ÖNDER ÇOBAN

INVESTIGATION OF SPERM DNA DOUBLE STRAND BREAK INCIDENCE IN IVF PATIENTS

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A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF HEALTH SCIENCES OF NEAR EAST UNIVERSITY

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In Partial Fulfillment of the Requirements for

The Degree of Master of Science

In

Medical Biology and Genetics

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THESIS APPROVAL

(Bu sayfa yerine, başarılı geçen Tez Sınavı sonrası sınav tutanağı ekinde yer alan Tez Onay sayfası gelecektir)

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Mol.Biol Önder Çoban

DEDICATION

To my family; wife, daughter and son

ABSTRACT

The aim of this study is to investigate the incidence of sperm DNA Double Strand Breaks (DSBs) in IVF patients and evaluate differences in fertile and infertile groups. To this aim, a total of 8240 sperm cells from 60 individuals were analyzed by using phosphorylation of gamma-H2AX as a DNA DSB marker. Results were analyzed for standard semen characteristics and morphology based on Kruger's Strict Criteria (normozoospermia, oligozoospermia, Asthenoteratozoospermia, Teratozoospermia, Oligoasthenoteratozoospermia) and fertility diagnosis (fertile, infertile). The results did not showed any differences in volume (p=0,786), count (p=0,258) and motility (p=0,932). Also, no statistical differences were found between sperm morphology (p=0.869), fertility (p=0,212) and DNA DBS frequency. Although no correlation found between DNA DSB in sperm cells and studied characteristic of study groups, mean DNS DSB frequency was similar with previous studies in the literature (~20%). as a conclusion; these results suggest that rate of DNA DSB in sperm cells is not a deterministic factor for seminal characteristics and infertility.

KEYWORDS*:* sperm DNA DSB, infertility, sperm morphology, gamma-H2AX

ÖZET

Bu çalışmanın amacı IVF hastalarında sperm çift sarmal DNA kırıklarının sıklığının araştırılması, kısır ve kısır olmayan erkeklerdeki oranların incelenmesidir. Bu amaçla; toplam 60 erkekte 8240 adet sperm bir DNA çift sarmal kırığı göstergesi olan gamma-H2AX fosforulasyonunun incelenmesi vasıtası ile analiz edildi. Sonuçlar temel semen karakteristikleri, Kruger in kesin kriterleri temel alınarak sperm morfolojisi (normozoospermia, oligozoospermia, Asthenoteratozoospermia, Teratozoospermia, Oligoasthenoteratozoospermia) açısından ve kısırlık tanısı (Kısır, kısır değil) açısından incelendi. Elde edilen souçlara göre semen hacmi ($p=0.786$), sperm sayısı ($p=0.258$) ve haraketliliği ($p=0.932$) arasında istatistiksel bir fark bulunamadı. Aynı zamanda sperm morfolojisi ($p=0.869$), kısırlık ($p=0.212$) ve DNA kırığı sıklığı arasında da bir fark bulunamadı. Çalışılan parametreler ile DNA çift sarmal kırıklıkları sıklığı arasında bir ilişki bulunamamasına rağmen, ortalama DNA kırıklıkları yüzdesi literatürdeki diğer çalışmalarla benzer bulundu (~20%). Sonuç olarak; elde edilen verilere göre spermlerdeki DNA çift sarmal kırıklarının, temel semen karakteristikleri ya da kısırlık durumu üzerinde belirleyici bir etkisinin olmadığı görüldü.

ANAHTAR KELIMELER: sperm DNA DSB, kısırlık, sperm morfolojisi, gamma-H2AX

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SYMBOLS AND ABBREVATIONS

WHO	World Health Organization
OAT	Oligoastenoteratospermia
SSB	Single Strand Break
DSB	Double Strand Break
DNA	Deoxyribonucleic acid
T CELLS	T lymphocyte
B CELLS	B lymphocytes
H2O2	Hydrogen Peroxide
HIV-1	Human İmmunodeficiency Virus-1
VPR	Viral Protein R
ROS	Reactive oxygen species
SAHA	suberoyl+anilide+hydroxamic acid
PARP	poly ADP ribose polymerase
DNA-PK	DNA-dependent protein kinase
H2AX	Subset of histone protein 2 A
H2A	Histone protein 2 A

H2B	Histone protein 2 b
H3	Histone protein 3
H4	Histone protein 4
H1	Histone protein 1
SQ	Sequence Motif
ATM	Ataxia Telangiectasia Mutated
ATR	ATM-Rad3-related
NHEJ	None Homogous End Joining
HR	Homologour Recombination
SSA	Single Strand Annealing
CO	Cross-over
NCO	None- Crossing Over
SDSA	Synthesis Dependent Strand Annealing
MLH1	MutL homolog 1, colon cancer protein
STR	Single Tandem Repeats
IVF	In Vitro Fertilization

AT	Ataxia Telangiectasia
ZGA	Zygotic Gene Activation
PBS	Phosphate Buffered Saline
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
HORMADs	HORMA Domain-Containing Protein
γ	Gamma

CHAPTER 1

INTRODUCTION

Infertility is a global health problem which affects approximately 15% of couples at reproductive age (Lewis 2015) and defined by World Health Organization ("WHO | Infertility," 2014) as inability to become pregnant or to be able to maintain pregnancy to live birth. There are two types of infertility named as primary and secondary. Primary infertility defined as inability to have a child in 1 to 2 years with regular attempts without contraception and seconder infertility is inability to have another child after one or more previous pregnancy (Lunenfeld and Van Steirteghem, 2004). According to recent data 20-30% of infertility caused by male, 20-35% female, 25-40% both male and female, and 10-20% unexplained factors (Ferlin et al., 2007).

Male infertility is one of the biggest causes of infertility with around 30% of reproductive age among infertile couples and it is generally evaluated by semen parameters such as volume, count, motility and other morphological assessments (Jungwirth et al., 2012). These parameters are appropriate to identify cause of male infertility if there are severe abnormalities such as oligozoospermia, asthenozoospermia, teratozoospermia, oligo-astheno-teratozoospermia (OAT) or azoospermia but they do not give certain information about cause of low embryo quality, implantation failures, birth defects and miscarriages which are mostly caused by chromosomal abnormality in offspring (Gonzalez-Marin et al., 2012). Although relations with chromosomal abnormalities and sperm morphology have been shown in some cases, there are some abnormalities, which do not affect morphological appearance of the sperms (Martin and Rademaker, 1988). Some of these abnormalities are mutations, aneuploidies, double or single strand breaks (SSB and DSB respectively) and DNA fragmentations and could be originated from oxidative stress (Li, Yang, and Huang, 2006), meiosis and chromatin re-modelling process during spermatogenesis (Aitken and De Iuliis, 2010). In addition to these, there are environmental factors such as toxins and anticancer drugs can cause abnormal sperm production and so infertility (M. Liu, Hales, and Robaire, 2014). In

spite of these, 10-15% cases still unexplained with routine diagnostic tests according to European Association of Urology Guidelines on Male infertility (Jungwirth et al., 2012).

One of the newest approaches for analyzing gamete quality is investigating DSBs, which was previously linked with fertilization failure and/or abnormal embryonic development (Kuo and Yang, 2008). There are different methods for investigating DSB such as 2D electrophoresis, comet assay and neutral elution but recent and most precise one is γH2AX assay. Detecting γH2AX is easy to apply and be able to show the location of DSB in intact cell with fluorescence labelling (Kuo and Yang, 2008).

In this study, we have aimed to investigate γH2AX incidences as a DSB marker in sperms and their possible correlation with sperm morphology.

1.1 Causes of DNA double-strand breaks (DSBs) formation

DNA double-strand break is one of the most biologically damaging chromosomal lesions that can be produced by ionizing radiation, chemical agents and also some endogenous processes such as DNA replication (Figure 1.1), programmed V(D)J recombination (Figure 1.2) and meiotic exchange which can jeopardize its physical integrity (Rogakou et al., 1999). This chromosomal integrity is necessary for appropriate segregation during meiosis and mitosis. Also, it disturbs chromosomal informational integrity and thus maintaining the function of cellular components. DSBs can lead cell cycle arrest, initiation of apoptosis and/or cell death if left unpaired because of losing genetic material. Moreover, it has been shown that incorrect repairing of DSBs can lead carcinogenesis with causing chromosomal mutations such as translocations, deletions and/or inversions (Rothkamm et al., 2003).

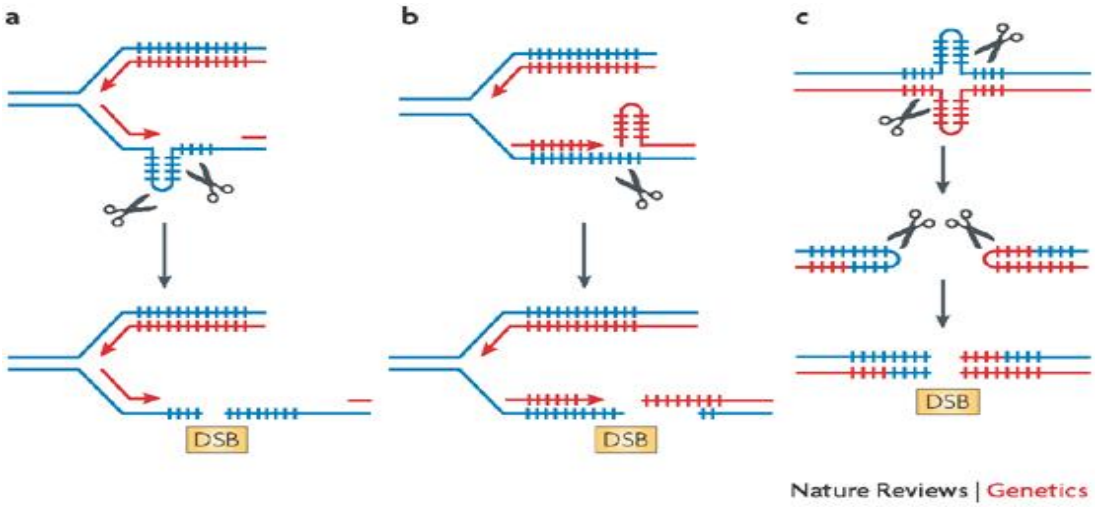

Figure 1.1 İllustration of double-strand break formation at fragile sites. a) Secondary DNA structures, such as hairpin or loop on lagging-strand can lead DSB by action of some nucleases (scissors). b) Secondary structures (a palindrome represented in the figure) in 5' end of an Okazaki fragments facilitates a brakeage on template strand. c) Secondary structures on both strands can form holiday junction structure. This structure can be converted to two hairpin-ended molecules and form DSB by action of MR(X)N (Aguilera and Gomez-Gonzalez, 2008).

