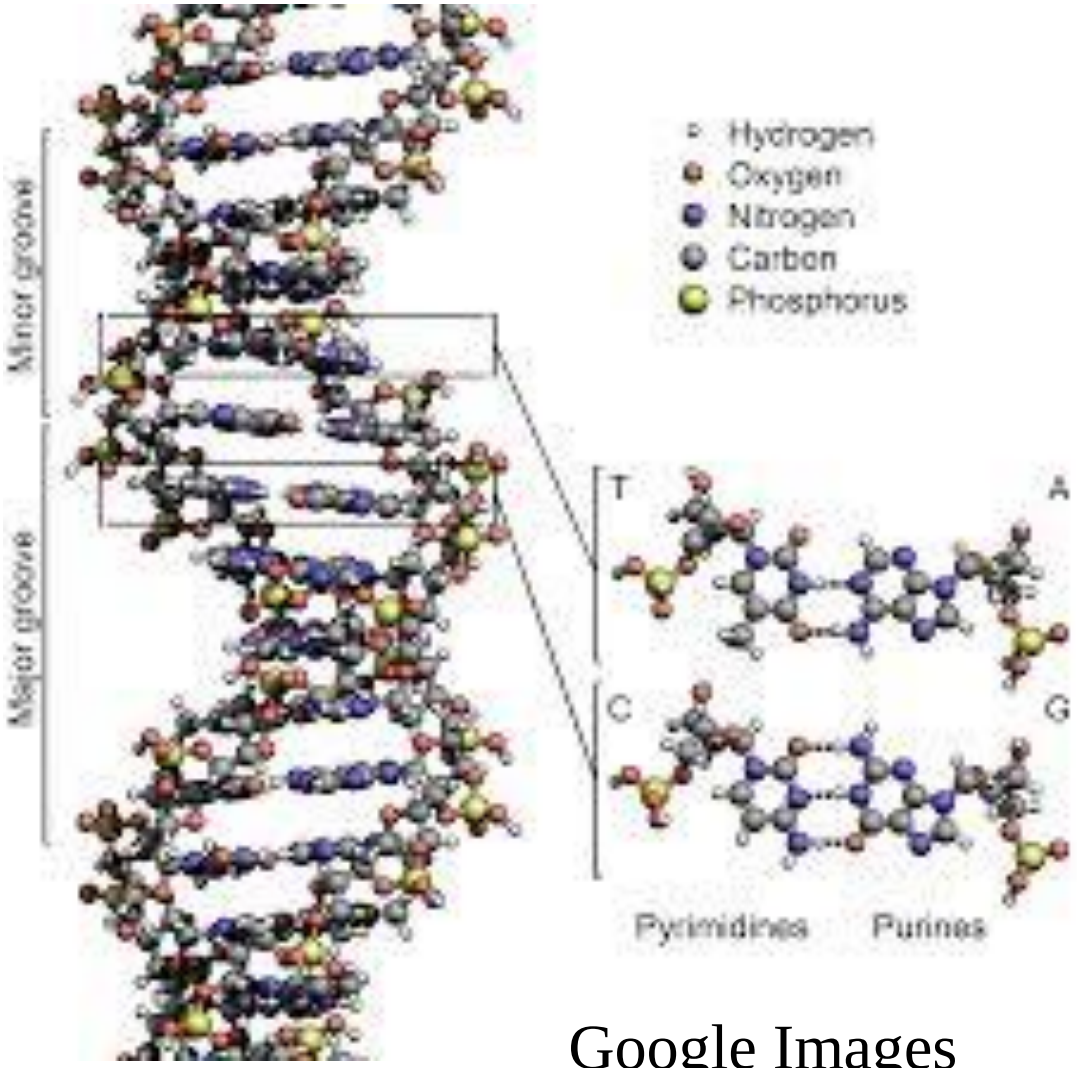
Outline Of The Talk

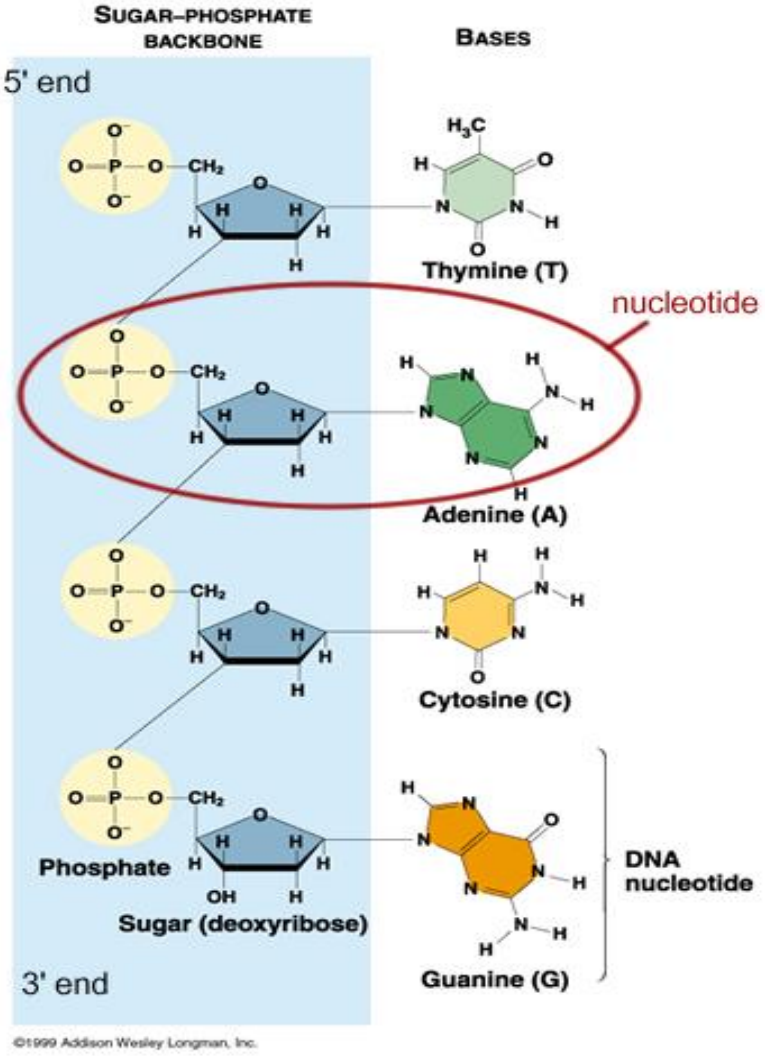
- Structure of DNA
- Changes in DNA to facilitate the nuclear matrix attachment and inclusion within the nucleus
  - Somatic cells and Gametes (sperm)
  - "Each of us has enough DNA to reach from here to the sun and back, more than 300 times. How is all of that DNA packaged so tightly into chromosomes and squeezed into a tiny nucleus?" Annunziato, A. (2008)
- Sperm nuclear DNA damage/fragmentation
- Tests to assess sperm nuclear damage
- Influence of sperm nuclear damage on reproduction

Outline Of The Talk

- Our experience with sperm nuclear damage
- Association of DNA damage and basic semen parameters
- Two brief case presentations
- Some thoughts on the utility of sperm nuclear DNA fragmentation assays in reproduction

DNA Structure and Points of Damage





Google Images

DNA loops and SCD

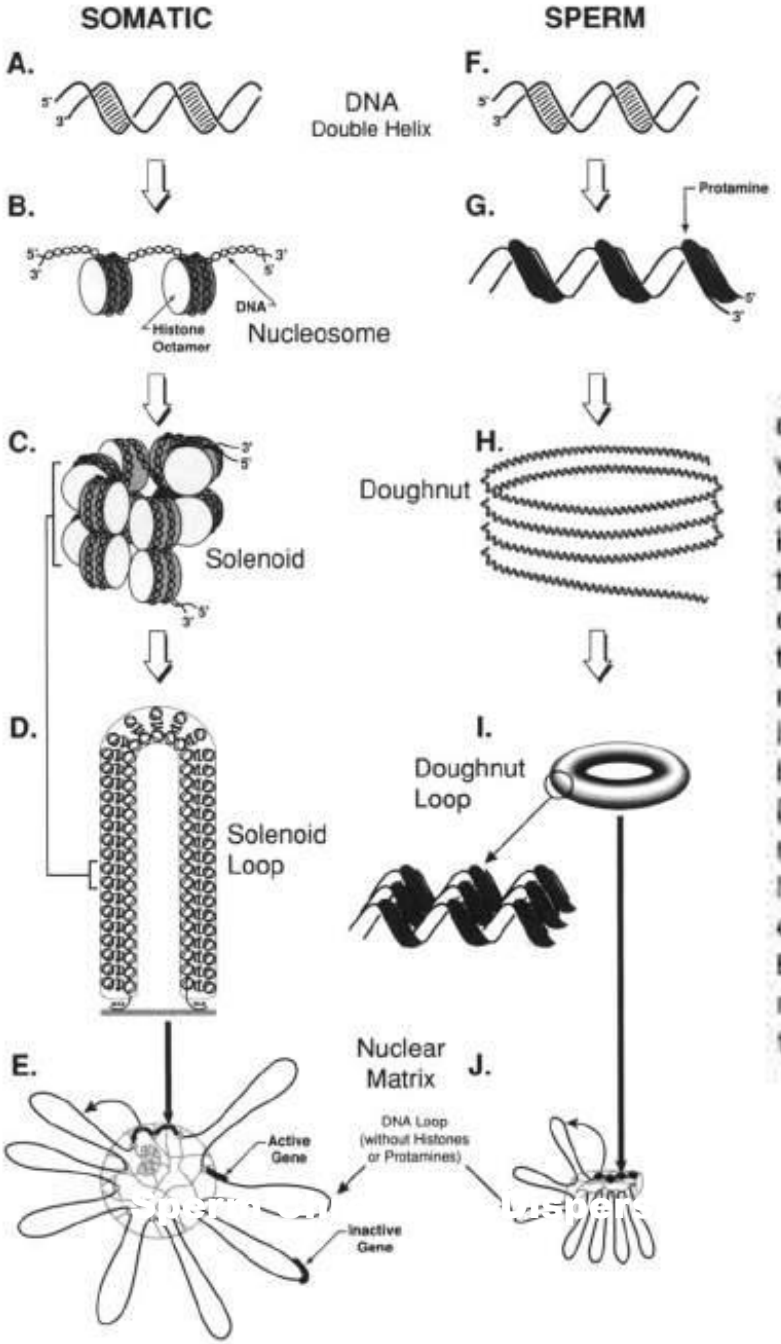
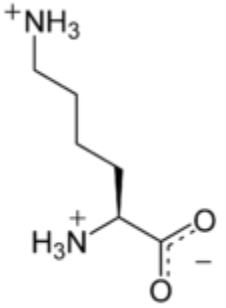


FIG. 4. Equivalent levels of DNA packaging in somatic and sperm chromatin based on the doughnut-loop model. In somatic cells, DNA (A) is wound twice around histone octomers into nucleosomes (B), which then coil into solenoids with six nucleosomes per turn (C). DNA in solenoid form is attached at intervals of about 60 kb to the nuclear matrix at their bases to form DNA loop domains (D). In the somatic cell, these solenoid loop domains are contained within the nucleus, but when the histones are extracted, they can be visualized outside the nucleus (E). Active genes are more closely associated with the somatic nuclear matrix than inactive genes. In the sperm nucleus, highly positively charged protamines bind to DNA lengthwise along the double helix, neutralizing the negative charges of the DNA (G). The protamine-bound DNA is coiled with a very slight bend in the protamine DNA complex into concentric circles (H). These circles of one loop then collapse into a doughnut (I) in which the neutral DNA protamine complexes are tightly packed together by Van der Waal's forces (I, inset). Each doughnut represents one DNA loop domain attached to the sperm nuclear matrix (J). The DNA loop domains of sperm nuclei are smaller than those of somatic cells.

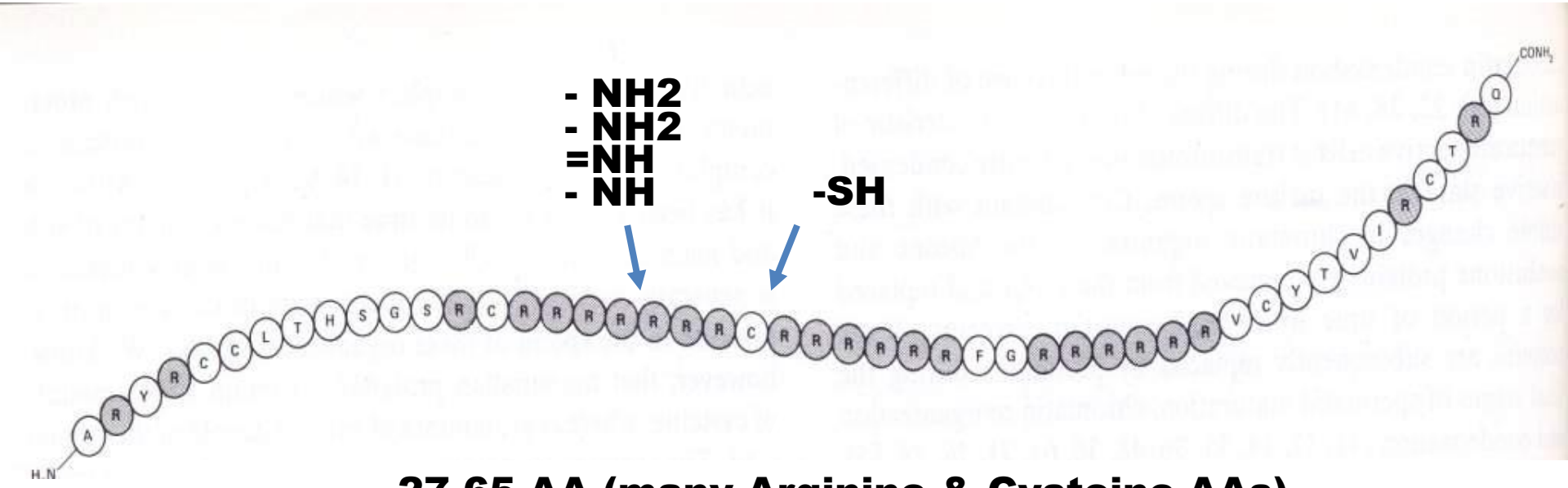
Lysines in histones



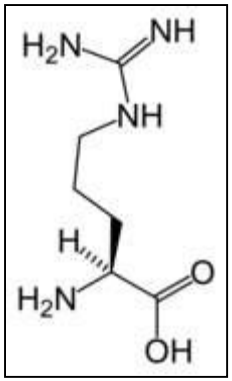
S. Ward, 1991

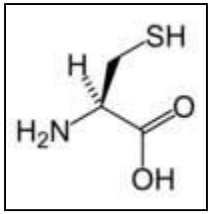
- What are the protamines?

Protamines, Rich in Arginine and Cysteine



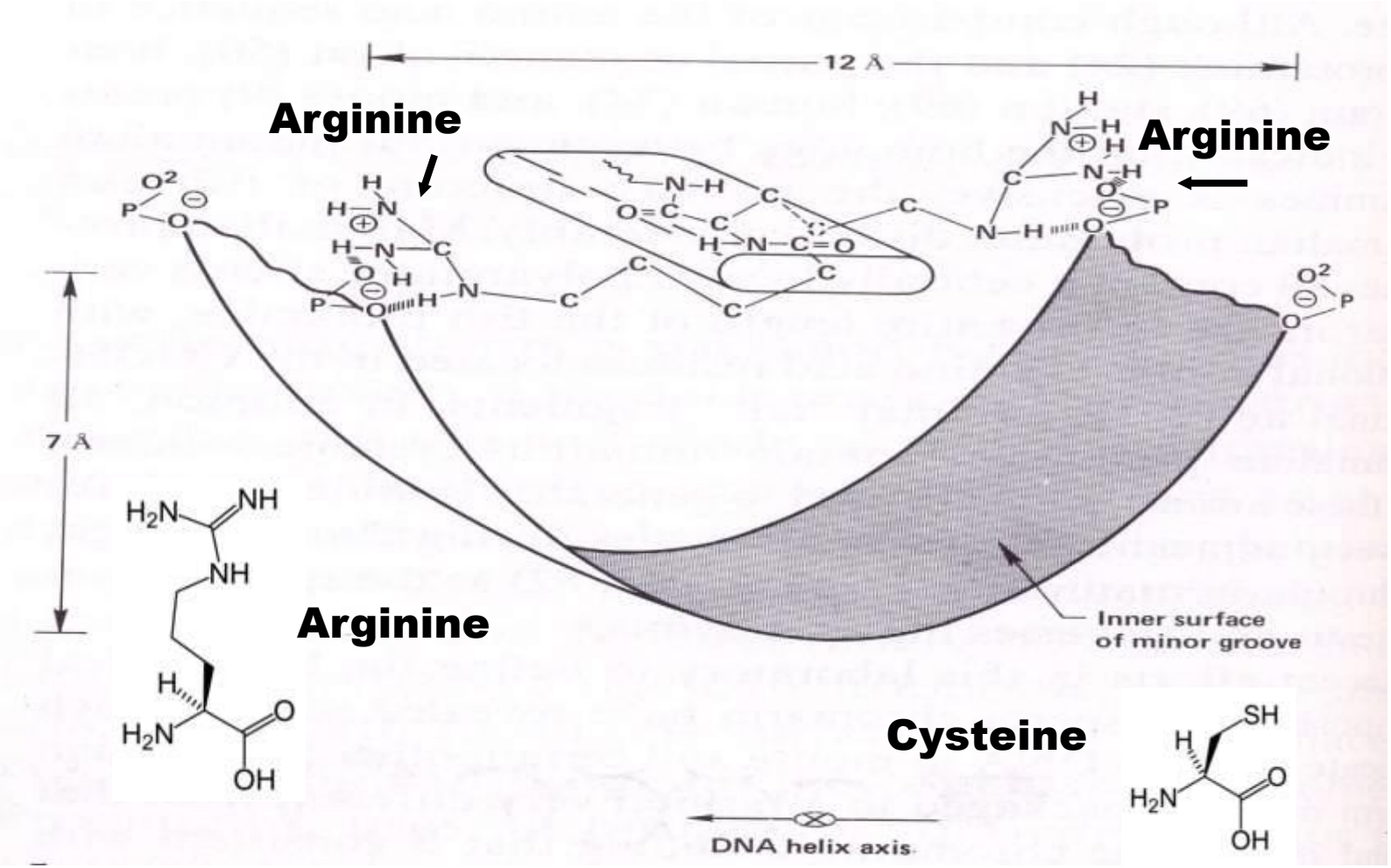
27-65 AA (many Arginine & Cysteine AAs)





Ref??

