New markers of male fertility

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Introduction

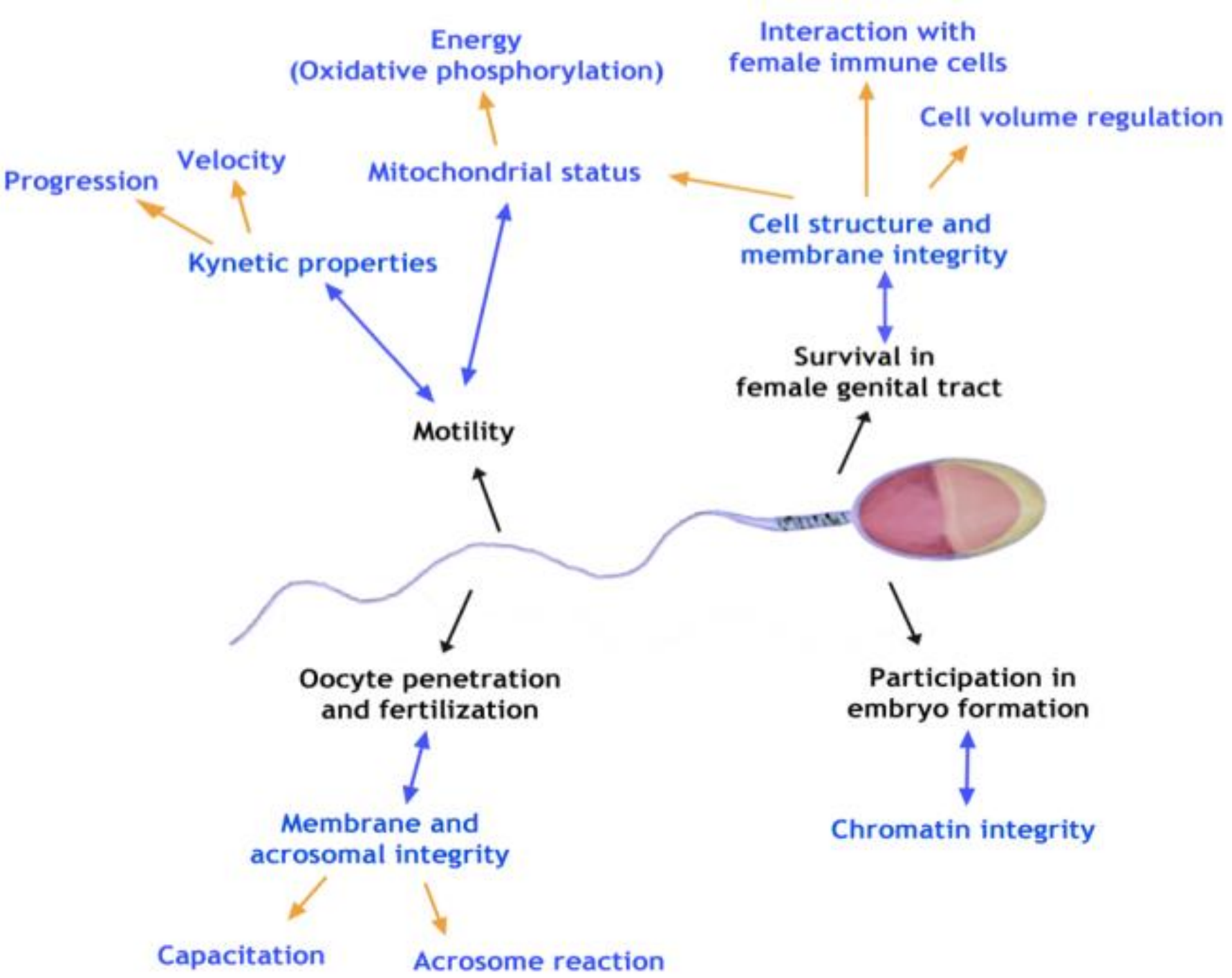
- Despite the continuing increase in the global population, it has been estimated that 10-20% of couples around the world cannot conceive. About 50% are male factor. On the other hand, idiopathic infertility can be a problem for clinicians to detect due to the lack of specific symptoms.
- So, the development of efficient tests and biomarkers for better diagnosis was started from last two decades. In fact, biomarker based technology allows for unbiased diagnosis of human male infertility and proper clinical treatment decision making.