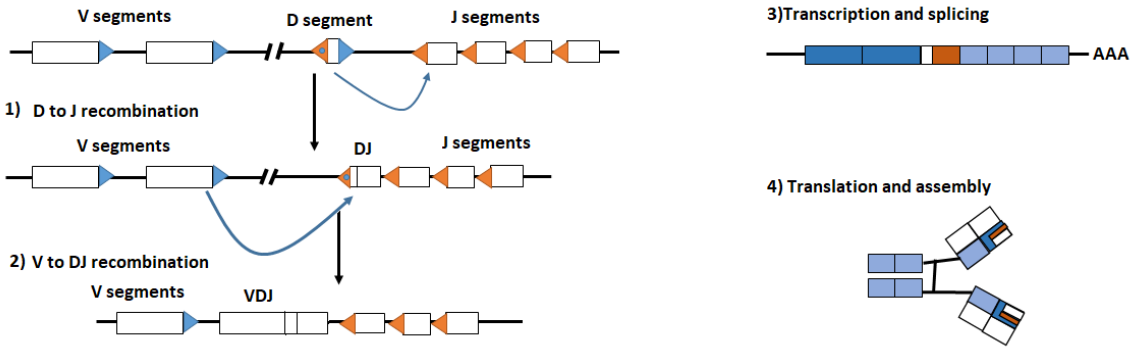

Figure 1.2 Representation of VDJ Recombination. VDJ recombination is a process that adaptive immune cells (T and B cells) randomly organize different gene segments (known as variable (V), diversity (D), Joining (J)) and creates unique antibodies. This process has two steps. Firstly, D and J segments fuse and form DJ sequence (1) then this combined segment fuse one of the V segments (2).VDJ sequence is transcribed together and added to the transcript by exon splicing (3). This recombine region is resembled in functional part of translated antibody (4).

1.1.1 Chemical and environmental factors

There are several chemical agents such as hydrogen peroxide (H2O2) and some chemotherapeutic drugs, which previously reported as DSB inducing agents. According to reports, most of these agents produce oxidative stress and induces ROS formations in tissue and lead cellular damage and double or single strand breaks on DNA (Bonner et al., 2008). As mentioned before these structural abnormalities can cause cell death or growth arrest, which can be used as treatment approach in cancer treatment. Therefore, some anticancer drugs designed to initiate DSBs in tumor cells to initiate cell death pathway as a treatment. As shown in table 1.1, some of these agents create DSB directly, while others make cellular damage that can cause DSB formation (Bonner et al., 2008).

Also, some environmental factors and their relation with DNA double or single strand break formation are known, such as cosmic or ionized radiation (Santivasi and Xia, 2014) and some contaminants create reactive forms of oxygen and lead breakage on

DNA. Also recently, some pathogens such as *HIV-1 Vpr* protein and *Helicobacter pylori*, were reported as DSB inducing agents (Tachiwana et al., 2006).

Table 1.1 Some known anticancer drugs and their role in DSBs formation

Agent	Direct/ Indirect	Mechanism of induction	Referans
Bleomycin	Direct	Metal ion-mediated oxidative cleavage	(Banath and Olive, 2003; Tomilin et al., 2001)
Camptothecins, indenoisoquinolines	Indirect	SSB to DSB conversion by replication	(Antony et al., 2007; Furuta et al., 2003)
Doxorubicin, etoposide, mitoxantrone, batracylin	Indirect	production of DSBs by trapping TOP2 cleavage complexes and causing ROS formation	(Kurz and Lees-Miller, 2004; Olive et al., 2004; Rao et al., 2007)
cytarabine, gemcitabine, hydroxyurea	Indirect	Collapse of replication fork	(Krynetskaia et al., 2008; Kurose et al., 2006)
cisplatin, temozolomide, aminoflavone,trabectedin	Indirect	DNA alkylation	(Meng et al., 2005; Nowosielska and Marinus, 2008; Pabla et al., 2008)
tirapazamine	Indirect	ROS production	(Olive et al., 2004)
5-Azacytidine, SAHA	Indirect	DNA methylation and inhibition of histone deacetylation	(Kiziltepe et al., 2007; Munshi et al., 2006)
PARP and DNA-PK inhibitors	Indirect	interfere with the repair of SSBs and DSBs that are caused by different agents	(Albert et al., 2007; Zhao et al., 2006)
UCN-01	Indirect	checkpoint inhibitor potentiating replication-associated DNA damage induced by TOP1 inhibitors cytarabine and gemcitabine	(Ewald et al., 2007)
TRAIL	Indirect	death receptor-mediated activation of DNA-PK	(Solier et al., 2009)
imatinib	Indirect	apoptosis induced by KIT and PDGF tyrosine kinase inhibition	(Liu et al., 2007)

1.1.2 Errors during spermatogenesis

Errors during meiotic recombination generally initiate cell death. It is known that some nucleases are having role in meiotic cross-over and important for DNA-DNA and DNA protein interactions. This interaction is high in mature sperms due to condensed form of DNA. Although its' molecular mechanism is not well known, Windt et al., (1994) showed that there is relation between highly cross linked chromatin and defective spermatozoa

DNA breaks are required for temporary relief of nucleosome to help binding of protamine. There is a step in chromatin packaging that topoisomerase II create and ligate DNA breaks after histone hyper-methylation. This step should be completed to restore DNA integrity in epididymis in order to prevent DNA breaks in ejaculated spermatozoa (Mengual et al., 2003).

Through end of the spermatogenesis up to 95% of histone are replaced by testes specific histone variants after hyper acetylation. Thereafter, transient proteins are replaced by protamine 1 (P1) and protamine 2 (P2). Normally, expression of P1 and P2 is equal and provide condensation of sperm DNA that lead blocking gene expression. It is known that inequality in P1 and P2 1:1 ratio is related with high DNA fragmentation, fertilization failure and decreased embryo quality (Gonzalez-Marin et al., 2012).

Due to lack of transcriptional and translational activity, sperm cells lose their ability to process programmed cell death in case of abnormal chromosomal status. Therefore, they form DNA fragmentation rather than apoptotic response and cell death (Sakkas et al., 2004).

1.1.3 Storage Temperature and Cryopreservation

There are several studies about sperm freezing temperature in various species. These studies revealed that level of sperm storage temperature shows different effect on different species. For example: temperature between 5^{o}C and 15^{o}C sustain DNA

integrity longer then above 20ºC on bulls, dogs and rabbits (Love et al., 2002) while below 37ºC reduces Sperm DNA stability and on elephants (Gonzalez-Marin et al., 2012). Although known features of sperm cryopreservation like detrimental effects on sperm structure and function, it is preferred for fertility preservation before medical therapy, surgical treatments and vasectomy. It is known that sperm cryopreservation is one of the factors that should be considered due to its role on sperm DNA fragmentation dynamics (Gosalvez et al., 2011).

1.1.4 Bacterial Infections

Although mechanism is not well understood, connection between infections that caused by *Cylamydia trachomatis* and *mycoplasma* and high sperm DNA fragmentation is known (Gallegos et al., 2008). In additionally *Cylamydia trachomatis* infections suggested to increases the immotile sperm percent in semen . One possible explanation could be that the level of tyrosine phosphorylation of sperm protein, which is important for sperm capacitation, increases by co-incubation with *Cylamydia trachomatis.* (Hosseinzadeh et al., 2000) Three years later (2003) same scientist confirmed it in another study by co-incubation of sperms with polysaccharides purified from bacteria (Hosseinzadeh et al., 2003).

Unlike other causes of sperm DNA fragmentation, studies also indicates that antibiotic treatment can reduce DNA fragmentation level as well as other semen characteristics that caused by bacterial infections (Gallegos et al., 2008).

1.2 DNA double strand breaks and γH2AX expression

Approximately 146 bp of DNA is wrapped around a core histone molecule, which contains four types of histone proteins known as H2A, H2B, H3 and H4, and forms nucleosome. H1, also known as linker histone, is located at outside of nucleosome and creates more compact form of chromatins by interacting with linker DNA (up to 80 bp) (Figure 1.3). There are various types of H2A proteins such as H2A1, H2A2, H2AX, H2AZ and more but only H2AX is unique in eukaryotes because of serine at position

139 and glutamine at position 140, also known as SQ motifs, in the highly conserved sequence of its carboxyl tail (Kuo and Yang, 2008).

