Origin of DNA damage in ejaculated human spermatozoa

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The molecular basis of many forms of male infertility is poorly defined. One area of research that has been studied intensely is the integrity of the DNA in the nucleus of mature ejaculated spermatozoa. It has been shown that, in men with abnormal sperm parameters, the DNA is more likely to possess strand breaks. However, how and why this DNA damage originates in certain males and how it may influence the genetic project of a mature spermatozoon is unknown. Two theories have been proposed to describe the origin of this DNA damage in mature spermatozoa. The first arises from studies performed in animal models and is linked to the unique manner in which mammalian sperm chromatin is packaged, while the second attributes the nuclear DNA damage in mature spermatozoa to apoptosis. One of the factors implicated in sperm apoptosis is the cell surface protein, Fas. In this review, we discuss the possible origins of DNA damage in ejaculated human spermatozoa, how these spermatozoa arrive in the ejaculate of some men, and what consequences they may have if they succeed in their genetic project.

The formation of mature spermatozoa is a unique process involving a series of meioses and mitoses, changes in cytoplasmic architecture, replacement of somatic cell-like histones with transition proteins and the final addition of protamines, leading to a highly packaged chromatin (Kumaroo et al., 1975; Goldberg et al., 1977; Pocchia, 1986). Mature mammalian spermatozoa contain high percentages of protamines, for example, human and mouse sperm nuclei contain more than 85% and 95% protamines in their nucleoprotein component, respectively (Gatewood et al., 1987; Bellvé et al., 1988; Debarle et al., 1995). In mice, protamines allow the mature sperm nuclei to adopt a volume 40 times less than that of normal somatic nuclei (Ward and Coffey, 1991).

In many mammals, spermatogenesis leads to the production of spermatozoa that appear highly homogeneous in form and function. However, in humans, it is apparent that there are large differences between the form and function of spermatozoa among males and within the ejaculate of an individual. Classically, analyses of the differences in spermatozoa among men have been measured by examining sperm concentration, motility and morphology. Although this analysis gives a broad clinical insight, it does not explain why and where differences originate.

For a number of years, many laboratories have concentrated on analysing differences in sperm populations by examining chromatin structure. These studies have shown that the major factor affecting chromatin packaging in ejaculated human spermatozoa appears to be linked to faulty or incomplete protamine deposition during spermiogenesis. In numerous studies, spermatozoa from infertile men were found to exhibit sperm chromatin anomalies related to the deposition of protamines (Balhorn, 1982; Foresta et al., 1992; Belokopytova et al., 1993; de Yebra et al., 1993). These anomalies range from altered ratios of protamine 1 and 2 (Balhorn et al., 1988; Belokopytova et al., 1993) to the complete absence of protamine (de Yebra et al., 1993).

During the 1990s, several groups have analysed the sperm nucleus further by examining the integrity of the DNA in mature human spermatozoa. This review summarizes the accumulated knowledge concerning DNA damage in mature human spermatozoa and how this may be related to male infertility. Furthermore, we will speculate on how and why DNA damage may originate in certain males and how it influences the genetic project of a mature spermatozoon.

DNA packaging in mammalian spermatozoa

The chromatin contained in the nuclei of mature mammalian spermatozoa is an extremely compact and stable structure. Sperm DNA must be organized in a specific manner (Fig. 1), which differs substantially from that of somatic cells, to achieve this unique condensed state (Pocchia, 1986; Ward and Coffey, 1991). This DNA organization not only permits transfer of the very tightly packaged genetic information to the egg, but also ensures that the DNA is delivered in such a physical and chemical form that the developing embryo can access the genetic information.

Ward (1997) has proposed a model for sperm DNA packaging on the basis of his work and that of other laboratories. This model depicts a mock assembly of chromosomes starting as long strands of DNA that are gradually packaged at four levels of organization within the mature spermatozoon: (i) chromosomal anchoring by the nuclear annulus, (ii) sperm DNA loop domain organization, (iii) protamine decondensation,
and (iv) chromosome organization. This model represents the intricacies of the organization of the sperm nucleus and supports the assumption that anomalies in the DNA would most likely extrapolate to anomalies in overall nuclear organization.