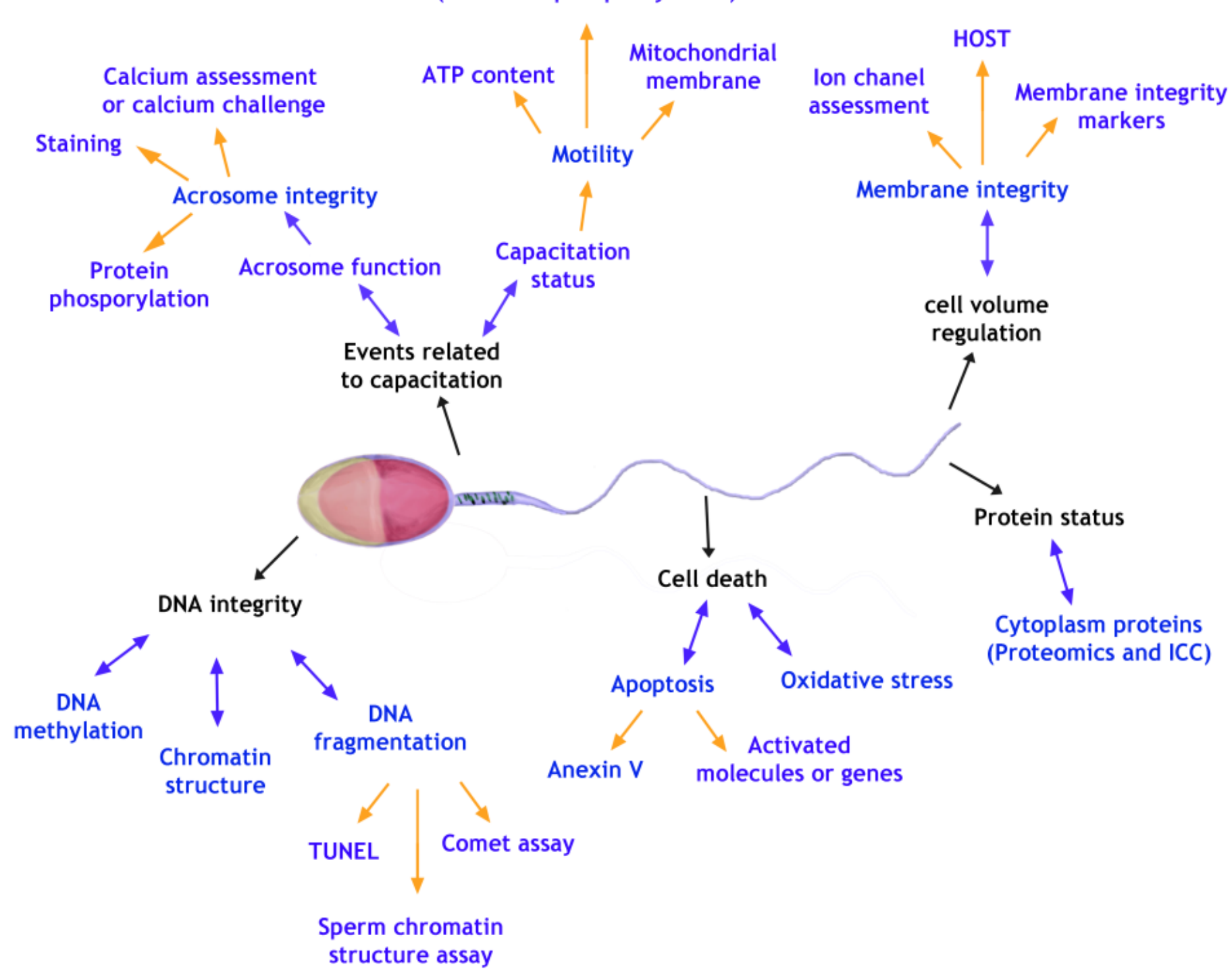
Types of classic assays for male fertility evaluations

1. Descriptive assay:	spermogram
2. Functional assays:	1)penetration of cervical mucus 2)binding of sperm to the zona pellucida 3)fusion of sperm with zona-free hamster oocyte 4)hypoosmotic swelling test
3. Immunological and bacteriological assays:	mixed agglutination test immunobead test sperm immobilization in cervical mucus Microbial culture
4. biochemical assays:	a-glucosidase, Carnitine, Zinc, Citrate,

New biomarkers of male fertility

- Although, the use of semen analysis in the clinical evaluation of male fertility is still a basic step, it is widely accepted that the information provided by conventional semen analysis is limited. In addition to well-known functional assays, other new functional and molecular markers which have developed are categorized into:
- **Sperm chromatin and DNA integrity biomarkers**
- **Oxidative stress biomarkers**
- **Apoptosis biomarkers**
- **Protein biomarkers (proteomics)**
- **Genetic biomarkers**
- **epigenetic biomarkers**


Energy
(Oxidative phosphorylation)
Interaction with
female immune cells
Cell volume regulation
Velocity
Progression
Mitochondrial status
Cell structure and
membrane integrity
Kynetic properties
Survival in
female genital tract
Motility
Oocyte penetration
and fertilization
Participation in
embryo formation
Membrane and
acrosomal integrity
Chromatin integrity
Capacitation
Acrosome reaction


Calcium assessment
or calcium challenge
Staining
Acrosome integrity
Protein
phosporylation
Acrosome function
ATP content
Mitochondrial
membrane
Motility
Capacitation
status
HOST
Ion chanel
assessment
Membrane integrity
markers
Membrane integrity
cell volume
regulation
Events related
to capacitation
Protein status
DNA integrity
Cell death
Cytoplasm proteins
(Proteomics and ICC)
DNA
methylation
Chromatin
structure
DNA
fragmentation
Apoptosis
Oxidative stress
Anexin V
Activated
molecules or genes
TUNEL
Comet assay
Sperm chromatin
structure assay

1) Sperm DNA damage markers

Sperm chromatin/DNA damage is associated with poor semen quality, poor outcomes after intrauterine insemination (IUI) and conventional IVF, impaired pre-implantation development, increased abortion (Talebi et al. 20012) and an elevated incidence of disease in the next generation.

sperm DNA damage assessments provide clinically valuable information about infertility problems and choosing a proper ART program. Therefore, it seems evaluation of sperm DNA/chromatin integrity could be regarded as a part of male infertility work-up.