Protamine Alignment in DNA Double Helix (minor groves)



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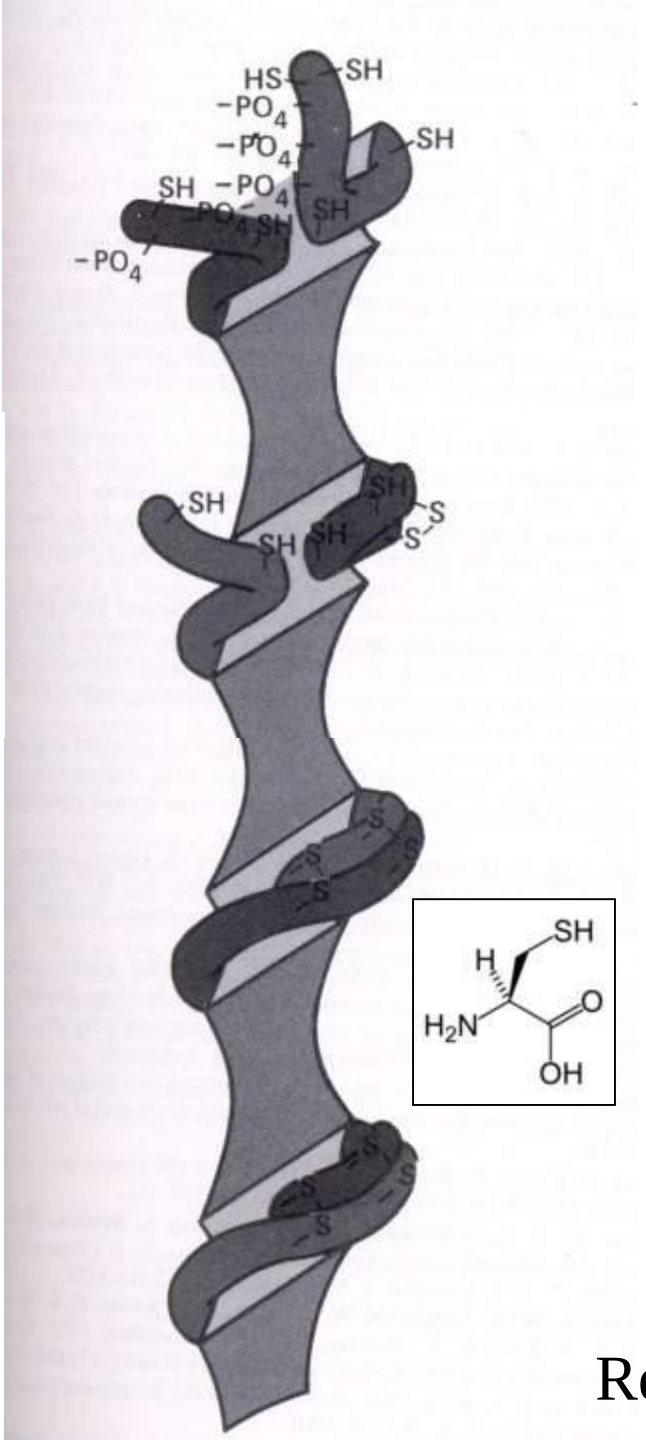
Protamine-DNA Complex in 3-D

What other property does the binding of protamines give the DNA molecule? Disulfide bond formation and further stabilization of the "now" compact molecules.



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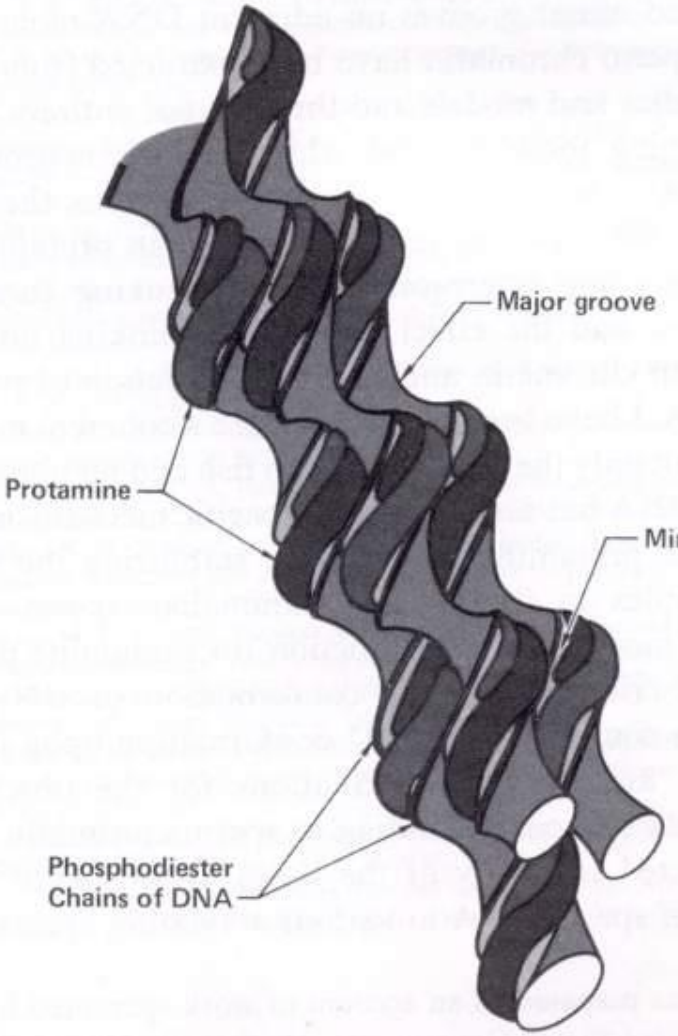
The consequence of protamine placement:

Tightly Packed Very Stable DNA (Charge stabilization, S-S)



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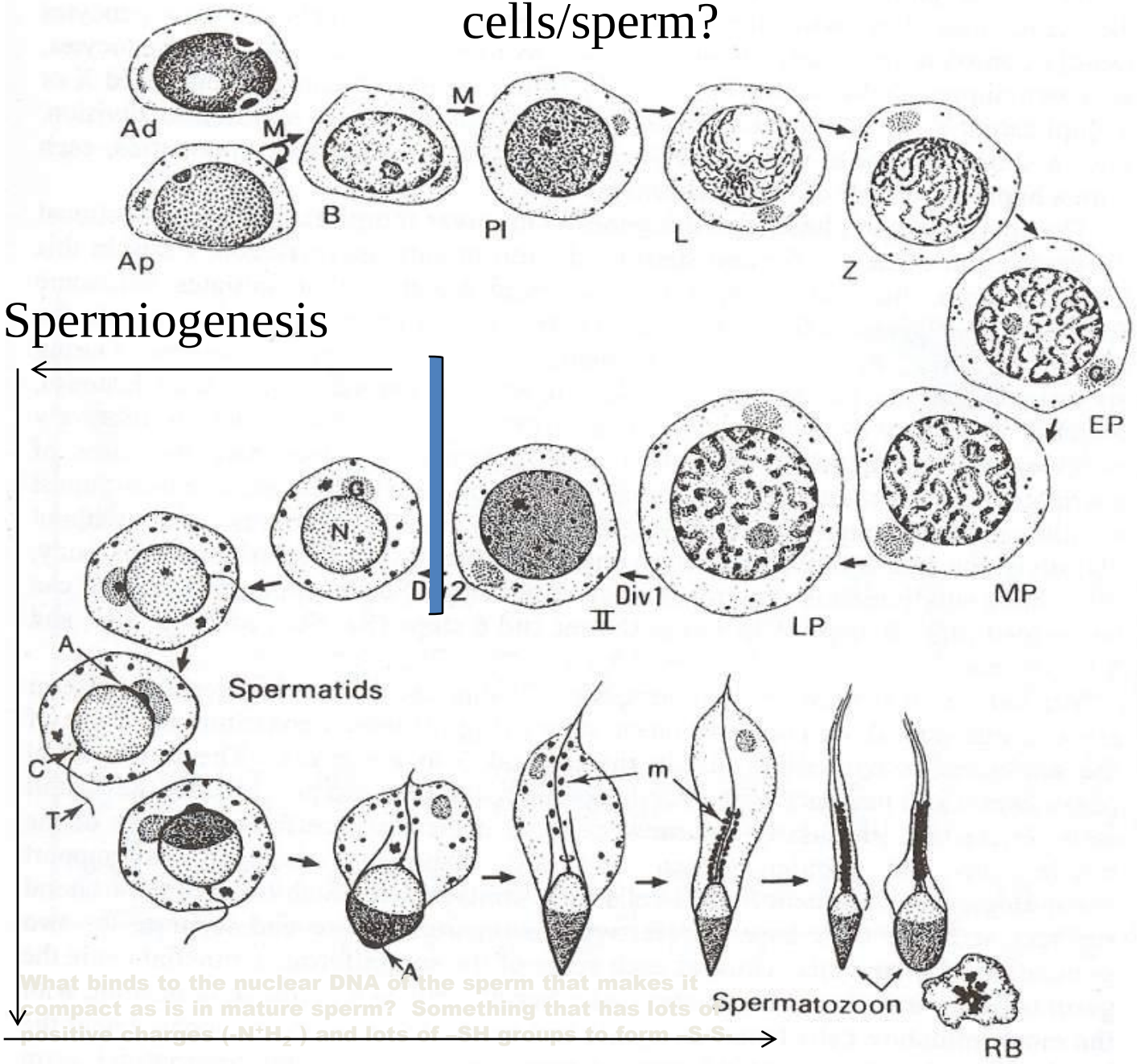
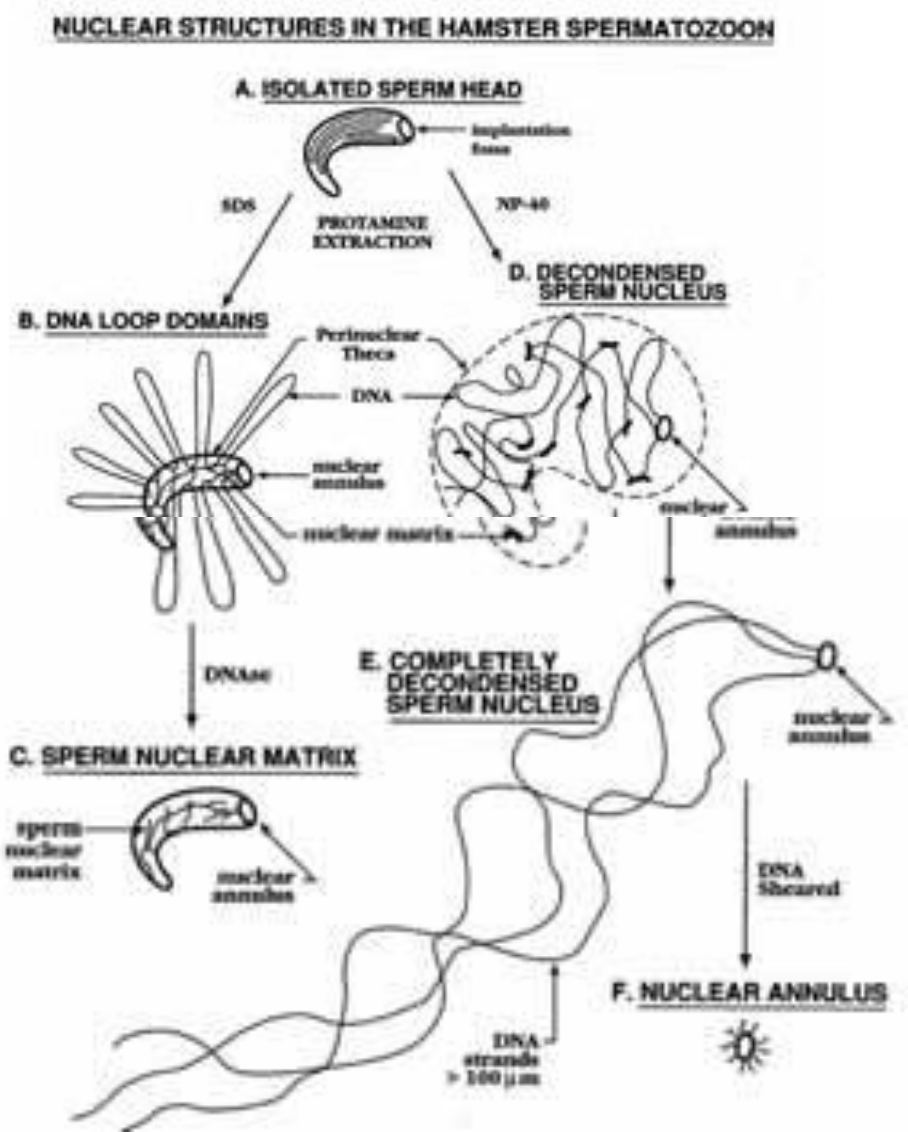
When the histones are replaced by protamines in germ cells/sperm?



Spermiogenesis

Textbook of Andrology, Springer, 2000

Positioning of a Single Chromosome in Sperm Nucleus



Ward, 1991

- With the compact structure described, how and where the sperm chromatin/DNA can be damaged?

DNA Fragmentation Can Be A Testicular Event

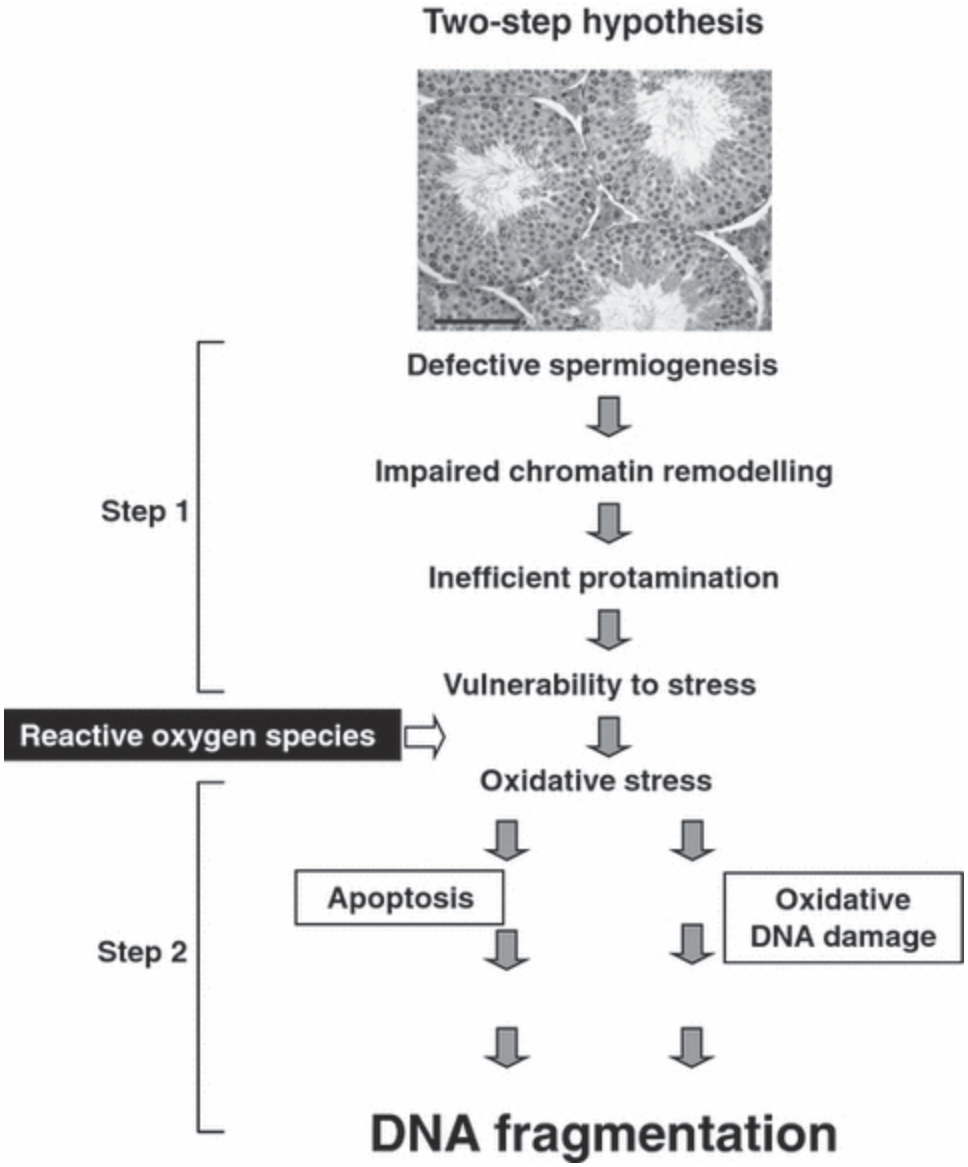
- DNA damage may still happen by factors like:
  - Protamine deficiency which can lead to the higher susceptibility of DNA to denaturation/instability
  - Oxidative stress (high ROS, low protective factors)
  - Unrepaired DNA breaks during chromatin remodeling
  - Abortive apoptosis during spermatogenesis (meiosis I)

