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The Amoroso Lecture*
The human spermatozoon – a cell in crisis?

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A great deal of evidence has accumulated in recent years to suggest that there has been a gradual increase in male reproductive pathology over the past 30–40 years, as evidenced by increased rates of testicular cancer and declining semen quality. The hypothesis is advanced that this phenomenon is causally related to the ability of male germ cells to generate reactive oxygen metabolites. When produced in low levels, such metabolites are thought to enhance sperm function by stimulating DNA compaction and promoting a redox-regulated cAMP-mediated pathway that is central to the induction of sperm capacitation. When produced in excessive amounts, the same metabolites stimulate DNA fragmentation and a loss of sperm function associated with peroxidative damage to the sperm plasma membrane. Free radical-induced mutations in the male germ line may also be involved in the aetiology of childhood cancer and recent increases in the incidence of seminoma. In light of these considerations, establishing the mechanisms for free radical generation by the male germ line and determining the factors that influence this activity are important objectives for future research in this area.

Introduction

The poor quality of the human ejaculate sets it apart from that of most, if not all, other mammalian species. Even in normal fertile specimens, as much as 50% of the ejaculated sperm population may be abnormally formed and a similar proportion may lack motility. In clinical terms, the impoverished nature of human ejaculate is reflected in the dominant role played by the male factor in the aetiology of human infertility, defective semen quality being the most frequently defined cause of this condition in humans (Hull *et al.*, 1985).

Not only is human semen quality poor, but there is a growing body of evidence that it is getting poorer. The first indication of this came from the meta-analysis of 14 947 normal men from 61 independent centres analysed by Carlsen *et al.* (1992). This study revealed an approximate halving of sperm concentrations (from 113 to 66×10^6 ml^{-1}) from 1938 to 1990. Clearly, this analysis is complicated by a wide range of confounding factors including a lack of standardization in the techniques used to monitor sperm concentration and the influences of age, race, geographical location and ejaculation frequency. Nevertheless, although this analysis has been revisited many times in an attempt to account for such sources of variation, the existence of a negative correlation between sperm concentration and time remains a difficult phenomenon to deny (Swan *et al.*, 1997). Moreover, this general trend has been independently confirmed in a number of separate data sets collected from France, Belgium, Greece, Denmark and the UK (Multigner and Spira, 1997). A careful analysis of semen donors to a sperm bank in Paris, for example, revealed a decrease in sperm counts from 89 to 60×10^6 ml^{-1} from 1973 to 1992 (Auger *et al.*, 1995). After taking into account all potential co-variates (year of birth, age and abstinence time) it was concluded that within this donor population, sperm concentration was declining by 2.6% per year. Similarly, analysis of semen donors in Edinburgh revealed a marked decline in semen quality in birth cohorts sampled over an 11 year period commencing in 1959 (Irvine *et al.*, 1996).

Even if sperm counts are regarded as an arcane and inherently unreliable means of monitoring semen quality, histological analysis of necropsy specimens has concluded that the incidence of men showing normal spermatogenesis has decreased in recent years (Pajarinen *et al.*, 1997). Additional evidence for a sudden change in germ cell function is also indicated by the alarming increase in the incidence of testicular cancer (largely germ cell tumours), which is now thought to be increasing at the rate of 2–4% per year in men under 50 years of age (Adami *et al.*, 1994).

Despite these data suggesting a recent deterioration in human testicular function, nothing is known about the pathological mechanisms involved. This review will consider the evidence indicating that oxidative stress is one of the central features of testicular dysfunction in men. This mechanism is not only an important contributory factor to

