Sperm DNA fragmentation tests

Ajyal 2nd international conference workshop

Topic outlines

1. Introduction
2. Sperm chromatin structure
3. Types of sperm DNA damage
4. Aetiology of sperm DNA damage
5. Mechanism of sperm DNA damage
6. Sperm DNA fragmentation tests
7. Indications & justification of sperm DNA Fragmentation tests

Beyond standard semen analysis:

An introduction

Introduction

- Although routine semen analysis is the initial investigatory tool for infertile couples and considered the gold standard test for assessment of male fertility, conventional semen parameters such as sperm count, motility, vitality, and morphology are inadequate to monitor sperm function and to be used alone as markers of fertility Potential (Lewis et al., 2007).

Introduction

- This is evidenced by the fact that about 6-27% of men having normal semen analysis will eventually become infertile, despite exclusion of female factor infertility in their wifes (Alaa hamada et al., 2011).
- Therefore, Sperm function tests can be used to distinguish between fertile and infertile men and to aid in showing the cause of male subfertility and in suggesting therapeutics.

Introduction

- Among vital Sperm function tests available in the andrology armamentarium are sperm DNA integrity tests.

Sperm chromatin structure

Sperm chromatin structure

- Chromatin of mammalian sperm has a unique structure that is highly organized, condensed, and compacted.

- This allows protection of the paternal genome during transport through the male and female reproductive tracts and its subsequent delivery to the ova in good condition.

Sperm chromatin structure

Organization of chromatin for packaging in the spermatozoon takes place at four different *levels (Ward W, Coffey D 1992):*

1.Chromosomal anchoring: the attachment of the DNA to the nuclear annulus.

2. Formation of DNA loop domains: DNA attaches to the newly added nuclear matrix.

Sperm chromatin structure

3. Histones replacement: histones are lost and replaced with transition proteins and subsequently with protamines, which are approximately half the size of histones.

4. Chromosomal positioning.

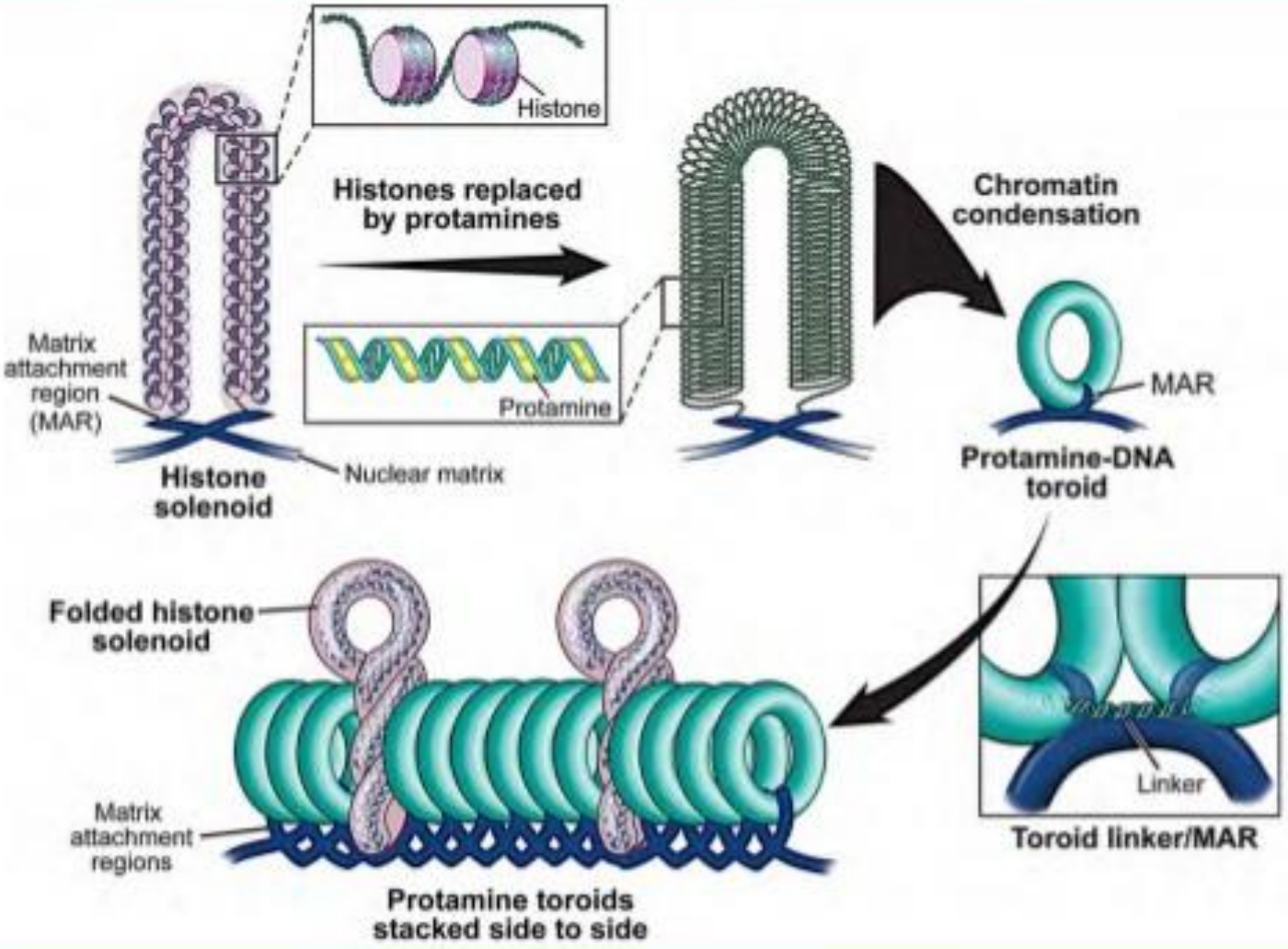
- Infertile men have been reported to have a higher histone to protamine ratio in their sperm chromatin *(Oliva R 2006)*.