DNA Fragmentation Can Also Be A Post-Testicular Event

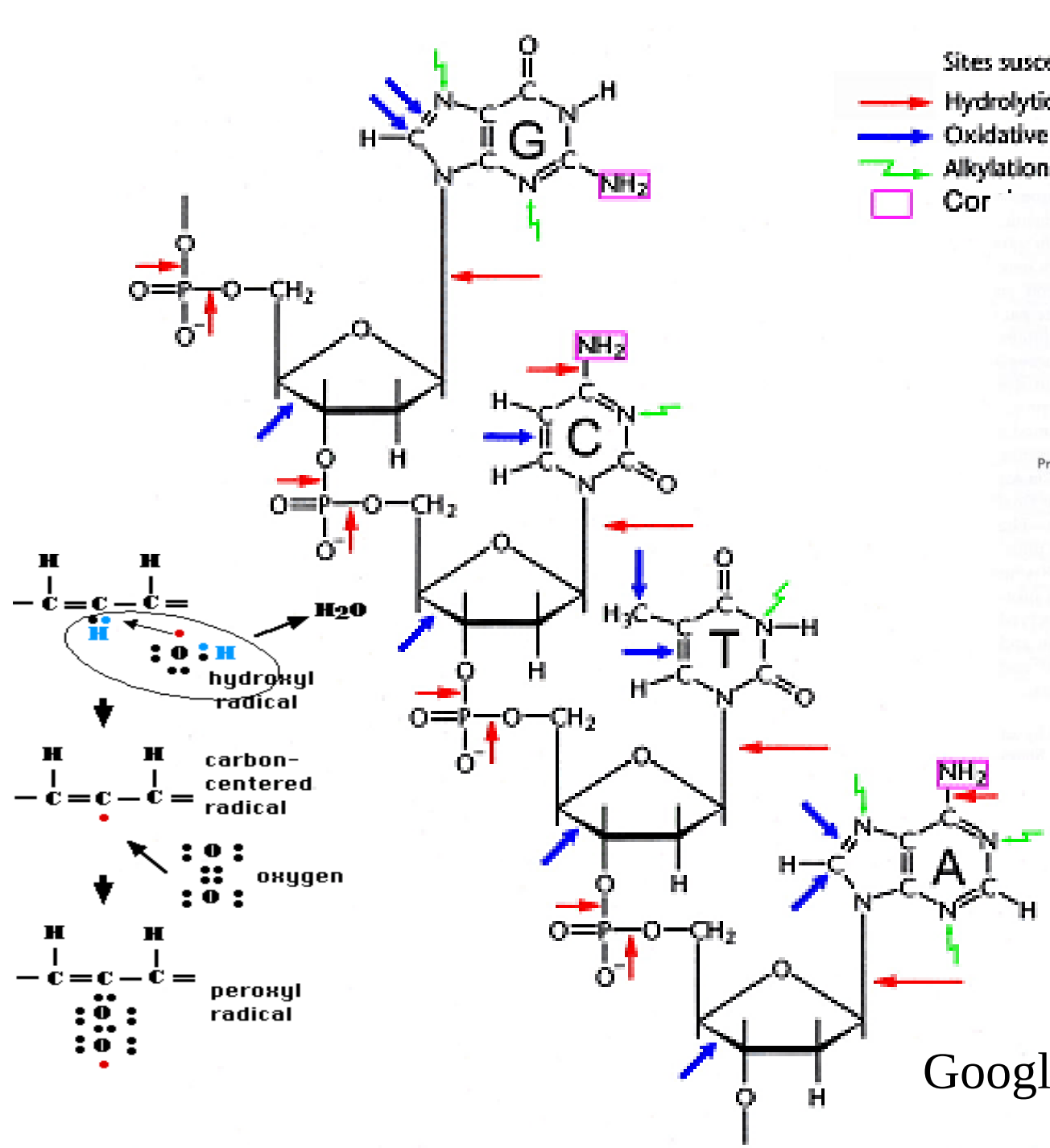
- As a post-testicular event often occurs in epididymis due to:
  - Unfavorable epididymal environment
  - Endogenous endonucleases, excess estrogens?
  - Caspases
  - Exogenous gonadotoxic agents
  - The reactive oxygen species (ROS)
    - The main sources of ROS in semen include leukocytes, abnormal/immature sperm, increased, scrotal temperature, varicocele, advanced male age , smoking, estrogens …

- Different theories and presentations of DNA damage are shown in the next several slides

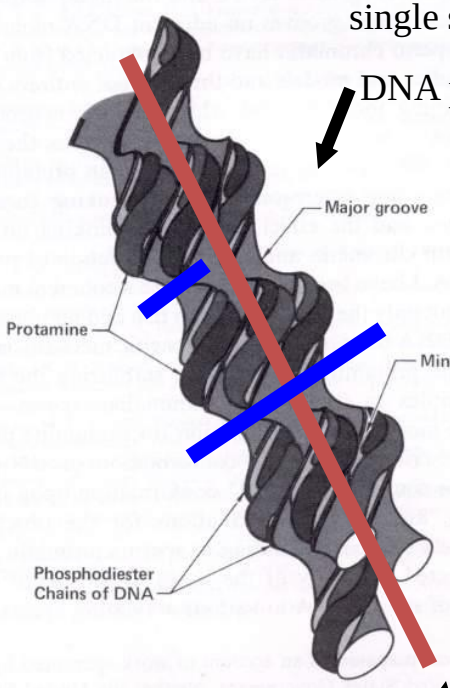
A two-step hypothesis of DNA damage in the male germ line



Aitken et al, 2009



DNA Fragmentation (Double or single stranded DNA pieces)



DNA denaturation

Google Image

What assays are used to assess sperm nuclear DNA status?

Method	Parameter Assessed	Method of Assessment
SCSA® (SDFA)	DNA Denaturation (heat/acid), nicks in DNA	Flow Cytometry
TUNEL	ss- & dsDNA (endog. nicks in the DNA molecule, fragmentation), terminal deoxynucleotidyl transferase	Fluorescent/Optical Microscopy, Flow Cytometry
Comet Assay	**Neutral:** Double stranded low molecular DNA Fragments (dsDNA) **Alkaline:** Double & single stranded low molecular DNA Fragments	Fluorescent Microscopy
Sperm Chromatin Dispersion (SCD)	Low molecular DNA Fragments	Fluorescent Microscopy
Acridine Orange (AO)	DNA Denaturation (acid), binding of AO to dsDNA (green) or ssDNA (yellow-red)	Fluorescent Microscopy, Flow Cytometry
Acidic aniline blue (AAB)	Nuclear Maturity (DNA protein composition, histones , lysines)	Optical Microscopy
Iodoacetamide	Nuclear DNA integrity/maturity	Fluorescent Microscopy

Method	Parameter Assessed	Method of Assessment
Toluidine Blue Stain	DNA Fragmentation	Optical Microscopy
Chromomycin A_3	Nuclear Maturity (DNA protein composition)	Fluorescent Microscopy
DNA Breakage Detection via FISH (Fernandez for SCD)	Single Stranded DNA Fragmentation (ssDNA)	Fluorescent Microscopy
***In Situ*Nick Translation**	Single Stranded DNA Fragmentation (ssDNA)	Fluorescent Microscopy, Flow Cytometry
8-OHdG Determination 8-Hydroxydeoxyguanosine	8-OHdG	HPLC
DNA Diffusion Assay	DNA Fragmentation using YOYO-1 stain	Fluorescent Microscopy
Gene-specific DNA Damage	β-globin, IGF-2, telomeric sequences	PCR

Tests to Determine Sperm Nuclear DNA Status

Initial Denaturation Step

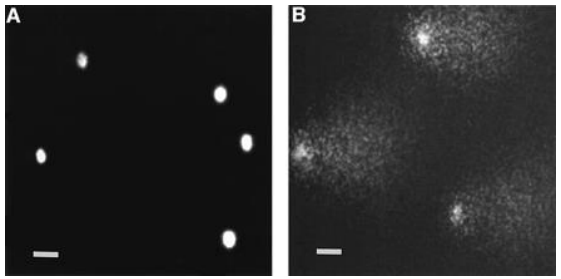
(DNA Susceptibility)

- **Sperm chromatin structure or stability assay (SCSA)**
- **Sperm chromatin dispersion assay (SCD or halosperm)**
- **Chromomycin A3**
- **DNA breakage detection (DBD) assay using FISH (DBD-FISH)**
- **COMET assay that utilizes acid or alkaline denaturation of sperm DNA**

No Denaturation Step

(Actual DNA Status)

- **TUNEL**
- **in situ-nick translation (ISNT)**
- **COMET assay carried out under neutral pH conditions**

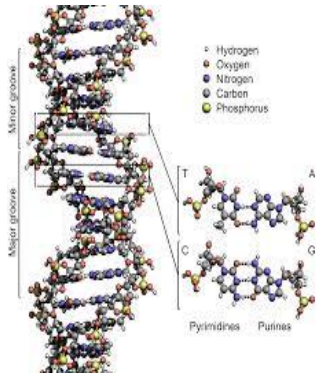


Principles Behind A Few Commonly Performed Sperm Nuclear DNA Assays

- TUNEL
- SCSA
- COMET
- Sperm Chromatin Dispersion Assay (SCD, Halosperm)
- Some of these assays assess DNA status as exists (physiological pH) within the sperm and some determine the DNA status and stability by treating the DNA with acids, bases or heat

Principles Behind the TUNEL Assay

- **Name:** Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)
- If sperm have damaged (fragmented) nuclear DNA, there will be many pieces of DNA with 3' and 5' ends within their nuclei
- If you have a compound (a nucleotide) that can bind to one of the DNA fragment ends (to the 3'-hydoxyl end) and this compound or nucleotide is attached to a fluorescent dye (FITC-conjugated), then we can read the percentage of sperm with damaged DNA. These cells exhibit strong green fluorescence (TUNEL positive)



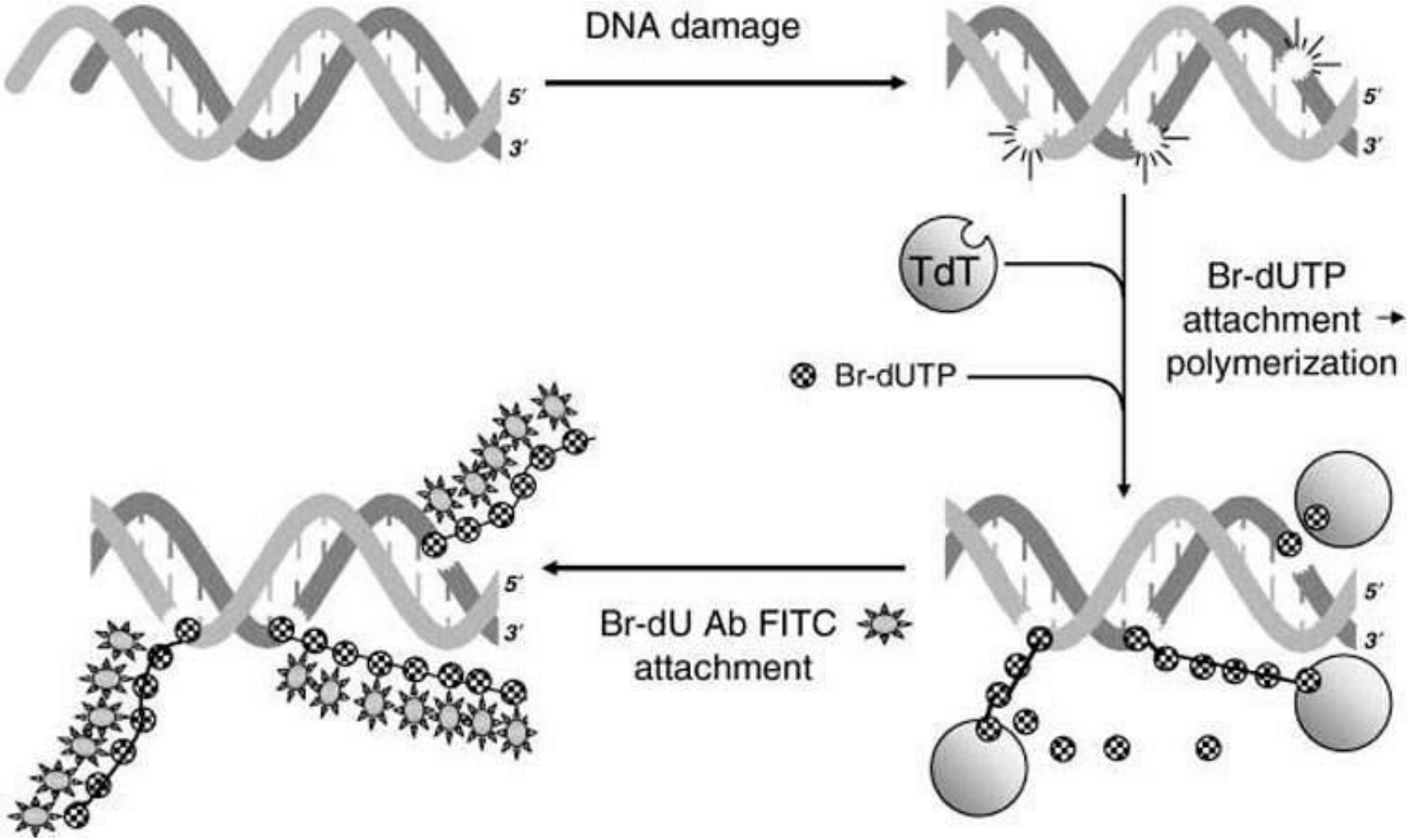


Figure 5.

Schematic illustration of DNA strand-break labeling by TdT-mediated Br-dUTP attachment to 3'OH ends and polymerization, followed by immunocytochemical (FITC) detection of BrdU using an antibody to d-UTP (Br-dU Ab FITC).

Cell Prolif. 2005 August; 38(4): 223–243.

dUTP= deoxyuridinetriphosphate; TdT= terminal deoxyribonucleotidyl transferase

Fresh or Frozen Sperm (native semen or motile sperm)

↓

Wash, adjust to a certain concentration, resuspend in a buffered medium with albumin (PBS, 1% HSA)

↓

Place the sample on a slide, fix with 4% paraformaldehyde, wash and permeabilize the membrane with 0.1% triton X-100 in citrate buffer

↓

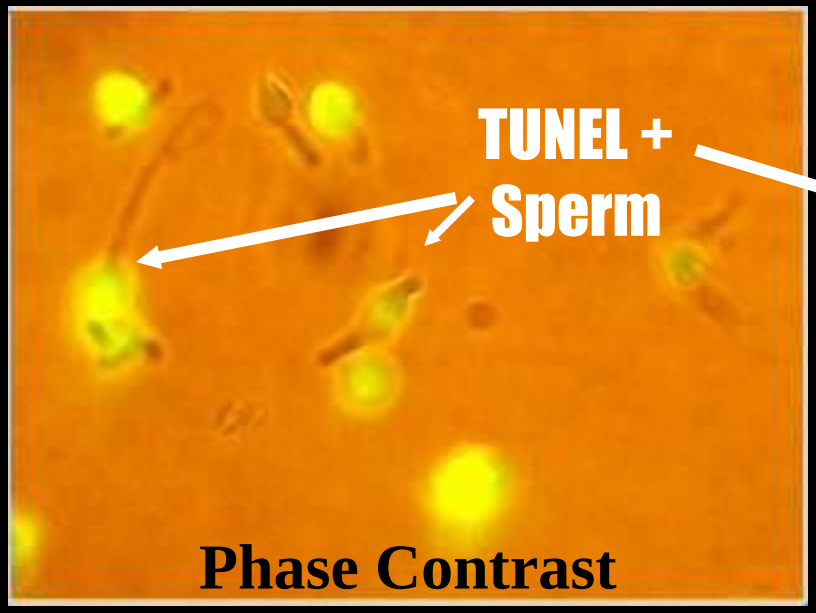
Wash, add TdT and Br-dUTP-FITC, wash.
dUTP-FITC binds to the 3'-OH sites of DNA stand breaks and fluoresces upon binding

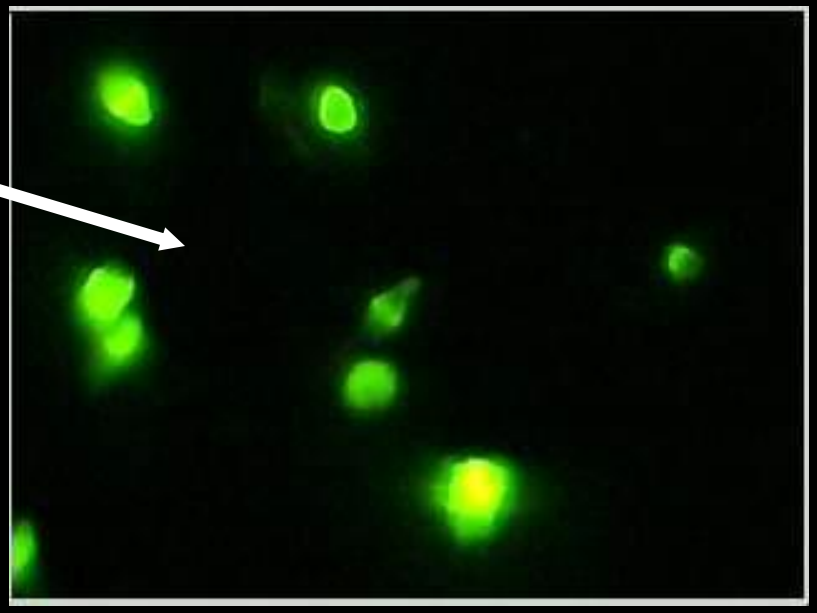
↓

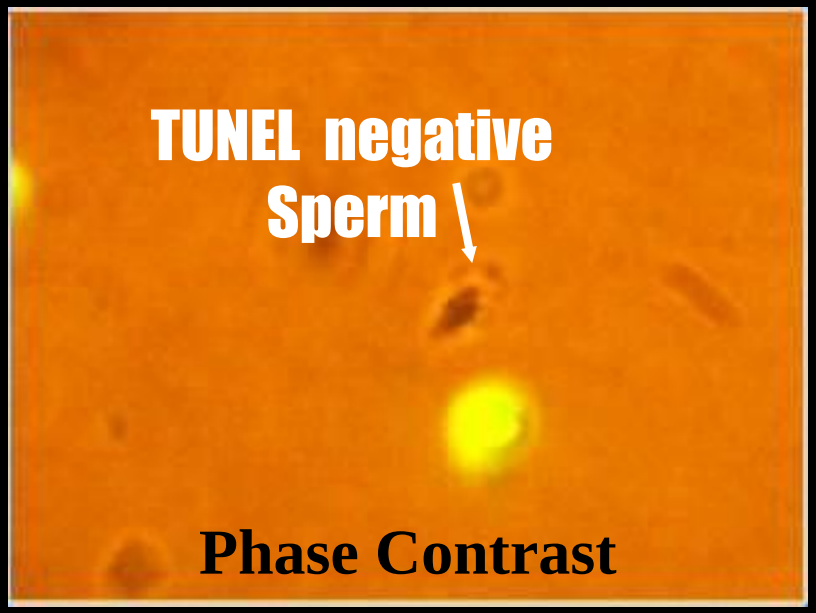
Read 100-500 sperm, using a fluorescent microscope