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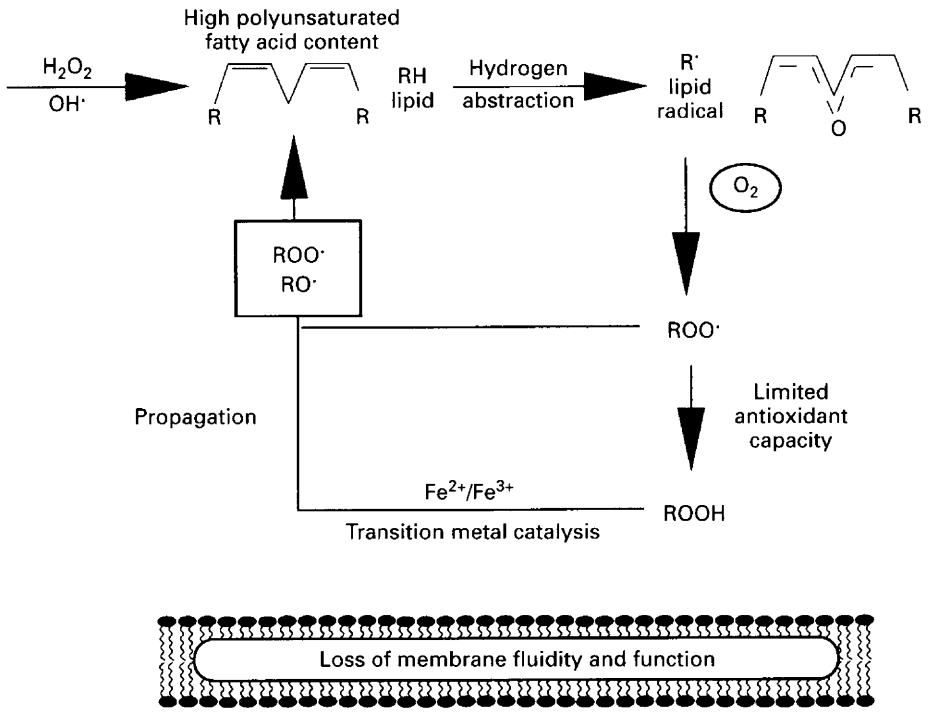

Fig. 1. Lipid peroxidation in human spermatozoa. This process is initiated by a free radical attack on the unsaturated fatty acids (RH) in the sperm plasma membrane leading to the abstraction of a hydrogen atom and the formation of a lipid radical (R•). The latter combines with oxygen to generate a peroxyl radical (ROO•) which is stabilized by conversion to the corresponding hydroperoxide (ROOH) by antioxidants or by abstraction of another hydrogen atom from an adjacent lipid. This creates another lipid radical and propagates the chain reaction. Transition metals such as iron and copper also propagate the peroxidative cascade by catalysing the breakdown of lipid peroxides to form alkoxyl and peroxyl radicals.

the aetiology of defective sperm function, but may also be involved in the induction of testicular cancer, considering the important role that free radicals are known to play in the genesis of neoplastic disease. An additional and unexpected conclusion from these studies is that free radical-mediated damage to the sperm genome may be associated with increased morbidity in the offspring, particularly male infertility involving deletions on the Y chromosome, and childhood cancer.

Oxidative stress and defective sperm function

The susceptibility of human spermatozoa to oxidative stress was first suggested as a cause of male infertility in a landmark paper by Jones *et al.* (1979). This study demonstrated that human spermatozoa contain a high concentration of polyunsaturated fatty acids, particularly docosahexaenoic acid with six double bonds per molecule. These double bonds are necessary to give the plasma membrane the fluidity it needs to participate in the membrane fusion events associated with fertilization. Notwithstanding their functional significance, such highly unsaturated fatty acids are also a potential source of danger to the spermatozoa because of their susceptibility to free radical attack. The latter results in hydrogen abstraction from a methylene group bis-allylic to the double bond and the initiation of a lipid peroxidation cascade. As a consequence of lipid peroxidation, the fatty acids lose their double bonds, the membranes lose their fluidity and the spermatozoa lose their function (Fig. 1).

A direct relationship between the generation of free radicals and defective sperm function was first indicated by studies demonstrating that the competence of human spermatozoa to fuse with the vitelline membrane of the oocyte was inversely proportional to the generation of reactive oxygen species (ROS) (Aitken and Clarkson, 1987). Particularly high ROS generation was observed in defective sperm populations isolated from patients with oligozoospermia, varicocoele and asthenozoospermia (Aitken *et al.*, 1989; Sharma and Agarwal, 1996). This oxidative stress was subsequently shown to originate from two distinct cellular sources, leucocytes and spermatozoa.

ROS production by leucocytes

The polymorphonuclear leucocytes that contaminate every human semen sample are powerful generators of ROS and appear to be spontaneously active in the native ejaculate (Aitken *et al.*, 1994, 1995a). Despite their competence for free radical generation, the presence of contaminating leucocytes

in human semen, even at concentrations exceeding the World Health Organization's threshold of normality of 1×10^6 ml^{-1}, does not appear to correlate with defective sperm function *in vivo* (Torffiinson *et al.*, 1993).