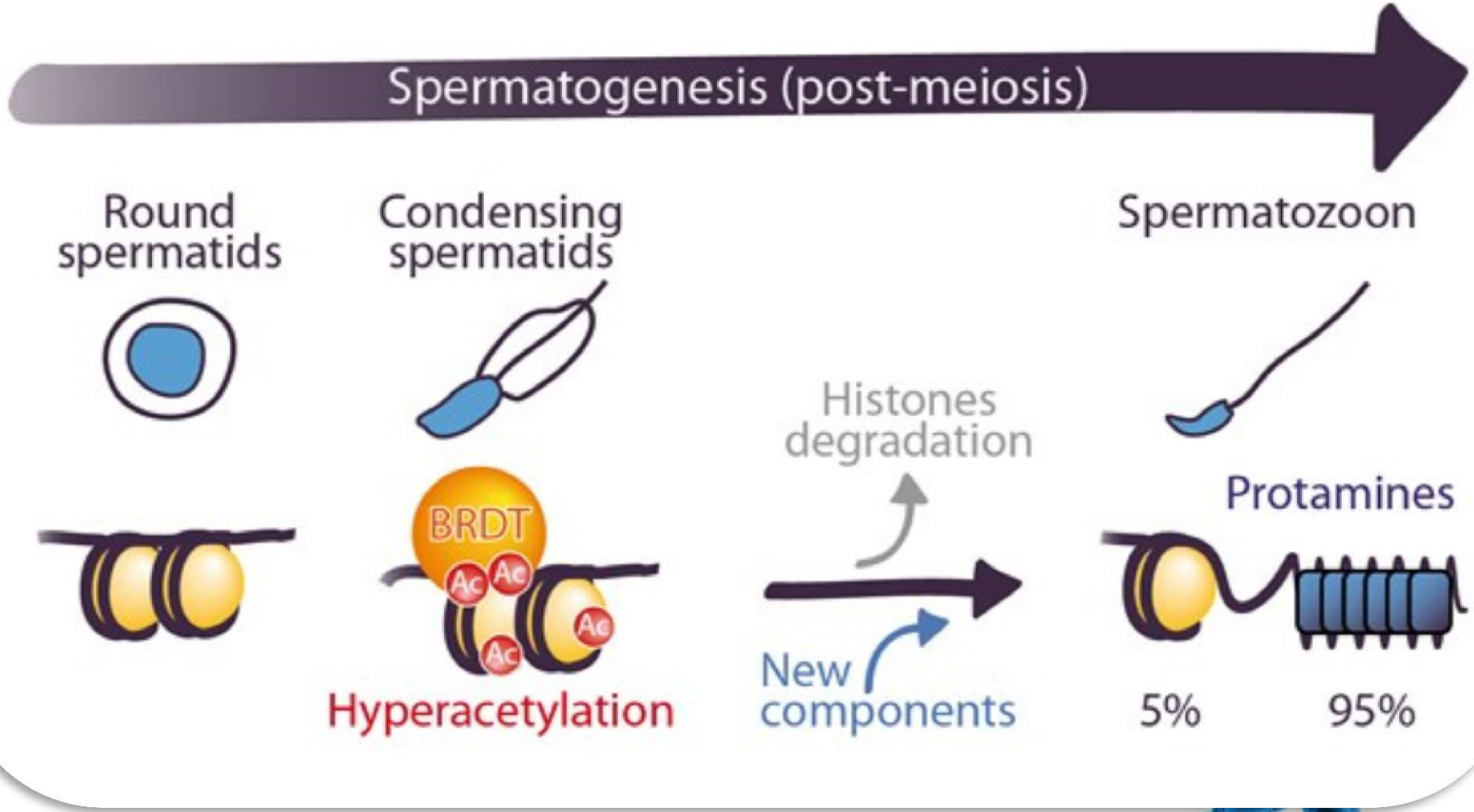
Sperm chromatin structure

- Human sperm also contain two types of protamines, **P1** and **P2**. **P2** protamines contain fewer cysteine groups and thus contain less disulfide crosslinks *(Corzett M et al., 2002)*.

- This theoretically leaves the DNA more susceptible to damage. It has been reported that **altered P2 expression is common in men with infertility** *(Carrell DT, Liu L, 2001)*.

Sperm Chromatin Compaction




Spermatogenesis (post-meiosis)
Round spermatids
Condensing spermatids
Spermatozoon
Histones degradation
Protamines
BRDT
Ac
Ac
Ac
Ac
Hyperacetylation
New components
5%
95%

Types of sperm DNA damage

Types of sperm DNA damage

1. **Damage to the actual DNA physical integrity** in the form of single-stranded or double-stranded DNA strand breaks.

2. **Nuclear protein defects** that may interfere with histone to protamine conversion and subsequent DNA compaction.

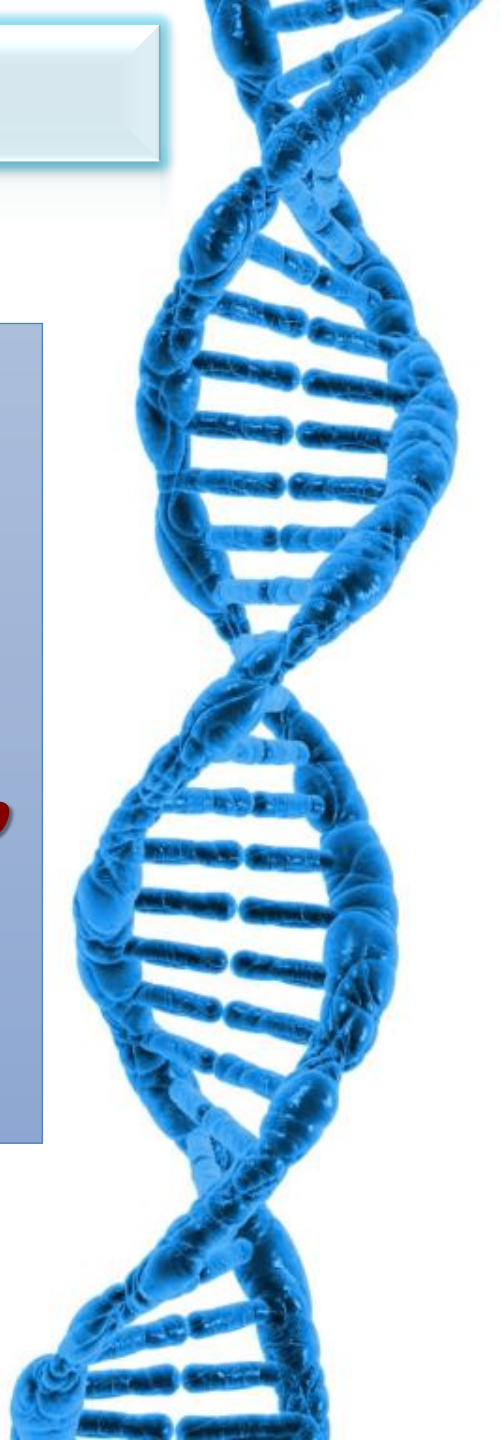
3. **Chromatin structural abnormalities** causing altered tertiary chromatin configuration.

Types of sperm DNA damage

- **Environmental stress, gene mutations, and chromosomal abnormalities can all disturb biochemical events that occur during spermatogenesis, which can ultimately lead to abnormal chromatin structure incompatible with fertility *(Evenson DP, 2002)*.**

Types of sperm DNA damage

Ova are able to repair sperm DNA damage to a certain extent. However, when sperm DNA damage is extensive, ovum may not have repair capacities to allow normal development (Genescà A et al, 1992).



