SPERM ANEUPLOIDY AND DNA INTEGRITY: A REVIEW

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ABSTRACT

Male factors leading to infertility account for at least half of all cases of infertility worldwide. The purpose of this review is to highlight the importance of sperm DNA integrity. A systematic literature search was performed up to January 2015 in order to determine the impact of sperm DNA integrity and of the techniques used to determine it. Only articles presenting sperm aneuploidy together with DNA fragmentation studies are discussed. We also discuss several causes and risk factors that have been identified as having detrimental effects on sperm genetic integrity. Aneuploidy and sperm DNA fragmentation (sDNAfrag) analyses show promising results in determining the sperm genetic status. However, more studies must be performed to develop a technique that can simultaneously verify the sperm DNA integrity and haploidy before introduction into routine clinical practice. Once sperm is subjected to the current technologies it cannot be immediately used in assisted reproduction treatments. However, recent studies have shown that an improved protocol of sperm selection can result in sperm with very low levels of sDNAfrag, rendering the risk of selection low.

Keywords: Sperm aneuploidy, sperm DNA integrity.

INTRODUCTION

The infertility onus has been increasing over recent decades and it is estimated to affect 15% of couples worldwide.[1] There is conspicuous evidence that male partners account for the aetiology of half the cases. The evaluation of male infertility is based on routine semen analysis, which measures both semen production and sperm quality. However, normal values of these parameters do not accurately mirror the fertilisation capability of the sperm. Moreover, there are numerous known causes of male infertility that this analysis provides no information about. One certain factor influencing male fertility is the integrity of sperm DNA.[2] Increasing concern regarding the transmission of genetic diseases through intracytoplasmic sperm injection (ICSI)[3] has spurred investigations into the genomic integrity of the male gamete. Possible sperm nuclear alterations include: A) abnormal chromatin structure; B) chromosomes with microdeletions; C) aneuploidies; and D) DNA strand breaks.[4]

Sperm DNA integrity is not evaluated routinely in an assisted reproduction technology (ART) laboratory, although it has been recognised as an important clinical parameter in infertile patients.[5] A variety of assays have been developed to measure sperm DNA fragmentation (sDNAfrag). While some approaches detect breaks in DNA strands, others assess the vulnerability of the DNA to denaturation.[6] Previous reports have indicated that natural pregnancy is impaired when increased levels of sDNAfrag are detected.[7,8] The threshold value is dependent on the technique used to determine sDNAfrag.[9,10] Nevertheless, it seems to have a prognostic value with regard to ART outcomes.[11,12] On the other hand, sperm aneuploidy has been evaluated by fluoresence *in situ* hybridisation (FISH) and is considered a major cause of pregnancy loss, aneuploid births, and developmental defects.[13] Recent reports

demonstrate a significant increase of the sperm aneuploidy rate in infertile men when compared with fertile counterparts, although this did not exceed 2% with regard to chromosomes X, Y, 18, and 21.[14,15] Due to the importance of these two parameters, sDNAfrag and aneuploidy, this review will focus on those studies that have evaluated both parameters within the same biological samples.

Controversial data have been obtained but, overall, studies provide clear evidence for a significant increase in both the rate of chromosome aneuploidy and the percentage of sDNAfrag in infertile patients when compared with fertile donors, as well as a positive correlation between the two parameters. Increases in both parameters have been reported in infertile patients with abnormal semen analysis results. Patients with low sperm counts (oligozoospermic) display a higher frequency of sperm aneuploidies and higher percentage of sDNAfrag,[16] although these are even higher in patients presenting with severe testicular damage[17] and in oligoasthenozoospermic men.[18]

Concerning morphology, teratozoospermic patients show an increase in sperm chromosome aneuploidy and sDNAfrag.[19-21] Rare sperm morphological alterations that affect <1% of infertile male patients have also been studied. Patients with the severe aberration of macrocephalic—multi-flagellated sperm syndrome exhibit high rates of aneuploidy.[22-25] Another rare condition termed globozoospermia, which is characterised by a lack of the acrosomal vesicle and associated structures, has returned conflicting results: some researchers did not find a correlation between globozoospermia and aneuploidy although they observed elevated percentages of sDNAfrag,[26,27] whereas others have demonstrated that globozoospermia is associated with increased sDNAfrag levels and increased frequencies of sex chromosome aneuploidy.[22,28-31] Increases in sperm aneuploidy and sDNAfrag were also reported for sperm with large-head vacuoles,[32] although others observed no clear link between these sperm features and this particular sperm abnormality.[33] The same observations were found in patients with severe-to-total asthenozoospermia presenting with dysplasia of the fibrous sheath and head abnormalities; these sperm showed high levels of aneuploidy and sDNAfrag.[34] It therefore seems that sperm aneuploidy and sDNAfrag rates are increased in infertile men with multiple morphological anomalies, regardless of the type of teratozoospermia.[19,21-23,25] In addition, increased sperm aneuploidy rates were observed in sperm with higher sDNAfrag in patients with each of the three major types of altered semen analysis values (count, morphology, and motility).[35]