Questions

- What will be a negative control for the TUNEL assay?
  - No dUTP in the mixture
- How about the positive control for the assay?
  - Add DNAse to break DNA strands







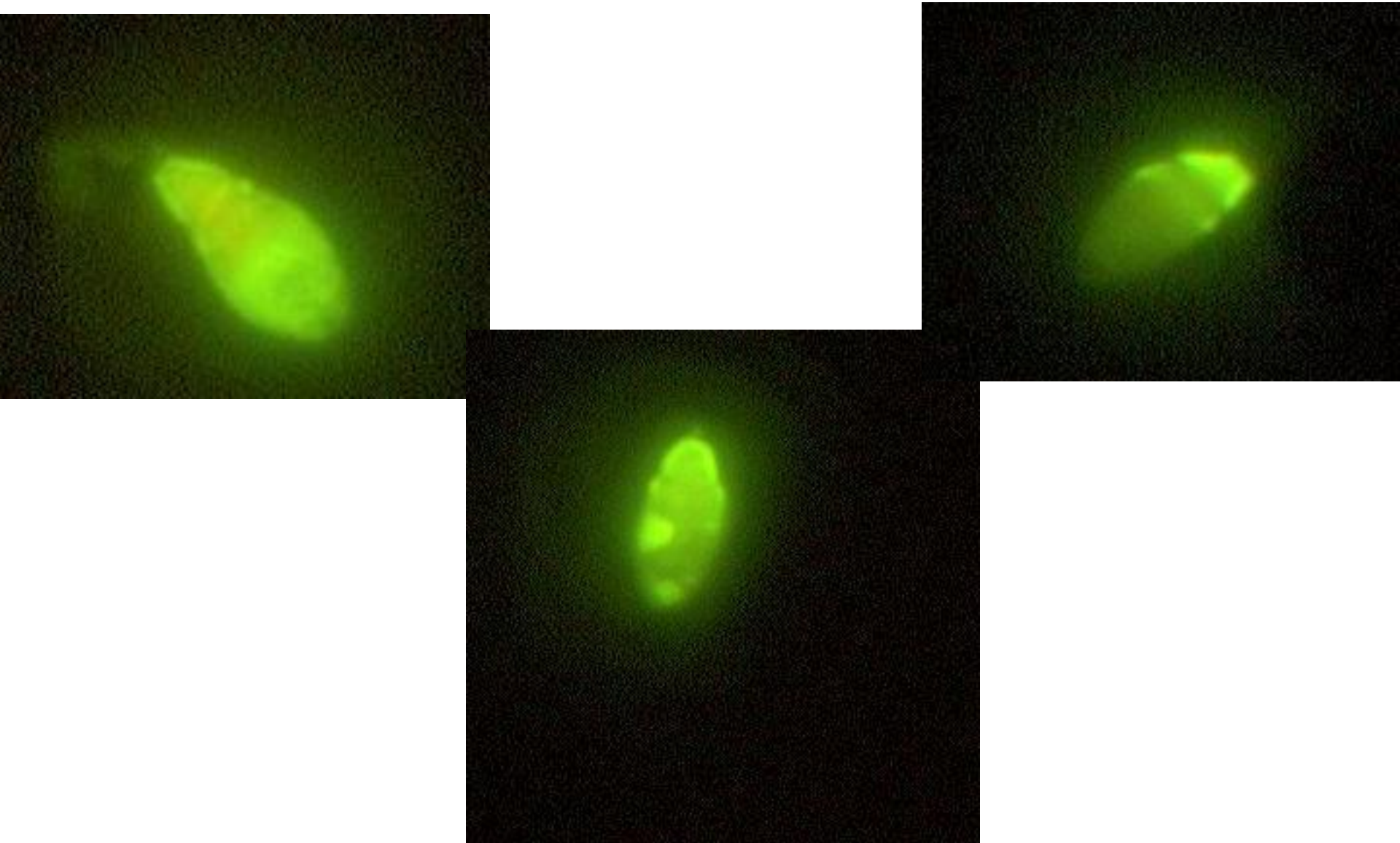
Calculation: (# of green/total sperm) x 100

More about the TUNEL Assay

- TUNEL can reveal both single and double stranded DNA damage
- However, it cannot quantify the magnitude of the damage in individual cells unless a technique develops that assesses the degree and the pattern of green fluorescence in sperm heads

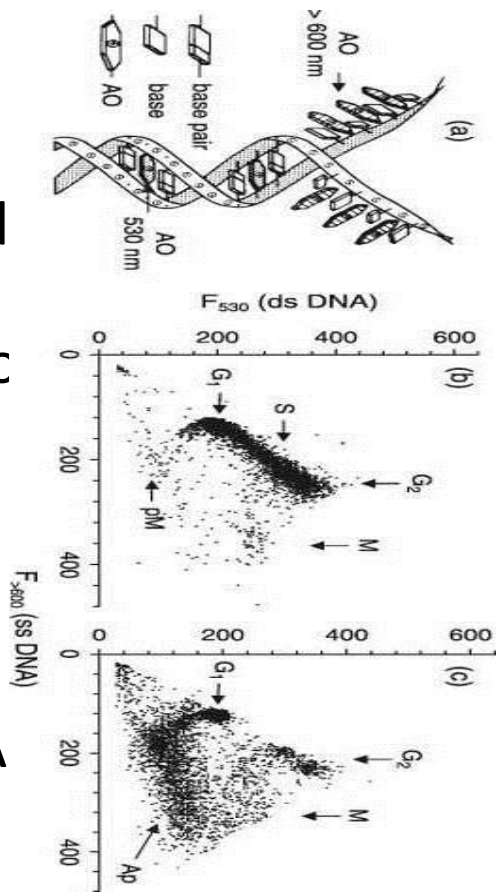
TUNEL: Patterns of DNA Fragmentation In Ejaculated Spermatozoa

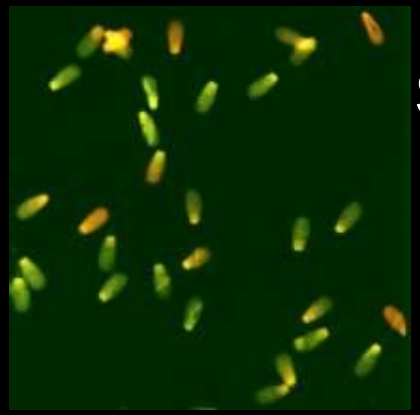
(Barroso et al, F@S, 2000, 2009)



Principles Behind the SCSA Assay

- Name: Sperm Chromatin Structure Assay
- If sperm is exposed to an acidic environment (i.e., pH of 1.2), DNA molecules with less than normal S-S bonds and other stabilizing factors, are denatured easily (become single stranded and unwind)
- Then, if a dye like acridine orange (AO) that binds tc the single stranded DNA is used, the dye binds to the denatured DNA and stains it orange/red
- Sperm with stable (sometimes called mature) DNA, do not have single stranded (ss) DNA and do not stain orange/red. They stain green because AO intercalates with the bases of double stranded DNA (intact DNA) as a monomer and emits green fluorescence at 530 nm
- With this assay a DNA Fragmentation Index (DFI) and High DNA Stainability (HDS) is calculated and reported





Stained Cells

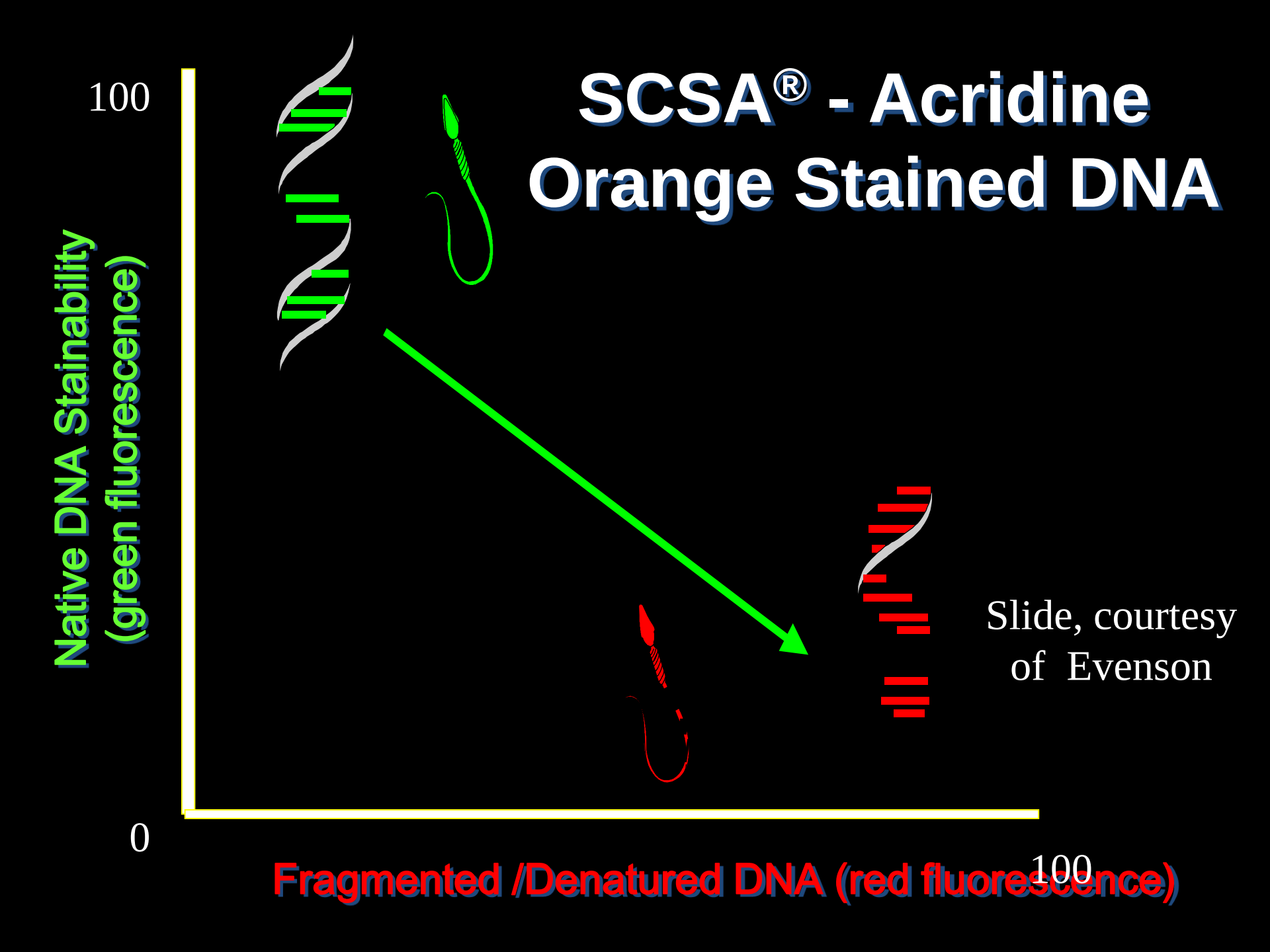
Photomultiplier Tube

Collecting Lens

Excitation Source (Laser Beam)

Slide, courtesy of Evenson

Flow Cytometry Determination of Green or Red Sperm


SCSA® - Acridine Orange Stained DNA
100
0
Native DNA Stainability (green fluorescence)
Fragmented /Denatured DNA (red fluorescence)
100
Slide, courtesy of Evenson

SCSA® Parameters

DFI = DNA Fragmentation Index

DFI = red fluorescence/ red + green

More pertinent for natural conception and IUIs rather than IVF or ICSI

HDS = High DNA Stainability =

sperm with defective DNA

Clinical Results of the SCSA®

Pregnant:
DFI of <10%

Measurement	DFI (%)	HDS (%)
1	6.8	5.0
2	8.3	5.4
mean	7.5	5.2
sd	1.1	0.2

Non-Pregnant

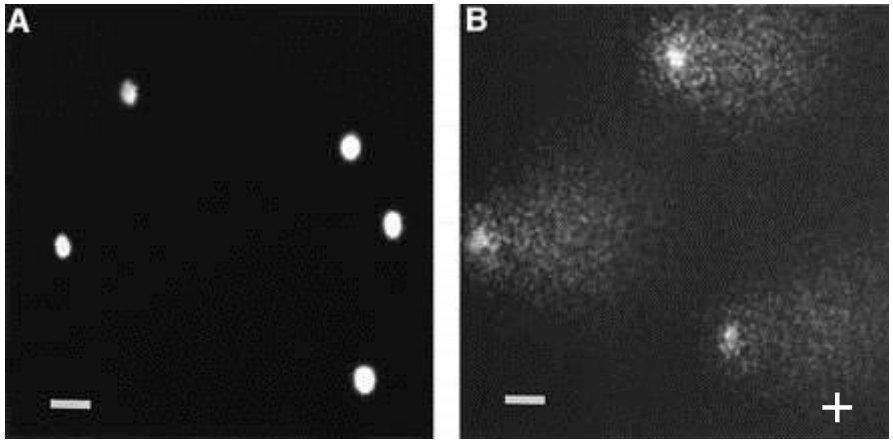
Measurement	DFI (%)	HDS (%)
1	64.9	6.4
2	64.9	7.2
mean	64.9	6.8
sd	0.0	0.4

Evanson, et al

NOTE: Published results have not been consistent. In general, DFI of >30% and HDS of >15% are indicative of subfertility SCSA's intra-individual coefficient of variation has been calculated as 30±21.5% (range: 0–130%)

The Principles Behind the Comet Assay

- The principle behind comet assay is that the negatively-charged broken DNA molecules are free to migrate in an electric field towards the anode (+ pole), with the shorter fragments moving faster. The pattern of migration produces a profile resembling the shape of a comet. Two main principles determine comet formation patterns: the size of DNA fragments and the number of fragments.

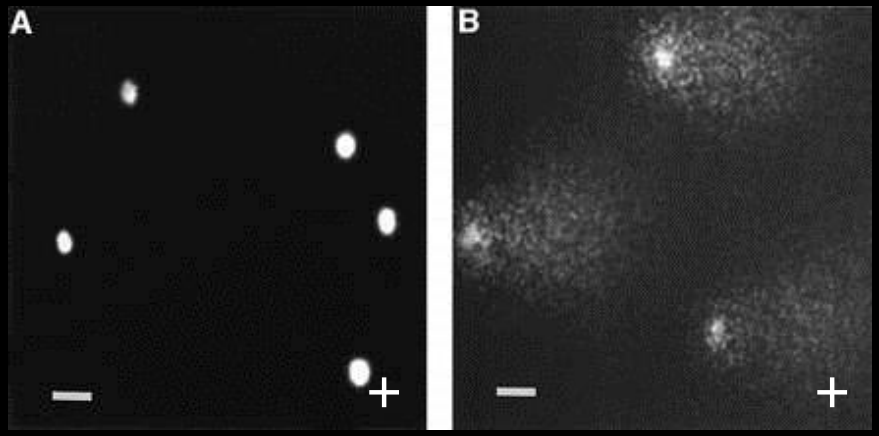


The Comet Assay: Methodology

- Performed at neutral and basic pH
- At neutral pH, it reveals only double stranded DNA breaks
- At basic pH, it detects both double and single stranded DNA breaks

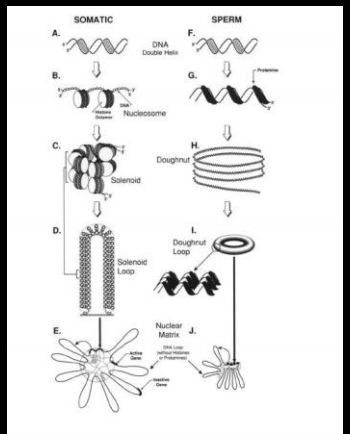
- The assay also is capable of measuring the magnitude of DNA damage
- The assay has not been standardized so techniques are different from lab to lab and the results cannot be compared due to this lack of standardization.

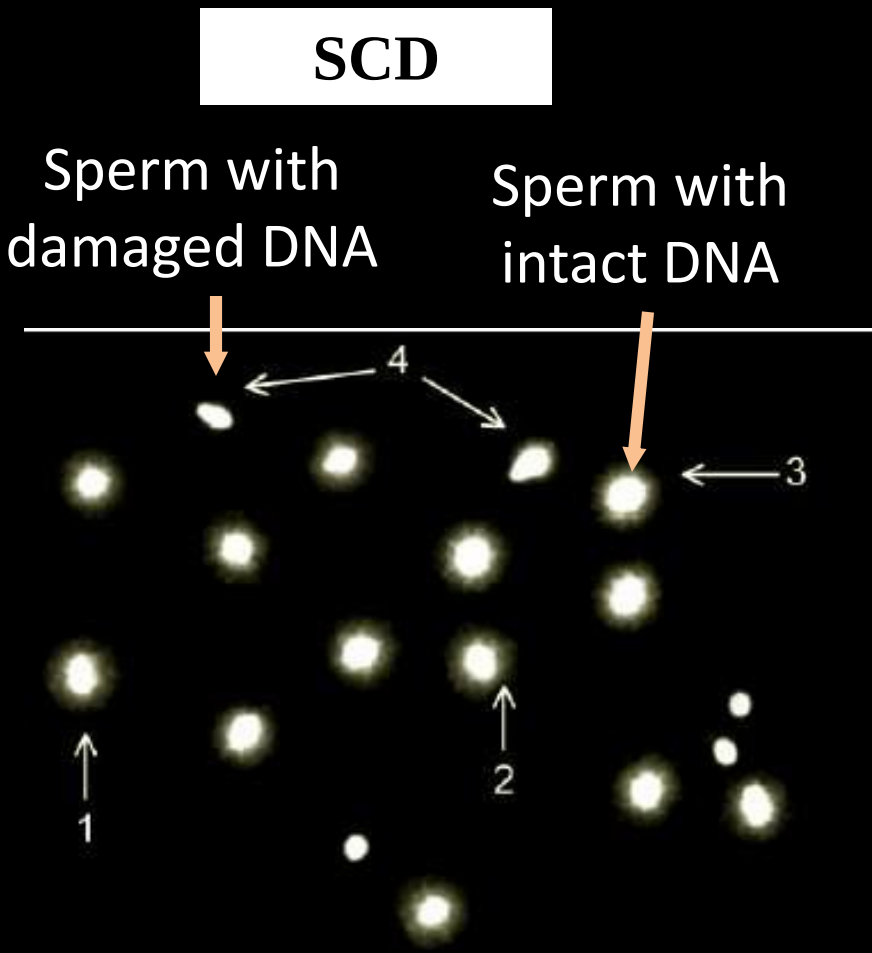
In sperm chromatin dispersion, SCD, assay, sperm with intact DNA appear fuzzy, whereas fuzziness in comet is indicative of damaged nuclear DNA



Sperm with intact DNA

Comet sperm with damaged DNA





Methodology: TUNEL Versus Other Assays of Sperm Nuclear DNA Status

- In the TUNEL assay, cells are fixed, permeabilized and their nuclear DNA status is assessed as is
- Cells are not treated with acids or bases
- In some other tests such as sperm chromatin structure assay, SCSA, or some versions of Comet assay, sperm are treated with acids or bases in order to assess the susceptibility of their nuclear DNA to denaturation. The more unstable the DNA (meaning less S-S bonds, less protamines, or more histones), the higher the level of denaturation.
- Some assess ssDNA, some dsDNA and some both

Are there any correlations among the most frequently performed assays of sperm nuclear DNA?