CHROMATIN / DNA ABNORMALITIES

- **Excessive histone**
- **Absence or deficiency of protamines (P1 , P2)**
- **Reduction in disulfide bonds formation**
- **Hypostabilized chromatin due to the zinc deficiency**
- **DNA fragmentation**
- **DNA denaturation**

DNA damages and fertility

Sperm DNA integrity may have a high predictive value for intrauterine insemination (IUI). For IVF and ICSI: IVF results are related to the sperm DNA damage but ICSI results are controversial (Ryan T. et al 2010)

High DNA fragmentation causes:

- Diminished sperm count, motility and morphology(Aoki, 2005 and Benechaib,2003)
- Decreased fertilization and implantation rate(Benchaib, 2003)(Muriel, 2006)
- Higher spontaneous abortion and unexplained recurrent abortion(Talebi, 2012, carrel, 2003)
- Decreased embryo morphology ar early cleavage stage(Virant-klun, 2002)
- Decrease pregnancy rate(Bungum, 2004)
- Failure to progress to the blastocyst stage in culture(Seli, 2004)
- Pre-implantation embryo loss(Harrouk, 2000)

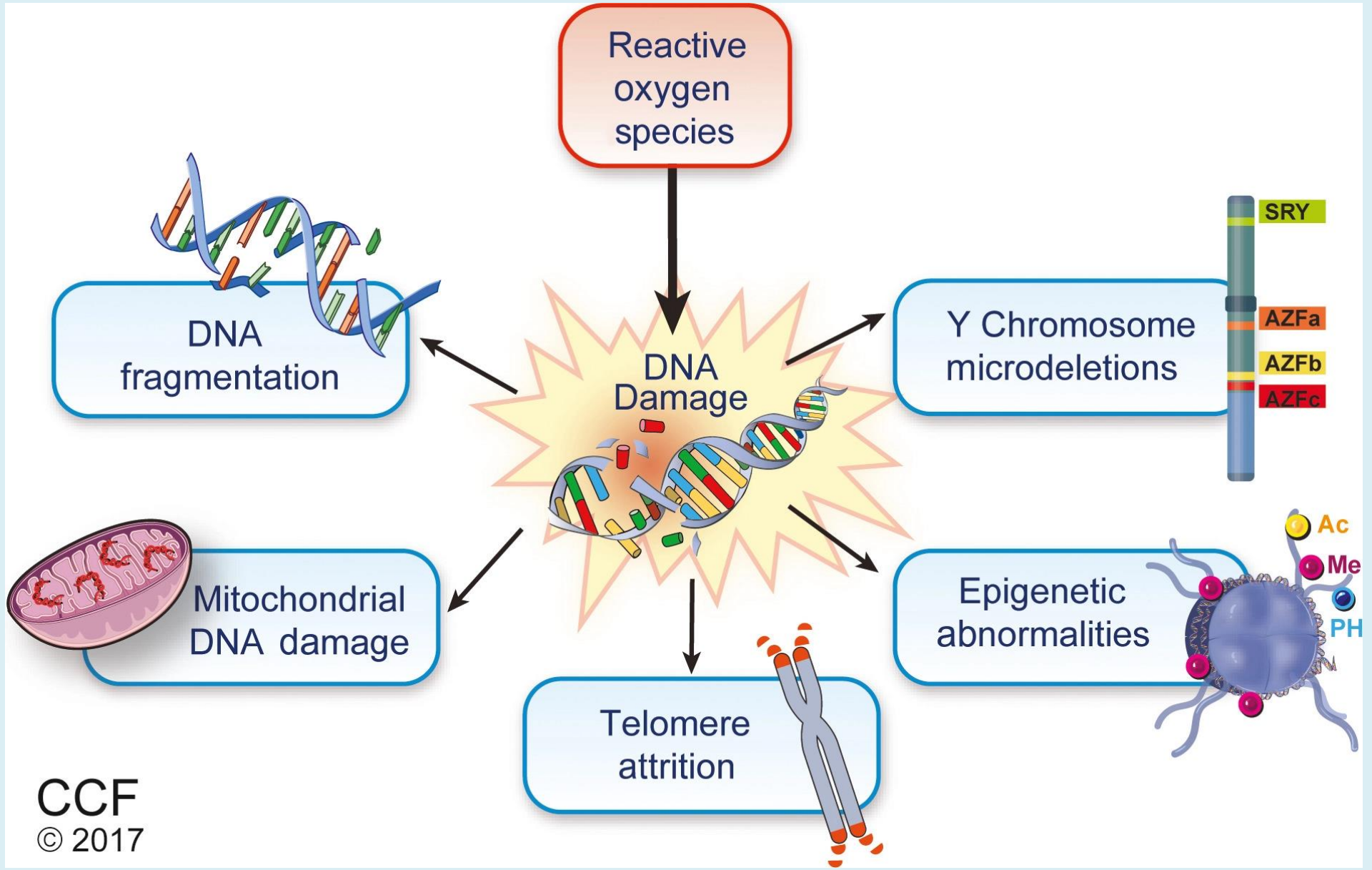
2) Oxidative stress (reactive oxygen species)