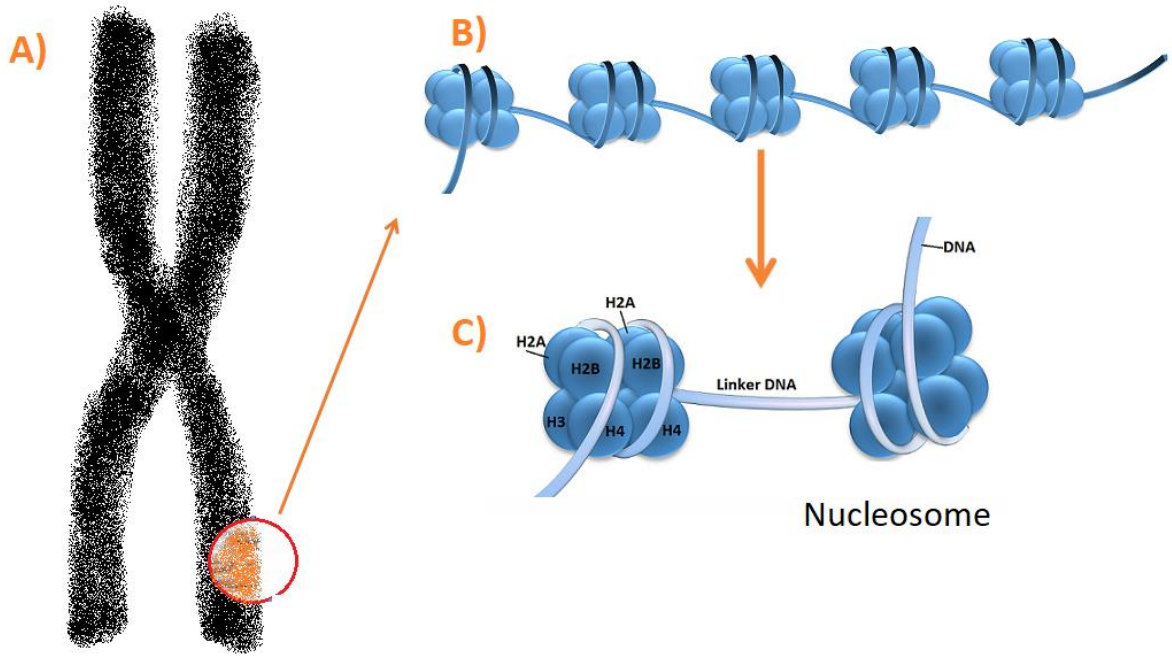

Figure 1.3 Nucleosome and organization of Histone proteins. DNA is wrapped twice around the histone octamer and form nucleosome. Nucleosome is consisted by histone proteins H2A, H2B, H3 and H4. H1 is located outside of the nucleosome and have role in linking nucleosomes to create solenoid formation (B, C).

Several recent studies showed that H2AX, which is a part of H2A histone protein family located in histone octamer in nucleosome, phosphorylated just after DNA damage (Antonelli et al., 2015; Kato et al., 2006). H2AX phosphorylation takes place by kinases like ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-PKCs, which are components in the phosphatidylinositol 3-kinase (PI3K) pathway. This newly phosphorylated H2AX protein at DSB sites called gamma-H2AX (γH2AX) which is the

first step for localization of DNA damage and initiation repairing process (Kuo and Yang, 2008).

In addition, it is accepted that disappearance of γH2AX loci related with DSB repair. However, number of γH2AX loci and their relation with DSB count is not clear. Even so, γH2AX is used as a biomarker for predicting individual response to certain treatments such as radiotherapy or ionizing radiation in cancer patients (Mariotti et al., 2013).

1.3 DNA double strand breaks (DSBs) repair mechanisms

DNA repair is a complex system that requires many enzyme and different pathways to maintain cellular genome. As mentioned earlier, many internal and external factors corrupt genetic information in living organisms. Thus, DNA repair system continuously scans the genome for abnormality and tries to fix by removing or repairing the damage. Employed pathway to repair DNA damage depends on type of the damage and cell cycle. If damage is not repairable, DNA repair machinery tries to minimize the errors as much as possible and ensure cell viability (Lindahl and Wood, 1999).

DNA repair pathways that listed in Reactome pathway database are base excision repair, DNA damage bypass, DNA damage reversal, DNA double strand break repair, nucleotide excision repair, Fanconi anemia pathway and mismatch repair (Reactome.org, Pathway: DNA repair, id:R-HSA-73894) Reviewed by Curtin (2012). In same database DSB repair mechanisms are detailed as DNA Double Strand Break Response, Homology Directed Repair (HR) and Nonhomologous End-Joining (NHEJ) (Rothkamm, K et al., 2003).

There are two distinct pathways called non-homologous end joining (NHEJ) and homologous recombination (HR) which are evolved to repair DNA DSBs after DNA Damage Response (DDR) (Iliakis et al., 2004)(Figure 1.4). Mostly, type of repair mechanism depends on cell stage and time of DNA damage. There are other options like activation of apoptotic pathway or toleration, if repair is not possible. Apoptotic pathway

leads cell death to prevent viability. It is possible to tolerate DNA damage, which may lead inheritance of mutation in next generation. In repairing pathway, HR needs undamaged sister chromatids which requires cells at S and G2 phase while NHEJ occurs at G1 phase (Pommier 2008)

NHEJ has role in modification and joining broken end of DNA without any or very little homology and generally creates small chromosomal aberrations such as deletions and/or insertions. In another words, NHEJ can repair DSBs by joining broken ends with leaving DNA sequence unrestored. NHEJ pathways occur at all phase of cell cycle but especially cells at G1 phase due to lack of homolog chromosomes. Cells at G1 phase use NHEJ mechanism before chromosomal replication, then chromosomal replication creates chromatid which can be used as a template for DSB repair (Mehta and Haber, 2014) (Figure 1.4). In addition, it is suggested that NHEJ can occur at S and G2 phases to fix defects in HR (Pommier 2008).

HR uses undamaged homologous sister chromatid to correct errors while repairing broken part of chromosome. There are different HR pathways to repair DSBs like Single-strand annealing (SSA) which allows formation of a deletion between homologous sequences near DSB. Other types depend on DSB detection and pairing broken part of DNA with homologous sequence of sister chromatid (Mehta and Haber, 2014) (Figure 1.4).

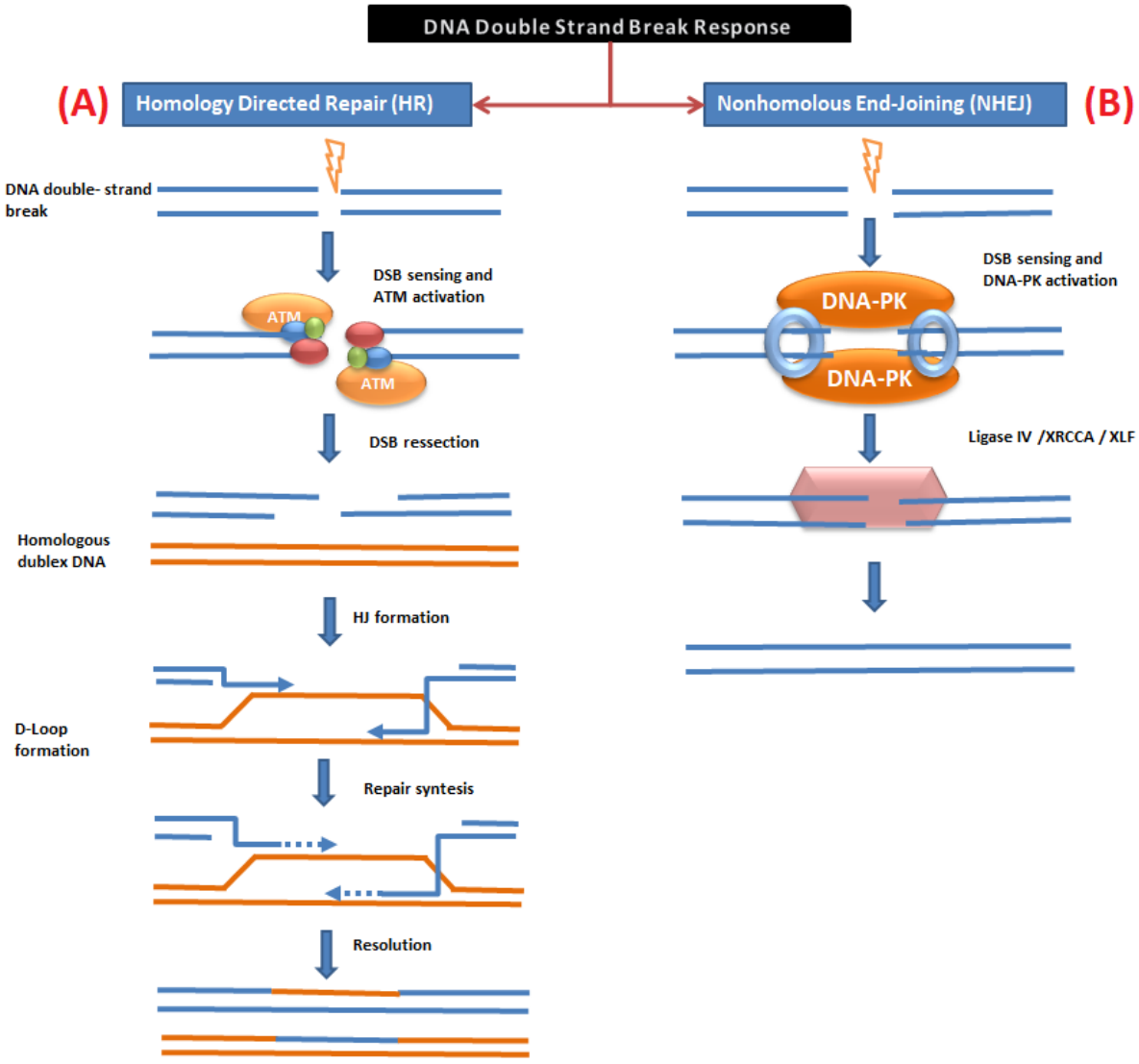

Figure 1.4 DNA DSB repair pathways. There are two main types of DNA repair pathways. Depending on cell type and cycle, one of them is employed for DNA repair. (A) Homologous Recombination (HR) uses a template to complete missing parts and repair the damage while (B) Non-homologous end joining (NHEJ) repairs damage without a template (Adapted from Lopez-Contreras and Fernandez-Capetillo, 2012).

1.3.1 During Spermiogenesis

Development of sperm from spermatogonia to mature sperm includes three distinct phases (Figure 1.5). In the first phase, spermatogonia (SSC) differentiate into primary spermatocytes (SPs) after several mitotic divisions. In the second phase, meiotic recombination take place and haploid spermatid form from spermatocytes. Third phase, also known as spermiogenesis, includes structural development and formation of mature spermatozoa from round spermatids (RS). Due to complex and remarkable genomic reorganization, development of gametes from precursor cells requires a unique process for chromatin remodeling. This process includes replacement of histone proteins with transition protein 1 and 2 and their replacement with protamines (P1 and P2) (Gonzalez-Marin et al., 2012).