**DNA damage in mature spermatozoa**

Sperm DNA has been analysed by a number of laboratories. In our laboratory, the integrity of the DNA in mature ejaculated human spermatozoa has been analysed using *in situ* nick translation. These experiments were performed by omitting endonuclease treatments, since, in the presence of pre-existing DNA endogenous nicks, the DNA polymerase I, by virtue of its 5’–3’ exonucleotytic activity can catalyse movement of the nicks along the double helix (Sambrook *et al*., 1989). Our results have described the percentage of spermatozoa possessing endogenous DNA nicks in the ejaculates of men and have shown a correlation with reduced fertility (Bianchi *et al*., 1993, 1996; Manicardi *et al*., 1995, 1996; Sakkas *et al*., 1996). In most cases, the spermatozoa of men with oligoasthenoteratozoospermia display a higher percentage of nicks.

The terminal deoxynucleotidyl transferase (TUNEL) assay has also been used to show the incidence of DNA fragmentation in human spermatozoa and a correlation between abnormal sperm chromatin packaging (poorly protaminated spermatozoa) and the presence of DNA strand breaks has been shown (Gorczyca *et al*., 1993a; Sailer *et al*., 1995; Manicardi *et al*., 1998). Sun *et al*. (1997), in a cohort of 298 patients, used TUNEL labelling and fluorescence-activated cell sorting to show a negative correlation between the percentage of DNA fragmentation and the motility, morphology and concentration of ejaculated spermatozoa. In 143 *in vitro* fertilization (IVF) samples, a significant negative association was reported between the percentage of spermatozoa with DNA fragmentation and the fertilization and embryo cleavage rates. Lopes *et al*. (1998) have also shown that the percentage of spermatozoa with DNA fragmentation was negatively correlated with fertilization rates obtained with intracytoplasmic sperm injection (ICSI).

The differences in the mode of action between nick translation and the TUNEL technique can be explained when considering that DNA polymerase, being primer- and template-dependent, cannot label blunt-ended or 5’-recessed DNA fragments, and that TUNEL, being template-independent, can label all types of fragments at the hydroxylated 3’ ends (Gold *et al*., 1994). However, data reveal that the two techniques cannot distinguish differences in the presence of endogenous DNA damage in human spermatozoa (Manicardi *et al*., 1998).

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**Fig. 1.** Levels of DNA packaging in sperm chromatin based on the doughnut loop model. Protamine binds to the DNA lengthwise along the double helix. The protamine-bound DNA is coiled into concentric circles. These circles of one loop then collapse into a doughnut into which the neutral DNA protamine complexes are tightly packed. Each doughnut represents one DNA loop domain attached to the sperm nuclear matrix. Single chromosomes are organized with centromeres located in the centre of the nucleus. Each chromosome is attached to the nuclear matrix at intervals of about 40 kb leading to DNA loop domain formation. (Adapted from Ward, 1993, 1997.)
The sperm chromatin structure assay (SCSA), which measures the susceptibility of DNA to heat- or acid-induced denaturation in situ, is also effective in identifying fertility potential (Evenson et al., 1980). However, accurate measurement of DNA damage in a single spermatozoon has proved difficult. Although techniques such as in situ nick translation and the TUNEL assay can be used to observe a single spermatozoon, there are limitations to the sensitivity of these techniques, and it is possible that many spermatozoa with damaged DNA escape detection. Other methods for assessing the DNA of a single spermatozoon, such as the Comet assay (single cell gel electrophoresis), are also being established (Hughes et al., 1996; Aravindan et al., 1997). This assay has been recognized in many cell lines to be one of the most sensitive techniques available for measuring DNA strand breaks (Collins et al., 1997). Aravindan et al. (1997) established a significant relationship between the Comet assay for human spermatozoa and the SCSA and TUNEL assays. The Comet assay has been used to examine the effect of various chemicals, such as food mutagens and oestrogenic substances, on spermatozoa in vitro (Anderson et al., 1997a,b) and may prove to be one of the more sensitive tests for examining DNA strand breaks in sperm chromatin.