ROS are a class of free radicals , which are highly reactive oxidizing agents

ROS effects on :

- **Membrane polyunsaturated fatty acids**
- **Phosphodiesters backbone of DNA**

ROS cause membrane disintegration , decrease sperm motility due to the rapid loss of intracellular ATP , decrease sperm viability and increase morphological defects and chromatin / DNA abnormalities .


Reactive oxygen species
DNA fragmentation
DNA Damage
Y Chromosome microdeletions
SRY
AZFa
AZFb
AZFc
Mitochondrial DNA damage
Epigenetic abnormalities
Ac
Me
PH
Telomere attrition
CCF
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- NOX 5
- NADPH oxidases (NOXs) are the main sources of hydrogen peroxide and superoxide anions.
- Role of NOX5 expression was assessed in sperm motility and ROS production in samples of Iranian infertile asthenozoospermic cases.
- It was reported that the level of NOX5 expression was significantly increased in asthenozoospermic cases compared with controls.
- NOX5 upregulation can induce ROS production and DNA damage in asthenozoospermic cases.
- There was a direct association between ROS generation and NOX5 expression in teratozoospermia.

3) Apoptosis markers

- Apoptosis is increased in spermatozoa of infertile men affected by cryptorchidism, infection or varicocele (talebi et al. 2011), drugs, alcohol (Talebi et al. 2013) and so on.
- 3 main apoptotic markers are membrane phospholipid asymmetry , changes in mitochondrial membrane potential and DNA fragmentation.

Apoptosis is related to:

- Poor sperm quality, Recurrent abortion, Ivf failure (Talebi et al. 2012).

- TNF α

- TNF-α is an apoptotic cytokine belong to the TNF family that produced by testicular germ cells and macrophages.
- The association between TNFR1 36A/G polymorphism and sperm abnormalities was assessed among a sub-population of Iranian subjects which showed significantly increased frequency of polymorphism in azoospermic infertile males.
- TNF-α upregulation was associated with sperm DNA chromatin abnormalities, impaired motility and reduced testosterone levels.

4) Protein biomarkers

The so-called sperm proteome contains everything the sperm needs to survive and function correctly.

Altered levels of proteins might be useful as reliable biomarkers of infertility. Some protein biomarkers have positive effects on sperm function and some of them have negative effects. In addition, there are several biomarkers for diagnosis of male fertility problems.

Protein biomarkers

- For example,
- Normal epididymis-express ECM1 and testis-express TEX101, which can differentiate OA, NOA and Sertoli cell–only syndrome from each other.
- P34H is a biochemical marker for the diagnosis of male infertility due to the inability of spermatozoa to interact efficiently with the zona pellucida.

Positive biomarkers

Examples:

- AIG2-like domain 1 that is four times more abundant in fertile men than infertiles.
- ATPase, Na^+/K^+ transporting β-3 polypeptide and cysteine-rich secretory protein 2 (CRISP2) are another positive biomarkers.
- Cyclin and protamine are considered as prognostic molecular markers for testicular sperm extraction in patients with azoospermia.