Aetiology of sperm DNA damage

Aetiology of sperm DNA damage

(A) Pathophysiologic conditions:

1. Varicocele *(Saleh et al., 2003).*

2. Leukocytospermia *(Alvarez et al., 2002).*

3. genitourinary infections *(Gallegos et al., 2008).*

4. Cancer *(Kobayashi et al., 2001).*

Aetiology of sperm DNA damage

(B) Enviromental factors:

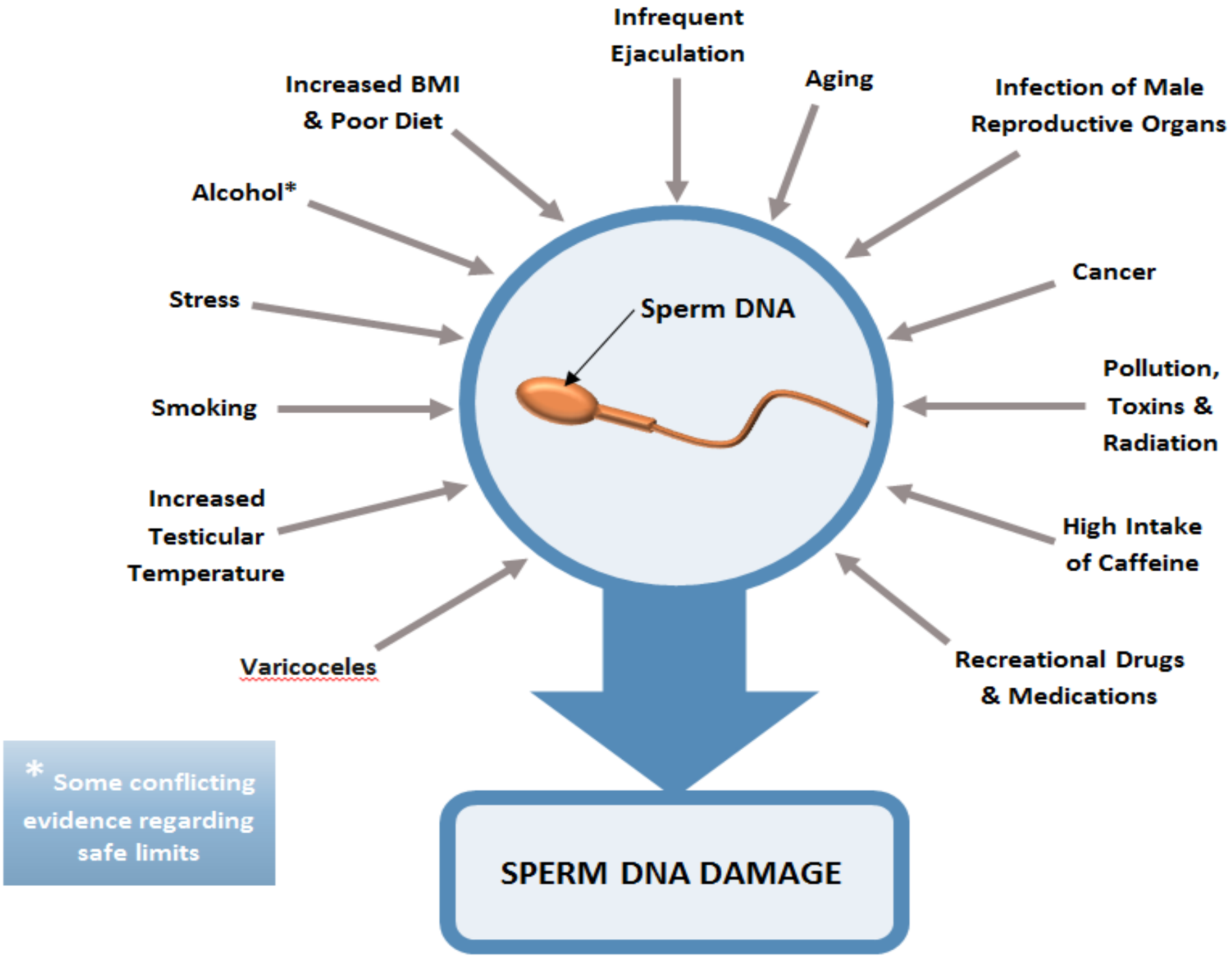
1. Cigarette smoking *(Potts RJ et al., 1999).*
2. Irradiation *(Arnon J et al., 2001).*
3. Chemotherapy *(Morris ID, 2003).*

(C) Iatrogenic:

1. sperm cryopreservation *(Labbe C et al., 2001).*
2. Sex sorting *(Gosálvez J et al., 2011).*

Aetiology of sperm DNA damage

↓ Abstinence →
↓ DNA fragmentation in the ejaculate?
(Gosálvez et al, 1992).


Infrequent Ejaculation
Increased BMI & Poor Diet
Aging
Infection of Male Reproductive Organs
Alcohol*
Cancer
Stress
Sperm DNA
Pollution, Toxins & Radiation
Smoking
Increased Testicular Temperature
High Intake of Caffeine
Varicoceles
Recreational Drugs & Medications
* Some conflicting evidence regarding safe limits
SPERM DNA DAMAGE

Mechanism of sperm DNA damage

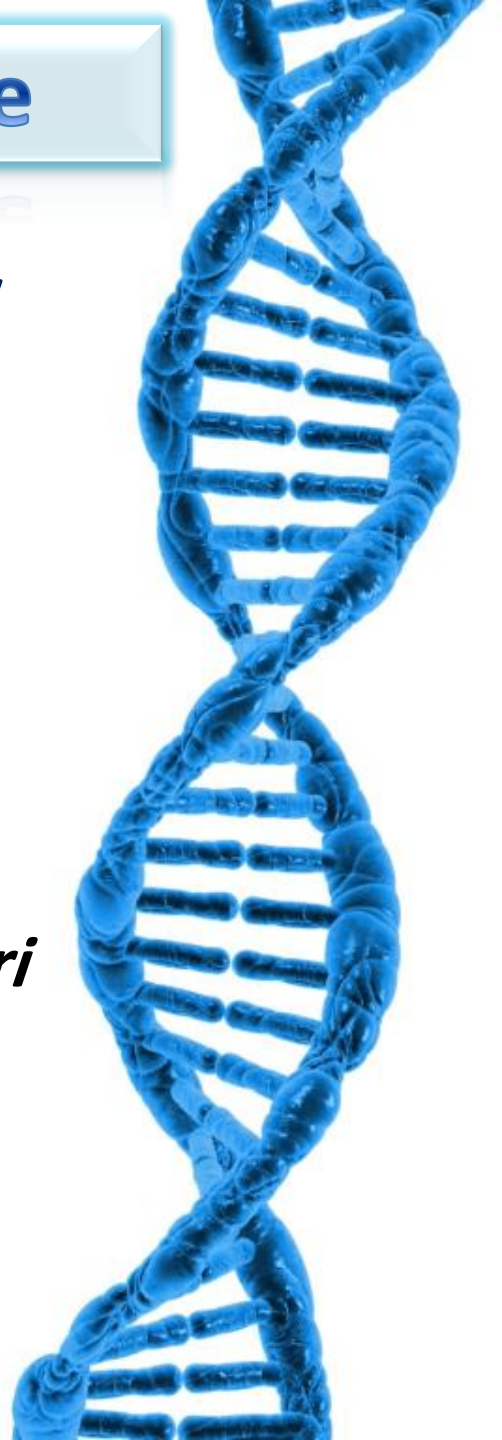
Mechanism of sperm DNA damage

(A) Chromatin packaging abnormalities:

- Chromatin remodeling is facilitated by the coordinated loosening of chromatin by histone hyper-acetylation as well as the enzyme DNA topoisomerase II (topo II) which produces temporary nicks in the sperm DNA to relieve torsional stress resulting from supercoiling.