Males of couples presenting with recurrent spontaneous abortion have also been evaluated for sperm aneuploidy and sDNAfrag, with studies showing increases in both parameters and sDNAfrag being significantly correlated with the percentage of sperm aneuploidy.[36] However, even though significant differences have been shown, there is no consensus regarding the correlation between these parameters and miscarriage[37] or their correlation with embryo chromosomal anomalies in these couples.[38] Unfortunately, patients showing significantly increased sperm aneuploidy rates were excluded from the embryo aneuploidy study.[38] Transmission of a damaged sperm cell to the oocyte has its risks and thus more studies in patients presenting with recurrent miscarriage or implantation failure should be performed and enlarged.

ICSI has become a powerful technique in overcoming male infertility. However, selection of the spermatozoon is necessary prior to the technique being attempted. Driven by the objective of improving embryological and clinical outcomes, non-invasive methods were developed to select for clinical use those sperm that are free of DNA damage. As the selection of sperm continues to be performed based on morphology and motility, sperm assortment under high magnification can improve this selection[39] and has also been shown to decrease sperm aneuploidy and sDNAfrag levels,[17] with this decrease being accentuated with hyaluronic acid treatment.[40]

In addition to the classic sperm preparation techniques, approaches such as magnetic-activated cell sorting (MACS) have also been applied.[41] Using semen from normozoospermic men, it has been demonstrated that density gradient centrifugation (DGC) and MACS can effectively decrease sperm aneuploidy rates and sDNAfrag levels. This study also showed that the decrease of both parameters was correlated, and that the decrease after MACS was more apparent.[42] Using MACS followed by DGC and swim-up can substantially reduce sDNAfrag levels, which was also true in cases with abnormal morphology, motility, vitality, and membrane integrity.[41] These results reinforce the need for more studies to be conducted in patients with altered semen parameters.

INFLUENCE OF CHROMOSOMAL ABNORMALITIES

Spermatogenesis is a complex biological process that can be influenced by chromosomal abnormalities. Although some of these anomalies impair spermatogenesis apparently due to germ cell degeneration,[43] others may license the achievement of spermatogenesis with sperm production. However, the presence of structural or numerical chromosomal abnormalities can interfere with normal spermatogenesis and cause male infertility due to abnormal conventional sperm parameters. Moreover, these patients may be at a higher risk of transmitting a chromosomal anomaly to their progeny. Due to this augmented jeopardy, and since conventional semen parameters do not provide information about the nuclear status of sperm, several studies in carriers of chromosomal abnormalities were performed in order to assess the male gamete risk of aneuploidy and DNA fragmentation. We have summarised the different chromosomal anomalies and their consequences on sperm aneuploidy and DNA fragmentation in Table 1.

Table 1: Effects of different chromosomal anomalies on sperm aneuploidy and DNA fragmentation.

Chromosomal anomaly	Carrier	Sample type	Effects on sperm aneuploidy and DNA fragmentation	Reference
47,XYY	Extreme OAT	Testicular biopsy	Two-thirds of the cells with an XYY constitution Aneuploidy rate (FISH): • 3.93% in round and elongated spermatids • 0.91% in late spermatids and spermatozoa High rate of germ cell degeneration High rates of DNA fragmentation (TUNEL) in spermatids and spermatozoa	44
9qh+++ polymorphism	Severe OAT	Ejaculate	Increased rates of disomy for Chr X, Chr Y, and Chr 18 (FISH) Increased DNA fragmentation (77.81%) (TUNEL)	45
t(7;8)(p12;p22) t(13;15)(q31;q26.2) t(6;8)(q27;q24.1) rob(13;14)(q10;q10)	Three reciprocal and one Robertsonian translocation	Ejaculate	Increased aneuploidy (FISH) Increased DNA fragmentation (TUNEL) 2-5-times higher proportion of spermatozoa with unbalanced Chr content and fragmented DNA than among those with normal balanced content	46
46,XY,t(3;6)(p24;p21.2),inv(8)(p11;2q21.2)	Normal spermiogram	Ejaculate	No difference regarding Chr aneuploidy rates (FISH) Increased DNA fragmentation (TUNEL)	47
46,XY	45 infertile men	Ejaculate	Chromosomally abnormal sperm cells more likely to display DNA fragmentation (SCDt) Lower sperm count and motility increased the percentage of chromosomally abnormal sperm (FISH)	48
46,XY,t(6;10;11)(q25.1;q24.3;q23.1)	Asthenozoospermic patient	Ejaculate	Five-fold increased level of aneuploidy of Chr 13, 15, 18, 21, 22, X, and Y (5.3-fold for disomy and 1.7-fold for diploidy) (FISH) No difference regarding DNA fragmentation (TUNEL)	49
Mosaicism 45,X and 47,XYY Mosaicism 45,X; 46,XY and 47,XYY	Normal spermiogram Teratozoospermia	Ejaculate	Significant increase in frequency of XY disomic and diploid spermatozoa (FISH) Significant increase in diploidy and autosomal aneuploidy (FISH)	50