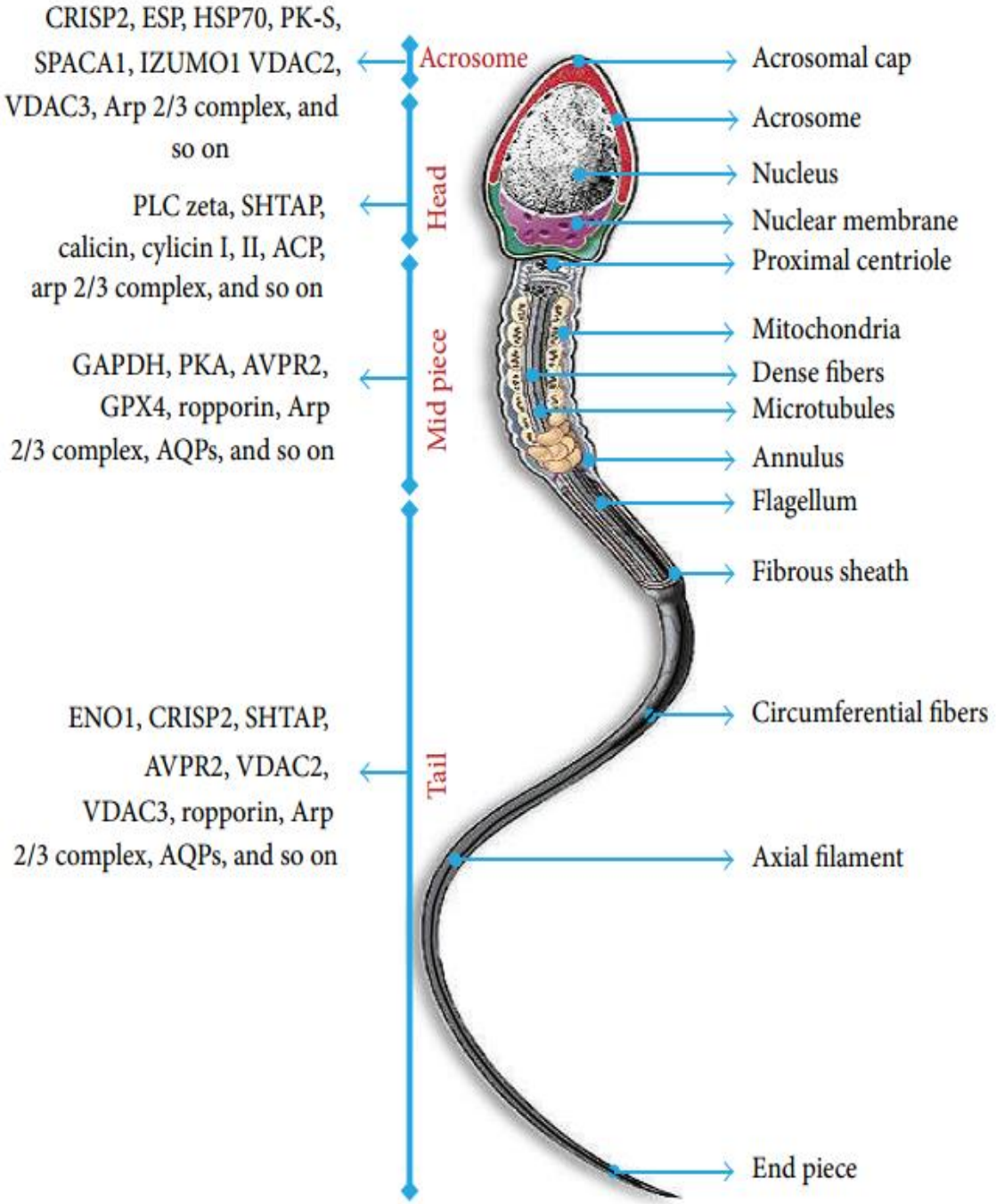
Negative biomarkers

Examples:

- Spermatid specific Thioredoxin 3 (SPTRX3/TXDC8), that is present in testicular round spermatids reduces chance of pregnancy by ART and increase recurrent miscarriage.
- Ubiquitin is found on the surface of defective spermatozoa after sperm passage through epididymis. Elevated semen ubiquitin correlates with reduced fertility outcomes and conventional semen parameters in humans and animals.

Negative biomarkers

- Voltage dependent anion channel 2 (VDAC2) may induce infertility with idiopathic asthenozoospermia.
- Ubiquinol-cytochrome-c reductase complex core protein 2 is associated with oxidative stress and ROS generation in mitochondria in spermatozoa and may induce male infertility.
- Cleaved PARP is a molecular marker of oxidative stress and apoptosis


CRISP2, ESP, HSP70, PK-S, SPACA1, IZUMO1 VDAC2, VDAC3, Arp 2/3 complex, and so on
Acrosome
PLC zeta, SHTAP, calicin, cylicin I, II, ACP, arp 2/3 complex, and so on
Head
GAPDH, PKA, AVPR2, GPX4, ropporin, Arp 2/3 complex, AQPs, and so on
Mid piece
ENO1, CRISP2, SHTAP, AVPR2, VDAC2, VDAC3, ropporin, Arp 2/3 complex, AQPs, and so on
Tail
Acrosomal cap
Acrosome
Nucleus
Nuclear membrane
Proximal centriole
Mitochondria
Dense fibers
Microtubules
Annulus
Flagellum
Fibrous sheath
Circumferential fibers
Axial filament
End piece

Energy Related proteins

Table 1. Energy Related Enzymes.