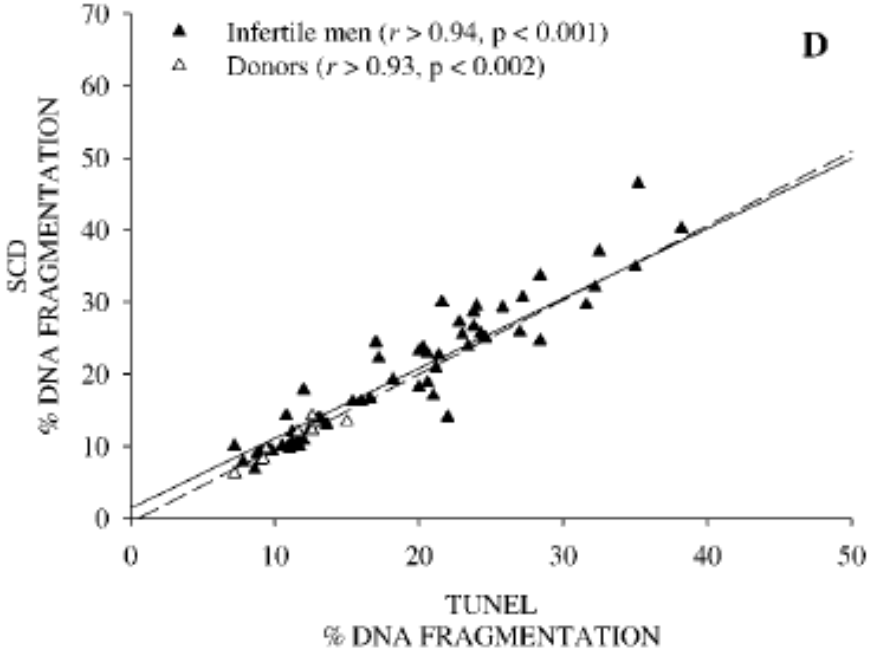
Chohan et al Compared:

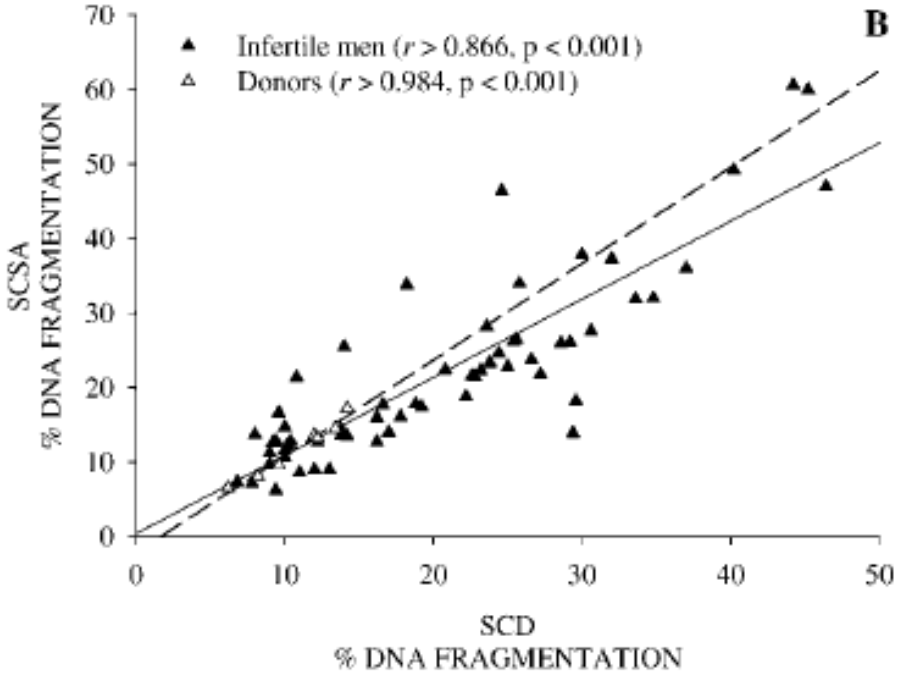
- TUNEL with
- SCSA® (SDFA) and
- SCD (sperm chromatin dispersion)
- AO

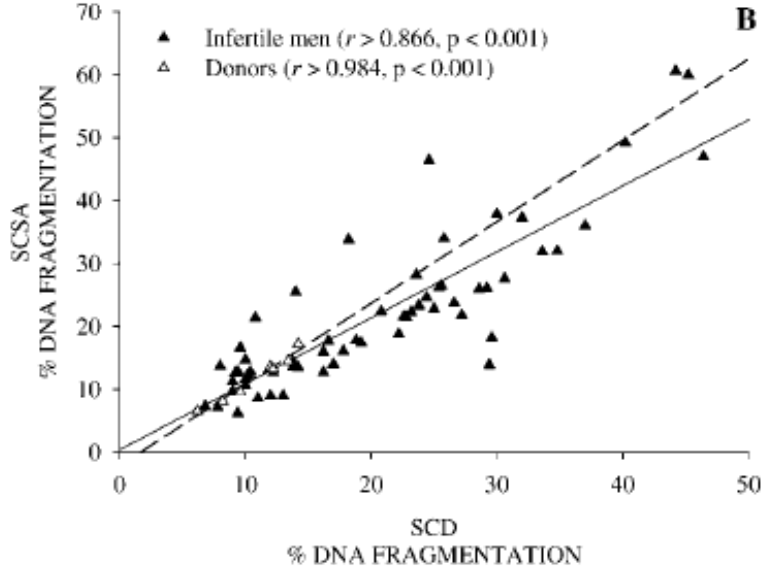
- 60 men attending the andrology lab and 7 fertile men
- Semen samples were washed only

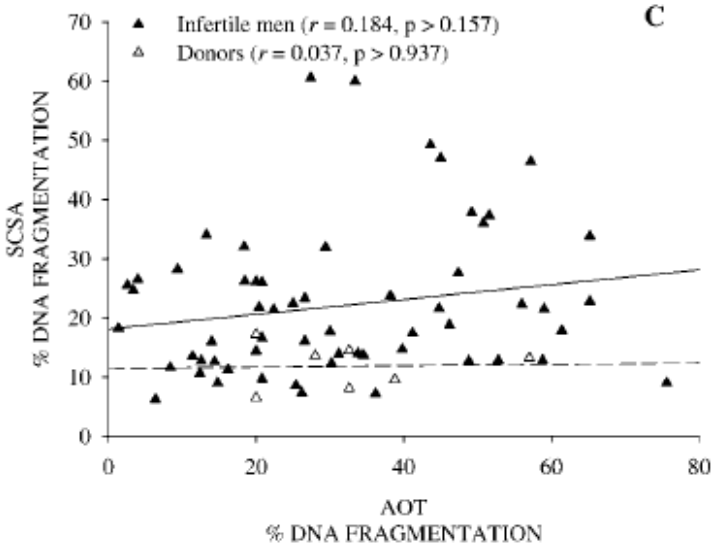
Chohan et al J Androl 2006

Chohan K. et al, J Androl, 27(1):2006


Infertile men ($r > 0.94$, $p < 0.001$)
Donors ($r > 0.93$, $p < 0.002$)
D
SCD
% DNA FRAGMENTATION
TUNEL
% DNA FRAGMENTATION


Infertile men ($r > 0.866$, $p < 0.001$)
Donors ($r > 0.984$, $p < 0.001$)
B
SCSA
% DNA FRAGMENTATION
SCD
% DNA FRAGMENTATION


Infertile men ($r > 0.866$, $p < 0.001$)
Donors ($r > 0.984$, $p < 0.001$)
B
SCSA
% DNA FRAGMENTATION
SCD
% DNA FRAGMENTATION


Infertile men ($r = 0.184$, $p > 0.157$)
Donors ($r = 0.037$, $p > 0.937$)
C
SCSA
% DNA FRAGMENTATION
AOT
% DNA FRAGMENTATION

*Comparison of sperm DNA fragmentation in infertile men and fertile donors; values are mean ± SEM; different superscript lowercase letters show statistical difference (P < .05) within rows; different superscript capital letters show statistical difference within columns (P < .05)**

	SCSA	TUNEL	SCD	AOT
Infertile men (n = 60)	22.0 ± 1.6^{aA}	19.5 ± 1.3^{aA}	20.4 ± 1.3^{aA}	31.3 ± 2.4^{bA}
Donors (n = 7)	11.8 ± 1.4^{aB}	11.1 ± 0.9^{aB}	10.8 ± 1.1^{aB}	32.7 ± 4.8^{bA}

Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay

Ribas-Maynou et al, 2013

Samples were frozen, then tested. All assays except neutral Comet assay were able to differentiate between fertile donors and infertile patients

Comet (alkaline): The best, with the threshold value of ~ 45% for infertility followed by
TUNEL: Threshold value for infertility ~ 20%
SCD: Threshold value for infertility ~ 23%
SCSA: Threshold value for infertility ~ 19%
Comet (alkaline): No predictive power (Threshold: 34%)

Table 2 Cut-off values with sensitivity and specificity obtained for each technique

Technique	*n*	Area*	Cut-off value (%)	Sensitivity	Specificity
Alkaline Comet	183	0.937	45.37	0.850	0.920
Neutral Comet	183	0.516	34.37	0.970	0.320
SCD test	123	0.869	22.75	0.730	0.918
SCSA	98	0.792	18.90	0.595	0.875
TUNEL	93	0.903	20.05	0.764	0.952

*Area below the ROC curve.

Ribas-Maynou et al, 2013

- What is reported in the literature about the influence of sperm nuclear damage on the outcome of ART?

Use of Sperm with Nuclear Damage May Result In:

- Poor embryonic development
- Decreased implantation
- Lower pregnancy rates
- Fetal mutations
- Recurrent pregnancy losses
- Increased risk of cancer in offspring

Sperm DNA Damage and Outcome of ART

Study	Assay	IVF, cases	ICSI, cases	FR	EQ	PR
Host, 2000	TUNEL	50	61	Dec., IVF	No Report	No Report
Tomlinson, 01	TUNEL	140	--	No change	No change	Decreased
Benchaib, 03	TUNEL	50	54	Dec., ICSI	--	Dec., ICSI
Henkel, 04	TUNEL	249	--	No change	No Report	Decreased
Seli, 04	TUNEL	49	--	No Report	Decreased	No change
Haung, 05	TUNEL	217	86	Decreased	No change	No change
Tomsu, 02	COMET	40	--	No change	Decreased	Decreased
Morris, 02	COMET	20	40	No change	Decreased	No Report
Larson, 03	SCSA	55	34	No change	No change	Decreased
Virro, 04 (blast)	SCSA	249	--	No change	Decreased	Decreased
Payne, 05	SCSA	46	54	Decreased	No Report	**Increased**
Zini, 05	SCSA	--	60	Decreased	Decreased	Decreased

Sperm DNA Damage and Outcome of ART

Study	Assay	IVF, cases	ICSI, cases	FR	EQ	PR
Bungum, 04	SCSA	109 IVF 131 IUIs	66	No Report	No Report	Higher with low DNA damaged sperm
Gosalvez, 13	Halosperm 24.8% semen; 17.5% SU	__	ICSI, Donor Oocytes	__	__	Decreased
Duran et al, 2002	TUNEL	IUIs , 119 patients 154 IUI cycles				No pregnancy with TUNEL > 12%
Alkhayal et al, 2013	SCSA, AAB, IAF	IUIs, 102 cycles, 15 fertile donors				Progressive motility and DFI <15% correlated

Problems with DNA Fragmentation Studies

Some Studies assessed DNA status in neat semen some in the motile fraction

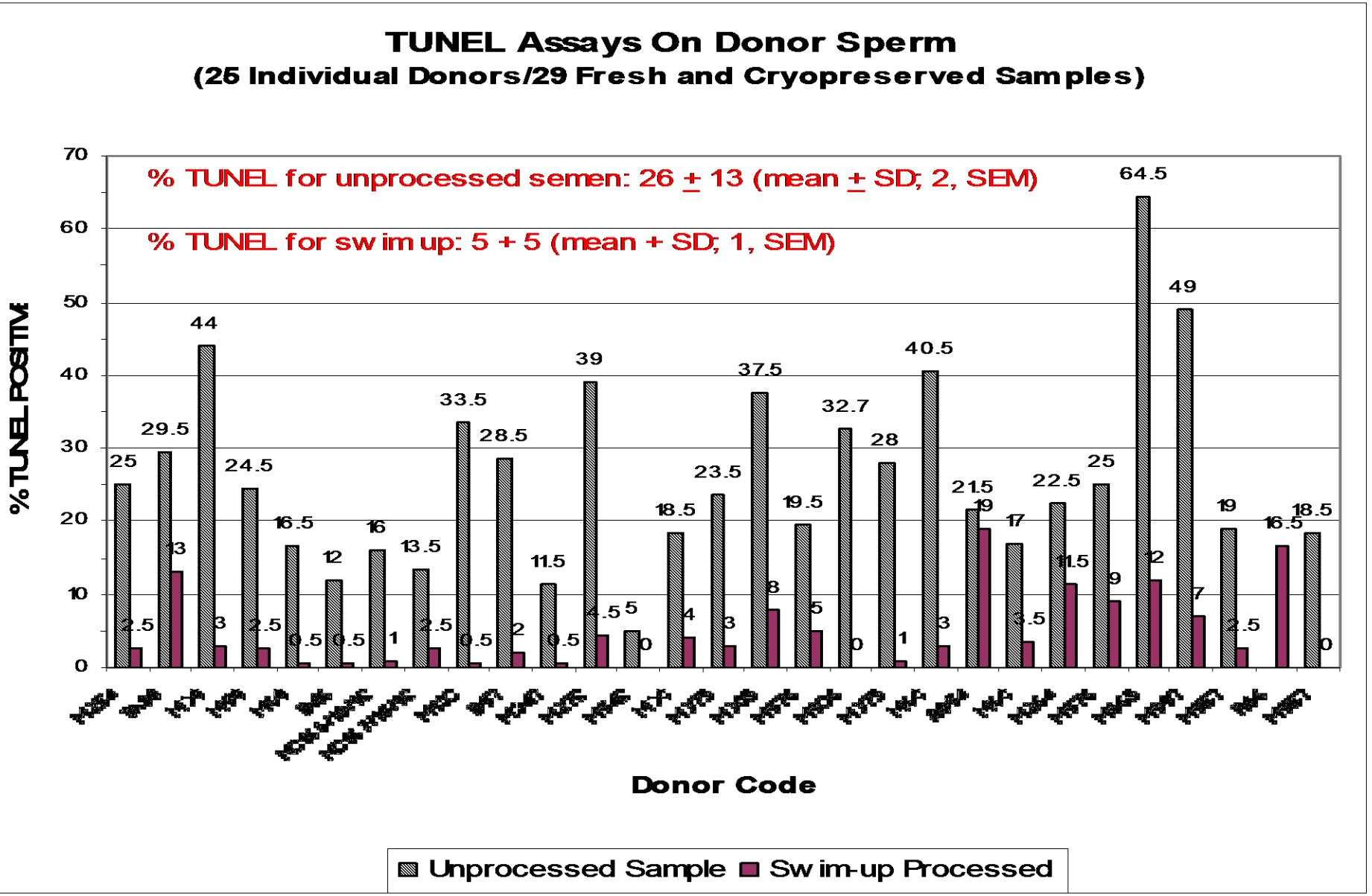
Ranges of DNA-fragmentation (reviewed in Sergerie *et al.*, 2005) have been very wide.