Chr: chromosome; OAT: oligoasthenozoospermia; FISH: fluorescence *in situ* hybridisation; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labelling assay; SCDt: sperm chromatin dispersion test.

Previous studies evaluated sDNAfrag and sperm aneuploidy in spermatozoa of infertile patients with both numerical and structural chromosomal abnormalities. The majority of the studies reported an increased incidence of aneuploidy in sperm with fragmented DNA compared with those with intact DNA. In this regard, although infertile males with a chromosomal abnormality showed a significant association between sperm chromosome abnormalities and sDNAfrag, they also presented with a wide spectrum of detrimental effects on the male fertility status. The conflicting results obtained by different authors (Table 1) may be due to the fact that different and variable numbers of chromosomes and different sDNAfrag detection techniques were investigated in these studies. Moreover, the number of sperm cells evaluated may also explain the different results. Due to these conflicting results, there is a need to further investigate the relationship between meiotic segregation, DNA fragmentation, and conventional sperm parameters using a full panel of chromosomes and the same sDNAfrag detection technique. Moreover, there is still the need to confirm these results in a larger number of carrier patients. Because these patients have a low probability of being able to conceive naturally, they should be enrolled in genetic counselling programmes in order to reduce the risk of genetically abnormal offspring and be advised about prenatal diagnosis and preimplantation genetic diagnosis.

INFLUENCE OF ANEJACULATION

Male infertility may also be due to the inability to ejaculate semen; despite producing sperm, some men are not empowered to expel it. This sexual disorder is commonly designated as anejaculation and can be caused by psychological or physical factors, with the latter occurring due to neurogenic or obstructive reasons. With the main goal of retrieving sperm for artificial insemination, several treatment options for men with anejaculation are available.[51,52] Studies have shown that therapies for the treatment of anejaculation due to spinal cord injury, such as penile vibratory stimulation, produce a decreased sperm concentration,[53] an increase in sperm aneuploidy rates of approximately 1.5-2.4-times for chromosomes 13, 18, and 21, and about 2.2-2.4-times for chromosomes X and Y, as well as an increase in the rate of sDNAfrag.[53,54] Although the use of a minimally invasive percutaneous vasal sperm aspiration procedure in these patients has been shown to increase sperm motility, these patients still exhibited poorer semen quality and higher rates of sDNAfrag and sperm chromosomal aneuploidies when compared with healthy donors.[53,54] However, the effect on sDNAfrag may be overcome by the use of testicular sperm aspiration;[55] although the same is not true for aneuploidy.[52]

INFLUENCE OF AGE

Another factor shown to impact semen quality and sperm genetic integrity is the age of the male. Unlike women, male fertility varies from man to man and age is not a good predictor, as men may experience spermatogenesis for up to 95 years.[56] The introduction of ART and innovative medicines for erectile dysfunction allow paternity for elderly men. However, with increasing paternal age, the amount and motility of sperm cells decreases, testicular histological architecture deteriorates, and, as well as decreased fecundity, there may also be an increased risk of transmitting a heritable disease to progeny. A recent review reports evidence that male ageing after 40 years is associated with decreased sperm concentration, motility, and fecundity, and with an increase in sperm aneuploidy, sDNAfrag, sperm DNA mutations and epigenetic changes, altered pregnancy, and offspring prone to autosomal diseases and several neurocognitive disorders.[57] To investigate the impact of paternal ageing on sDNAfrag and sperm chromosomal abnormalities, several experiments have been conducted. Comparing testicular tissue and semen from 35 men aged 65-102 years, investigators observed a 1.29% increase in the aneuploidy rate of postmeiotic cells only when spermiogenesis was arrested, but no influence when complete. Furthermore, sDNAfrag did not seem to increase with age. Therefore, it was concluded that advanced male age does not represent any specific risk.[56] Another study in 97 non-smokers aged 22-80 years found no association between age and frequency of aneuploidy. Nevertheless, these authors disclosed a 5-fold increase in sDNAfrag, with a negative correlation to sperm motility and a positive correlation to sexual abstinence.[58] In a more recent report comparing 140 infertile patients aged 24-76 years with 50 men with proven fertility and aged 25-65 years, authors observed that male age did not affect sperm morphology, motility, sDNAfrag, or disomy. However, increasing male age was associated with a decrease in semen volume and sperm vitality,

and with an increase in sperm concentration and sperm diploidy.[21]