Mechanism of sperm DNA damage

- These temporary nicks are then normally repaired by this same enzyme, topo II, prior to completion of spermiogenesis and ejaculation. However, if these nicks are not repaired, DNA fragmented sperm may be present in the *ejaculate* *(Muratori M et al., 2006).*



Mechanism of sperm DNA damage

(B) Reactive oxygen species:

- A positive correlation was reported between sperm DNA fragmentation and ROS *(Saleh et al., 2002).*

Mechanism of sperm DNA damage

- Major sources of ROS in semen are leukocytes and the sperm themselves, particularly immature sperm with cytoplasmic retention and abnormal head morphology characterized by retention of residual cytoplasm *(Ollero et al., 2001).*

Mechanism of sperm DNA damage

- Both leukocytospermia and retention of residual cytoplasm within sperm have been associated with increased sperm DNA damage, likely secondary to increased level of ROS produced by these cells *(Alvarez et al., 2002)*.

Mechanism of sperm DNA damage

(C) Abortive Apoptosis:

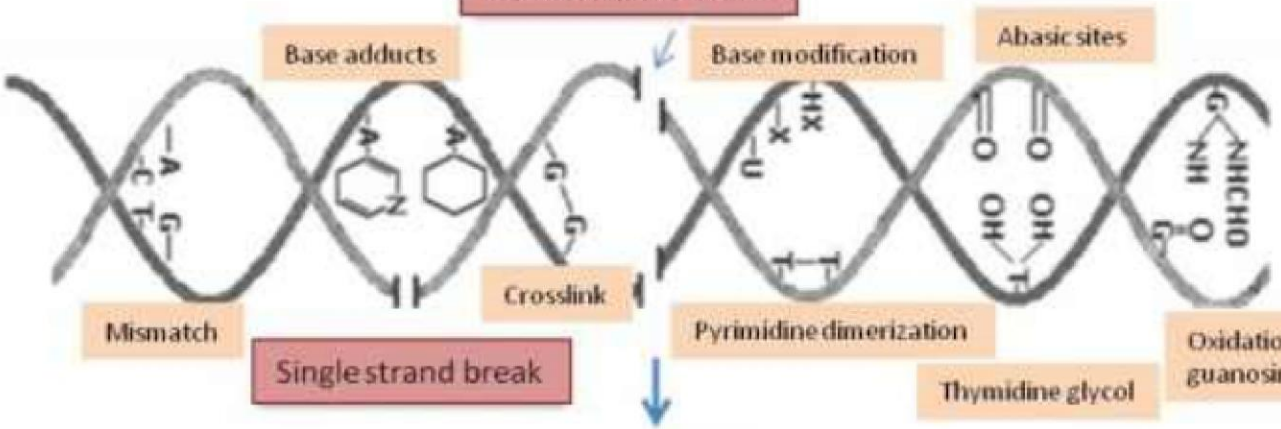
- As male germ cells transform into highly differentiated spermatozoa, they progressively lose their capacity to undergo programmed cell death in the form of apoptosis since these cells are transcriptionally and translationally silent.

Mechanism of sperm DNA damage

- Instead of engaging in a complete apoptotic response leading to cell death, differentiating haploid germ cells are thought to undergo a restricted form of this process leading to DNA fragmentation in the nucleus whereas retaining the capacity to differentiate into mature functional spermatozoa that may still be capable of fertilization *(Sakkas D et al., 2004).*

Main DNA damage

-Intrinsic factors
-Extrinsic factor



Sensors

Transducers

Effectors

DNA Repair

Spermatids
Oocyte

No Repair

de novo mutations

Cell cycle arrest

Stress response

Apoptosis

Tests of sperm DNA integrity*

* Tables were adapted from *"Sperm chromatin structure and male fertility: biological and clinical aspects" (J. Erenpreiss et al., 2006)*

** Figures were adapted from *"Sperm chromatin assessment "(Ashok Agarwal, 2004)*

Technique	Assay principle	Detection method	Advantages	Disadvantages	Clear clinical levels
I. Chromatin structural probes					
1. SCSA	The SCSA relies on the fact that abnormal sperm chromatin has a greater susceptibility to the physical induction of partial DNA denaturation *in situ*. The extent of DNA denaturation following heat or acid treatment is determined by measuring the metachromatic shift from green fluorescence (AO intercalated into double-stranded nucleic acid) to red fluorescence (AO associated with single stranded DNA)		- Quantitative detection of sperm with DNA breaks and sperm with nuclear immaturity - Extensively standardized - High statistical robustness - High intra- and inter-lab repeatability	Needs flow cytometer and dedicated software	Yes
2. AO test	The monomeric AO bound to native DNA fluoresces green, whereas the aggregated AO on denatured DNA fluoresces red	Fluorescence microscopy	Inexpensive Simple	- Heterogeneous slide staining - Necessity to evaluate slides shortly after staining (fading) - Inter-labvariability not tested	No

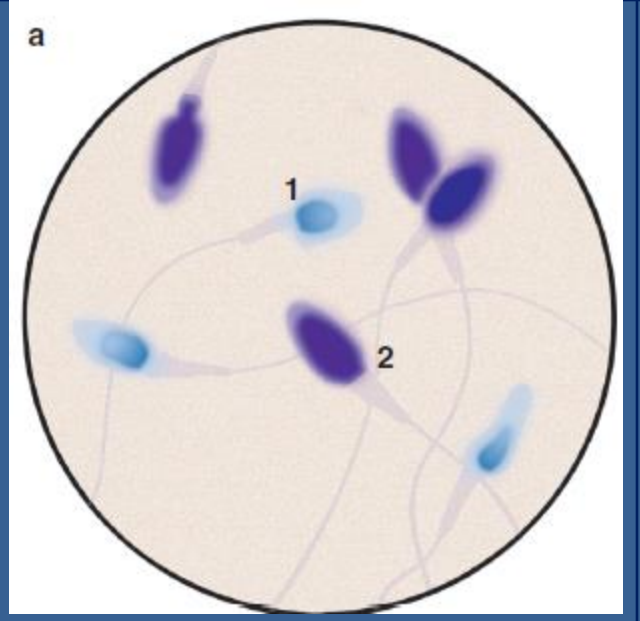
Technique	Assay principle	Detection method	Advantages	Disadvantages	Clear clinical levels
3. Acidic Aniline blue (Histone specific)	Histone-rich nuclei of immature spermatozoa are rich in lysine and will take up the blue stain.	Bright field microscopy	Inexpensive simple	Heterogeneous slide staining nter-lab variability not tested	No
4. Chromomycin –A3 (Protamine Competitive)	Chromomycin A3 and protamines compete for the same binding sites in the DNA. Therefore, high CMA3 fluorescence is a strong indicator of the low protamination state of spermatozoa	Florescence microscopy	Inexpensive simple	Inter-lab variability not tested	No
5. Toluidine blue	The stain becomes heavily incorporated in the damaged dense chromatin	Bright field microscopy image cytometery	Inexpensive simple Correlates well with SCSA and TUNEL assays	Inter-lab variability not tested	No