Several studies, working with SCSA, TUNEL and Comet assays, attempted to establish threshold values with which success or failure of natural conception and assisted reproduction treatment could be predicted (see next slide)

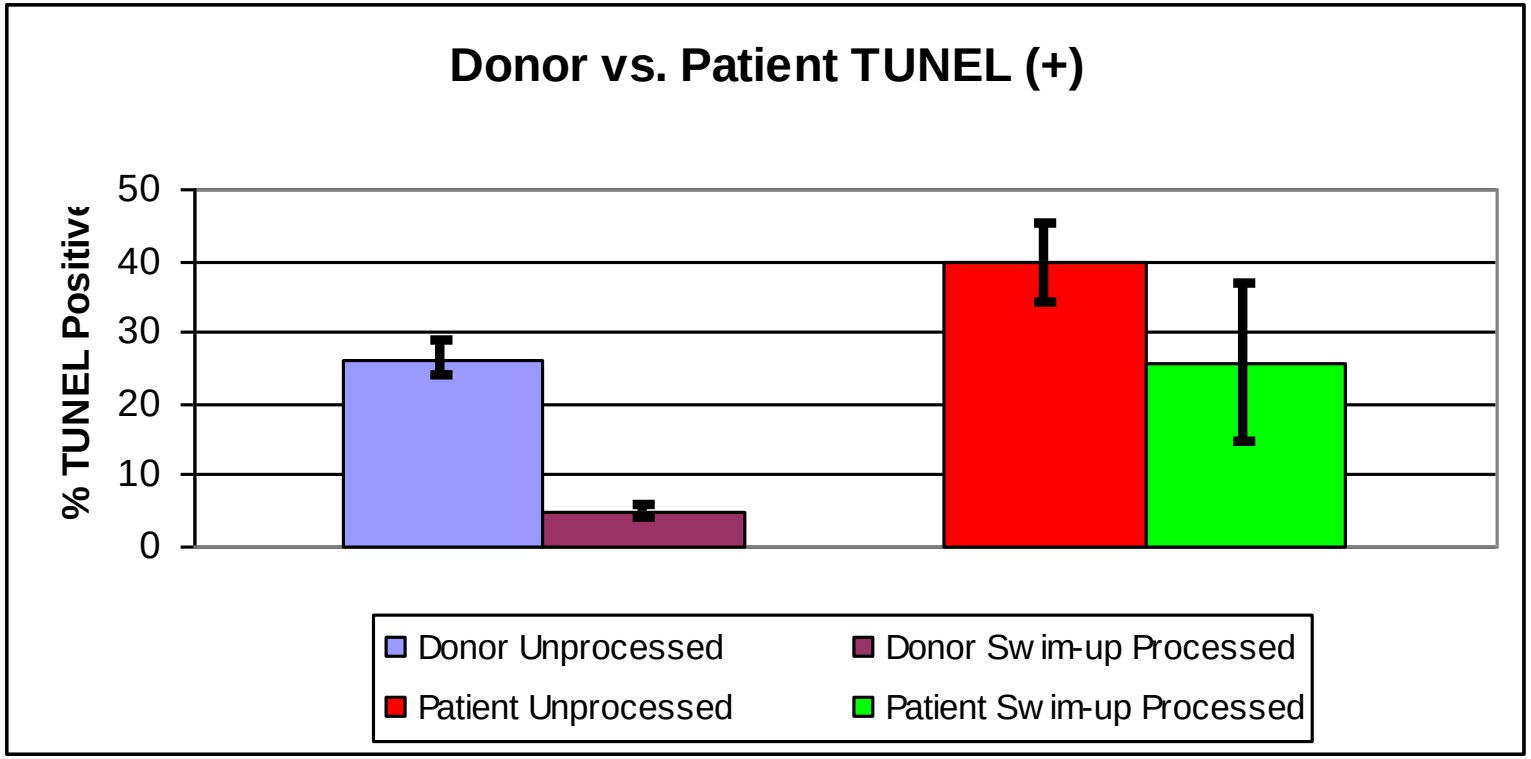
The values recommended in different studies showed a high degree of variability with a clear relationship with the type of assay used and the type of assisted reproduction treatment considered

Discriminating threshold values of SCSA & TUNEL suggested for prediction of ART outcome

Study	Threshold	DNA Method	ART Method
Duran, 02	12%	TUNEL (Mic.)	IUI
Benchaib, 03	18%	TUNEL (Mic.)	ICSI
Henkel, 03	24.3%	TUNEL (Mic.)	ICSI
Henkel, 04	36.5%	TUNEL (Mic.)	IVF
Larson-Cook, 03	27% (DFI)	SCSA (Flow)	IVF/ICSI
Virro, 04 (blast) Same group as L-C	30% (DFI)	SCSA (Flow)	IVF/ICSI
Greco, 05	>15%	TUNEL (Mic.)	ICSI (n=29); 2 preg., no term preg., prev. >=2 ICSI failure
Hazout, 06	30%	TUNEL (Mic.)	ICSI
Our Internal Study, 2010, sperm donors	26% washed 5% swim-up	TUNEL (Mic.)	--
Gosalvez, 2013	24.8%, washed 17.5%, SU	Halosperm	ICSI



In our donor population, The mean for TUNEL positive sperm in semen was 26% (27± 13) and for the donor motile sperm was 5%



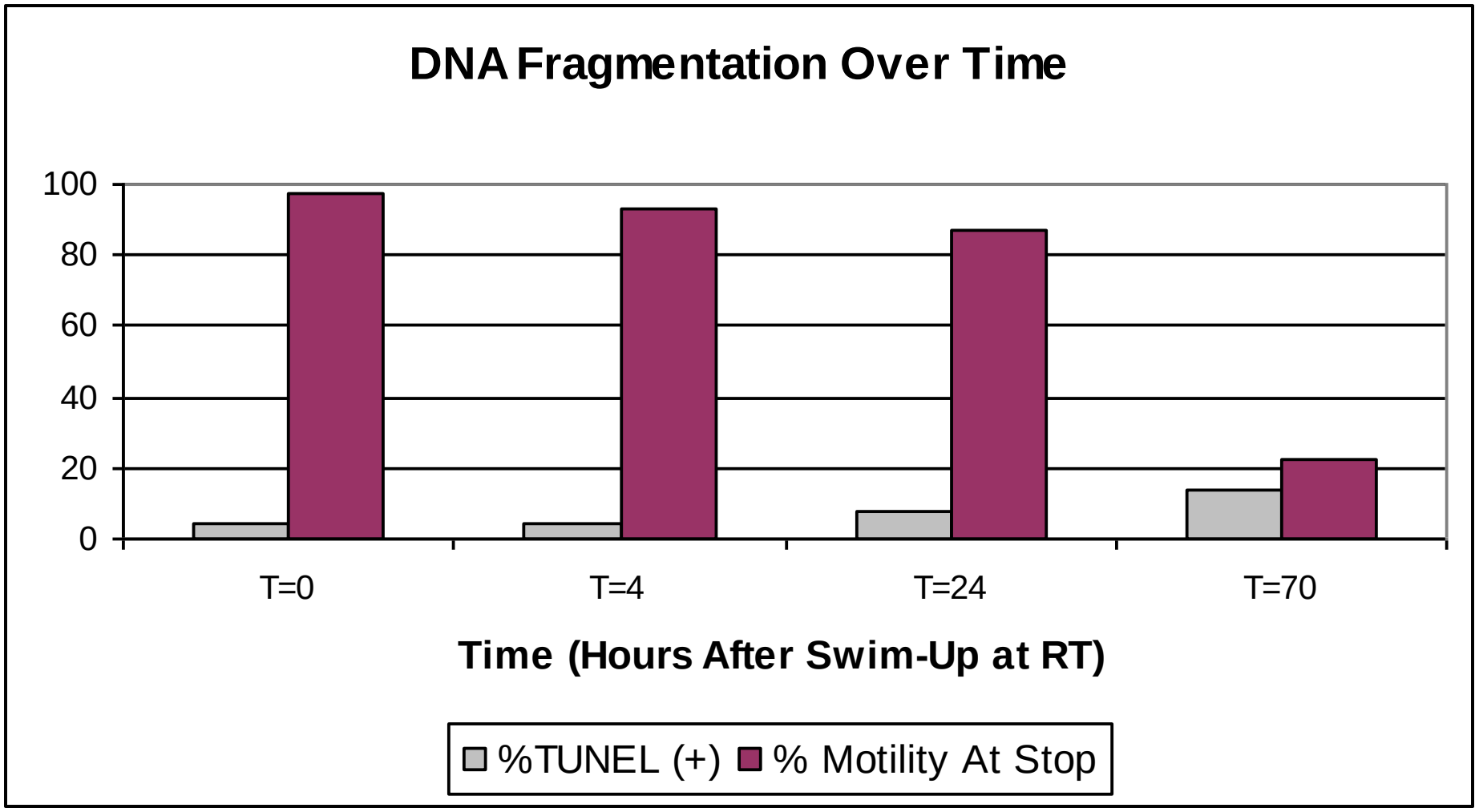
28 Sperm Donors

- **5 Fresh**
- **10 ICI Cryopreserved**
- **14 IUI-ready Cryopreserved**

20 individual Patients

- **6 Post-cancer therapy**
- **14 Infertility Patients**

Our Study: Motile sperm fraction from patients (green) had significantly higher TUNEL + sperm than the donors' (maroon)



Our small study: DNA fragmentation does not increase significantly upon storage of swim up sample for up to 70 hours at room temperature (n=3)

TUNEL in Post-Chemo Patients

Patient's Initials	TUNEL Semen % +	TUNEL Swim up % +
SC	69.5%	21.5%
NH	62%	34%
AP	56%	12.5%
SY	16%	2.8%
SH	30.5%	0%
MS (organ transplant)	17%	2%
NH (pre-cancer treatment)	89%	90%

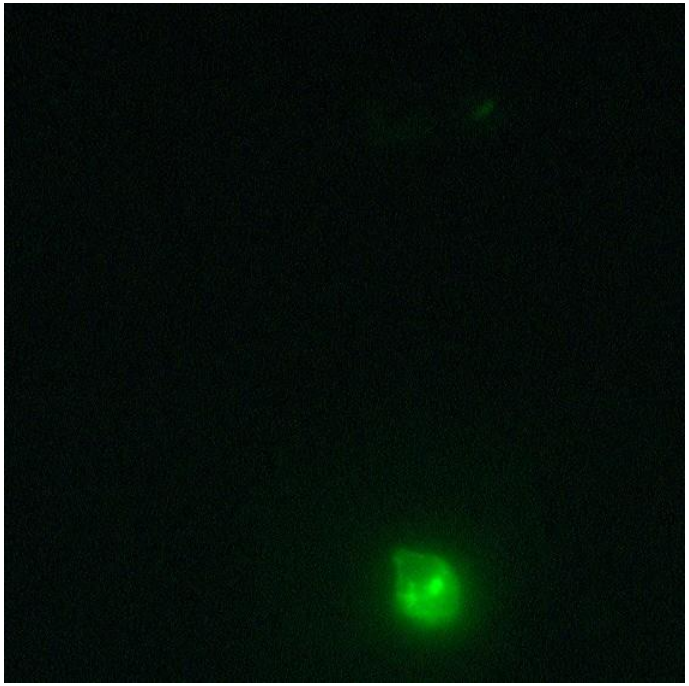
Our Small Study: Some patients who resumed sperm production post chemotherapy had low TUNEL and some had high in their motile sperm fractions. The wife (29 years old) of the one with the low TUNEL, SY, had positive clinical pregnancy.

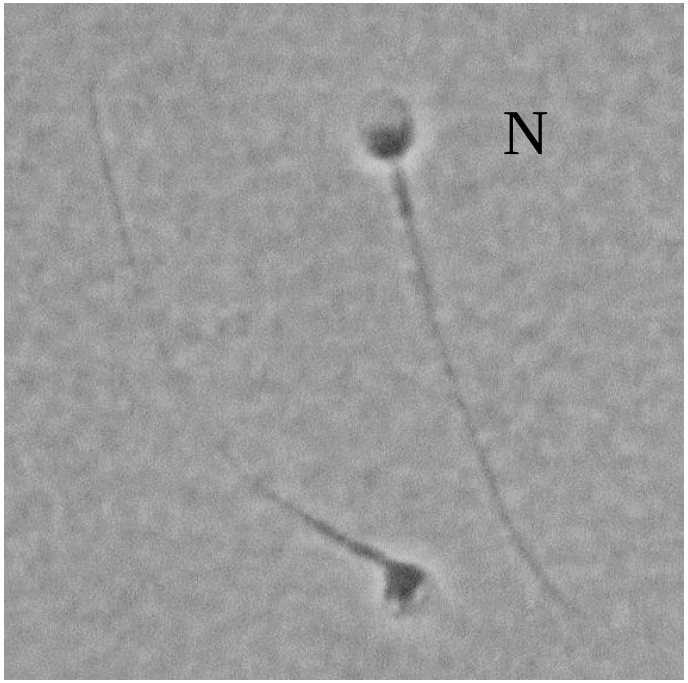
Does DNA Fragmentation Relate to Major Semen Parameters?

- Published Studies

- Our Studies
  - Sperm morphology and DNA fragmentation (TUNEL)
  - Sperm morphology, DNA fragmentation and embryo quality

- Next several slides show that the morphology of sperm selected for ICSI may not necessarily gurantee that the sperm has intact nuclear DNA

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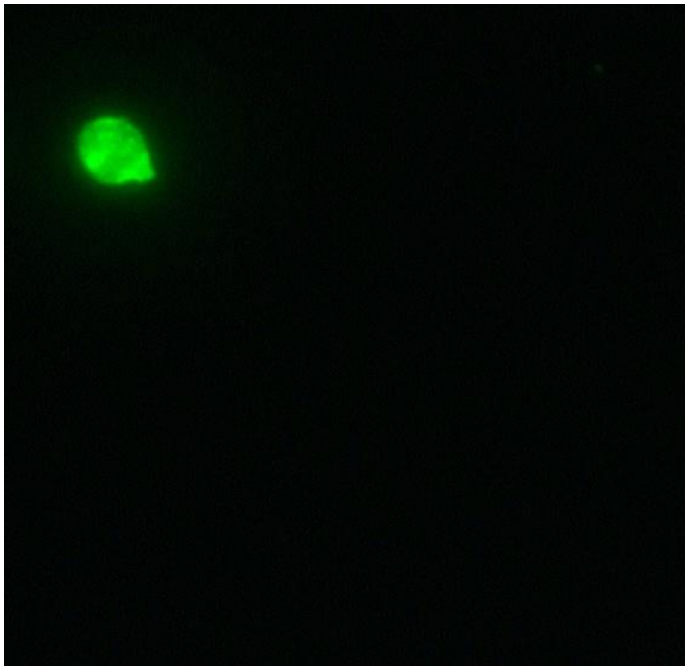
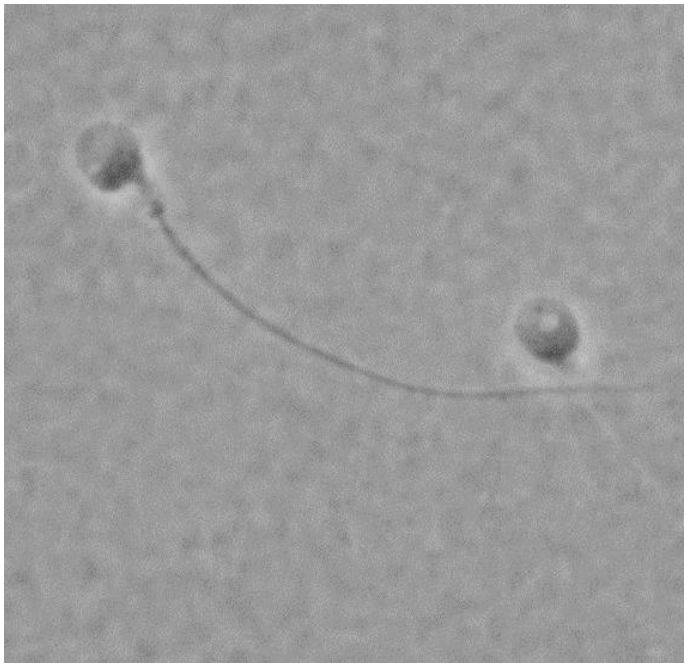
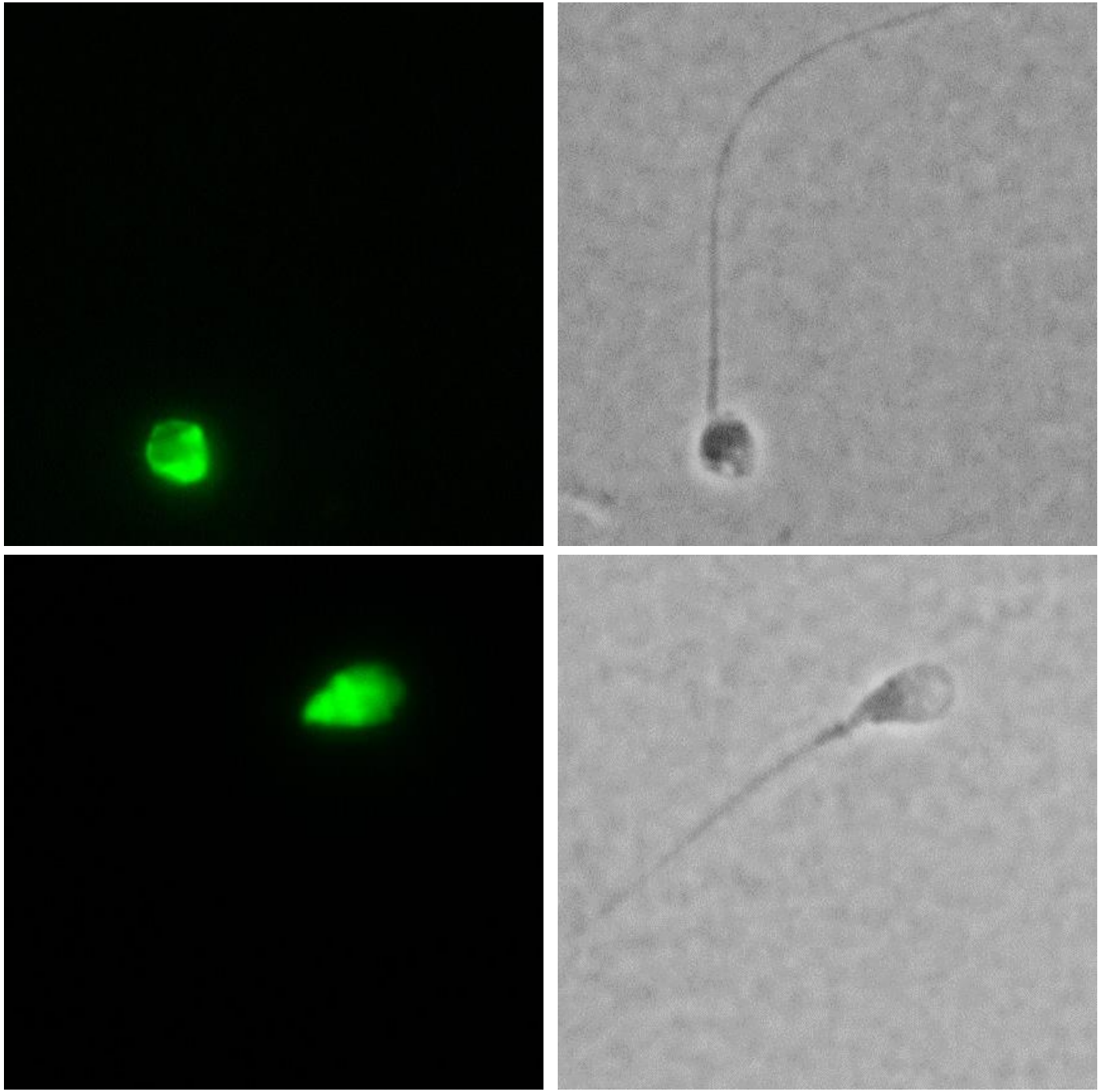
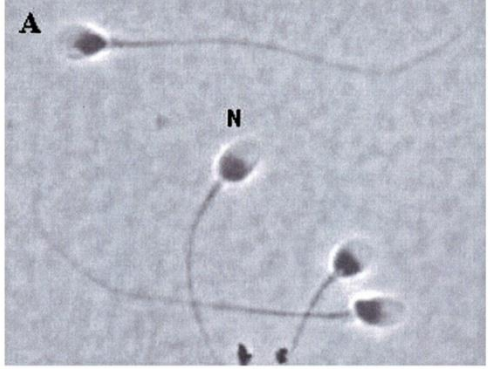
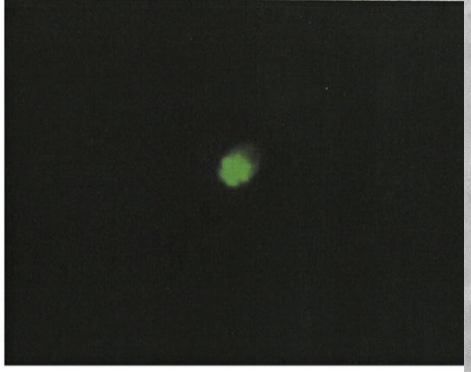
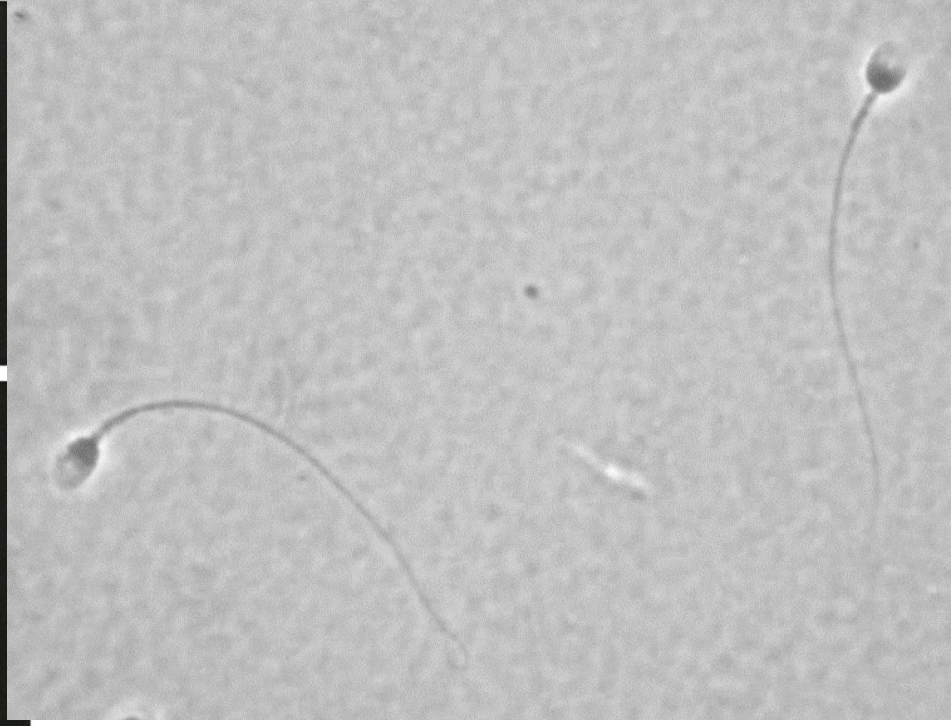