INFLUENCE OF EXTERNAL FACTORS

Male infertility may be innate for several reasons, but may also be acquired. The major risk factors associated with acquired male infertility include medical treatments and medicines, lifestyle habits, and environmental and occupational influences.[59] Several therapies also appear to play an important role in acquired male infertility due to their gonadal toxicity.[60] The threat of infertility due to these causes is related to the risk factor, amount, and time of exposure (Table 2). Dividing cells are the preferential target of these risk factors, which makes them very injurious to male spermatogenesis. Because mitosis of spermatogonia and meiosis of spermatocytes ensue during the course of adult life, these processes are susceptible to the permanent or temporary effects of these risk factors. Even though there is much evidence in the literature for the overall effect of these risk factors on the male reproductive function, only a few studies have looked into their possible simultaneous consequences on sperm aneuploidy and sDNAfrag; a summary of these studies can be found in Table 2.

Table 2: Adverse effects of various aneugenic agents with respect to sperm aneuploidy and DNA fragmentation.

Type of exposure	Aneugenic agent	Effects on sperm aneuploidy and DNA fragmentation	Study type	Reference
Occupational	Carbaryl	No relation to semen parameters although some not significant morphological defects Increased Chr X and Chr Y disomy, increased Chr 18 disomy, increased frequency of Chr X, Chr Y, and Chr 18 nullisomy (FISH) Increased sperm DNA fragmentation (TUNEL)	*In vivo*	61
	Ionising radiation	Decreased motility and viability, and increased morphological abnormalities No significant incidence of sperm aneuploidy (FISH) Increased sperm DNA fragmentation (TUNEL and SCSA) Increased hypermethylated sperm (immunodetection of 5-methylcytosine)	*In vivo*	62
Environmental	Seasonal air pollution with reactive polyaromatic hydrocarbons	Reduced sperm motility and normal morphology Increased risk of sperm aneuploidy (FISH) Increased DNA denaturation (SCSA)	*In vivo*	63
	Episodic air pollution	No relation to semen parameters No association with increased sperm aneuploidy (FISH) Increased sperm DNA fragmentation (SCSA)	*In vivo*	64
	Perfluorinated compounds	Alteration of sperm parameters Increased disomy and diploidy (FISH) Increased sperm DNA fragmentation (TUNEL)	*In vivo*	65
Medical treatment	Cancer and antineoplastic therapy (chemo and/or radiation)	Increased rate of structural and numerical Chr abnormalities Long-lasting-to-permanent sperm DNA fragmentation	Review	66
	Hormonal (FSH)	Improves sperm parameters of oligozoospermic patients Reduces aneuploidy Reduces sperm DNA fragmentation	Review *In vivo*	67

FISH: fluorescence in situ hybridisation; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labelling assay; SCSA: sperm chromatin structure assay; Chr: chromosome; FSH: follicle-stimulating hormone.

With regard to lifestyle habits such as alcohol consumption and smoking, there is a lack of reports on these two parameters. Cigarette smoke and alcohol are considered aneugenic agents.[68] However, there are conflicting results concerning DNA integrity, with some authors showing an increase in sDNAfrag[69] whereas others observed no effect.[70] It is therefore necessary to further study sperm aneuploidy and sDNAfrag in the same pool of patients with excessive alcohol consumption and smoking. Little is acknowledged about the origins of human sperm aneuploidy and sDNAfrag, particularly with regard to the impact of environmental and occupational exposures. Although all of the studies included in this review (Table 2) seem to be consistent with respect to occupational and environmental factors increasing sDNAfrag, the same cannot be said about its association with sperm aneuploidy because inconsistent results have been obtained.

Cancer treatment has been improving over recent decades, with many patients now experiencing long-term survival.[71] With this rise in life expectancy, concern over the quality of life for the surviving patients is a fundamental matter. Efforts have been made to preserve male reproductive function.[72] Meanwhile, sperm aneuploidy rates and sDNAfrag indices may afford means of evaluating genomic damage that might prove useful in genetic counselling efforts.[73] The authors of a review on this topic state that both chemotherapy and radiotherapy augment the rate of structural and numerical chromosome abnormalities, and patients who could preserve or restore their fertility status present with long-lasting-to-permanent sDNAfrag.[66] Nonetheless, broader investigations are necessary for each anti-cancer agent and on the variety of compounds used in combination with those agents that theoretically protect the male reproductive function. Gonadotrophins have been used empirically for the treatment of idiopathic male infertility, and have been shown to improve ART outcomes.[74] The impact of this treatment on sperm genomic integrity was considered and researchers found that hormonal treatment with follicle-stimulating hormone improved sperm parameters and reduced sperm aneuploidy and sDNAfrag in oligozoospermic patients.[67] Albeit preliminary, this study opens doors for future investigations.

