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Review Article

DNA Fragmentation in Spermatozoa: A Historical Review

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Abstract

Fragmentation has been extensively studied for more than a decade and its relevance for infertility is well known. In the forties, the uniqueness of the spermatozoa protein complex, which stabilizes the DNA, was discovered. In the fifties and sixties, the connection between unstable chromatin structure and subfertility was investigated. In the seventies, the impact of induced DNA damage was investigated. During the eighties and nineties, the molecular techniques advanced and several methods for detecting DNA fragmentation in spermatozoa emerged. A possible association between DNA fragmentation in spermatozoa and pregnancy loss was investigated in the zeroes spurring the need for a therapeutical tool for these patients. This gave rise to an increased interest in the aetiology of DNA damage. The present decade continues within this area of research.

In spite of half a century of research within the area, this analysis is not yet routinely implemented into the fertility clinics. The underlying causes are multiple. The abundance of methods has impeded the need for a clinical significant threshold. A patent inhibits the implementation of one of the most promising methods. Furthermore, myriads of reviews and meta-analyses with studies using different assays for analysis of DNA fragmentation, different clinical Artificial Reproductive Treatment (ART), different definitions of successful ART outcome and small patient cohorts have been published, are blurring the picture.

Even though the area of DNA fragmentation in spermatozoa is highly relevant in the fertility clinics, the need for further studies focusing on standardization of methods and clinical implementation persists.

ABBREVIATIONS

ART: Artificial Reproductive Treatment; TTP: Time To Pregnancy; IUI: Intrauterine Insemination; IVF: *in vitro* Fertilization; ICSI: Intracytoplasmic Sperm Injection; SCSA: Sperm Chromatin Structure Assay; AO: Acridine Orange; DB: Double stranded; SS: Single Stranded; DFI: DNA Fragmentation Index; HDS: High DNA Stainability; TUNEL: Terminal deoxynucleotidyl transferase Nick End Labelling; DBD-FISH: DNA Breakage Detection-Florescence *in situ* Hybridization; SCD: Sperm Chromatin Dispersion; ROS: Reactive Oxygen Species; MACS: Magnetic Activated Cell Sorting; HA: Hyaluronic Acid; AOT: Acridine Orange staining Technique.

INTRODUCTION

Paternal contribution to the fertilization and to the development of healthy offspring is of vital importance. There have been reports of an increased risk of autism, leukaemia and cancer in offspring from fathers with increasing age or fathers with increased level of DNA fragmentation due to smoking. Furthermore, some spontaneous dominant genetic diseases, epilepsy and some birth defects are linked to paternal contribution [1]. A number of studies involving DNA fragmentation of spermatozoa have reported an association between an increase in DNA fragmentation in the spermatozoa and subfertility. Comparing studies of fertile and infertile males have shown that the amount of DNA damage is significantly higher in the infertile group [2-6]. An abnormal chromatin packing is more recurrent in men with normospermia undergoing ART treatment than in fertile men [5]. If the man has increased DNA fragmentation in the spermatozoa, a prolonged Time To Pregnancy (TTP) [3], an increased risk of a missed abortion [7-11], and a significantly reduced chance of *in vivo* fertilization of the partner have been suggested [2,12-15]. When seeking fertility treatment, DNA

fragmentation in the spermatozoa also seems to be of vital importance when planning the course of treatment. A study included 131 couples seeking fertility treatment by intra uterine inseminations (IUI). Twenty-three of the male patients had an increased amount of DNA fragmentation followed by a pregnancy rate of 4% in their partner [13]. A later study including 387 cycles showed that the pregnancy rate dropped to 3% if the level of DNA fragmentation exceeded 30% [14]. In a smaller Danish study including 48 couples, no pregnancies were observed in couples, where the male DNA fragmentation exceeded 27% [16]. Until now, no clear association between increased amount of DNA fragmentation and fertilization rate after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI)has been established [8,14]. However, it may affect the clinical pregnancy rate. It thus seems that an increase of DNA fragmentation primarily affects *in vivo* fertility, either by reducing natural conception or by a significant reduction in successful intrauterine inseminations. It is estimated that up to 20% of males with semen parameters otherwise suitable for IUI treatment present with a DFI > 30%, and on this basis the authors behind this study recommend that IVF or ICSI being the first choice of treatment if the amount of DNA fragmentation exceeds 30% [12,17].