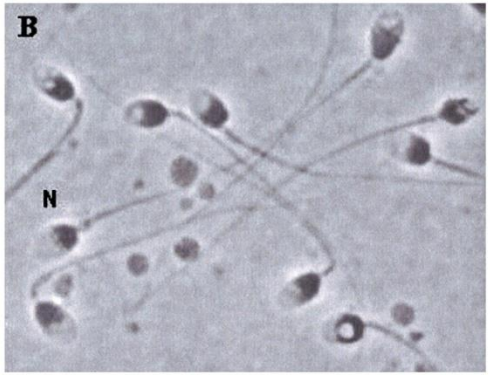


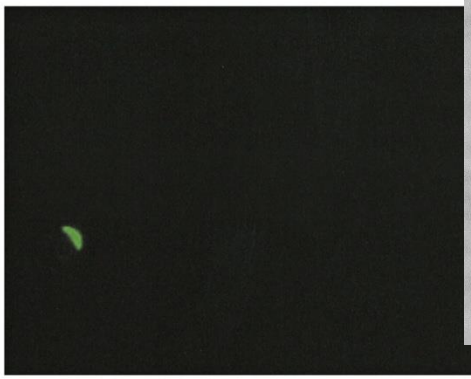


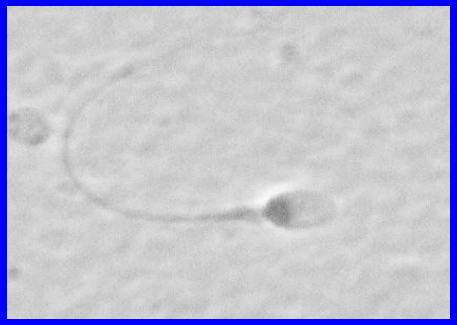


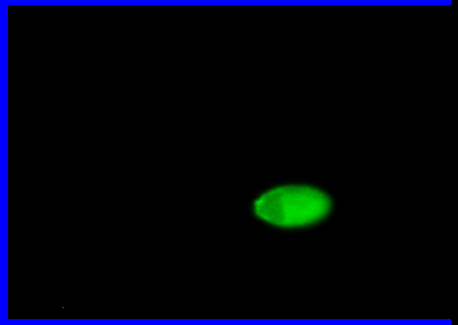


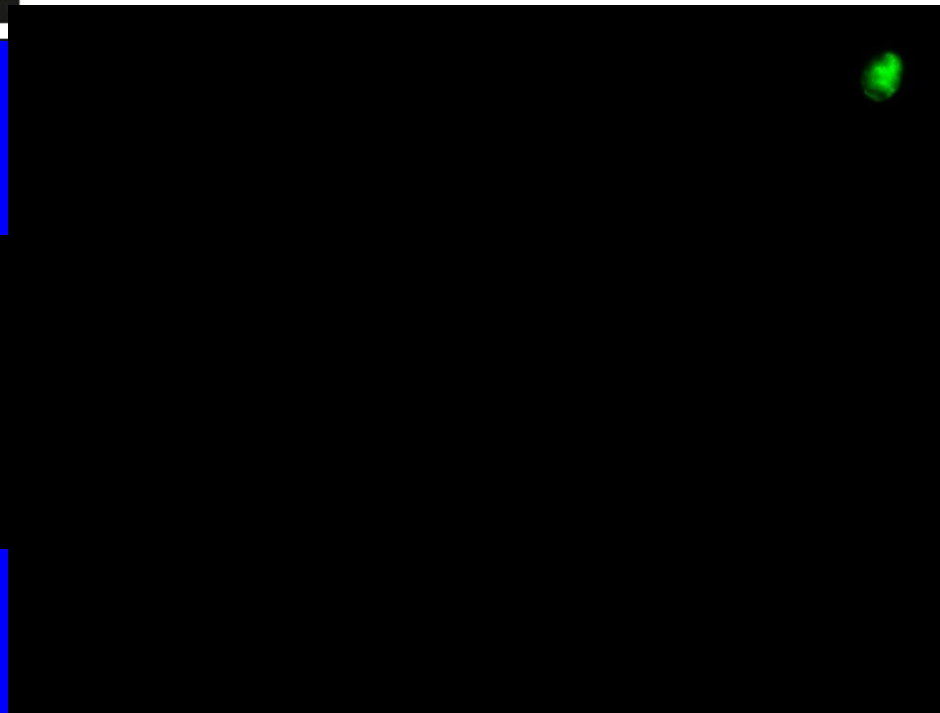






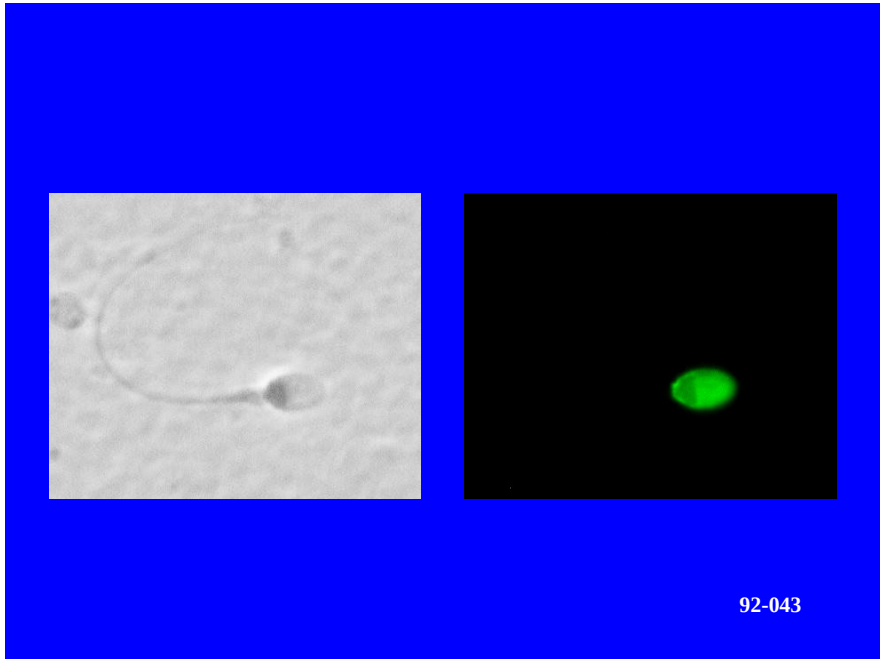


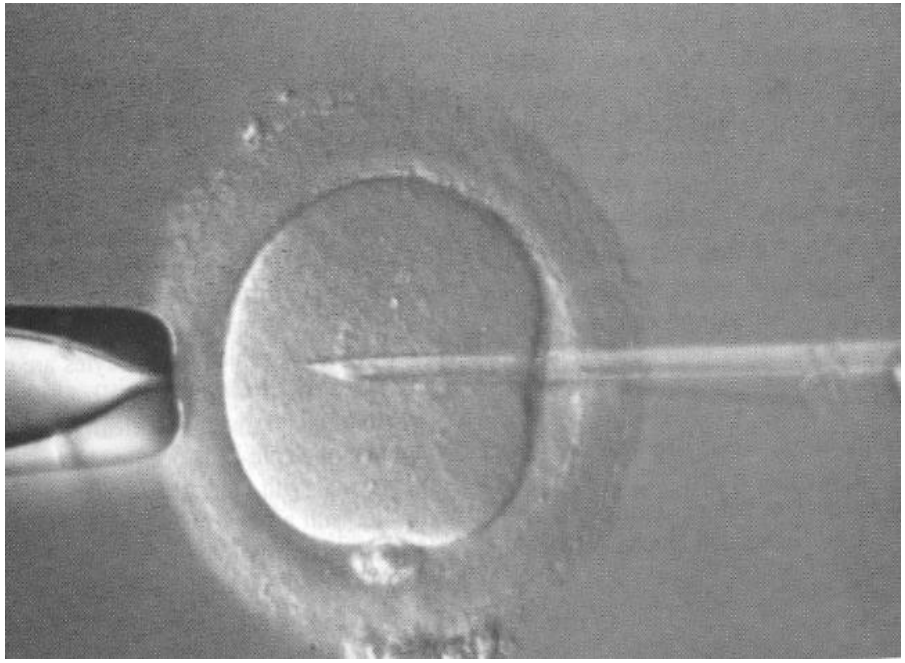




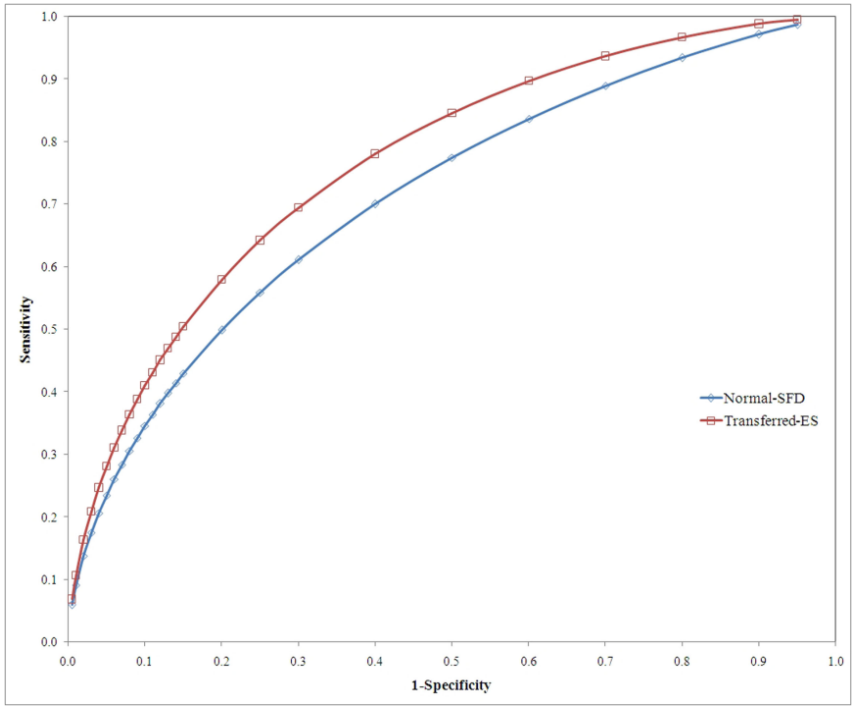
92-043

What is the impact of the proportion of morphologically normal sperm with DNA fragmentation on embryo quality and ICSI outcome?



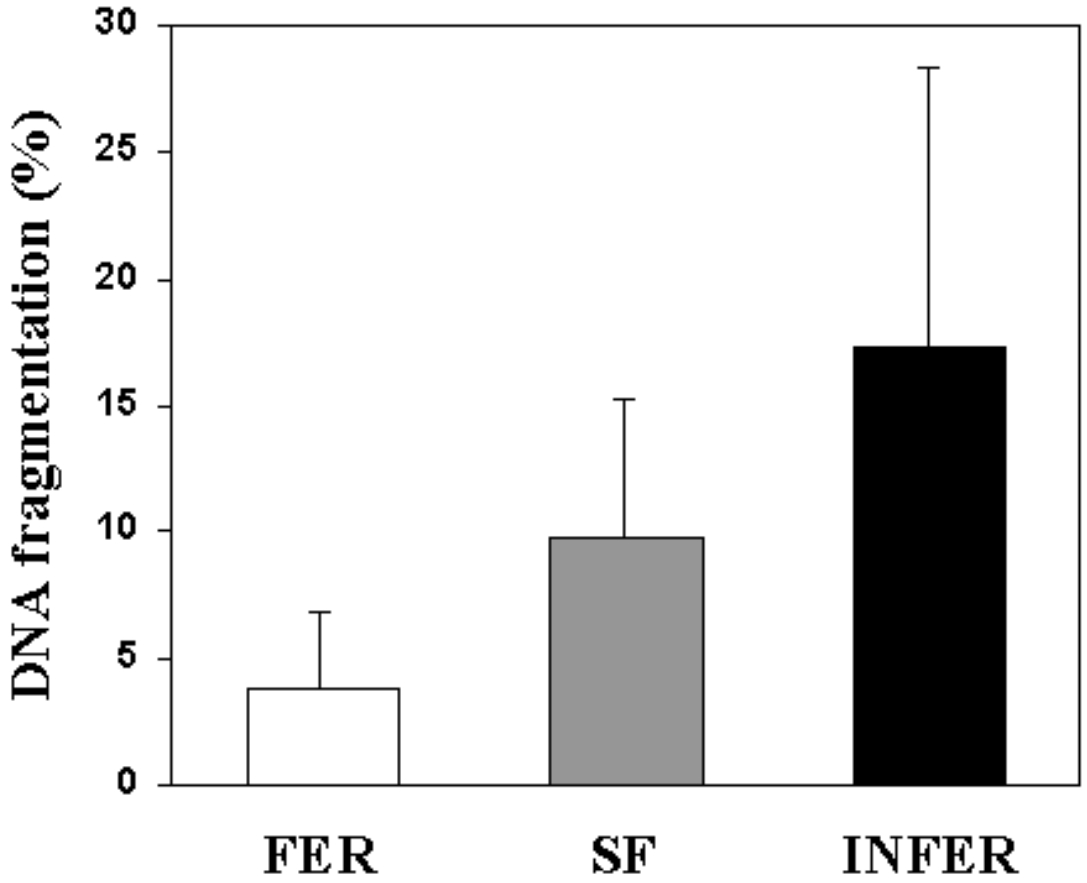


If the % of normal-SFD was ≤ 17.6 %, the likelihood of pregnancy was 3.5 times higher



Parameter	Area Under the Curve	95% Confidence Intervals	P	Cut-off Point	Sensitivity	Specificity	Positive Likelihood Ratio	Negative Likelihood Ratio	Positive Predictive Value	Negative Predictive Value
Normal-SFD	0.70	0.53-0.84	0.02	≤17.6%	61.5	82.6	3.5	0.5	66.7	79.2

DNA Fragmentation (TUNEL) In Fertile, Subfertile and Infertile Men



Normal sperm with DNA fragmentation: 0% (0/4 cases). 30% (1/5 cases), 43% (10/10 cases)

OUR TUNEL REPORTS

- Information about basic semen parameters
- DNA fragmentation in neat semen
- DNA fragmentation in the motile sperm fraction

ANDROLOGY LABORATORY

RECEIVED MAY 02 2012

Wife: 37

EASTERN VIRGINIA MEDICAL SCHOOL
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CLIA Certificate # 49D0723270

Laboratory Director:
Mahmood Morshedi, Ph.D., HCLD

Clinical Director:
Sergio Oehninger, M.D., Ph.D.