Exposure to potential reproductive risk factors can affect sperm cells by either inducing breaks in the DNA or affecting sperm chromosomes, altering both their number and structure. However, more epidemiological studies should be conducted, especially taking into account the evaluation of semen quality both prior to and following exposure. In addition to the external risk factors presented here, numerous others may affect sperm DNA integrity and may need to be disclosed and studied. Moreover, it is necessary to take into account the transience of lifestyles and other types of exposure, which may mean that sperm damage can vary over an individual's lifetime.

INFLUENCE OF CRYOPRESERVATION

Along with the improvement of ART, preservation of sperm has been a widely used procedure (mainly for sperm donation)[75] for men undergoing vasectomy or at risk of azoospermia.[76,77] However, cryopreserved sperm from infertile men displayed greater DNA fragmentation and decreased motility and fertility compared with that of fertile donors, when used *ad infinitum* after cryopreservation, with the longest use reported after 28 years of storage.[78] Damaged sperm was also revealed to be likely to be less cryoresistant.[79] Cryopreservation in liquid nitrogen has been commonly used in ART. However, the risks of its contamination are now being appreciated and solved with the use of closed cryopreservation straws. In a study carried out in a cohort of 30 healthy donors, sperm was cryopreserved using the conventional protocol with liquid nitrogen and lyophilisation. This research revealed that both methods decreased sperm viability, motility, and morphology, and did not induce any change in aneuploidy and diploidy rates. Moreover, no statistically significant difference in sDNAfrag was observed before or after lyophilisation. Despite this observation, a statistically significant decrease in sDNAfrag after cryopreservation in liquid nitrogen was detected. The authors suggested combined studies with different physical (warming regimens) and chemical (antioxidants and zinc in the cryopreservation media) exposure environments.[80]

Many factors may have varied side-effects on male fertility, and so fertility preservation is the only option for fatherhood in several types of patients. Therefore, it is of utmost importance to continue and extend studies on the integrity of cryopreserved sperm, but also to not forget prepubescent patients whose only option is to preserve the germinal tissue. Studies on the ability of the oocyte repair system to restore sperm-

contributed imperfections should also be pursued, even after cryopreservation. The clinical impact of increased levels of sperm aneuploidy and sDNAfrag should also be taken into account in further studies, as correlations may be an adjunct in predicting ART outcome.

CONCLUSION

ARTs have improved in the preceding decades, which has pushed newborn rates to their highest percentage ever. For a couple to be infertile, it is usually due to a combination of both partners' fertility, which brings the issue of gamete quality to the fore. Apart from the female's contribution, studies of males and male gametes must be properly addressed before the treatment is initiated. In this report we have discussed the possible relationship between sDNAfrag and sperm aneuploidy in several causes of infertility. Although there seems to be a trend towards a positive correlation, the studies that addressed both parameters are debatable. One of the reasons for the conflicting results may be the use of different methods to evaluate sDNAfrag and sperm aneuploidy, which may produce different results. Regarding sperm aneuploidy, FISH appears to be a reliable method to measure sperm aneuploidy rates.[81] However, the limitation lies in the fact that only 2-4 of the 23 human chromosomes are analysed. These studies would probably benefit from the use of probes for multiple chromosomes. Some incongruity in results may also be the consequence of not evaluating both parameters at the same time in the same sperm cell or sample, which is necessary to definitely endorse a clear link between the two parameters. There seems to be no doubt that the most severe underlying reason for male infertility is the risk of sperm containing damaged DNA. This cell may not eventually be repaired by the oocyte, which will decrease ART outcomes or transmit an anomaly to the embryo.

Despite not having been the purpose of this review, we cannot fail to notice throughout all of the literature reviewed that, while numerical and structural alterations to chromosome segregation are likely to arise during meiosis, sDNAfrag may occur at any point during spermatogenesis. Unlike spermatogonia, spermatocytes (due to DNA breaks during chromosome recombination) and early spermatids (due to chromatin remodelling) display a high DNA repair ability, whereas mature spermatids and testicular sperm do not exhibit such a need.[82] Moreover, sDNAfrag seems to be related to different nuclear regions, which are either related to the type of chromosomes with gene-rich areas or to the amount of histones and protamines.[41]

More studies should be conducted, both in testicular tissue and ejaculate, in order to reveal if increased frequencies of sDNAfrag are due to abortive spermatogenesis. The presence of sperm with damaged DNA may be a failed attempt by the germ cells to complete apoptosis.[83] Besides sperm nuclear domains and abortive spermatogenesis, another point of view is that a low rate of aneuploid sperm coincides with a high rate of spermatocyte-I degeneration.[44] The emerging data therefore seem to support the idea that both sDNAfrag and aneuploidy are correlated not only with the severity of the male infertility but also with poorer ART outcomes.