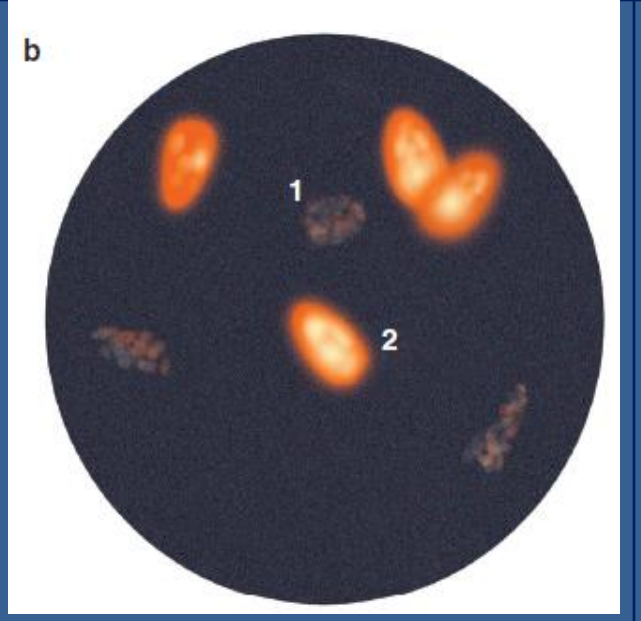
Technique	Assay principle	Detection method	Advantages	Disadvantages	Clear clinical levels
II. Direct methods for assessment of fragmented sperm DNA					
1. TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling assay)	Quantifies the incorporation of dUTP at breaks in double-stranded DNA in a reaction catalyzed by terminal deoxynucleotidyl transferase	- Bright field microscopy - Fluorescence microscopy - Flow cytometry	**Sensitive exclusively for DNA DSBs and SSBs** Correlates well with other assays like SCSA, TB and COMET	- Relatively expensive and labour Consuming - High intra-assay variability, inter-lab variability	No
2. In situ nick translation assay	Quantifies the incorporation of biotinylated dUTP at single-stranded DNA breaks in a reaction catalyzed by the template-dependent enzyme, DNA polymerase I	Fluorescence microscopy	Relatively simple	Lack of sensitivity compared with other sperm assays	No

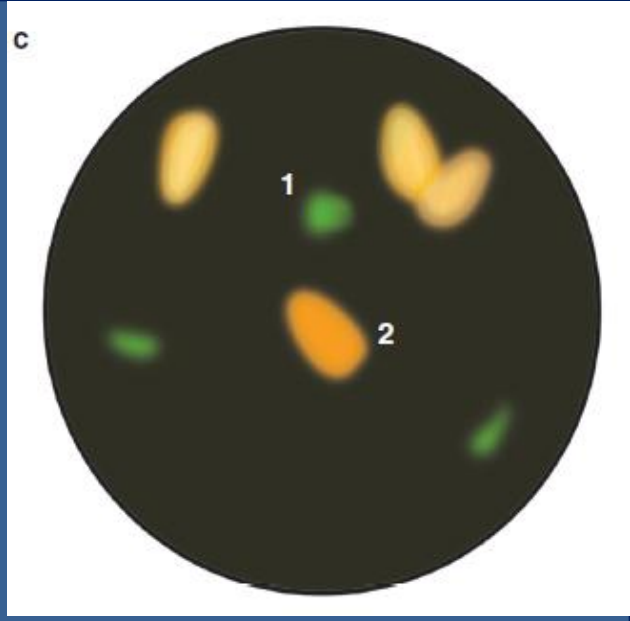
Technique	Assay principle	Detection method	Advantages	Disadvantages	Clear clinical levels
3. COMET assay	Quantifies DNA SSBs and DSBs, using electrophoresis of DNA-fluorochrome-stained single sperm cells	Fluorescence microscopy	**High level of sensitivity (alkaline COMET)**	- Time consuming - Requires computer-assisted image analysis - High inter-assay variability - Difficult to standardize	No
4. Sperm nuclear matrix stability assay	Determines the high level DNA organization or aberrations in the sperm nuclear matrix's ability to organize the DNA into loop-domains	Fluorescence microscopy	Relatively simple and inexpensive	Preliminary stage, not extensively validated	No

Technique	Assay principle	Detection method	Advantages	Disadvantages	Clear clinical levels
5. Sperm chromatin dispersion test	If spermatozoa with nonfragmented DNA are immersed in an agarose matrix and directly exposed to lysing solutions, the resulting deproteinized nuclei (nucleoids) show extended halos of DNA dispersion as monitored by fluorescent microscopy. The presence of DNA breaks promotes the expansion of the halo of the nucleoid	Bright field microscopy Fluorescence microscopy	- Relatively simple and inexpensive - Reliable - Reproducible - Correlates well with SCSA	Less sensitive than SCSA, TUNEL and COMET	NO

Technique	Assay principle	Detection method	Advantages	Disadvantages	Clear clinical levels
III. Combinations of tests					
1. TUNEL and COMET		Fluorescence microscopy	Improved assessment of male infertility	Difficult application in routine andrology lab	No
2. COMET and long PCR	Detect boths DNA strand breaks (COMET) and mitochondrial DNA deletions (PCR)		Associated with pregnancy in ICSI	Difficult application in routine andrology lab	No







(A)toluidine blue:

(1) mature sperm heads

(2) Immature sperm heads

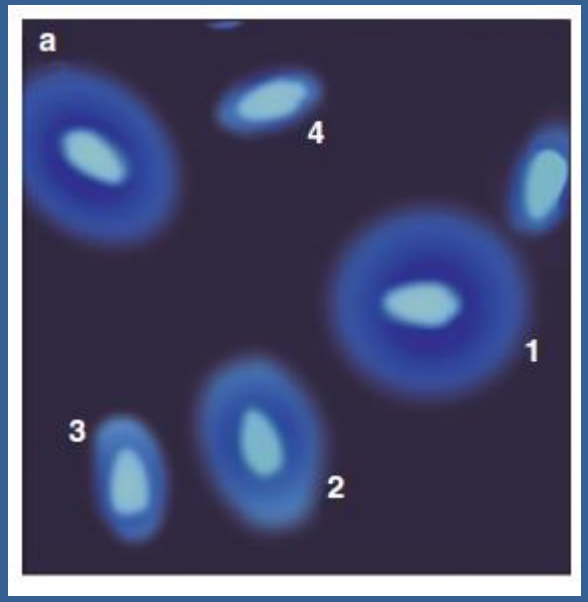
(B)