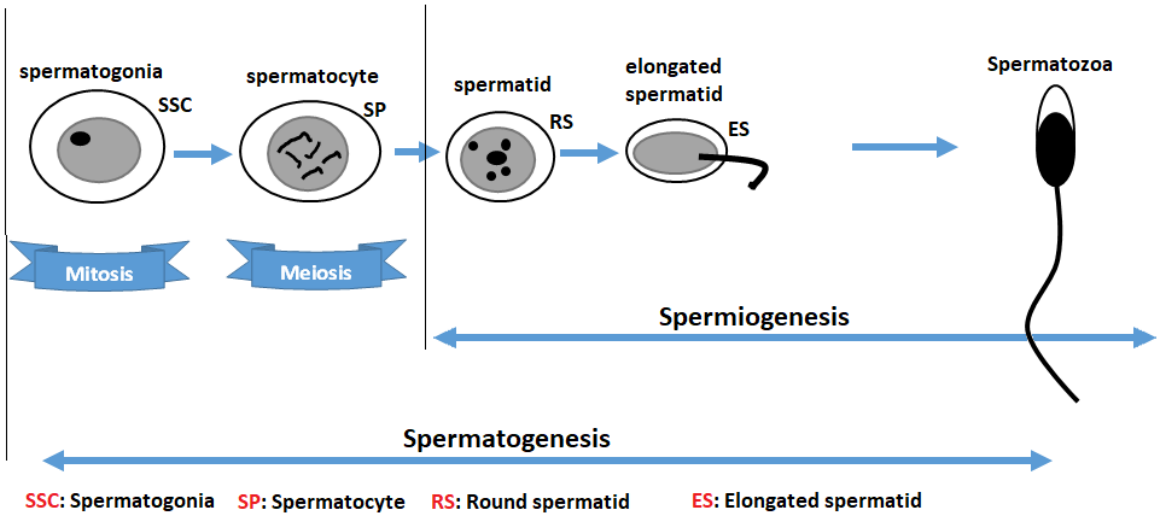

Figure 1.5 Phases of spermatogenesis from spermatononia (SSC) to mature spermatozoa. Process starts with mitotic proliferation of spermatogonia (diploid). Afterward, primary and secondary spermatocytes (SPs) form as a result of first meiotic division. Second spermatocytes turn into round spermatids and differentiation steps starts with elongation and continue until formation of fully matured spermatozoa.

Spermatogenesis includes all steps from primary germ cells to mature spermatids while spermiogenesis from round spermatid to matured spermatozoa (adapted from Gonzalez-Marin et al., (2012)).

1.3.2 During fertilization and embryonic development

It is known that sperm with structural chromosome abnormalities has ability to fertilize oocytes and pursue embryonic development (Jiang, L.-Y et al., 2015). If damage is too high and is not repaired by oocyte DNA repair system, development of the embryo may stop or not be able to implant in the uterus. Furthermore, it may cause miscarriage in early stage of pregnancy (Shamsi et al., 2011) Also, incomplete repair of DNA damage in oocytes results partial deletions or sequence errors in the embryo. Fortunately, oocytes are able to repair most of the DNA damages and provide normal embryonic development (Brandriff and Pedersen, 1981).

The DNA repair components in oocytes (RNAs and proteins) have role in chromatin remodeling process and maintenance of the chromatin integrity during fertilization. There are many genomic events that occur during the first cell cycle after fertilization. For example, completion of meiosis and chromosome de-condensation (both paternal and maternal). New programming of parental chromosomes takes place to compose embryonic genome by complex chromatin remodeling events in order to start embryonic development (Baarends et al., 2001).

Oocytes are able to repair most of the DNA damages that originated from spermatogenesis during pronuclear formation, DNA replication and nuclear fusion events (Aguilera and Gomez-Gonzalez, 2008). It is believed that repair capacity of the oocytes is depend on maternal mRNAs and proteins that produced before ovulation in the oocyte. Also, it has been previously shown that mammalian genes that related with DNA repair are active in the early stage of development (Derijck et al., 2008). This repair activity and high level of related gene transcripts (mRNA) in oocytes and embryos provides templates to repair pathways before embryonic genome activation. Both HR and NHEJ mechanisms available in mature oocytes and blastocysts but mostly HR is

preferred due to error-free property when compared to NHEJ (Derijck et al., 2008). Although both pathways can repair DSBs, it is still not well understood how cells choose whether HR or NHEJ pathways before repair.

DNA damage in sperms may appear in the zygote genome after the first metaphase. Unlike oocytes, DNA repair in zygote occurs by both HR and NHEJ pathways. In general, somatic cells use NHEJ to repair DSBs while embryonic cells use HR (Derijck et al., 2008). It is known that HR and NHEJ mechanisms are active during different part of the cell cycle (HR during S/G2 and NHEJ in G1 phases). Therefore, the phase of cell cycle is important for pathway choice.

1.3.3 Repair during and after Implantation

Cell cycles during embryonic development are fast. Phases of mitotic cell cycle is include G1, S, G2 and M. DNA replication occurs in S phase so it is longer in embryonic cells when compared with somatic cell division. On the other hand, G1 and G2 phases are short due to necessity of rapid cell division rather than cell growth. Therefore, HR is mostly preferred DSB repair mechanism in the blastocysts. According to previous studies, preimplantation death is common in many animals due to lacking necessary DNA repair enzymes. This information supports the importance of DNA repair system during rapid cell division at cell differentiation and proliferation. Similar with early embryonic development, many genes that related with DNA repair system active after implantation of mammalian embryos (Baarends et al., 2001).

In summary, DNA damage in zygote, embryo and blastocysts originated from sperm can be repaired and may have no influence on fetal development. Today, there are several tests, that can detect DNA damage in sperm but it is not possible to make prediction about their “reparability” (Gonzalez-Marin et al., 2012).

CHAPTER 2

2.1 DSB detection methods

There are several types of DNA damages and most of them originate from male gametes (González-Marín et al., 2012). It is known that most of the DNA fragmentation in ejaculated sperms includes double (DSB) and single strand breaks (SSB) and can be detected by various methods such as TUNEL, Comet assay, Sperm Chromatin Structure Assay (SCSA) or Sperm Chromatin Dispersion (SCD) methods.

2.1.1 TUNNEL

Single or double strand breaks on DNA creates free 3'-hydroxyl termini. TUNEL (TdT-mediated dUTP-biotin nick end labeling) assay use this biochemical hallmark of DNA breaks and provide visualization of cells containing fragmented DNA by labeling free 3' end of fragment. In this method, staining procedure works with interaction of enzyme terminal deoxynucleotidyl tranferase (TdT) and labeled dUTP complex with free 3'-hydroxl termini of fragmented DNA. Steps in the test are as follows: TUNEL reaction, staining by fluorescent label and analyze by Flow Cytometry or fluorescence/confocal microscopy after counterstaining (Deryk, 2011).

TUNEL is a nonspecific test and all free 3' hydroxyl end of the fragments in the samples are stained whether caused by DNA repair mechanism, apoptosis, mechanical forces or gene transcription. Therefore, it is mainly used as DNA fragmentation tests and a method for identifying apoptotic Cells when used with additional specific pathways (Deryk, 2011).

2.1.2 Comet assay

The comet assay is an easy but very sensitive method for detecting DNA damage in the cell. Application of the method includes embedding cells into agarose on a microscope slide, lysis of the cells and electrophoresis. Lysis steps includes treatment the sample with high salts which remove histones and leaves the DNA attached to nuclear matrix without nucleosome. During electrophoresis only damaged DNA migrates and forms a

pattern known as comet tail. In this method, it is possible to get frequency of the damage by evaluating percentage of migrated DNA in agarose. Its effectiveness increases with use of lesion specific endonucleases to study specific type of DNA damages such as SSB and/or DSB. Also it is possible to investigate DNA repair capacity in specific cell type by using artificial damaging agents and evaluating results at different time periods. Another advantage of the comet assay is ability to examine DNA damage at singe gene level by combining this method with FISH by using specific probes designed for the certain DNA sequence (Deryk, 2011).

2.1.3 Sperm Chromatin Structure Assay (SCSA)

This method is based on decondensation of sperm nuclear DNA and staining with acridine orange (AO). Briefly, method includes; denaturation of DNA at sites of strand breaks by using acid (pH 1.20) and analyze by flow cytometry fallowed by AO staining. In molecular level, AO molecule enters into the dense sperm chromatin and fuse into double stranded DNA that seen as green light under blue laser. In case of DNA breaks, AO clusters on single stranded DNA and this new complex collapses. This event leads metachromatic shift of green to red appearance under blue laser light (Deryk, 2011).

2.1.4 Sperm Chromatin Dispersion (SCD) Test

Unlike other test, SCD shows undamaged DNA instead of DNA fragmentation. It is not based on analyzing colors or fluorescence intensity therefore it is easy to apply and evaluate with light microscopy. Due to loop form of sperm chromatin in mammalian sperm cells, chromatin will open into coils under molecular torsion if not damaged. DNA loops are spread and produce a shape called halos originated from central core. Sperms with undamaged DNA create big halos while sperms with fragmented DNA small or no halo. It is possible to apply FISH on previously SCD test applied sperm samples. This makes it easy to study sperm DNA fragmentation and chromosomal abnormalities simultaneously (Deryk, 2011).

CHAPTER 3

3.1 DSBs and infertility

3.1.1 In gametogenesis

DNA DSBs can occur during meiosis and lead infertility and miscarriages through abnormal gametes and so embryo development (Collins and Jones, 2016).

Gametogenesis is a highly conserved process and includes homologous recombination by programmed DNA DSBs formation due to valuable genetic exchange during prophase 1 while ensure protecting cell from genomic damage that risk for cell survival. It is believed that meiotic DSBs through genome is not random and concentrated at regions called DSB hot spots (around 10 to 40 thousand in mammalian genome). Because distribution of DSBs hot spots not random although not universally conserved, it can be used as a marker for meiosis in many organisms (Cooper T. J. et al., 2016).