As a final remark, the genetic integrity of sperm assessed through sDNAfrag and sperm aneuploidy analysis should remain the standard semen analysis. Meanwhile, although selecting sperm for ART based on MACS-DGC-swim-up[41] will reduce the risk of selecting damaged sperm based on their morphology and motility, patients will remain predisposed and susceptible to changes in the genomic integrity of the sperm DNA, and therefore should be offered genetic counselling or, alternatively, should seek prenatal diagnosis and/or preimplantation genetic diagnosis due to the risk of conceiving offspring with genetic anomalies.

REFERENCES

1. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th edition. Available at: http://whqlibdoc.who.int/publications/2010/9789241547789_eng.pdf. Last accessed: 6 June 2015.

2. Practice Committee of the American Society for Reproductive Medicine. The clinical utility of sperm DNA integrity testing: a guideline. Fertil Steril. 2013;99(3):673-7.

3. Barroso G et al. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum Reprod. 2000;15(6):1338-44.

4. Hofmann N, Hilscher B. Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. Hum Reprod. 1991;6(7):979-82.

5. Agarwala A, Allamaneni SS. The effect of sperm DNA damage on assisted reproduction outcomes. A review. Minerva Ginecol. 2004;56(3):235-45.

6. Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros and cons. J Androl. 2009;30(3):219-29.

7. Evenson DP et al. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl. 2002;23(1):25-43.

8. Lewis SE, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell Tissue Res. 2005;322(1):33-41.

9. Robinson L et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012;27(10):2908-17.

10. Simon L et al. Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment. Hum Reprod. 2014;29(5):904-17.

11. Benchaib M et al. Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. Fertil Steril. 2007;87(1):93-100.

12. Brahem S et al. Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. Urology. 2011;78(4):792-6.

13. Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet. 2001;2(4):280-91.

14. Tempest HG, Martin RH. Cytogenetic risks in chromosomally normal infertile men. Curr Opin Obstet Gynecol. 2009;21(3):223-7.

15. Harton GL, Tempest HG. Chromosomal disorders and male infertility. Asian J Androl. 2012;14(1):32-9.

16. Óvári L et al. Double probing individual human spermatozoa: aniline blue staining for persistent histones and fluorescence in situ hybridization for aneuploidies. Fertil Steril. 2010;93(7):2255-61.

17. Garolla A et al. High-power microscopy for selecting spermatozoa for ICSI by physiological status. Reprod Biomed Online. 2008;17(5):610-6.

18. Liu CH et al. DNA fragmentation, mitochondrial dysfunction and chromosomal aneuploidy in the spermatozoa of oligoasthenoteratozoospermic males. J Assist Reprod Genet. 2004;21(4):119-26.

19. Tang SS et al. Aneuploidy and DNA fragmentation in morphologically abnormal sperm. Int J Androl. 2010;33(1):e163-79.

20. Chatzimeletiou K et al. Semen analysis by electron and fluorescence microscopy in a case of partial hydatidiform mole reveals a high incidence of abnormal morphology, diploidy, and tetraploidy.

Fertil Steril. 2011;95(7):2430.e1-5.

21. Brahem S et al. The effects of male aging on semen quality, sperm DNA fragmentation and chromosomal abnormalities in an infertile population. J Assist Reprod Genet. 2011;28(5):425-32.

22. Brahem S et al. Cytogenetic and molecular aspects of absolute teratozoospermia: comparison between polymorphic and monomorphic forms. Urology. 2011;78(6):1313-9.

23. Brahem S et al. Detection of DNA fragmentation and meiotic segregation in human with isolated teratozoospermia. J Assist Reprod Genet. 2011;28(1):41-8.

24. Brahem S et al. Study of aneuploidy rate and sperm DNA fragmentation in large-headed, multiple-tailed spermatozoa. Andrologia. 2012;44(2):130-5.

25. Perrin A et al. Study of aneuploidy and DNA fragmentation in gametes of patients with severe teratozoospermia. Reprod Biomed Online. 2011;22(2):148-54.

26. Vicari E et al. Globozoospermia is associated with chromatin structure abnormalities: case report. Hum Reprod. 2002;17(8):2128-33.

27. Taylor SL et al. Complete globozoospermia associated with PLCζ deficiency treated with calcium ionophore and ICSI results in pregnancy. Reprod Biomed Online. 2010;20(4):559-64.

28. Dam AH et al. Globozoospermia revisited. Hum Reprod Update. 2007;13(1):63-75.

29. Brahem S et al. Analysis of sperm aneuploidies and DNA fragmentation in patients with globozoospermia or with abnormal acrosomes. Urology 2011;77(6):1343-8.