That leucocytospermia is lacking in clinical significance is possibly a reflection of the powerful antioxidant properties of the seminal plasma. This complex biological fluid protects spermatozoa from the flow of reactive oxygen metabolites emanating from the leucocyte population by means of small molecular mass free radical scavengers such as vitamin C and uric acid, thiols such as ergothionine, and ROS-metabolizing enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Jones *et al.*, 1979; Zini *et al.*, 1993; Aitken and Fisher, 1994). The presence of such antioxidants is necessitated by one of the most unique features of spermatozoa, the lack of a substantial cytoplasmic space. Since the cytosol is normally a major site for the storage of antioxidant enzymes, spermatozoa are extremely reliant on extracellular sources of antioxidant protection, both during the storage of these cells in the epididymis and after their release into the ejaculate.

Although leucocyte contamination may not have a major impact on male fertility *in vivo* (Aitken, 1997), these cells do influence fertilization rates *in vitro*. In this situation, spermatozoa are washed free from the protective environment provided by the seminal plasma and resuspended in simple culture media that are generally devoid of antioxidant factors and may even be supplemented with transition metals (milligram quantities of copper and iron in the case of Ham's F10) that promote the lipid peroxidation pathways leading to impaired sperm function (Gomez and Aitken, 1996). It is for this reason that leucocyte contamination of human sperm suspensions has a negative impact on fertilization *in vitro* (Krausz *et al.*, 1994; Sukcharoen *et al.*, 1995). The addition of antioxidants to the culture media would be a rational means of counteracting this source of damage and improving fertilization rates in the context of assisted conception therapy (Aitken *et al.*, 1996; Baker *et al.*, 1996).

ROS production by spermatozoa

It is necessary to remove contaminating leucocytes to detect ROS generation by human spermatozoa. This can be readily achieved with magnetic beads coated with a monoclonal antibody directed against the common leucocyte antigen, CD45 (Aitken *et al.*, 1996). With the aid of this technique, human sperm suspensions can be generated that are effectively leucocyte free. When a suitable agonist such as the 12-myristate 13-acetate phorbol ester (PMA) is added to such cell suspensions, a free radical signal is generated that originates from the spermatozoa (Fig. 2). The intensity of this signal is inversely proportional to the functional competence of the spermatozoa. The median PMA-induced ROS signal produced by the spermatozoa of oligozoospermic patients is around two log orders of magnitude greater than that generated by spermatozoa from fertile donors (Aitken *et al.*, 1992). Intriguingly, the amount of PMA-induced ROS generation by such purified sperm populations is very

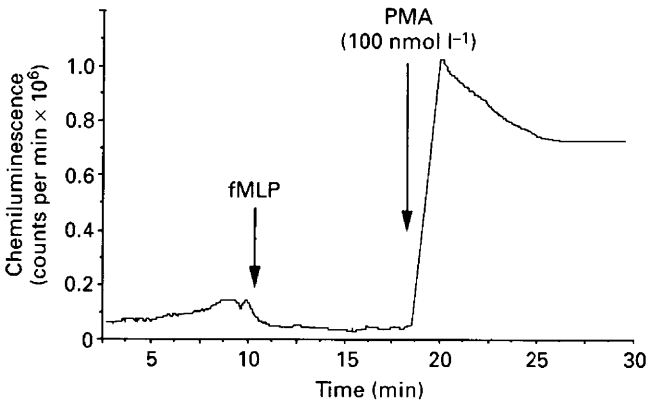

Fig. 2. Induction of reactive oxygen species generation by spermatozoa with 12-myristate 13-acetate phorbol ester (PMA). The sperm suspension was treated with anti-CD45-coated magnetic beads to remove all detectable traces of leucocyte contamination. Leucocyte-specific agonists such as formyl-methionyl-leucyl phenylalanine (fMLP) do not elicit free radical signals, but the addition of PMA stimulates an oxidative burst by the spermatozoa.

tightly correlated with the quality of the original semen sample, as reflected in the movement characteristics and morphology of the spermatozoa as well as sperm count (Gomez *et al.*, 1998). Such results emphasize that the capacity of human spermatozoa to generate ROS in response to PMA is reflective of some underlying defect in the fundamental process of spermatogenesis which impacts upon different attributes of semen quality.

The positive correlations observed between PMA-induced free radical signals and markers of the cytoplasmic space such as creatine kinase and glucose-6-phosphate dehydrogenase, suggest that the link between semen quality and ROS generation lies in the presence of excess residual cytoplasm (Gomez *et al.*, 1996). Thus, when spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa released during spermiation are thought to be immature and functionally defective (Huszar *et al.*, 1997).