Whether the DNA damage in spermatozoa is representative of single- or double-stranded DNA breaks will also be of interest. Single-stranded lesions should be repaired in the oocyte upon fertilization and so should not be lethal. However, if a fertilizing spermatozoon possesses single stranded DNA breaks of significant size, these may prove difficult for the oocyte to repair and may lead to failure in either the fertilization process or later in development.

**Origin of DNA damage in mature spermatozoa**

In humans, it is clear that the population of spermatozoa in an ejaculate can be highly heterogeneous. Unfortunately, this appears to be more evident in patients whose sperm parameters fall below normal WHO values (WHO, 1992). The positive relationship between poor sperm parameters and DNA damage in spermatozoa points to inherent problems in spermato genesis in specific patients. Two theories have been proposed to explain the phenomenon of why there are anomalies in the DNA of ejaculated human spermatozoa.

The first theory arises from studies performed in animal models and is linked to the unique manner in which mammalian sperm chromatins is packaged. Endogenous nicks in DNA have been shown to be present normally at specific stages of spermiogenesis in rats and mice, and are thought to have a functional significance (McPherson and Longo, 1992, 1993a,b; Sakkas et al., 1995). In the rodent species, endogenous nicks are evident during late spermiogenesis (step 12–13 rat spermatids) but are not observed once chromatin packaging is completed (McPherson and Longo, 1993a; Sakkas et al., 1995). Therefore, the presence of nicks is greatest during the transition from round to elongated spermatids in the testis and occurs before the completion of protamination in maturing rat and mouse spermatozoa (McPherson and Longo, 1992, 1993a,b; Sakkas et al., 1995).

In considering the remodelling of chromatin, McPherson and Longo (1992, 1993a,b) postulated that chromatin packaging may necessitate endogenous nuclease activity to create and ligate nicks that facilitate protamination. They proposed that the endogenous nuclease, topoisomerase II (topo II), may play a role in both creating and ligating nicks during spermiogenesis. These nicks are thought to provide relief of torsional stress and to aid chromatin rearrangement during the displacement of histones by protamines (McPherson and Longo, 1992). Chen and Longo (1996) have also shown that changes in DNA topo II expression and localization patterns are consistent with the involvement of topo II in mediating DNA modifications and chromatin changes during rat spermatogenesis. Therefore, the presence of endogenous nicks in ejaculated spermatozoa indicates incomplete maturation during spermiogenesis. This hypothesis is supported by observations that the presence of DNA damage in mature spermatozoa is correlated with poor chromatin packaging due to underprotamination (Gorczyca et al., 1993a; Manicardi et al., 1995; Sailer et al., 1995).

The second theory proposes that the presence of endogenous nicks in ejaculated human spermatozoa is characteristic of programmed cell death, as seen in apoptosis of somatic cells, and functional elimination of possibly defective germ cells from the genetic pool (Gorczyca et al., 1993a,b).