Together, these studies provide important insight into the significance of DNA fragmentation in the spermatozoa when treating couples for infertility.

With this in mind - why is sperm DNA fragmentation testing not a standard diagnostic tool in the treatment of the male fertility patient?

The journey regarding DNA fragmentation in spermatozoa has been long and began more than half a century ago.

In 1946 Pollister and Mirsky discovered that a large part of the protein complexes surrounding the DNA in trout sperm was not composed of histones but of protamines [18], and in 1955 Alfert found that the protamines replace the histones after meiosis in the maturation of the salmon spermatozoa [19]. Today it is estimated that only 5-15 % of the chromatin in the spermatozoa consist of histones and the major part consists of protamines [20]. After the discovery of the double helix in 1953, the interest of investigating the structure of the human chromatin in spermatozoa escalated. Already in 1963, Getz off recognized that the chromatin was altered in subfertile men. The techniques available at the time left him to measure the quantity of the chromosomes, which he found to be very stable in fertile men and to vary significantly in subfertile men [21].

During the seventies, an increasing interest in a possible association between exposure of DNA damaging agents and a possible reduction in fertility emerged. This focus could be influenced by the political and societal tension concerning the nuclear advancements during the previous decade. In 1970 Ringertz et al., used an assay where bull spermatozoa were heated and the denaturation of the DNA was detected using acridine orange followed by microfluoriemetry. They realized that the spermatozoa expressed an increased stability during the Spermiogenesis [22]. A decrease in epididymal sperm count and weight of the testis was observed in mice after exposure to irradiation. Additionally, an increased pre-implantation loss was observed in the female mice [23].

In the eighties, the technology for molecular biology advanced. Evenson et al., developed a flow cytometric assay for detection of DNA fragmentation in spermatozoa [24]. He called the assay Sperm Chromatin Structure Assay (SCSA) and took out a patent on the name. The assay is based on the detection of DNA fragmentation by flow cytometry after denaturation of the spermatozoa by acid and subsequent staining with the fluorescent cationic dye Acridine Orange (AO). AO attaches to the DNA in the ratio of approximately two AO molecules per phosphate group [25]. When the laser from the flow cytometer illuminates the cells, AO fluoresces with a green emission when bound to double stranded (db) DNA and a red emission when bound to singe stranded (ss) DNA. Furthermore, the flow cytometer measures forward scatter and side scatter. Usually a total of 5,000-10,000 cells areanalysed. DNA Fragmentation Index (DFI) is described as the percent wise ratio of red florescence [26-28]. SCSA also measures High DNA Stainability (HDS), which is believed to be an expression of the amount of immature spermatozoa. However, the association to infertility is not clear-cut [13,26].

In the eighties, the single cell gel electrophoresis was also developed and refined and in the nineties, the comet assay showed that spermatozoa from infertile men were more susceptible to induced damage than spermatozoa from fertile men. In the comet assay, 200-300 cells are covered with agarose gel and subsequently lysed. If the DNA is embedded with breaks, the super coiling of the DNA is released allowing the DNA to migrate towards the anode. This migration leaves a comet-like tail and the intensity of the fluorescence of the tail relates to the number of DNA breaks [29,30].

In the nineties, terminal deoxynucleotidyl transferase nick end labelling (TUNEL) of human spermatozoa was developed. In this assay a terminal deoxynucleotidyl transferase labels the DNA strand breaks with fluorescent dUTP nucleotides. This assay can be performed using flow cytometry and microscopy. Both the neutral comet assay and the TUNEL assay are considered “direct” assays as they measure actual DNA strand breaks whereas some of the other assays developed measure the DNA susceptibility to denature or a differentiated binding of a dye to ds- or ssDNA [31,32].

In the zeroes, other methods for determination of DNA fragmentation appeared. The DNA Breakage Detection-Florescence *in situ* Hybridization (DBD-FISH) was developed for human spermatozoa. Here the DNA is transformed into ssDNA by an alkaline unwinding solution, the proteins are removed and the DNA is made accessible to hybridization [33]. The Sperm Chromatin Dispersion (SCD) test, also developed in this decade, detects spermatozoa with increased amount of fragmented DNA by identifying the lack of a halo of dispersed DNA loops after acid denaturation and removal of nuclear proteins [34]. Two years later, an advanced SCD test was developed as a kit, Halosperm® [35]. SCD assays use microscopy for the detection of DNA breaks.