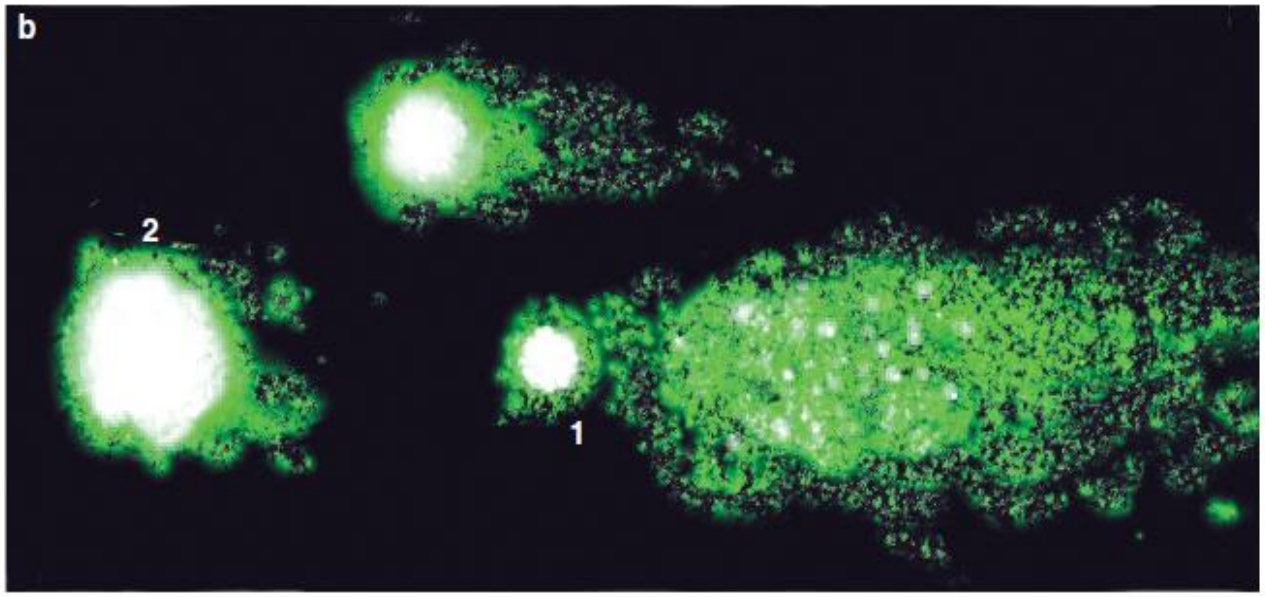
DNA breakage detection–fluorescence *in situ* hybridization (DBD–FISH) labeling with a whole genome probe (red fluorescence), demonstrating extensive DNA breakage in those nuclei that are intensely labeled.

(C) Acridine orange

(1) Native DNA

(2) Denaturated DNA





(A) Sperm chromatin dispersion test :

Sperms without DNA fragmentation

(1) Large sized halo

(2) Medium sized halo

Sperms with DNA fragmentation

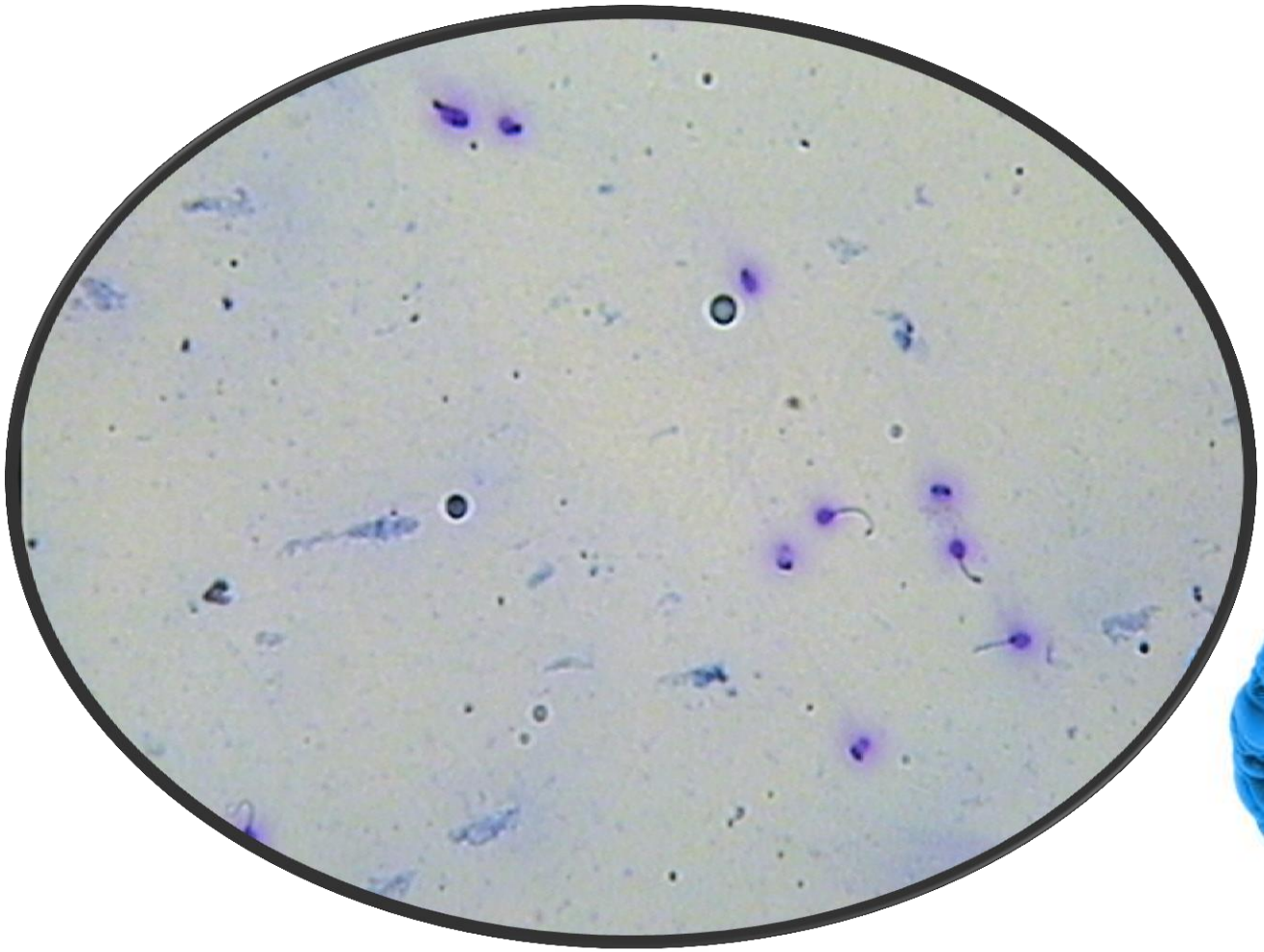
(3) Small halo

(4) No halo

(B) COMET assay

(1) Damaged DNA

(2) Undamaged DNA



Clinical applications of sperm DNA damage testing

1. Diagnosis of male infertility.

2. Predict chances of natural pregnancy.

3. Predict outcomes of ART methods.

4. Predict pregnancy loss after ART methods.

5. Selection of best ART method.

6. Sperm DNA damage testing and cancer patients

1. Diagnosis of male infertility:

Unexplained infertility is unexplained no more

- Several studies have demonstrated increase sperm DNA damage among males with unexplained infertility.