**Apoptosis during spermatogenesis**

Spermatogenesis is the dynamic process of germ cell proliferation and differentiation from stem spermatogonia to mature spermatozoa. In mammalian testes, germ cells expand clonally through many rounds of mitosis before undergoing the differentiation steps that result in mature spermatozoa. This clonal expansion is excessive, requiring that there is a mechanism to match the numbers of germ cells with the supportive capacity of the Sertoli cells. In a number of animal models, overproliferation of early germ cells is tempered by selective apoptosis of their progeny (Allan et al., 1992; Tapanainen et al., 1993; Bartke, 1995; Billig et al., 1995; Sinha Hikim et al., 1997; Furuchi et al., 1996; Rodriguez et al., 1997). Testicular germ cell apoptosis occurs normally and continuously throughout life. One factor postulated to be implicated in sperm apoptosis is the cell surface protein, Fas (Lee et al., 1997). Fas is a type I membrane protein that belongs to the tumour necrosis factor–nerve growth factor receptor family, and mediates apoptosis (Suda et al., 1993; Kramer et al., 1994; Schulze-Osthoff, 1994). Binding of Fas ligand (Fasl) or agonistic anti-Fas antibody to Fas kills cells by apoptosis (Suda et al., 1993). In mice and rats, it has been shown that, in the normal state, Sertoli cells express Fasl and signal the killing of Fas-positive germ cells, limiting the size of the germ cell population to numbers they can support (Lee et al., 1997; Rodriguez et al., 1997). In addition, after injury, FasL expression of Sertoli cells increase to reach a new equilibrium state that matches the reduced capacity of the dysfunctional Sertoli cells with fewer germ cells. Thus, upregulation of Fas in germ cells is seen as a self-elimination process for cells that are destined to die because of inadequate support. The role of Fas during spermatogenesis in humans is unclear. However, there are a number of indications that apoptosis occurs during spermatogenesis in humans. The observation that mature ejaculated human spermatozoa are positive for the TUNEL assay has lead to the theory that apoptosis is occurring (Gorczyca et al., 1993a; Baccetti et al., 1996; Sun et al., 1997; Lopes et al., 1998; Manicardi
Baccetti et al. (1996) described certain ultrastructural features indicative of apoptosis in human spermatozoa, while Hadziselimovic et al. (1997) showed that there was increased apoptosis in the testes of patients with testicular torsion.

In our studies, we have examined whether ejaculated human spermatozoa express Fas. Spermatozoa from a series of patients were labelled with anti-Fas conjugated to fluoroscein isothiocyanate (FITC) and 104 spermatozoa were passed through a fluorescence-activated cell sorter. A clear increase in Fas positivity has been found in many men who have sperm parameters lower than normal (Fig. 2). However, why do ejaculated sperm exhibit these apoptotic features when they should be eliminated? In mice, in which apoptosis occurs via a Fas-mediated system (Rodriguez et al., 1997), mature spermatozoa rarely show any signs of DNA damage (Sakkas et al., 1995) or Fas positivity (D. Sakkas, unpublished). In men with normal sperm parameters, the percentage of Fas-positive spermatozoa is low. However, in men with reduced sperm parameters, the percentage of Fas-positive spermatozoa can be as high as 50% (Fig. 2). When the percentage of spermatozoa positive for Fas is plotted against sperm concentration, it becomes evident that men with decreased sperm concentrations in their ejaculate have a greater chance of expressing high concentrations of Fas-positive spermatozoa.

These results indicate that, in these subfertile men, spermatozoa that have been earmarked to undergo apoptosis escape this process. The above evidence suggests that the correct clearance of spermatozoa via apoptosis is not occurring. Therefore, production of ejaculated spermatozoa that possess apoptotic markers (such as Fas positivity and DNA damage) indicate that, in some men with abnormal sperm parameters, an ‘abortive apoptosis’ has taken place. This finding suggests that spermatozoa showing for example abnormal morphological forms, irregular biochemical function (Huszar et al., 1997) or nuclear DNA damage have failed to be eliminated owing to an abortive apoptotic mechanism (Fig. 3).

The failure to clear Fas-positive spermatozoa may be due to misfunction at various levels. First, as has been reported in other animal models, apoptosis during spermatogenesis limits any excess in the number of germ cells developing so that the supportive capacity of the Sertoli cells is not inhibited. Since it has been shown that the Sertoli cells can limit this overproliferation via synthesis of FasL, it could be postulated that, in oligozoospermic men, in whom spermatogenesis is reduced, sufficient spermatozoa may not be produced to trigger this action by the Sertoli cells. Thus, even though a spermatogonium may be Fas-positive, it may escape activation to undergo apoptosis. Fas-positive spermatozoa may also occur because of problems in activating Fas-mediated apoptosis. These problems could be inherent to a particular patient or may be due to lack of synchronization between apoptosis and spermatogenesis, in which case, even though apoptosis has been initiated, the spermatozoa will go through spermiogenesis and fail to complete apoptosis (Fig. 3). This hypothesis may explain why patients with abnormal sperm parameters also possess a higher percentage of spermatozoa containing DNA fragmentation and abnormal spermatozoa that display signs of apoptosis.