Programmed DSBs are induced by the evolutionarily conserved meiosis-specific topoisomerase-II-like Spo11 endonuclease, which provides substrate for recombination. These breaks is recognized by HR repair system (including phosphorylation of H2AX to form γH2AX by ATM) and cleaved. Recombination occurs by inter homologue interaction including pairing with contribution of proteins DMC1, RAD51, MSH4, MSH5 and HORMADs. There are two distinct main recombination pathways, cross over (CO) and Non-crossover (NCO). These pathways produce newly organized chromosomes. For example, CO pathway produces CO products at metaphase1 as chiasmata while NCO which is also known as synthesis dependent strand annealing (SDSA), gene conversion. During these processes, missegregated chromosomes can cause DNA damage (may lead unbalanced translocations) in the daughter cells. (Mehta and Haber, 2014).

It has been previously showed that, testis expressed gene 15 (TEX15) involved in DSB repair pathway by helping DNA repair proteins to bind location of DSBs (mutL

homolog 1 (MLH1), mutL homolog 3 (MUTL3) and MutLy). In other words, meiotic recombination failures in case of functionless TEX15 due to impaired localization of DNA repair proteins. In addition to this testis expressed gene 11 (TEX11) is essential gene for completing crossover, even synapsis established. Absence of MLH1 and MLH3 proteins leads to abnormality in mismatch repair and cause infertility in both male and females. Therefore, TEX11, TEX15, MLH1 and MLH3 genes are important during infertility investigations (Zhang et al., 2015).

3.1.2 DSB at fertilization

Fertilization starts by the sperm binding on egg cell surface and fusing with egg plasma membrane. This event results with a new diploid cell that contains genetic codes from both parents. Fertilization is not just mixing the parental genetic materials also induces various mechanisms that are essential for further development. Furthermore, this process activates the egg and triggers completion of oocyte meiosis. In mammals, DNA from oocyte and sperm form two haploid nuclei in zygote (fertilized egg) and enter S phase than replicate itself while migrating towards each other. When they meet, zygote enters M phase and nuclear envelopes breakdown. After that, condensed paternal and maternal chromosomes align on a same spindle and two new identical embryonic cells are formed by completion of mitosis (Cooper G. M., 2000).

In mammals, it has been suggested that male gametes are more likely to carry higher rate point mutations, STR (Singe Tandem Repeats) and structural abnormalities when compared to oocytes thus abnormal structured genetic contribution to newly formed zygote after fertilization is not similar between sperm and oocytes (Derijck et al., 2008). There are some zygote specific properties related with DNA DSBs repair. For instance: (1) there is no transcription dependent translation so, it is based on mRNAs and proteins present in the oocyte. (2) Remodeling of sperm originated chromatin by topoisomerase 2 leads short term DSBs. (3) there is no known G1-S checkpoint which means cell cycle starts after gamete fusion. (4) There is epigenetic differences in physically separated male and female chromatin. Lastly, in the *de novo* chromosome aberrations, zygote

shows a tendentious behavior on chromosome type when compared to chromatid type. After fertilization, chromatins from both parents hold one of the pronuclei, after that DNA repair occurs like a maternal origin because of proteins and mRNAs present in oocyte. DNA DSBs repair is performed by either NHEJ or HR system (Derijck et al., 2008).

3.1.3 DSB in embryogenesis

Zygote formation is the first and essential step for development because its role in coordination of cell division and gene activation to promote embryo for cell differentiation and future embryonic development. According to previous studies, embryonic transcription in humans starts before first cleavage and reach large scale before embryonic compaction. Cleavage cycles in mammalian embryos considered as slow when compared to other vertebrates (Jukam et al., 2017). The first cleavage takes 18 to 36 hours to finish, and fallowing divisions occurs every 12-24 hours up to blastocyst stage. After several days, mammalian embryo hatches and implantation event take place in to the uterus (Jukam et al., 2017). Because of faster cell division cycles when compared to somatic cells, DNA DSBs are particularly dangerous at embryogenesis, which leads abnormal chromosomal segregation into daughter cells. DSBs do not equally distribute during cellular division, thus broken chromosomes will not inherited evenly by daughter cells through mitosis, which leads mosaicism. However, there are several cell cycle checkpoints to block DNA replication in case of chromosomal abnormality, such as G1/S checkpoint for DNA replication, the intra S checkpoint for initiating DNA replication and G2/M checkpoint for starting mitosis. But in disorders like human syndrome ataxia telangiectasia (AT), DSB initiated cell cycle checkpoints cannot works. Therefore, AT cells are highly sensitive to external mutagenic factors like ionizing radiations (Gent, Hoeijmakers and Kanaar, 2001).

Previously , zygote and embryos were analyzed for presence of DSBs after fertilization it has been shown that DNA DSB repair starts during zygote gene activation (ZGA) and continue at early cleavage stage (Xiao et al., 2012).

CHAPTER 4

Material and methods

4.1 Study design

In this prospective study, sperm samples from men who attended to have a baby by using reproductive treatment in British Cyprus IVF Hospital used. Patients informed about the study by an informed consent form (APPENDIX A) and their samples were used after their approval. In addition, information about their medical history and risks factors that related with their lifestyle were collected with a survey (APPENDIX C) and patients who are using regular medicine or have previous chemical exposure excluded from study due to possible effect on sperm quality. Samples were selected in different diagnostic groups (Normospermia, oligospermia. Teratospermia, etc). Sperm samples from fertile men who have a child and attended to our clinic for family balancing have selected for control group.

4.2 Sperm preparation

Sperm samples were donated for this study by patients who attend to the private fertility clinic for assisted reproduction treatments. Fresh sperm samples were obtained by masturbation after 3 days abstinence except testicular sperms.

Sperm samples were evaluated by using Makler® Counting Chamber (Origio, ref: SEF-MAKL, Denmark) and basal values recorded. Morphological analyses were performed according to Kruger's Strict Criteria (Kruger et al., 1988). To separate sperm cell from ejaculate, two layer (%90, %50 of PureSperm®100, BioCare, Ref: PS100-250, UK) method was used (Figure 4.1). 1,5ml of ejaculate was added on top of the layer and centrifuged for 20 minutes at 1500 rpm (181g). Pellet was aspirated and re-suspended with sperm washing solution (Irvine sci, Cat. Id: 9983, USA) in order to remove gradient solution (Puresperm), and centrifuged for 5 minutes at 1200 rpm than supernatant discarded. Pellet was washed three times with PBS for 5 minutes at 1200rpm. After that,

sperm concentration was adjusted to ~5 million/ml and 50µl of sample was dropped onto polylysine-coated slides (Thermo Fisher Scientific, Cat No: 10143265, USA) and left to dry at room temperature. Thereafter, sperm cells were fixed with %4 paraformaldehyde for 30 minutes at room temperature. Lastly, slides were kept at +2, +8 C until staining procedure.

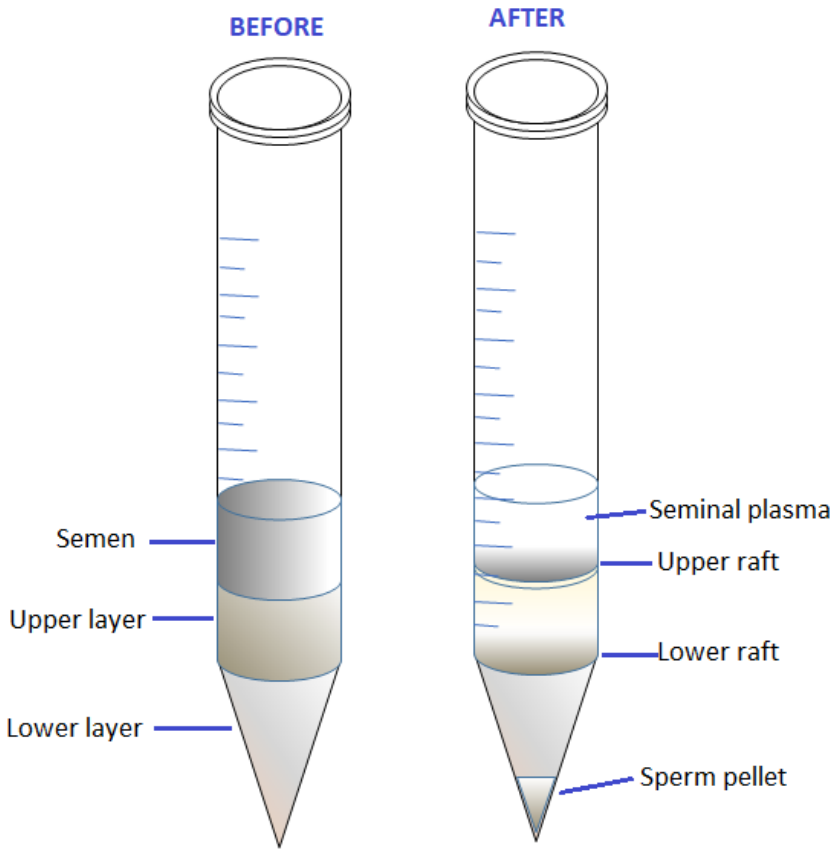

Figure 4.1 Illustration of three layers density Gradient method before and after centrifugation. Immotile and/or dead sperms, epithelial cells, debris, leucocytes and bacteria stay between upper and lower rafts. Lower raft contains immature and senescent sperm. Motile sperms accumulate in lower layer and selected motile population in sperm pellet.

4.3 Sperm Freezing and Thawing

Before freezing, sperm was washed as described above and 0.8 ml washed sperm samples was added into the cryovial (Cat no: V7759, Sigma Aldrich. USA). Freezing medium (Quinn's Advantage™ Sperm Freeze, Ref: ART-8022, Denmark) was added to the cryovial dropwise and mixed gently after ever drop in order mix sperm cells and freezing solution. After 1:1 ratio freezing media added, mixture was left equilibrium for 3 minutes. Vials transferred to liquid nitrogen vapor for 30 minutes and transferred to the final storage address in liquid nitrogen.

To thaw frozen sperms, vials transferred to the 30-32 C water bath. Thawed samples diluted with sperm washing medium (Quinn's™ Sperm Washing Medium, Ref: ART-1006, Denmark) and centrifuged at 1200 rpm for 5 minutes. This step was repeated twice due to remove freezing media from the sample.