30. Perrin A et al. Molecular cytogenetic and genetic aspects of globozoospermia: a review. Andrologia. 2013;45(1):1-9.

31. Vozdova M et al. Total globozoospermia associated with increased frequency of immature spermatozoa with chromatin defects and aneuploidy: a case report. Andrologia. 2014;46(8):831-6.

32. Perdrix A et al. Assessment of acrosome and nuclear abnormalities in human spermatozoa with large vacuoles. Hum Reprod. 2011;26(1):47-58.

33. Boitrelle F et al. Large human sperm vacuoles observed in motile spermatozoa under high magnification: nuclear thumbprints linked to failure of chromatin condensation. Hum Reprod. 2011;26(7):1650-8.

34. Ghedir H et al. Meiotic segregation and sperm DNA fragmentation in Tunisian men with dysplasia of the fibrous sheath (DFS) associated with head abnormalities. J Assist Reprod Genet. 2014;31(9):1167-74.

35. Muriel L et al. Increased aneuploidy rate in sperm with fragmented DNA as determined by the sperm chromatin

dispersion (SCD) test and FISH analysis. J Androl. 2007;28(1):38-49.

36. Carrell DT et al. Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss. Obstet Gynecol. 2003;101(6):1229-35.

37. Bellver J et al. Y chromosome microdeletions, sperm DNA fragmentation and sperm oxidative stress as causes of recurrent spontaneous abortion of unknown etiology. Hum Reprod. 2010;25(7):1713-21.

38. Bronet F et al. Sperm DNA fragmentation index does not correlate with the sperm or embryo aneuploidy rate in recurrent miscarriage or implantation failure patients. Hum Reprod. 2012;27(7):1922-9.

39. Henkel R. Sperm preparation: state-of-the-art–physiological aspects and application of advanced sperm preparation methods. Asian J Androl. 2012;14(2):260-9.

40. Mongkolchaipak S, Vutyavanich T. No difference in high-magnification morphology and hyaluronic acid binding in the selection of euploid spermatozoa with intact DNA. Asian J Androl. 2013;15(3):421-4.

41. Bucar S et al. DNA fragmentation in human sperm after magnetic-activated cell sorting. J Assist Reprod Genet. 2015;32(1):147-54.

42. Vendrell X et al. Correlation between aneuploidy, apoptotic markers and DNA fragmentation in spermatozoa from normozoospermic patients. Reprod Biomed Online. 2014;28(4):492-502.

43. Patrizio P et al., "Chromosomal and genetic abnormalities in male Infertility," Oehninger AC, Kruger TF (eds.), Male Infertility. Diagnosis and treatment. Chapter 16 (2007), London: Informa UK Ltd, pp. 239-54.

44. Rives N et al. From spermatocytes to spermatozoa in an infertile XYY male. Int J Androl. 2005;28(5):304-10.

45. García-Peiró A et al. Sperm DNA integrity and meiotic behavior assessment in an infertile male carrier of a 9qh+++ polymorphism. J Biomed Biotechnol. 2011;2011:730847.

46. Perrin A et al. Aneuploidy and DNA fragmentation in sperm of carriers of a constitutional chromosomal abnormality. Cytogenet Genome Res. 2011;133(2-4):100-6.

47. Ferfouri F et al. Sperm FISH analysis of a 46,XY,t(3;6)(p24;p21.2),inv (8)(p11;2q21.2) double chromosomal rearrangement. Reprod Biomed Online. 2012;24(2):219-23.

48. Enciso M et al. Increased numbers of DNA-damaged spermatozoa in samples presenting an elevated rate of numerical

chromosome abnormalities. Hum Reprod. 2013;28(6):1707-15.

49. Olszewska M et al. Sperm FISH and chromatin integrity in spermatozoa from a t(6;10;11) carrier. Reproduction. 2014;147(5):659-70.

50. Nguyen MH et al. A study of aneuploidy and DNA fragmentation in spermatozoa of three men with sex chromosome mosaicism including a 45,X cell line. Hum Fertil (Camb). 2014;29:1-4.

51. Barros A et al. Birth after electroejaculation coupled to intracytoplasmic sperm injection in a gun-shot spinal-cord injured man. Arch Androl. 1998;41(1):5-9.

52. Burrello N et al. Chromosome analysis of epididymal and testicular spermatozoa in patients with azoospermia. Eur J Hum Genet. 2002;10(6):362-6.

53. Qiu Y et al. Sperm chromosomal aneuploidy and DNA integrity of infertile men with anejaculation. J Assist Reprod Genet. 2012;29(2):185-94.