Whereas the presence of DNA fragmentation in ejaculated human spermatozoa may be explained by apoptosis taking place during spermatogenesis, the initial theory that the presence of endogenous nicks indicates that incomplete endogenous nuclease activity creates and ligates nicks during spermiogenesis (McPherson and Longo, 1992) may still hold. As stated above, a spermatid that has initiated apoptosis may fail to activate its endogenous nuclease activity completely or default in the ligation of nicks in the DNA. The evidence points to an abortive apoptosis taking place in many males that exhibit sperm parameters that are below normal. We hypothesize that, in certain males, this abortive apoptosis appears to fail in the
total clearance of spermatozoa that are earmarked for elimination by apoptosis. Therefore, the subsequent population of ejaculated spermatozoa present an array of anomalies representative of the characteristics observed in cells that are in the process of apoptosis.

Consequences of DNA damage in human spermatozoa

It has been established that there are some anomalies in the DNA of ejaculated spermatozoa. However, the consequences of this DNA damage during fertilization and embryo development are unknown. The increased presence of these anomalies in males with abnormal sperm parameters puts the population of patients being treated by assisted reproductive technologies, in particular ICSI, at the greatest risk.

Whether DNA-damaged spermatozoa can impair the process of fertilization or embryo development is not clear. Studies by Robaire and co-workers have indicated that damage to sperm DNA may be linked to an increase in early embryo death. They showed that treatment of male rats with cyclophosphamide had little effect on the male reproductive system, but resulted in single-strand DNA breaks in the cauda epididymal spermatozoa and altered the decondensation potential of spermatozoa (Qiu et al., 1995a,b). Similar treatment protocols using cyclophosphamide produced an increase in postimplantation loss and malformations (Trasler et al., 1985, 1986, 1987) and were transmissible to the next generation (Hales et al., 1992).

In humans, failed fertilized oocytes injected with spermatozoa from patients with a large number of endogenous DNA nicks in their sperm population contain more condensed spermatozoa (Sakkas et al., 1996). This finding indicates that DNA-damaged spermatozoa selected for ICSI may impede the completion or initiation of decondensation, leading to a failure of fertilization. Lopes et al. (1998) have shown that men with a sperm population containing >25% DNA damage are more likely to experience a fertilization rate of <20% after ICSI. In addition, in humans, ICSI patients have a lower percentage of embryos that form blastocysts when compared with patients undergoing routine IVF (Shoukir et al., 1998).

The use of ICSI has heightened the risk that spermatozoa containing damaged DNA may participate in the development of an infant. Whether spermatozoa possessing damaged DNA will fail in their project to contribute to a viable offspring at the time of fertilization, embryo development or fetal development is not clear.

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**Fig. 3.** The role of Fas during spermatogenesis in men with (a) normal and (b) abnormal (oligospermic, asthenospermic and teratospermic) sperm parameters.
Conclusions

The observation that ejaculated human spermatozoa possess DNA damage raises numerous problems relating to why and how these spermatozoa arise in the ejaculate of some men and what consequences they have if they succeed in their genetic project. The chance of such spermatozoa contributing their damaged DNA to subsequent generations has been heightened with the advent of ICSI, although our understanding of what ensuing problems may arise is poor. The present aim is to further improve our understanding of the molecular basis of male infertility. In doing so, we will need to broaden our knowledge of the effect abnormal spermatozoa have on fertilization and embryo development and avoid the inappropriate use of ICSI.

This work was supported by the Fonds National Suisse and Consiglio Nazionale delle Richerche (CNR, Italy).

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