From the late zeroes and into the tenths the focus concerning DNA fragmentation shifted from development of methods to the aetiology of sperm DNA fragmentation and the implications of fertility treatment with spermatozoa with increased amount of DNA fragmentation.

Several studies added to the viewpoint that fertility treatment

with intrauterine inseminations had very low chance of resulting in pregnancy if the DFI in the spermatozoa was increased. Furthermore, studies were beginning to show that even though the implantation rate after IVF and ICSI was not affected by increased amount of DNA fragmentation in the spermatozoa, the risk of early pregnancy loss was increased in these couples [36-40].

In 2005, Greco et al., showed that ICSI with testicular sperm resulted in a significantly higher clinical pregnancy rate compared with ICSI where ejaculated sperm was used. This provided one of the first treatment options for male fertility patients with increased amount of DNA damage in the spermatozoa. This study also gave insight to the aetiology of DNA damage as at least a part of the DNA damage seemed to appear after the spermatozoa have left the testis [41]. Recently, both Esteves et al., and Pabuccu et al., achieved similar results and in 2015, Zini concluded that testing for DNA fragmentation should be a part of the male infertility diagnosing [42-44].

As the research in the area expanded, several studies have shown that the origin of DNA fragmentation can be very diverse. A link between increased DNA fragmentation and inadvertent effects during the spermiogenesis, increased amount of oxidative stress, sperm collection methods, storage temperature, varicocele, bacterial infections, age, temperature of the testes and reaction to medicine and more has been seen [45]. It is thus possible that the damage to the DNA happens in multiple steps. This has probably contributed to the blurred picture of DNA fragmentation. Initially the DNA might be subjected to denaturing events during the spermatogenesis such as nicks in the backbone of the DNA or poor packaging of the chromatin during the replacement of histones. Subsequently, the already weakened DNA is more susceptible to external stressors such as medication, temperature, Reactive Oxygen Species (ROS) [46,47].

The role of antioxidants has been studied extensively in several areas in the last decade, possibly due to the easy access to oral supplements. Regarding spermatozoa, ROS are believed to play a part in the presence of DNA fragmentation. ROS play a positive role in several crucial functions such as proliferation and differentiation of cells. However, a pathogenic effect can occur when the balance between ROS and antioxidants is disturbed which can result in an excess of ROS, for example in the reproductive tract or in the seminal plasm. Several studies have shown that antioxidants can have a positive impact on some of the primary seminal parameters [48-50]. Supplements of some antioxidants distributed to men with increased DFI have previously shown a significant reduction in DFI and an increase in the clinical pregnancy rate [51]. However, the overall effectiveness of antioxidants remains controversial. This is mainly due to non-standardized assays for determination of ROS or antioxidant capacity, diversity in methods for determination of DNA fragmentation, lack of distinction between direct and indirect antioxidants and inadequate data on fertilization and pregnancy rates [52].

In the present decade, the magnetic activated cell sorting (MACS) technique was enhanced. It was first described in the zeroes concerning fertility treatment [53]. Recent research has shown that the relevance for optimizing ICSI remains controversial [54,55]. A novel method where spermatozoa with increased amount of DNA fragmentation are separated by fluorescence-activated cell sorting has recently been presented. The spermatozoa are stained using a YO-PRO staining technique and the researcher showed that it is possible to separate the dead spermatozoa and the spermatozoa with increased amount of DNA fragmentation, thereby optimizing the sperm sample [56]. The next step is to investigate the clinical relevance of this method. Another novel method being investigated in the present decade is the possibility of detecting damage in spermatozoa by a synthetic oligopeptide binding to damaged DNA. The non-binding end of the oligopeptide consists of a rhodamine B dye. There was seen a correlation of the amount of DNA damage detected with this method and the more classical methods such as SCD, comet and TUNEL [57]. One of the future aims for the two ladder methods is the possibility to preserve the fertility potential in the spermatozoa with low DNA fragmentation in order to increase the chance of fertilization. Furthermore, investigators have studied the hyaluronic acid (HA) binding technique. Hyaluronic acid surrounds the oocyte only allowing spermatozoa with sufficient expression of specific receptors to fertilize it. It seems that there is an inverse association between sperm HA binding and chromosomal abnormalities in the spermatozoa [58]. A study showed that HA binding test increased the chance of selecting a spermatozoon with a low amount of DNA fragmentation [59], and commercial kit has been developed [60]. However, in a recent meta-analysis it was not found that HA binding test increases fertilization rates after ICSI [61], and further research in the area is thus needed for this test to have relevance in the fertility clinics. Research continuously seems to focus on the possible association between DNA fragmentation in spermatozoa and recurrent pregnancy loss [62-65]. An increasing interest in which types of fragmentations are present in the DNA [66], which enzymes are effected by DNA fragmentation [67-69], and how DNA fragmentation can be reduced in cryopreservation [70-72], has supervened. Substantial amounts of reviews and meta-analysis have been published, many of them imploring further studies with a controlled, randomized study population and more sensitive assays [73-76].