54. Qiu Y et al. Quality of sperm obtained by penile vibratory stimulation and percutaneous vasal sperm aspiration in men with spinal cord injury. J Androl. 2012;33(5):1036-46.

55. Moskovtsev SI et al. Testicular spermatozoa have statistically significantly lower DNA damage compared with ejaculated spermatozoa in patients with unsuccessful oral antioxidant treatment. Fertil Steril. 2010;93(4):1142-6.

56. Dakouane M et al. [Aging and spermatogenesis: an histologic, cytogenetic and apoptosis study]. Gynecol Obstet Fertil. 2005;33(9):659-64.

57. Zitzmann M. Effects of age on male fertility. Best Pract Res Clin Endocrinol Metab. 2013;27(4):617-28.

58. Wyrobek AJ et al. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. Proc Natl Acad Sci U S A. 2006;103(25):9601-6.

59. Petraglia F et al. The changing prevalence of infertility. Int J Gynaecol Obstet. 2013;123 Suppl 2:S4-8.

60. Ethics Committee of the American Society for Reproductive Medicine. Fertility preservation and reproduction in patients facing gonadotoxic therapies: a committee opinion. Fertil Steril. 2013;100(5):1224-31.

61. Xia Y et al. Genotoxic effects on spermatozoa of carbaryl-exposed workers. Toxicol Sci. 2005;85(1):615-23.

62. Kumar D et al. Semen abnormalities, sperm DNA damage and global hypermethylation in health workers occupationally exposed to ionizing radiation. PLoS One. 2013;8(7):e69927.

63. Perreault SD et al. Integrating new tests of sperm genetic integrity into semen analysis: breakout group discussion. Adv Exp Med Biol. 2003;518:253-68.

64. Rubes J et al. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. Hum Reprod. 2005;20(10):2776-83.

65. Governini L et al. Chromosomal aneuploidies and DNA fragmentation of human spermatozoa from patients exposed to perfluorinated compounds. Andrologia. 2014. [Epub ahead of print].

66. De Palma A et al. Effects of cancer and anti-neoplastic treatment on the human testicular function. J Endocrinol Invest. 2000;23(10):690-6.

67. Valenti D et al. Follicle-stimulating hormone treatment in normogonadotropic infertile men. Nat Rev Urol. 2013;10(1):55-62.

68. Robbins WA et al. Effect of lifestyle exposures on sperm aneuploidy. Cytogenet Genome Res. 2005;111(3-4):371-7.

69. Anifandis G et al. The impact of cigarette smoking and alcohol consumption on sperm parameters and sperm DNA fragmentation (SDF) measured by Halosperm. Arch Gynecol Obstet. 2014;290(4):777-82.

70. De Bantel A et al. Simultaneous vitality and DNA-fragmentation measurement in spermatozoa of smokers and non-smokers. Cytometry B Clin Cytom. 2015;88(2):120-4.

71. Howlader N et al. (eds). SEER Cancer Statistics Review, 1975-2011. Available at: http://seer.cancer.gov/csr/1975_2011/. Last accessed: 6 June 2015.

72. Robinson RD, Knudtson JF. Fertility preservation in patients receiving chemotherapy or radiotherapy. Mo Med. 2014;111(5):434-8.

73. Choy JT, Brannigan RE. The determination of reproductive safety in men during and after cancer treatment. Fertil Steril. 2013;100(5):1187-91.

74. Attia AM et al. Gonadotrophins for idiopathic male factor subfertility. Cochrane Database Syst Rev. 2013;8:CD005071.

75. Lansac J, Royère D. Follow-up studies of children born after frozen sperm donation. Hum Reprod Update. 2001;7(1):33-7.

76. Sá R et al. Cryopreservation of human testicular diploid germ cell suspensions. Andrologia 2012;44(6):366-72.

77. De Sanctis V, Ciccone S. Fertility preservation in adolescents with Klinefelter's syndrome. Pediatr Endocrinol Rev. 2010;8 Suppl 1:178-81.

78. Feldschuh J et al. Successful sperm storage for 28 years. Fertil Steril. 2005;84(4):1017.

79. Kopeika J et al. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum Reprod Update. 2014;21(2):209-27.

80. Gianaroli L et al. DNA integrity is maintained after freeze-drying of human spermatozoa. Fertil Steril. 2012;97(5):1067-73.e1.

81. Balasuriya A et al. Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa. Reprod Biomed Online. 2011;22(5):428-36.

82. Mennella MRF, "Mammalian spermatogenesis, DNA repair, poly(ADP-ribose) turnover: the state of the art," Storici F (ed.), DNA repair – on the pathways to fixing DNA damage and errors. Chapter 12. (2011), Rijeka: InTech, pp. 235-54.

83. Sakkas D et al. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Exp Cell Res. 1999;251(2):350-5.