Enzyme name	Access code	Enzyme Code	Symptoms	Protein regulation	Location	Reference
Isocitrate dehydrogenase subunit α (IDH- α)	P50213	EC=1.1.1.41	Asthenozoospermia	Down	Mitochondria	[22]
Phosphoglycerate mutase 2	P15259	EC=5.4.2.1	Asthenozoospermia	Up	Cytosol	[22]
Triose phosphate isomerase (TPIS)	P60174	EC=5.3.1.1	Asthenozoospermia	Up	Cytosol	[20, 22]
Triose phosphate isomerase (TPIS)	P60174	EC=5.3.1.1	Globozoospermia	Down	Cytosol	[23]
Glutamate oxaloacetate transaminase-1	P17174	EC=2.6.1.1	Asthenozoospermia	Up	Cytosol	[22]
Fumarate hydratase precursor	P07954	EC=4.2.1.2	Asthenozoospermia	Up	Mitochondria & Cytosol	[21]
Cytochrome c oxidase subunit 6B	Q7L1R4		Asthenozoospermia	Down	Mitochondria	[21]
Glycerol kinase, testis specific 2 (GKP2)	Q14410	EC= 2.7.1.30	Asthenozoospermia	Up	-	[20]
Succinyl-CoA:3-Ketoacid co-enzyme A transferase 1 (OXCT1) precursor	P55809	EC=2.8.3.5	Asthenozoospermia	Up	Mitochondria	[20]
Glycealdehyde-3-phosphate dehydro-genase, testis specific (GAPD-S)	Q64467	EC=1.2.1.12	Knock-out Genotype Mice		Cytosol	[24]

Structural Proteins

Table 2. Flagella related proteins.

Protein name	Access code	Symptoms	Protein regulation	Location	Reference
Outer dense fiber protein 2 (ODF2)	Q5BJF6	Globozoospermia & Asthenozoospermia	Down	Flagella	[22, 23]
Tektin 1 (TEKT1)	Q969V4	Asthenozoospermia	Down	Flagella	[20]
Septin 4 (SEPT4)	O43236	Asthenozoospermia	Down	Annulus	[26]
Testis anion transporter 1 (Tat1)	Q96RN1	Asthenozoospermia	Down	Annulus	[26]
Secretory actin-binding protein (SABP)	P12273	Asthenozoospermia	Up	Midpiece	[50]
Tubulin beta-2C chain (TUBB2C)	P68371	Asthenozoospermia	Down	Flagella	[20]
Isoform 1 of tubulin α-2 chain	-	Globozoospermia	Down	Flagella	[23]
Isoform 2 of tubulin α-2 chain	-	Globozoospermia	Down	Flagella	[23]
Similar to α-tubulin	-	Globozoospermia	Down	Flagella	[23]
α-tubulin isotype H2-α	P68366	Globozoospermia	Down	Flagella	[23]

Sperm-Zona Binding Proteins

Table 4. Sperm-Zona Binding Proteins.

Protein name	Access code	Location	Zona Receptor (binding moiety)	Reference
β1,4-galactosyltransferase 1 (GalT)	P15535	Apical Region	ZP3 (N-acetyl glucosamine)	[3]
Lactadherin (SP47/ SED1)	P21956	Apical Region	ZP3 (Sialylated & Sulfated carbohydrate)	[31]
Hepatic lectin R2/3 (rHL-2)	P08290	Head & Flagella	ZP3 (Galactose moiety)	[31]
Spermadhesin (AQN-3)	P24020	-	ZP3 (Carbohydrate moiety)	[33]
Angiotensin-converting enzyme (ACE)	P09470	Cell Membrane	-	[33]

Sperm-Oolemma Penetration Related Proteins

Table 5. Sperm-Oolemma Penetration Related Proteins.

Protein name	Access code	Location	Reference
Sperm inner acrosomal membrane protein (IAM38)	Q2PMM0	Inner acrosomal membrane	[36]
Zona-pellucida binding protein 2 (ZPBP2)	Q6X784	Inner acrosomal membrane	[36]
Zonadhesin	Q28983	Inner acrosomal membrane	[31]
Equatorin (MN9)	B7SXT5	Integral membrane protein	[36]
IZUMO family members	-	Integral membrane protein	[36]
Fertilin subunit beta (ADAM 2)	Q99965	Integral membrane protein	[38]
Cyritestin (ADAM 3)	Q62287	Integral membrane protein	[40]
CRISP1	Q03401	Equatorial segment in capacitated sperm	[36]
CRISP2	P16563	Inner acrosome membrane	[36]
ERp57	P30101	Acrosome, tail and after acrosome reaction in equatorial segment	[45]
Sperm lysozyme-like protein 1 (mSLLP1)*	Q9D9X8	Equatorial part of acrosome reacted sperm	[29]
Spermatozoa acrosome membrane-associated protein 1 (SPACA1)	Q9HBV2	Equatorial part of capacitated sperm	[23]
Guanylyl cyclase receptor G	Q6TL19	Acrosome cap & equatorial segment	[60]

Nuclear Proteins

Protamine 1 and protamine 2 are the most abundant sperm nuclear proteins.

In infertiles, the P1/P2 ratio (which is 1 in fertile individuals), is increased as a result of under-expression of P2. In this case, low fertility is a result of DNA fragmentation.

Protamine deficiency may correlate to fertilization following ICSI, because of premature chromosomal condensation(PCC)(Nasr-Esfahani, 2007).

The PRM2 mRNA transcript is significantly decreased in patients with spermatogenic disorders, maturation arrest and Sertoli cell–only syndrome.