- It was concluded that significant increase in SCSA-defined DNA damage can be found in sperm from infertile men with normal standard sperm parameters *(Saleh et al., 2002)*.

- **Therefore, sperm DNA damage analysis may reveal a hidden abnormality of sperm DNA in infertile men classified as idiopathic based on apparently normal standard sperm parameters *(Saleh et al., 2002).***

2. Predict chances of natural pregnancy:

*A screening Test for First Pregnancy Planners?**

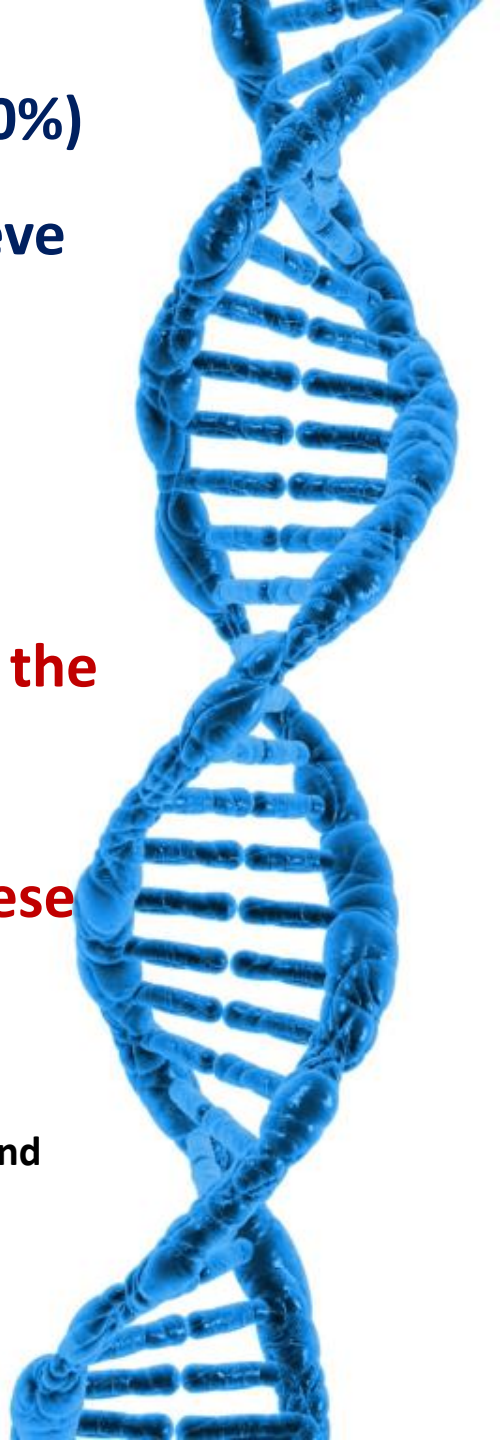
- The relationship between sperm chromatin/DNA damage and pregnancy outcomes (natural, IUI, IVF and ICSI) has been examined by systematic reviews and meta-analyses.

- The data from these studies show that sperm DNA damage is associated with a reduced probability of natural pregnancy.

- The analysis of data predicts that in populations with an overall pregnancy rate of 53% (at 6–12 months of follow-up), the pregnancy rate is 17% when there is a positive test for sperm DNA damage and is 58% when the test result is normal.

- However, prevalence of a positive test is low (<10%) and 17% of couples with a positive test will achieve a Pregnancy.

- Therefore, clinicians may choose to test first pregnancy planners, but they should understand the predictive value and limitations (e.g., sensitivity, specificity) of the sperm DNA test and discuss these issues with the patients.



* "Sperm Chromatin: Biological and Clinical Applications in Male Infertility and Assisted Reproduction" A. Zini and A. Agarwal (eds.), 2011

3. Predict outcomes of ARTs*

	IUI	IVF	ICSI
Studies included	one valid IUI study (Bungum M et al., 2007).	More than 20 studies	more than 20 studies
Conclusion	sperm DNA damage is associated with significant reduction in the IUI pregnancy rate	sperm DNA damage is associated with a modest but significant reduction in the IVF pregnancy rate.	Sperm DNA damage is not related to ICSI pregnancy rates
Analysis of data	in populations with an IUI pregnancy rate of 20%, a positive test for sperm DNA damage predicts the pregnancy rate to be 3% and a normal test result predicts the pregnancy rate to be 24%.	In populations with an overall IVF pregnancy rate of 33%, a positive test for sperm DNA damage predicts the IVF pregnancy rate to be 23% and 34% if the test is negative.	sperm DNA testing is not clinically valuable in predicting ICSI outcomes
Recommendation	*Couples with high levels of sperm DNA damage should proceed to ICSI*	*Couples with high levels of sperm DNA damage should proceed to ICSI*	
Limitations	additional studies are needed		

** Adapted from "Sperm Chromatin: Biological and Clinical Applications in Male Infertility and Assisted Reproduction" A. Zini and A. Agarwal (eds.), 2011*