4.4 Immunofluorescent staining

Slides were washed two times with PBS for five minutes than blocked with blocking solution (1% BSA, 0.5% triton X) for 30 minutes at room temperature. During blocking period, antibodies were diluted with blocking solution. Fallowed by washing slides with PBS three times for five minutes, incubation with primer antibody (Anti-phospho-Histone H2AX (pSer139) antibody produced in rabbit, Sigma, Cat.No: H5912, USA, 1:100) was performed overnight at 4C. Slides were washed with PBS with 0,05% Tween 20 and incubated with secondary antibody (Alexa Flour® 488 goat ant,-rabbit IgG (H+L), LifeTechnologies, USA, Cat.No: A11008, 1:400) for 2 hours at room temperature in dark. Lastly, ProLong® Gold Antifade Reagent with DAPI (ThermoFisher, USA ,Ref:936935)) was applied as counterstaining and mounting media and covered with coverslip and kept in refrigerator (+2, +8 C) until analyzing with fluorescence microscope (adapted from Mariotti et al., 2013).

4.5 Data analyzes

Statistical analyzes were done by using SPSS 15.0 for windows. In descriptive statistics; number and percentage for categorical variables, mean, standard deviation, minimum and maximum for numerical variables were used. Relationships between numerical variables analyzed by Pearson Correlation and Spearman Correlation tests depending on parametric test requirements. Comparisons of numerical variables between independent groups were performed by Mann Whitney U test due to lack of parametric test requirements. Deterministic factors were analyzed by Linear Regression test. $P<0,05$ was considered as statistical alfa significance level.

CHAPTER 5

Results

5.1 General characteristics of the study groups

In this study, 8540 sperm cells in 60 samples obtained from participants were analyzed. All sperm samples were evaluated for standard semen parameters and morphology based on Kruger's Strict Criteria (Kruger et al., 1988). Sperm sources were young healthy sperm donors (8 samples (13,3 %)) and male partners (52 samples (86,7%)). Mean male age was 43,4±6,3 ranging from 31 to 60 years. 9 of the samples were frozen and analyzed after thawing procedure. Diagnostic distribution of the samples were normozoospermia (24 (40.0%)), oligozoospermia (4 (6.7%)), Asthenoteratozoospermia (1 (1.7%)), Teratozoospermia (21 (35.0%)) and Oligoasthenoteratozoospermia (10 (16.7%)). Average values of volume, total motility, mot+4, mot+3, mot+2, percent of Kruger morphologic score and percent of yH2AX were 2,8(±1,1), 51,4(±42,8), 54,2(±22,3), 8,2(±8,3), 27,3(±14,5), 18,6(±8,1), 3,0(±2,0) and 19,3(±8,2) respectively. Additionally, 21 sperm samples were obtained from fertile men and used as a control group (details are listed in Table 5.1).

Table 5.1 Descriptive statistics in study groups

Sperm Source	Donor	8 (13,3)
	Partner	52 (86,7)
Male Age		43,4±6,3 (31-60)
Infertility	Infertile	39 (65,0)
	Fertile	21 (35,0)
Fresh/Frozen	Frozen	9 (15,0)
	Fresh	51 (85,0)
Diagnosis	Normozoospermia	24 (40,0)
	Oligozoospermia	4 ()6,7
	Asthenoteratozoospermia	1 (1,7)
	Teratozoospermia	21 (35,0)
	Oligoasthenoteratozoospermia	10 (16,7)
Volume (ml)		2,8±1,1 (0,7-5)
Sperm Count (mil/ml)		51,4±42,8 (0,3-250)
Total Motility		54,2±22,3 (5-90)
Mot+4		8,2±8,3 (0-35)
Mot+3		27,3±14,5 (0-65)
Mot+2		18,6±8,1 (2-40)
Kruger %		3,0±2,0 (0-7)
γH2AX (%)		19,3±8,2 (0-53,3)

5.2 Detection of γH2AX loci in human sperm

Due to highly condensed form of sperm DNA, evaluating γH2AX loci by immunoblotting is hard. There were many artefacts and γH2AX expression out of nucleus (in acrosome and neck of the sperm possibly because of defected mitochondrial DNA) (Image 5.1). Therefore, we have only included clear γH2AX loci in calculation by filtering specific color in order to eliminate false positive results (Image 5.2 and Image 5.3).

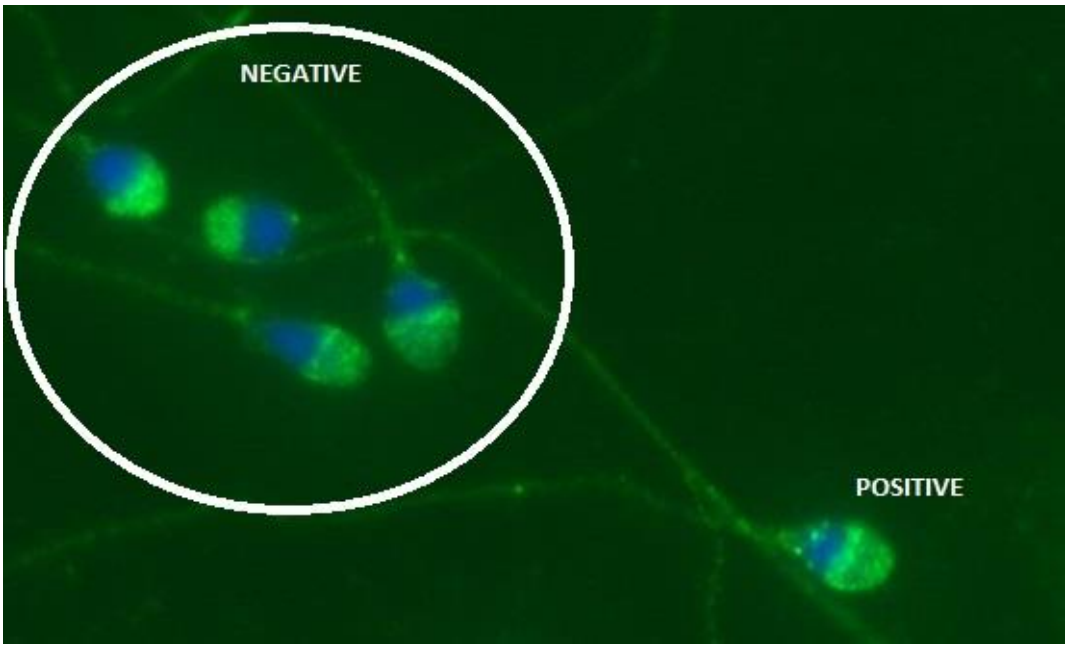

Figure 5.1 Examples of acrosomal artefacts and γH2AX positive sperm. γH2AX loci can be seen in nucleus (Right bottom of the frame. γH2AX (green) , DAPI (blue)). Cells in white circle are examples of γH2AX negative sperms.

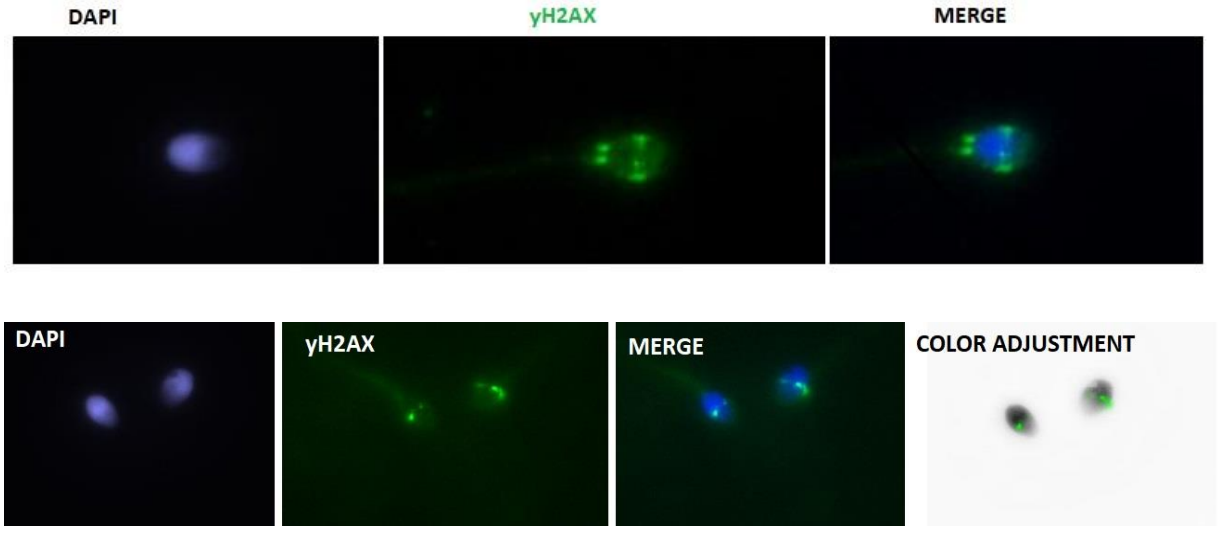

Figure 5.2 Immunofluorescence staining of γH2AX loci in human sperm. Picture shows an example of DNA DSB positive sperm. DAPI (Blue), γH2AX (green).