In figure (1), an illustrative view of the historical development of DNA fragmentation in spermatozoa is presented.

DISCUSSION AND CONCLUSION

In spite of half a century´s research and the widely accepted conviction that infertility and DNA fragmentation in spermatozoa are linked, this diagnostic tool is not yet standard care in the fertility clinics. Several issues contribute to this. The lack of uniformity in assays for analysis of DNA fragmentation and thereby absence of a clear clinical threshold, a myriad of studies using different assay, different clinical ART and diverse outcomes and small patient cohorts.

Bungum et al., estimates that 40 % of all cases of unexplained infertility can be related to increased amount of DNA damage. Furthermore, it is speculated that even a moderate increase in DFI (between 20-30% by SCSA) can give rise to a prolonged TTP – information that the treating physician can employ when counselling fertility patients and planning the treatment course [17].

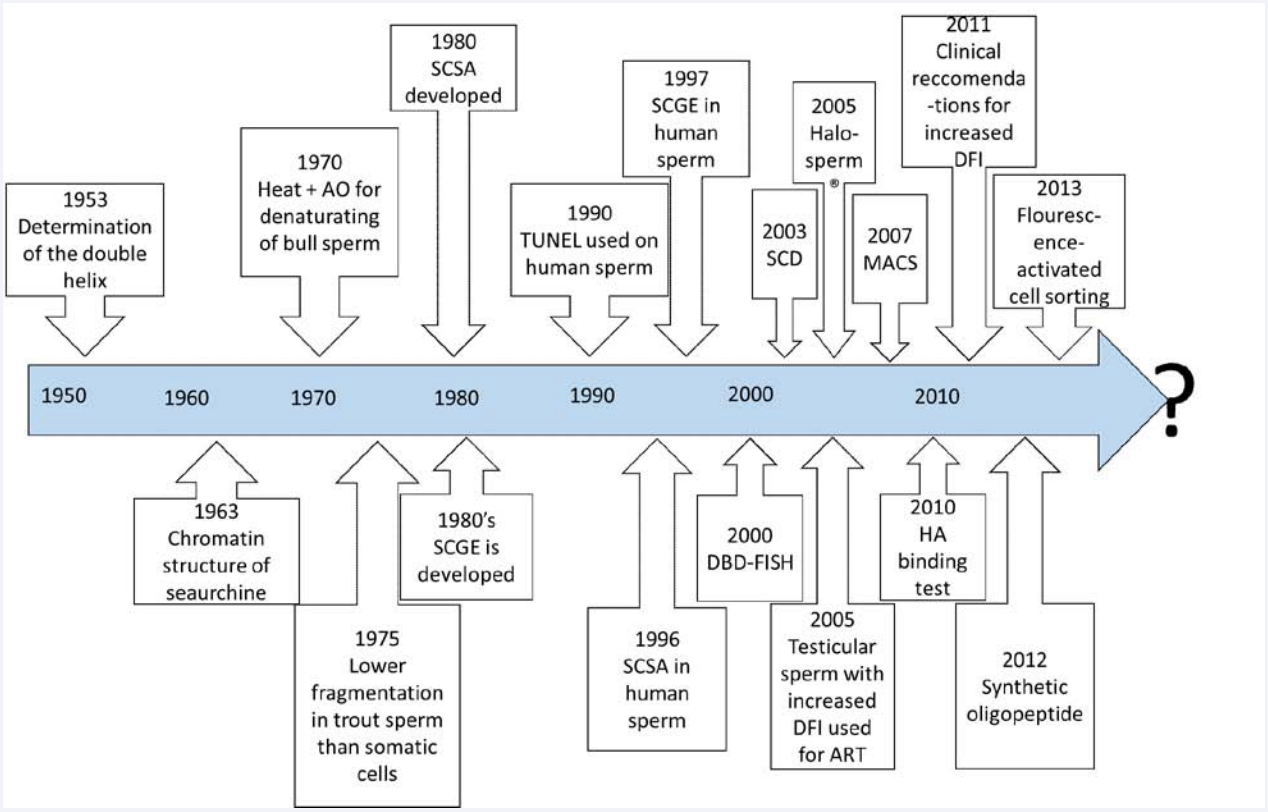

Figure 1 An illustrative view of the landmarks and development in methods concerning DNA fragmentation in spermatozoa.