4. Predict pregnancy loss after ARTs:

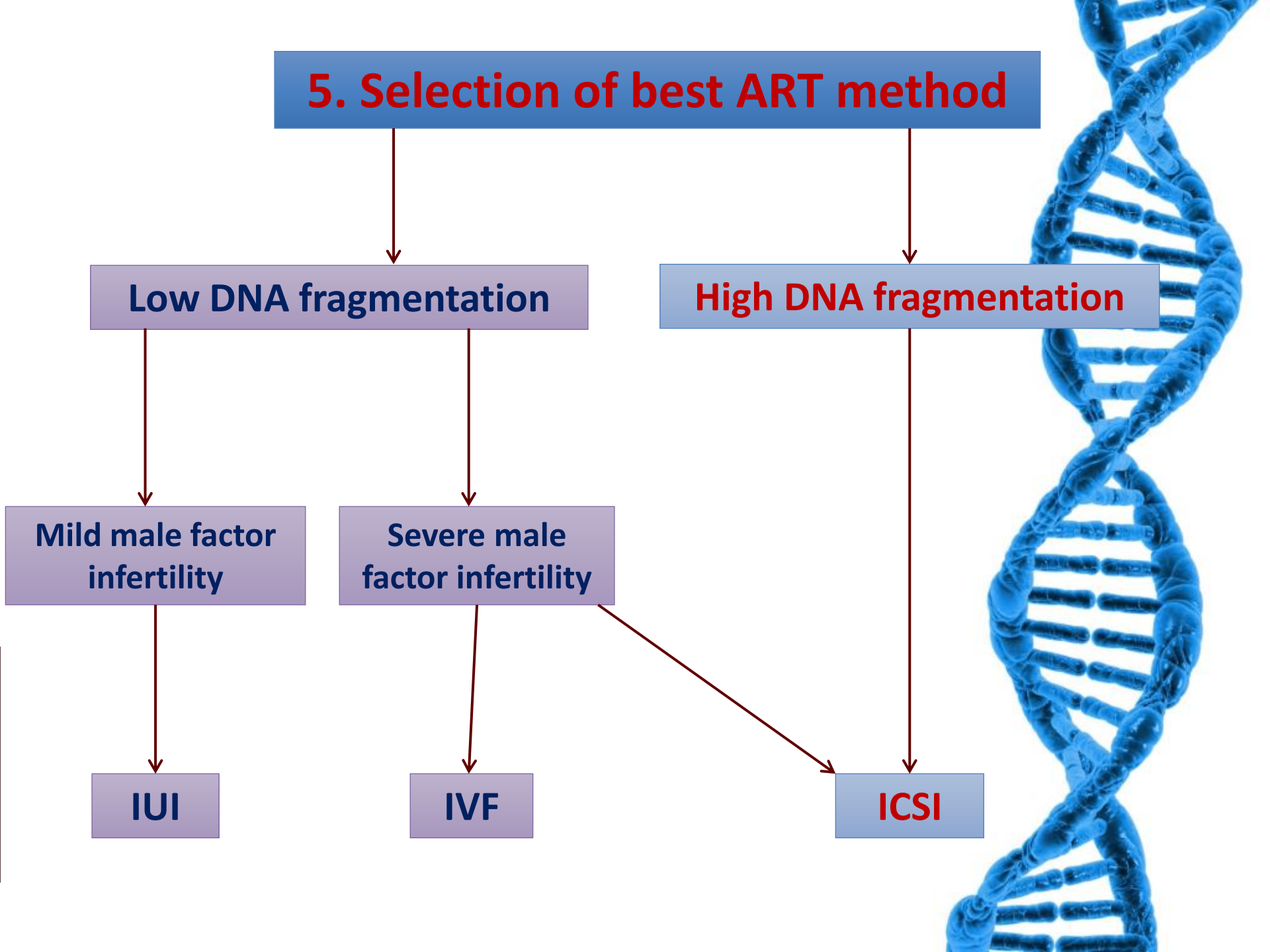
- **Sperm DNA damage is associated with a significantly higher rate of pregnancy loss after IVF or ICSI**

"Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis" ***(Zini et al., 2008).***

- Data derived from these studies (PPV and NPV) indicate that in populations with an overall rate of pregnancy loss of **18%**, the rate of pregnancy loss is estimated at **37%** when the test is positive and **10%** when it is negative.

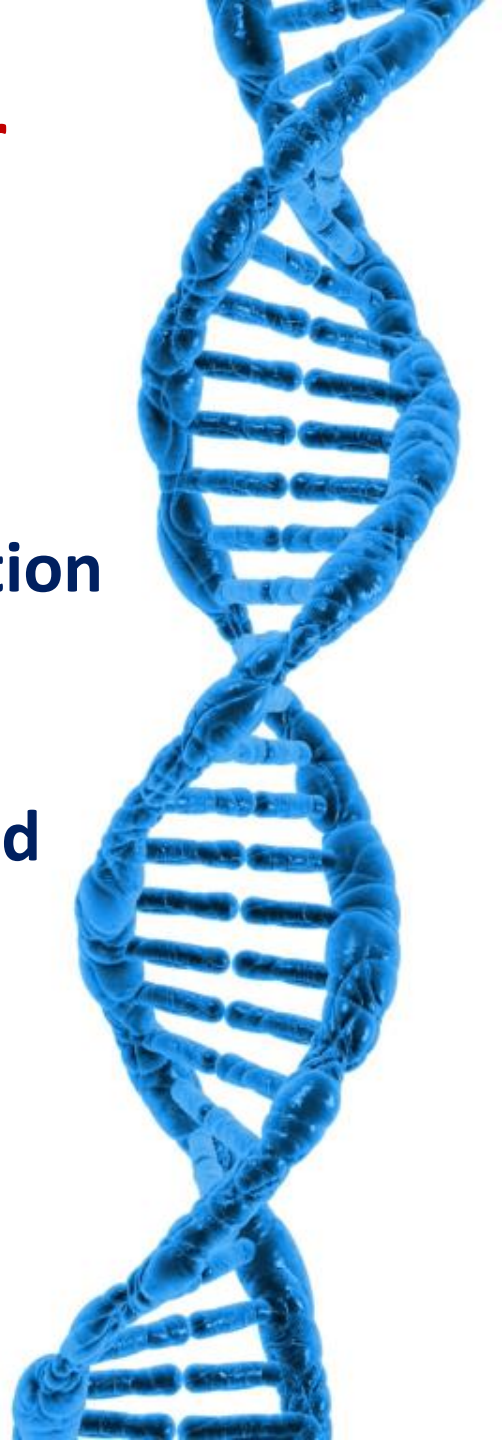
- Although the effect of DNA damage on pregnancy loss should be discussed with patients prior to undergoing ART, many couples will proceed with these treatments regardless of sperm DNA test results and the impact on pregnancy loss.

5. Selection of best ART method



6. Sperm DNA damage testing and cancer patients:

- Patients with cancer are often referred to sperm banks before chemotherapy, radiation therapy, or surgery is initiated. Although pregnancies and births have been reported using cryopreserved sperm from patients with cancer, these semen samples have decreased fertilization potential.



- **The extent of DNA damage may help to determine how semen should be cryopreserved before therapy begins.** Specimens with high sperm concentration and motility and low levels of DNA damage should be preserved in relatively large aliquots that are suitable for intrauterine insemination (IUI). If a single specimen of good quality is available, then it should be preserved in multiple small aliquots suitable for IVF or ICSI *(Kobayashi et al., 2001)*.

The clinical utility of sperm DNA integrity testing: a guideline

The Practice Committee of the American Society for Reproductive Medicine
American Society for Reproductive Medicine, Birmingham, Alabama

Question	Answer
Does the DNA integrity test predict male fertility with natural conception?	Insufficient evidence (Level C)
Does the DNA integrity test predict pregnancy with intrauterine insemination (IUI)?	Insufficient evidence (Level C)
is DNA fragmentation predictive of pregnancy with in vitro fertilization (IVF)?	Insufficient evidence (Level C)
Is DNA fragmentation predictive of pregnancy with IVF and intracytoplasmic sperm injection (ICSI)?	Insufficient evidence (Level C)
Is DNA fragmentation predictive of pregnancy loss?	Insufficient evidence (Level C)


Thank you