Peripheral Proteins

- Some sperm proteins are peripheral i.e. produced outside the sperm and then attach to the sperm. These proteins might originate from testicular tissues (seminiferous tubules), epididymis and accessory glands. It has been demonstrated that some of these proteins are fertility related based on different expression levels in fertile and infertile individuals

Peripheral Proteins

protein	Location or secreted by	function
Epididymosome	epididymal epithelium	sperm maturation and fertility
Eppin	epididymis	human sperm acrosome reaction
guanylyl cyclase receptor-G (hGC-G)	testis	zona binding
Glycosaminoglycans	Epid. Testis and others	capacitation, acrosome reaction, and sperm-oocyte penetration

5) Genetic markers

it has been suggested that up to 50% of infertility in humans can be attributed to genetic abnormalities.

- Karyotype anomalies and Y-chromosome microdeletions, SNPs and DNA damage represent important genetic causes of spermatogenic failure.

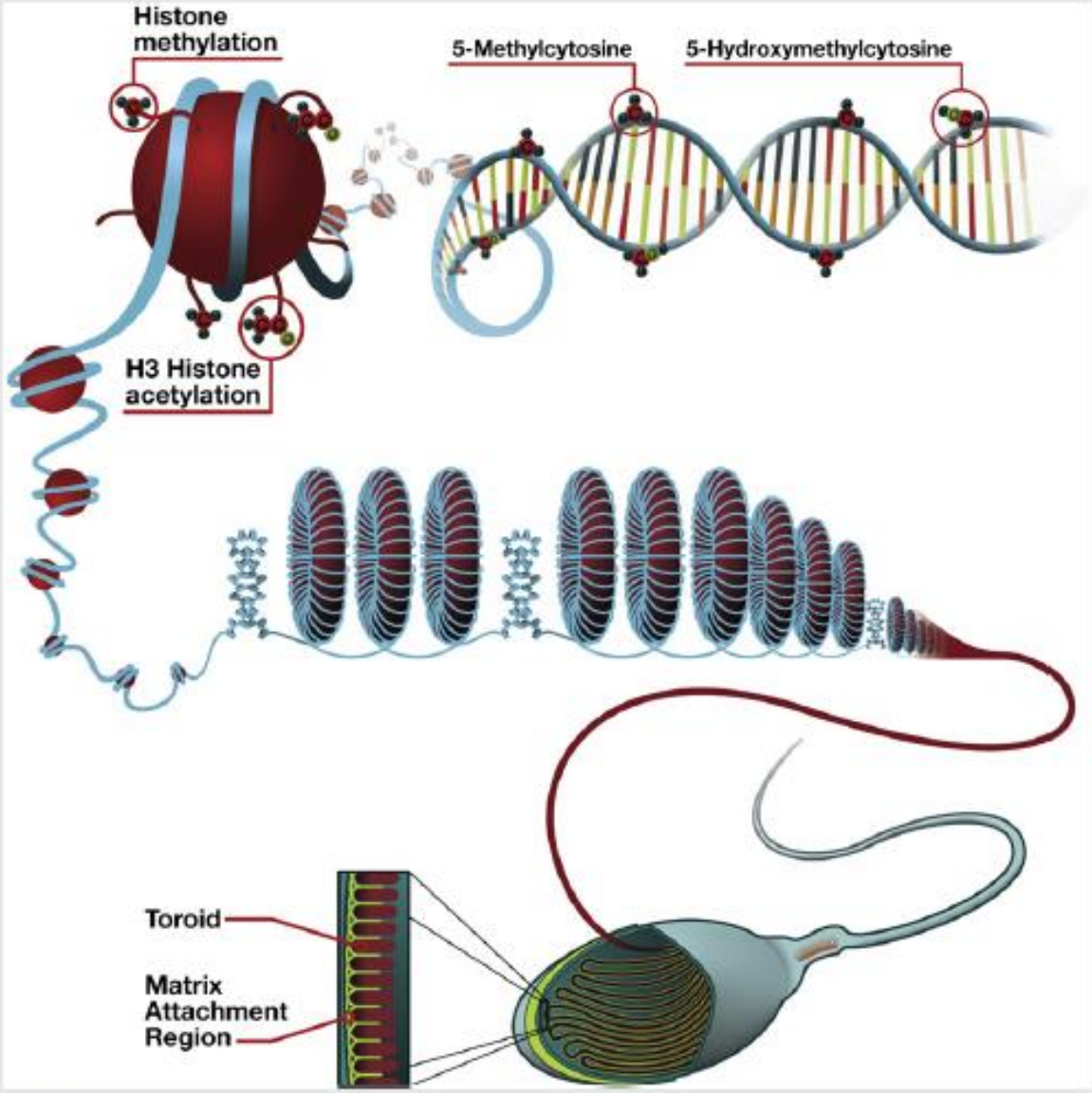
*Genetic abnormalities cause spermatogenic failures, diminished spermogram, fertilization and pregnancy rates and finally anomalies in next generation.