DNA Fragmentation (TUNEL) REPORT

Referring Physician: Silvina Bocca, MD, PhD Specimen Date: 03/26/2012 Test Date: 4/19/2012

Patient: [redacted]
Patient SSN: [redacted]
Patient DOB: [redacted]
Medical Record Number: [redacted]

Spouse: [redacted]
Spouse SSN: [redacted]
Spouse DOB: [redacted]
Medical Record Number: [redacted]

Specimen Number: [redacted]
Time Collected: [redacted]
Time Analyzed: [redacted]
Collection Method: MASTURBATION

Date of Last Emission: 03/22/2012
Location of Collection: Collection Room
Collection Complete (Y/N) Yes

PARAMETER	RESULT	REFERENCE RANGE
SEMEN DATA		
Volume	1.6	2.0-5.0 mL
Odor	Spermine	Spermine
Color	Opalescent	Whitish, Gray, Opalescent
Viscosity	Normal	Normal
Liquefaction	Complete	Complete in 30 minutes
pH	8.3	Basic ≥ 7.6
Agglutination	None	None
Round Cells	≤ 1 million/mL semen	≤ 1 million/mL semen
SPERM DATA (Computer Assisted)		**Number of Sperm Analyzed: 453**
Concentration	4.7×10^6/mL	20-200 million/mL
Percent Motility	55.3%	≥ 50% progressive
Rapid: 33% Medium: 12%	Slow: 20%	
Mean Velocity	39.5 μm/s	≥ 25 micrometers/second
Mean Linearity	45%	35-79% Circular to Straight Line
Motile Sperm/Ejaculate	4.2×10^6	25-250 million/Ejaculate
Motility Index	21.8	≥ 10 (% motile x mean velocity)
Viability (eosin)	97.8%	≥ 75% live from non-motile cells

Comments: Sperm concentration and motility have been checked twice.

TUNEL RESULTS — **Number of Sperm Analyzed: 800**

Unprocessed Sample (Semen Washed 2X in HTF (0.5% HSA)) **Number of Sperm Evaluated: 400**

Percent Sperm TUNEL Positive: **93%** **Reference Value (known fertile donors): ≤26%**
Morphologically Normal Sperm (by phase contrast microscopy): 2%
TUNEL NEGATIVE Morphologically Normal Sperm: 20% (20% of the 2% normal morphology = 0.4%)
TUNEL POSITIVE Morphologically Normal Sperm: 80% (80% of the 2% normal morphology = 1.6%)
Motility prior to processing for TUNEL: 43.9%

Swim-up Processed Sample **Number of Sperm Evaluated: 400**

Percent Sperm TUNEL Positive: **87%** **Reference Value (known fertile donors): ≤5%**
Morphologically Normal Sperm (by phase contrast microscopy): 3%
TUNEL NEGATIVE Morphologically Normal Sperm: 33% (33% of the 3% normal morphology = 1%)
TUNEL POSITIVE Morphologically Normal Sperm: 67% (67% of the 3% normal morphology = 2%)
Motility prior to processing for TUNEL: 89.7%

Note: Positive and negative controls are run with each TUNEL assay. Test results are reported only if controls perform as expected.

Date report prepared: 04/19/2012 End of **FINAL** Report Signature:

Two Case Studies

- Case #1

DNA Fragmentation (TUNEL) REPORT

Referring Physician: Silvina Bocca, MD, PhD Specimen Date: 03/26/2012 Test Date: 4/19/2012

Patient: [redacted]
Patient SSN: [redacted]
Patient DOB: [redacted]
Medical Record Number: [redacted]

Spouse: [redacted]
Spouse SSN: [redacted]
Spouse DOB: [redacted]
Medical Record Number: [redacted]

Wife: 37

Specimen Number: [redacted]
Time Collected: [redacted]
Time Analyzed: [redacted]
Collection Method: MASTURBATION

Date of Last Emission: 03/22/2012
Location of Collection: Collection Room
Collection Complete (Y/N) Yes

PARAMETER	RESULT	REFERENCE RANGE
SEMEN DATA		
Volume	1.6	2.0-5.0 mL
Odor	Spermine	Spermine
Color	Opalescent	Whitish, Gray, Opalescent
Viscosity	Normal	Normal
Liquefaction	Complete	Complete in 30 minutes
pH	8.3	Basic ≥ 7.6
Agglutination	None	None
Round Cells	≤ 1 million/mL semen	≤ 1 million/mL semen
SPERM DATA (Computer Assisted)		**Number of Sperm Analyzed: 453**
Concentration	4.7×10^6/mL	20-200 million/mL
Percent Motility	55.3%	≥ 50% progressive
Rapid: 33% Medium: 12%	Slow: 20%	
Mean Velocity	39.5 μm/s	≥ 25 micrometers/second
Mean Linearity	45%	35-79% Circular to Straight Line
Motile Sperm/Ejaculate	4.2×10^6	25-250 million/Ejaculate
Motility Index	21.8	≥ 10 (% motile x mean velocity)
Viability (eosin)	97.8%	≥ 75% live from non-motile cells

Comments: Sperm concentration and motility have been checked twice.

TUNEL RESULTS **Number of Sperm Analyzed: 800**

Unprocessed Sample (Semen Washed 2X in HTF (0.5% HSA) **Number of Sperm Evaluated: 400**

Percent Sperm TUNEL Positive: **93%** **Reference Value (known fertile donors): ≤26%**
Morphologically Normal Sperm (by phase contrast microscopy): 2%
TUNEL NEGATIVE Morphologically Normal Sperm: 20% (20% of the 2% normal morphology = 0.4%)
TUNEL POSITIVE Morphologically Normal Sperm: 80% (80% of the 2% normal morphology = 1.6%)
Motility prior to processing for TUNEL: 43.9%

Swim-up Processed Sample **Number of Sperm Evaluated: 400**

Percent Sperm TUNEL Positive: **87%** **Reference Value (known fertile donors): ≤5%**
Morphologically Normal Sperm (by phase contrast microscopy): 3%
TUNEL NEGATIVE Morphologically Normal Sperm: 33% (33% of the 3% normal morphology = 1%)
TUNEL POSITIVE Morphologically Normal Sperm: 67% (67% of the 3% normal morphology = 2%)
Motility prior to processing for TUNEL: 89.7%

lote: Positive and negative controls are run with each TUNEL assay. Test results are reported only if controls perform as expected.

)ate report prepared: 04/19/2012 End of **FINAL** Report Signature: [redacted]

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Clinical Director:
Sergio Oehninger, M.D., Ph.D.

Wife: 37

TESE DNA Fragmentation (TUNEL) REPORT

Referring Physicians: Victor Brugh, MD
Silvina Bocca, MD, PhD
Specimen Date: 9/27/2012
Test Date: 10/12/2012

Patient:	[redacted]	Spouse:	[redacted]
Patient SSN:	[redacted]	Spouse SSN:	[redacted]
Patient DOB:	[redacted]	Spouse DOB:	[redacted]
Medical Record Number:	[redacted]	Medical Record Number:	[redacted]

Specimen Number: [redacted] (Retrieved on 9-27-12)
Collection Method: Surgical
Type of specimen: TESE
Location of Collection: Physician's Office

TUNEL RESULTS	Number of Sperm Analyzed: 200

Note: Due to the fact that TESE samples typically recover low numbers of sperm, the results enumerated below should be interpreted with caution.

However, this sample had a considerable number of well formed sperm.

71% of these sperm were TUNEL negative. Of the sperm observed, 7% appeared to be normal-looking and TUNEL negative. 64% of the sperm had abnormally shaped heads.

Reference Value (Ejaculated sperm, known fertile donors): ≥74% TUNEL NEGATIVE

Note: Positive and negative controls are run with each TUNEL assay. Test results are reported only if controls perform as expected.

Date report prepared: 10/12/2012 End of **FINAL** Report Signature:_____

For this case, the following questions should have been asked

1. With the fact that she was 37, what would have been her contribution to the failure?
2. Was the quality of oocytes (i.e., inability to repair DNA damage and/or to contribute to the development of the embryo) a major factor in the failure?
3. If the question to #2 above is yes, was TESE necessary?
4. Occasionally, we have noted that DNA fragmentation level in the ejaculated semen/sperm may change. Would running the TUNEL assay for the ejaculated sperm during the time of ICSI (i.e., just prior to ICSI) have been useful?
5. What would have been the best suggestion to this couple? Would you have suggested accepting a donor egg? With TESE or without?

Case #2

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Laboratory Director:
Mahmood Morshedi, Ph.D., HCLD

Clinical Director:
Sergio Oehninger, M.D., Ph.D.

Wife: 32

DNA Fragmentation (TUNEL) REPORT

Referring Physician: Silvina Bocca, MD, PhD Specimen Date: 02/06/2012 Test Date: 02/21/2012

Patient:	[redacted]	Spouse:	[redacted]
Patient SSN:	[redacted]	Spouse SSN:	[redacted]
Patient DOB:	[redacted]	Spouse DOB:	[redacted]
Medical Record Number:	[redacted]	Medical Record Number:	

Specimen Number: [redacted]

A semen analysis was not ordered, however, concentration and motility were within normal parameters, similar to previous analyses for this patient.

TUNEL RESULTS **Number of Sperm Analyzed: 400**

Unprocessed Sample (Semen Washed 2X in HTF (0.5% HSA) **Number of Sperm Evaluated: 200**

Percent Sperm TUNEL Positive: **92.5%**
Morphologically Normal Sperm (by phase contrast microscopy): 2.5%
TUNEL NEGATIVE Morphologically Normal Sperm: 20%
TUNEL POSITIVE Morphologically Normal Sperm: 80%

Swim-up Processed Sample **Number of Sperm Evaluated: 200**

Percent Sperm TUNEL Positive: **74.0%**
Morphologically Normal Sperm (by phase contrast microscopy): 2.5%
TUNEL NEGATIVE Morphologically Normal Sperm: 57%
TUNEL POSITIVE Morphologically Normal Sperm: 43%

Note: ***Positive and negative controls are run with each TUNEL assay. Test results are reported only if controls perform as expected.***

Date report prepared: 02/21/2012 End of **FINAL** Report Signature:_____

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Laboratory Director:
Mahmood Morshedi, Ph.D., HCLD

Clinical Director:
Sergio Oehninger, M.D., Ph.D.

DNA Fragmentation (TUNEL) REPORT

Referring Physician: Silvina Bocca, MD, PhD Specimen Date: 06/21/2012 Test Date: 06/28/2012

Patient:	[redacted]	Spouse:	[redacted]
Patient SSN:	[redacted]	Spouse SSN:	[redacted]
Patient DOB:	[redacted]	Spouse DOB:	[redacted]
Medical Record Number:	[redacted]	Medical Record Number:	

Specimen Number:	[redacted]		
Time Collected:	[redacted]	Date of Last Emission:	06/14/2012
Time Analyzed:	[redacted]	Location of Collection:	Collection Room
Collection Method:	MASTURBATION	Collection Complete (Y/N)	Yes

PARAMETER	RESULT	REFERENCE RANGE
SEMEN DATA		
Volume	1.5	2.0-5.0 mL
Odor	Spermine	Spermine
Color	Opalescent	Whitish, Gray, Opalescent
Viscosity	Normal	Normal
Liquefaction	Complete	Complete in 30 minutes
pH	8.3	Basic ≥ 7.6
Agglutination	None	None
Round Cells	≤ 1 million/mL semen	≤ 1 million/mL semen
SPERM DATA (Computer Assisted)		**Number of Sperm Analyzed: 2037**
Concentration	46.0 x10^6/mL	20-200 million/mL
Percent Motility	63.0%	≥ 50% progressive
Rapid: 50% Medium: 11%	Slow: 2%	
Mean Velocity	51.9 µm/s	≥ 25 micrometers/second
Mean Linearity	43%	35-79% Circular to Straight Line
Motile Sperm/Ejaculate	43.5 x10^6	25-250 million/Ejaculate
Motility Index	24.3	≥ 10 (% motile x mean velocity)
Viability (eosin)	94.8%	≥ 75% live from non-motile cells
Comments: Sperm concentration and motility have been checked twice.		
TUNEL RESULTS		**Number of Sperm Analyzed: 800**

Unprocessed Sample (Semen Washed 2X in HTF (0.5% HSA)) **Number of Sperm Evaluated: 400**

Percent Sperm TUNEL Positive: **67%** **Reference Value (known fertile donors): ≤26%**

Morphologically Normal Sperm (by phase contrast microscopy): 0%

TUNEL NEGATIVE Morphologically Normal Sperm: 0% (NO NORMAL FORMS OBSERVED)

TUNEL POSITIVE Morphologically Normal Sperm: 0% (NO NORMAL FORMS OBSERVED)

Motility prior to processing for TUNEL: 59.8%

Swim-up Processed Sample **Number of Sperm Evaluated: 400**

Percent Sperm TUNEL Positive: **7.5%** **Reference Value (known fertile donors): ≤5%**

Morphologically Normal Sperm (by phase contrast microscopy): 1%

TUNEL NEGATIVE Morphologically Normal Sperm: 50% (50% of 1% normal morphology = 0.5%)

TUNEL POSITIVE Morphologically Normal Sperm: 50% (50% of 1% normal morphology = 0.5%)

Motility prior to processing for TUNEL: 99%

Note: ***Positive and negative controls are run with each TUNEL assay. Test results are reported only if controls perform as expected.***

Date report prepared: 06/29/2012 End of **FINAL** Report Signature:

Wife: 32

The Value Of Sperm Nuclear DNA Assessment In Assisted Conception: Some Thoughts

- Different methodologies has hampered our judgment about the value of the tests
- Tests give only the percentage of cells with damaged DNA. They do not reveal the extent of the damage in the sample being used.
- Setting thresholds is misleading as may vary in various sites, patients, ART methods. They can be used as a guide.

- We often do not ask if the DNA fragmentation is the sole or partial cause of the problem
- Focusing on the sperm DNA status, have negated the contribution of the oocyte to the success or to the failure

Tesarik states that “It is possible that the variation reported for the relationship between the extent of sperm DNA fragmentation and the outcome of ART is at least partly due to variable ability of the oocyte to repair the existing damage?”

Tesarik, 03-04, DNA Damage (TUNEL, microscopy) in 4 Groups

- Those achieving term pregnancy in their first ICSI attempt
- Those not achieving term pregnancy in their first ICSI attempt
- Those achieving term pregnancy in their third ICSI attempt after two previous failures
- Those not achieving term pregnancy in their third ICSI attempt after two previous failures

TUNEL values in successful and unsuccessful ICSI attempts after two previous failures (Group B) as compared with patients undergoing their first ICSI attempt (Group A)

	% TUNEL Positive Sperm	
	Group A (1st attempt, n= 343)	**Group B** (3rd attempt, n= 36)
Term Pregnancy	10.3 ± 1.4[b]	6.9 ± 1.3[b]
No Term Pregnancy	19.2± 3.3[c]	17.8 ± 3.4[c]

Data are mean ± SD; [b]P: < 0.05 (Student's t-test); [c]P: ns (Student's t-test)
% of TUNEL + was higher in those with no pregnancy irrespective of the attempt
Men whose wives had pregnancies at 1st ICSI had higher TUNEL results, 10.3% compared to men who were successful at 3rd attempt (Group A vs. B).

Sperm DNA fragmentation may have different clinical significance in couples with a history of previous failures of assisted reproduction treatment as compared with couples without such a history. Have the oocytes which possess oocytes operative nucleotide excision repair capacity performing their duties in group A with pregnancies?

Tsarik, et al, 04, 05

TUNEL values in successful and unsuccessful ICSI attempts performed with the patients' own oocytes (Group A) as compared with attempts performed with donated oocytes (Group B)

	% TUNEL Positive Sperm	
	Group A: Pt's oocytes n= 268	**Group B: Donor oocytes n= 281**
Woman's age[b]	34.7± 3.2[c]	21.8 ± 1.1[c]
Term Pregnancy	6.4 ± 1.4[d]	11.8 ± 2.9[d]
No Term Pregnancy	13.0 ± 2.8[c]	13.7 ± 3.2[e]

Data are mean ± SD; [b]Age of the female from which oocytes were retrieved
[c]*P* < 0.001 (Student's t-test); [d]*P* < 0.001 (Student's t-test); [e]*P:* ns (Student's t-test)

TUNEL results were higher in patients who did not achieve pregnancy, irrespective of the age of the women from which oocytes were retrieved. Oocytes from young donors enables the establishment of a term pregnancy with higher percentages of DNA-fragmented spermatozoa as compared with attempts performed with oocytes coming from the significantly older patient wives population.

Tesarik et al, 04, 05

Treatments to Reduce the Impact of Sperm DNA Damage

- ICSI using surgically retrieved testicular sperm instead of ejaculated ones (Tesarik, 04; Greco, 05)
- ICSI with ejaculated sperm after 2 months of oral antioxidant treatment (Greco, 05)
- ICSI with sperm selected with the use of a high-magnification optical system (high-magnification ICSI) (Hazout, 06)
- Oral antioxidant therapy??

Oral Antioxidant Therapy

- 64 patients with TUNEL >15%
- Randomly assigned to therapy or placebo
- 1 g vitamin C and 1 g vitamin E daily for 2 months
- Following therapy, TUNEL decreased significantly
- Then, 38 men were given the same treatment before ICSI, 29 (76%) showed significant decline in TUNEL and the ICSI outcome:
  - Before treatment: PR = 6.9%; IR = 2.2%
  - After treatment: PR = 48.2%; IR = 19.2%
  - No difference in FR, CR and EQ

- Sort of using the same sperm evaluated for DNA damage, it is not possible to know the extent of DNA damage in an individual sperm fertilizing the oocyte
- It is debatable whether any one of these tests is more preferable than the others to optimize clinical decision-making
- Finally, The ASRM, after meta-analysis of eligible studies, concluded that there is no proven role for routine DNA fragmentation testing in the evaluation of infertility

- Regardless of what has been published, the importance of sperm unclear DNA status assays cannot be disputed.

- We must learn how to utilize the results obtained particularly in conjunction with the female associated factors

END!