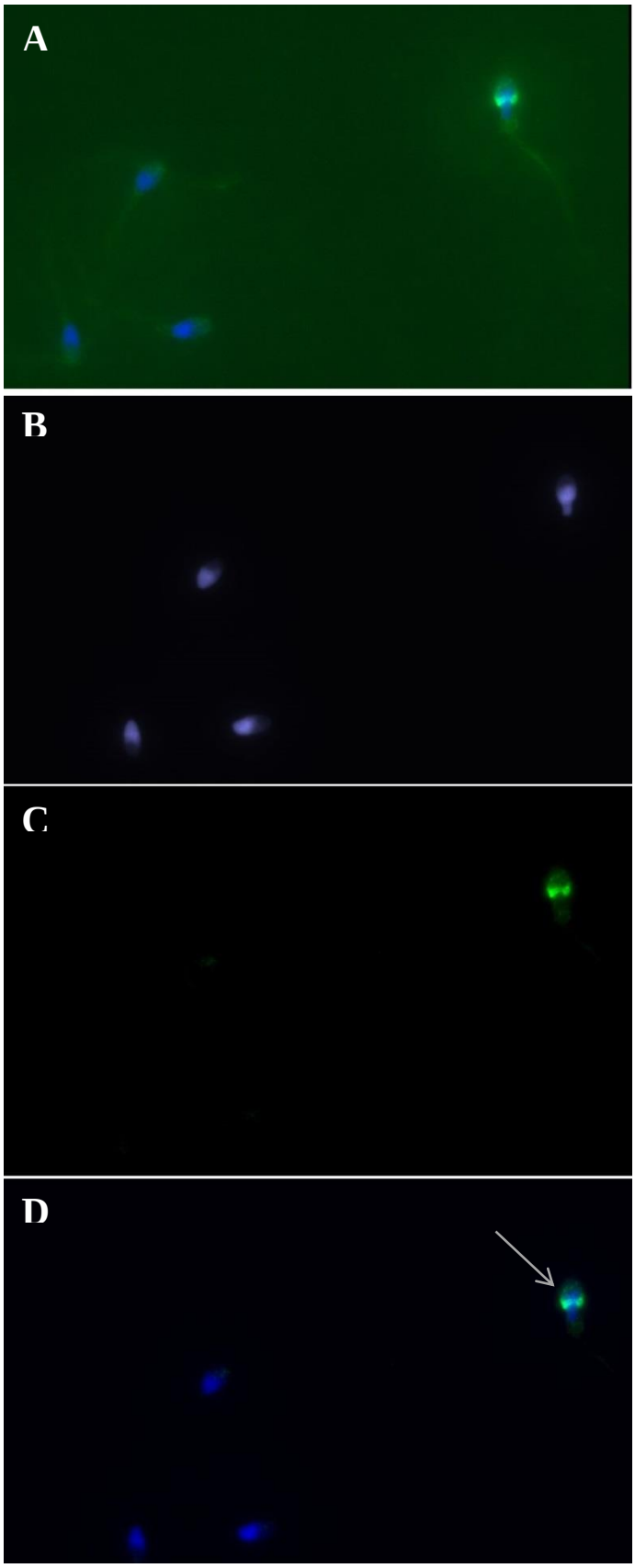

Image 5.3 Appearance of γH2AX loci after immunoblotting. A) Image before color adjustment. B) DAPI (Blue) C) γH2AX (green) D) Merged image. γH2AX positive sperm can be seen in right top of the frame

According to results, no significant correlation between level of γH2AX expression and male age (p=0.786), semen volume (p=0.258), sperm count (mil/ml) (p=0.519), total motility (p=0.932) and progression (+4, +3, +2) were found. Moreover, our data showed that, there were no correlation between sperm morphological score (Kruger %) and DNA DSB frequency (P=0.844) (Table 5.2).

Tablo 5.2. Correlation between Level of γH2AX expression and semen parameters

	γH2AX (%)	
	r	p
Male Age	-0,039	0,786
Volume (ml)	0,157	0,258
Sperm Count mil/ml	-0,085	0,519
Total Motility	0,011	0,932
Mot+4	0,019	0,883
Mot+3	-0,094	0,477
Mot+2	-0,082	0,532
Kruger %	-0,026	0,844

5.3 Donor or Partner sperm and DSB relation

As a sperm source, there were frozen donor samples from donation program and fresh and frozen samples from autologous cycles (Partner). Data is analyzed depending on these characteristics and results showed that percentage of γH2AX positive sperm in partner group was higher than sperm donor group ($p<0,014$) although all donor samples were frozen and no correlation found between frozen and fresh samples ($p=0.072$) (Table 5.3 and Figure 5.4).

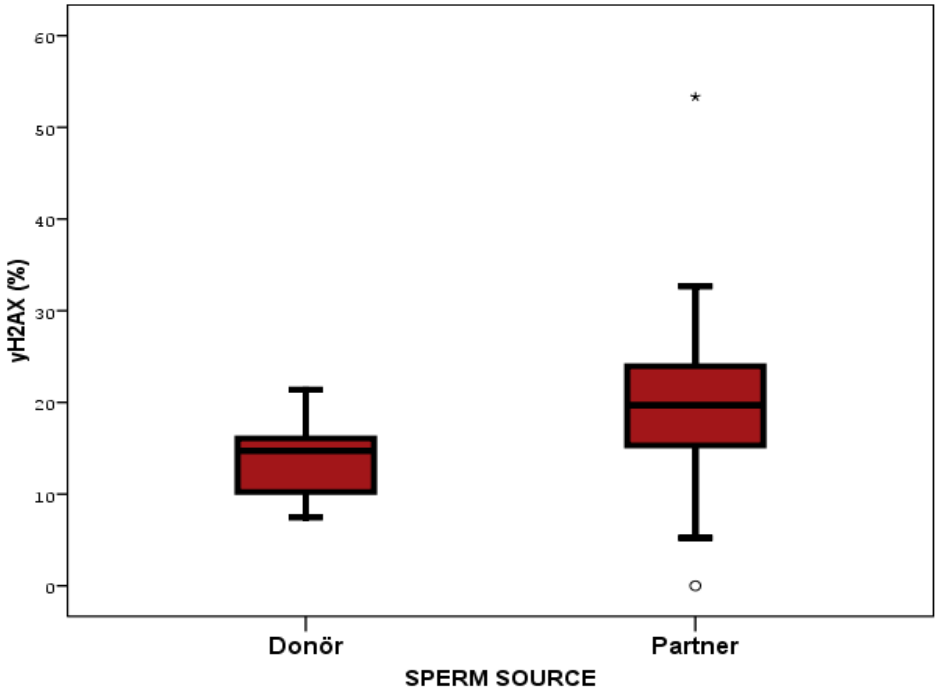

Figure 5.4 Sperm source and DSB relation. (**Donor**: Sperm donors from sperm bank, **Partner**= spouse). Sperm donor samples have significantly lower sperm DNA DSB value when compared with partner's.

5.4 DNA DSB and Infertility

Our samples were including fertile and infertile males and results showed that there were no statistically significant correlation between fertile and infertile samples (P=0.212) (Table 5.3).

5.5 DNA DSB and male diagnosis

Groups of Male diagnosis depending on seminal characteristics were created according to description in material and methods part. In these groups, only Asthenoteratozoospermia was excluded from statistical analyze due to small sample size. Expression of γH2AX (DNA DSB) in Normozoospermia, Oligozoospermia, Teratozoospermia, Oligoasthenoteratozoospermia groups were found equal (P=0.869) (Table 5.3). In other words, no correlation found between γH2AX expression and male diagnosis. Because male diagnosis was based on semen volume, sperm count, motility and progression as well as sperm morphology, similar DNA DSB frequency among groups was also important to represents overall score on the diagnostic parameters.

Table 5.3 Relation between DNA DSB frequency and sources of sperm, Infertility and male diagnosis

		yH2AX (%)	
		Ort.±SD (Median)	p
Fresh/Frozen	Frozen	15,7±7,1 (15,1)	0,072
	Fresh	20,0±8,3 (19,5)	
Sperm Source	Donor	13,9±4,6 (14,7)	0,014
	Partner	20,2±8,4 (19,7)	
Infertility	Infertile	20,4±9,2 (19,8)	0,212
	Fertile	17,3±5,7 (16,7)	
Diagnosis	Normozoospermia	18,9±7,2 (18,2)	0,869
	Oligozoospermia	21,0±6,8 (18,8)	
	Asthenoteratozoospermia*	19,0	
	Teratozoospermia	18,0±8,0 (18)	
	Oligoasthenoteratozoospermia	22,5±11,7 (18,9)	

*Not included in analyze

5.6 Factors that determine level of yH2AX percent

We have built a model in order to study sperm parameters and their possible role on level of yH2AX expression. As detailed in table 5.4, none of the sperm parameters such as volume, count, motility, progression (Mot+4, Mot+3, Mot+2) and morphology are able to determine the yH2AX level. In addition, fresh or frozen status of the sperm was not a deterministic factor for DNA DSB frequency (0,890).

Table 5.4. Linear Regression Analyze for deterministic factors for yH2AX(%)

		B	Beta	p
Enter Method	Fixed	20,294		
	Fresh/Frozen	0,797	0,022	0,890
	Volume (ml)	1,563	0,209	0,186
	Sperm Count mil/ml	-0,019	-0,103	0,538
	Total Motility	-0,404	-1,128	0,370
	Mot+4	0,366	0,377	0,459
	Mot+3	0,388	0,707	0,363
	Mot+2	0,229	0,227	0,640
	Kruger %	-0,185	-0,046	0,811

CHAPTER 6

Discussions and Conclusion

Phosphorylation of histone H2AX is one of the most important event that triggers recognition and repair pathways after breaks in chromosomes (Jiang, Xu, and Price, 2010) and specific to DSBs formation (Takahashi and Ohnishi, 2005). It is used as a marker for determination and localization of DNA DSBs. Because of this specific characteristic, it has been accepted as a good candidate for research in different areas such as drug target to control cell cycle in cancer and investigation of DNA repair mechanism. In addition, there are studies about structural DNA abnormalities in gametes and its relation with infertility. Although, morphological appearance of gametes and their relation with infertility are well studied, their association with chromosomal status of gametes not fully understood.

There are different methods to detect DNA damage and each of them has particular characteristics. Some of them are fast and easy to apply but not specific to certain types of damage. For example COMET assay is able to distinct type of DNA breaks (DSB or SSB) depending on method used in denaturation step while others not. In addition to this, some methods have problems during application. As an example: AO is absorbed by glass and cause difficulties during microscopic evaluation and rapid fading of AO fluorescence leads need for fast measurement of the samples and causes rushes (Evenson, 2016). Another difference between tests is their effectiveness in specific subject. In a comprehensive study, Ribas-Maynou et al., (2013) showed that natural comet assay is not useful to compare fertile and infertile men when compared with TUNNEL, SCSA and SCD tests. As mentioned previously, immunofluorescence labeling of the sperm cells is effective due to its' specify to DNA DSB and ability to show break site as well.