Genetic abnormality	Phenotype
CFTR mutation	Obstructive azoospermia due to congenital absence of the vas deferens
Robertsonian translocations	Azoospermia–severe oligospermia
Reciprocal translocations	Azoospermia–severe oligospermia
Klinefelter syndrome	Azoospermia–severe oligospermia
azoospermia factor (AZF) deletion of the Y chromosome	severe spermatogenic defects

6) Epigenetic markers

- Epigenetic mechanisms involve several layers of regulation of gene expression, such as

1. DNA methylation which is regulated by DNA methyltransferases (DNMTs)
2. histone modifications like Methylation that is monitored by histone methyltransferases [HMTases]
3. chromatin remodeling
4. histone variant composition
5. small noncoding RNA



Chromatin remodeling and epigenetic modifications in human sperm. DNA methylation is the first line of epigenetic modification of chromatin through methylation of position of cytosines found in CpG dinucleotides. An intermediate step in demethylation is the formation of 5-hydroxymethylcytosine residues, which are also observed in mature sperm. DNA is bound to histone octamers with unique modifications that present a second level of regulation of gene transcription. Most histones are removed from the elongating spermatid and replaced with protamines that result in a higher order of DNA packaging and silence gene expression. Retained histones are interspersed between protamine toroids and may be bound to matrix attachment regions, which facilitates replication of loop domains in the embryo.

Carrell. Epigenetics of the male gamete. Fertil Steril 2012.

List of the genes/proteins important for epigenetic modifications.

Genes/proteins	Function	Reference
MTHFR	Maintains the pool of methyl donors	[7]
DNMT1, DNMT3A, DNMT3B	DNA methylation	[24–26]
DNMT3L	Required for DNMT3A2 activity	[29]
SWI/SNF, ISWI	Chromatin remodeling	[22,23]
Jhdm2a	Chromatin remodeling	[34]
Suv39h1	Histone methylation	[31,32]
G9a	Histone methylation	[33]
LSD1-domain proteins, JmjC-domain proteins	Histone demethylation	[34]
HATs	Histone acetylation	[35]
MYST	Histone acetylation	[36]
HDACs	Histone deacetylation	[35]
SIRT1	Histone deacetylation	[37]
MUTp	Histone phosphorylation	[38]
NHK-1	Histone phosphorylation	[39]
MSK1, MSK2	Histone phosphorylation	[40]
PKA	Histone phosphorylation	[41]
HR6B	Histone ubiquitylation	[16]
E1 SUMO-activating enzyme 1, E1 SUMO-activating enzyme 2, UBC9	Histone sumoylation	[18]
CTCF	Interacts with differential methylated regions	[54,55]
BORIS	Interacts with demethylases	[54,55]

List of aberrant epigenetic modification reported in male infertility.

Genes/proteins	Aberration and male infertility	Reference
MTHFR	DNA hypermethylation results in poor semen quality and infertility	[83,84]
PAX8, NTF3, SFN, HRAS	DNA hypermethylation associates with poor sperm concentration, motility and morphology	[80]
JHM2DA	Knockout results in loose packaging of DNA and may cause infertility	[34]
IGF2, H19	Low methylation associates with low sperm concentration	[88]
RASGRF1	Hypermethylation at the imprinted locus associates with poor semen parameters	[53]
GTL2	Hypermethylation at the imprinted locus associates with poor semen parameters	[87]
PLAG1, D1RAS3, MEST	Hypermethylation at the imprinted loci associates with poor semen parameters	[80]
KCNQ1, LIT1, SNRPN	Hypermethylation at the imprinted loci associates with poor semen parameters	[96]

Rajender et al. / Mutation Research (2011)

Aberrant epigenetic regulation, male infertility and embryonic development

- Sperm epigenetic environment plays a role in establishing epigenetic marks in the embryo, thus aberrant epigenetic regulation in spermatogenesis has a profound effect on both male fertility and embryonic development.
- For example Poor sperm concentration, motility and morphology were associated with broad DNA hypermethylation across a number of loci (Houshdaran et al. 2007)

Sperm RNA

- Although spermatozoa do not have translational activity, these cells have a wide variety of RNAs. The synthesis of these transcripts takes place at the late stages of spermatogenesis and they will delivered to the oocyte during fertilization.
- The profiling of spermatozoal RNAs as clinical biomarkers of human male infertility has been investigated by several groups.
- Microarray profiling and RNA sequencing assay technologies have been used to investigate transcription levels of spermatozoa (Jodar et al., 2013).

Types of sperm RNA

Small noncoding RNAs (sncRNAs) are approximately 20–30 nucleotides in length and include:

microRNAs (miRNAs), small-interfering RNAs (siRNAs), tRNA, rRNA and piwi-interacting RNAs (piRNAs).

These molecules are functional in many gene expression and regulation processes (Liu et al., 2015). The miRNAs have critical regulatory functions in gene expression at the post-transcriptional level via various epigenetic mechanisms. miRNAs also play critical roles in mammalian gonadal function during both gametogenesis and differentiation of germ cell (Gunes, Metin Mahmutoglu, et al., 2016).


Thanks for you´r attention