One of the obstacles regarding DNA fragmentation in spermatozoa is the difference in the methods used to asses this value. Reviews and meta-analysis compare outcomes of fertility treatment across methods, which impede the progress of implementing the analysis in the fertility clinics. When comparing outcomes of the different methods there seems to be a correlation between SCSA, TUNEL and SCD with regard to levels of sperm DNA fragmentation. However, the Acridine Orange staining Technique (AOT), where DNA fragmentation is determined after acoloration with acridine orange and a microscopic evaluation, does not seem to have a clinical significance for fertility testing. Even though SCSA also uses AO for the coloration of the DNA, the evaluation by microscopy versus flow cytometer seems to be of crucial importance. Additionally, a study has shown that the neutral comet assay fails to distinguish between fertile donors and infertility patients. It does however relate to the risk of miscarriage. The alkaline comet assay seems to have a moderate correlation with the three previously mentioned methods for analysis. When the predictive value of male infertility is assessed, it seems that the alkaline comet assay has the highest sensitivity followed by the TUNEL, SCD and SCSA analysis and subsequently the neutral comet assay. This could explain the moderate correlation between the alkaline comet assay and the TUNEL, SCD and SCSA [77,78].

A two-step model for the development of DNA fragmentation in spermatozoa has been suggested. In step one, an error in the spermatogenesis weakens the DNA and impair the chromatin remodelling resulting in spermatozoa with low levels of nuclear protamine. In the second step, the vulnerable DNA is susceptible to oxidative stress. The aetiology of the fragmentation is not yet fully understood, however the causes are believed to be multi factorial and to include both endogenous, such as error in the spermiogenesis, as well as exogenous exposures, such as environmental, lifestyle and health. As mentioned in the introduction the biological implications of increased amount of DNA fragmentation in the spermatozoa are considerable and include increased risk of miscarriage or a number of pathogenic conditions in the offspring [1,79].

When relating to DNA fragmentation and infertility, the salient point is implementation in the fertility clinic. It is essential that this analysis is practicable in the daily work. Furthermore, uniformity and reproducibility across laboratories are of crucial importance.

At this point comparison studies between methods have concluded that SCSA, where DFI is measured by flow cytometer and analysed by SCSA software, is the most reproducible and uniform method, whereas the comet assay lacks a clear threshold and the methodology can change among laboratories. Furthermore, the method suffers by the fact that the evaluation of DNA fragmentation is estimated in only 200-300 cells. In addition, the method is labour-intensive. The TUNEL assay requires extensive preparation of the spermatozoa before analysis can be performed and there is currently a lack of a strict protocol thus inhibiting implementation as a diagnostic tool in a clinical setting. SCSA has a strict protocol developed in 1980 and has been used for fertility assessment in both animal and

human spermatozoa. The analysis by flow cytometer allows for evaluation of 10.000 spermatozoa in a short period allowing for a more robust analysis [26,48,80,81].

Bungum et al., suggested in 2011 a clinical recommendation where patients with DFI ≥ 30% measured by SCSA should be referred directly to IVF/ICSI treatment [17].

Currently, there are two major drawbacks when using SCSA. The software is patented, expensive and relatively unavailable and the analysis requires the investment in a flow cytometer. In conclusion, there is an urgent need for an analysis of DNA fragmentation that is stable, uniform and with a clear cut-off. It must be available for the fertility clinics in order to make progress in the field of DNA fragmentation in spermatozoa as a diagnostic tool in the fertility clinics.

The predictive value of the analysis of DNA fragmentation in spermatozoa is often criticized. As infertility is the couple's problem, one has to consider the fertility of the female as well. One single test of gamete dysfunction from just one partner of the couple cannot predict the outcome of the fertility treatment. Determination of DNA fragmentation is not a replacement of current diagnostic tools used when assessing the fertility of a couple. However, it is a valuable supplement adding independent information about the gamete status of the male partner.

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