In this study, we have focused on rate of DNA DSB in sperm cells and differences in distribution depending on seminal parameter and infertility. In this respect, the results

are compared in group of semen parameters, male age, diagnosis, infertility and fresh/frozen status.

We have used immunofluorescence staining method during the experiment to get information about localization of DNA breaks as well as its presence. Interestingly, some particular patterns such as high yH2AX expression in neck of the sperm are observed. This might be caused from DNA damage in mitochondrial DNA that highly localized in the neck of the sperm cells. Additionally, number of loci which is associated with number of DSB was taken in to consideration during decision about DSB positive cell to have clearer comparison, we have include sperm with clear signals and more than one yH2AX loci in order to avoid false positive results. This can be considered as threshold value like in most experiments. For example, Zhong et al., (2015) calculated 18.55% threshold value for yH2AX positive sperm to define infertility by comparing infertile and control groups. In same study, they give percentage of yH2AX as 14.39% for control and 23.48% for infertile groups. Similarly, we have found that percentage of yH2AX in our study groups were 20.4 %(±9,2) in infertile, 17.3%(±5,7) fertile and 19.3% (±8,2) overall.

Although, there is no specific report about DSBs in sperms and its association with paternal ages, accumulation of DNA damage in somatic cells depending on age is known (Velegzhaninov et al., 2015). However, in our study no statistical correlation was found between rate of DNA DSB in sperms and paternal ages (p=0,786). Similarly, Gonzalez-Marin et al., (2012) reports that rate of DNA fragmentation in sperm is not affected by male age. This might be explained by unique properties of the germ cells. In the literature, there are many reports about semen parameters and their relation with infertility, DNA fragmentation and aneuploidy. Nevertheless, it is also acceptable that sperm DNA fragmentation is an independent variable that gives additional information about the quality of the sperm and pregnancy outcome (Evenson, 2016). It is well known that DNA damage parameters are reversibly proportional to sperm low values. On the other hand, there is no evidence that structural DNA DSB is always related with seminal characteristics (Winkle et al., 2009). Particularly, there is no exact relation

between structural genetic condition of the sperm cells and their morphological appearance (Sun, Ko and Martin, 2006, In accordance with previous studies we have not found significant correlation between γH2AX (%) and semen parameters such as volume (p=0,258), count (p=0,519), motility (p=0,932) and progression. In addition, no correlation found with diagnosis (Normozoospermia, Oligozoospermia, Teratozoospermia, Oligoasthenoteratozoospermia).

In a recent study, Lu et al., (2018) analyzed factors that related with human Sperm DNA fragmentation index (DFI) by using sperm chromatin structure assay (SCSA) and reported that sperm DFI is positively related with age, abstinence period, semen volume and morphology. However, as explained before SCSA test is not specific to DNA DSB and has lower sensitivity when compared with γH2AX (Garolla et al., 2015). Therefore, our findings, which suggest no correlation between sperm DNA DSB and seminal characteristics, can be precious due to its specify to DNA DSB. In addition to this, our results allow us to conclude that evaluating sperm morphological appearance cannot be use as a possible predictive marker for DNA DSB level in sperm.

Infertility is a complex health problem that may be caused by various external and internal factors. Therefore, it is not always easy to make precious conclusion about a single factor and its' relation with infertility. For example: It is known that there are repair mechanisms which are active during and after fertilization (Derijck et al., 2008). Thus, quality of oocyte is also important during evaluating effect of sperm DNA DSB on infertility (Brandriff and Pedersen, 1981). Generally, studies in literature assume that structural DNA abnormalities in sperm may be related with male infertility (Evenson, 2016). In our study we have not found statistical correlation between sperm DNA DSB and infertility although majority of the studies in literature suggest otherwise. But unlike other studies that calculate a threshold value to predict relationship between sperm DNA damage and infertility, we have included all cases that show more than two gamma-H2AX loci. In other words, minimum threshold was set to two DNA damage loci in sperm cells as a predictive marker for reproductive outcome instead of all cases. .

Although, level of altered DNA in human Sperm and its relation with rate of miscarriages and infertility have been shown previously (reviewed by Robinson et al., 2012), we have found that DNA DSB incidences among fertile and infertile couples not statistically differ although infertile group has slightly higher rate. This might be explained by specific data about DNA DSB, higher sensitivity of γH2AX test and accepted lover threshold because of similar mean sperm DNA DSB rate, which is 20.4% for infertile groups like in previous studies (Robinson et al., 2012).

In light of our findings, we conclude that DNA double strand breaks in human sperm is not related with infertility and/or semen characteristics itself and must be evaluated together with specific investigation such as structural DNA abnormalities and morphology connection or repair capacity of oocyte and embryos in order to make precise inference.

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Appendix A. Informed Consent Form (For The Patients / Participants)

BİLİMSEL ARAŞTIRMA İZİN FORMU

Araştırmacı(lar)
Onder Coban
Çalışmanın başlığı
IVF Hastalarında sperm DNA kırıklarının sıklığının araştırılması

Lütfen bu formu dikkatlice okuyup doldurunuz. Eğer bu çalışmaya katılmak isterseniz, size uygun olan seçeneği işaretleyip tarih bilgisi ile imzalayınız. Eğer anlamadığınız birşey olursa ve bilgi almak isterseniz lütfen sorun.

- Çalışma bana araştırmacı tarafından yeterli şekilde sözlü ve yazılı olarak anlatıldı. **EVET / HAYIR**
- Çalışmanın şunları içerdiğini anladım: Sperm örneğinin değerlendirilmesi ve katılımcının medical durumu **EVET / HAYIR**
- Bu çalışmadan istediğim zaman hiç bir mazeret göstermeksizin ayrılabileceğimi, bunun daha sonraki tedavimi hiçbir şekilde etkilemeyeceğini anladım. **EVET / HAYIR**
- Benim hakıımdaki bilgilerin gizlilik içinde korunacağını ve bu çalışmayla ortaya çıkacak hiçbir yazılı dökümanda yayınlanmayacağını anladım. **EVET / HAYIR**
- Bu çalışma sürecinde gelişmelerin British Kıbrıs Tüp bebek hastanesi ve Yakındoğu üniversitesi genetic departmanındaki diğer araştırmacılarla tartışılbileceğini anladım **EVET / HAYIR**

Özgür irademle bu çalışmaya dahil olmayı kabul ediyorum. Bu formun bir kopyası tarafıma verilmiştir.

Imza: ……

Tarih:……
…

Appendix B: Ethic committee approval

British Cyprus IVF ethics committee approves this study with reference number 01042015-06.

Appendix C: Questionnaire for research participants

BRITISH CYPRUS IVF

Bilimsel araştırma Anket Formu

	Erkek	Kadın
Ad Soyad:		
Doğum Tarihi:		
Kimlik veya Pasaport Numarası		
Telephone:		
Address:		

Lütfen aşağıdaki soruları cevaplayınız.

1- Cinsel perhiz süreniz kaç gün?
2- İşiniz dolayısı ile çok sık seyahat ediyormusunuz? Ne sıklıkla?
3- İş seyahatlerinizde herhangi bir kimyasal madde ile çalışıyormusunuz?
4- Sürekli kullandığınız bir ilaç var mı?
    a. Varsa ne zaman başladınız ve ne amaçla kullanıyorsunuz?
5- Geçen 3 ay içerisinde ciddi bir rahatsızlık veya yüksek ateş geçirdiniz mi?
6- Aşağıdaki organlardan herhangi birinde enfeksiyon geçirdiniz mi?
    a. Böbrek
    b. Testis
    c. Mesane
7- İdrar yaparken ağrı yada yanma hissi yaşıyormusunuz?
8- Boşalma sırasında yanma ağrı yada yanma hisssi oluyormu?
9- Testis bölgesinde ağrı hissi yaşıyormusunuz?
10- Alkol kullanıyor musunuz? Ne sıklıkla?
11- Son 24 saat içerisinde alkol kullandınız mı? ne kadar?
12- Sigara kullanıyormusunuz? Günde kaç adet?
13- Daha once spermiyogram testi yaptırdınız mı? Varsa bir kopyasını verebilirmisiniz?
14- Daha once yapılan spermiyogram testine bağlı olarak herhangi bir ilaç kullandınız mı?
15- Daha once spermiyogram testine bağlı olarak cerrahi bir operasyon geçirdiniz mi?

Teşekkürler

İmza

Tarih:

Published Article during preparation of the thesis

Journal
Systems Biology in Reproductive Medicine ›
Latest Articles

Research Communication

Evaluation of the impact of sperm morphology on embryo aneuploidy rates in a donor oocyte program

Onder Coban, Munevver Serdarogullari, Zehra Onar Sekerci, Ekrem Murat Bilgin & **Nedime Serakinci**

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ABSTRACT

This study investigates the correlation between sperm morphology and the incidence of embryo aneuploidy in an oocyte donation program. A total of 1,165 embryos from 103 patients have been analyzed by fluorescent *in situ* hybridization (FISH) for numerical abnormalities in chromosome numbers 13, 18, 21, X, and Y. Data has been evaluated in five groups according to sperm morphology, which has been assessed according to the Kruger's strict criteria. The results did not show any difference in paternal ($p = 0.878$), maternal ($p = 0.873$), and donor ages ($p = 0.871$), sperm counts ($p = 0.782$) and motility ($p = 0.124$), and fertilization rate ($p = 0.080$) among the groups. However, total aneuploidy rate ($p < 0.001$) and its derivatives (trisomy $p = 0{,}042$, monosomy $p = 0{,}004$) differed significantly and they were reversibly correlated with sperm morphology (rho correlation test; total aneuploidy $p < 0.001$, trisomy $p < 0.001$, monosomy $p = 0.004$). Therefore, these results suggested that diminished sperm quality is correlated to the aneuploidy rate in preimplantation embryos.

Abbreviations: FISH: fluorescence *in situ* hybridization; ICSI: intracytoplasmic sperm injection; HCG: human chorionic gonadotropin

KEYWORDS: Donor egg, FISH, gamete aneuploidy, preimplantation genetic screening (PGS), sperm morphology

Planned articles from this thesis

1- **Title:** Investigation of DNA DSB incidence in human sperm and its association with infertility: γH2AX as a biomarker