The retention of residual cytoplasm by human spermatozoa is positively correlated with free radical generation via mechanisms that are thought to be mediated by the cytosolic enzyme glucose-6-phosphate dehydrogenase. This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn controls the intracellular availability of NADPH. The latter is used as a source of electrons by spermatozoa to fuel the generation of reactive oxygen metabolites by an enzyme system with the characteristics of an NADPH oxidase (Aitken *et al.*, 1997; Richer *et al.*, 1998). Therefore, when the cytosolic space is increased through the retention of excess residual cytoplasm, the resultant greater activity of glucose-6-phosphate dehydrogenase leads to the excessive generation of NADPH and hence to the over-production of ROS. The latter then initiate a lipid peroxidation cascade and precipitate a loss of sperm function (Fig. 3).

The physiological purpose of the sperm NADPH oxidase

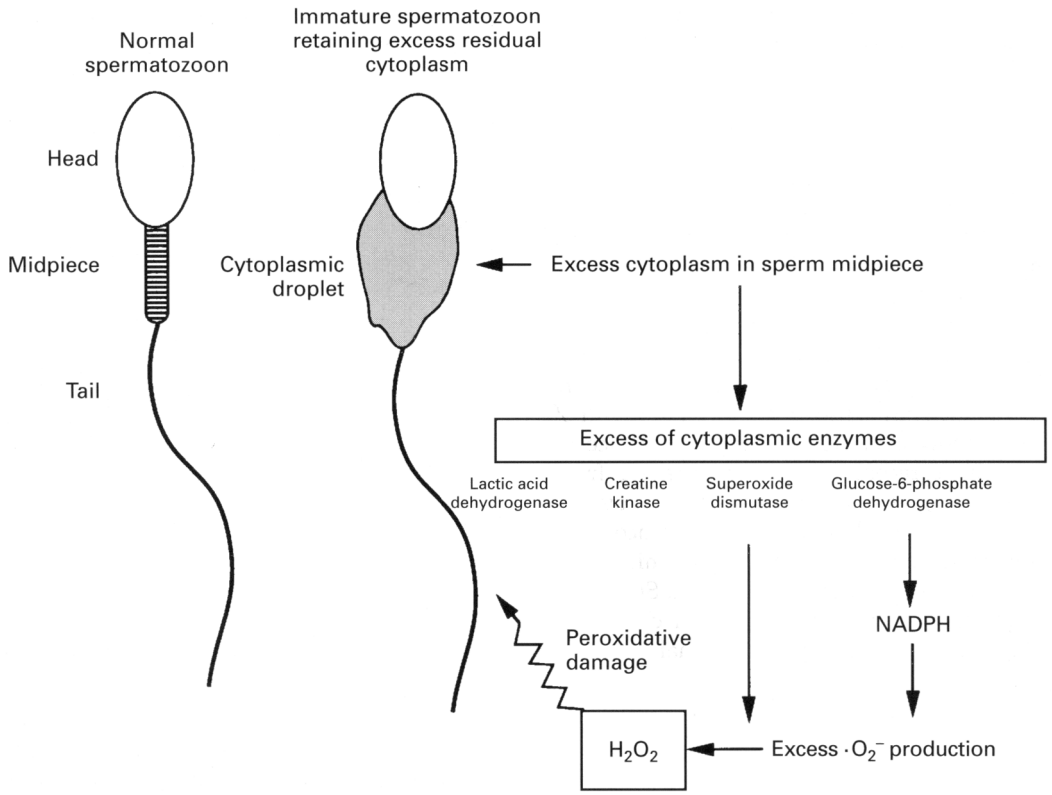

Fig. 3. Proposed mechanism for the increased production of reactive oxygen species (ROS) by defective spermatozoa. Impaired spermiogenesis results in the release of immature defective spermatozoa with excessive amounts of residual cytoplasm. This defect results in the presence of an abnormally high cellular content of many cytoplasmic enzymes including lactic acid dehydrogenase, creatine kinase, superoxide dismutase and glucose-6-phosphate dehydrogenase (G-6-PDH), all of which are positively correlated with defective sperm function. The presence of excessive G-6-PDH stimulates the production of NADPH, which in turn fuels the excessive generation of ROS and the induction of peroxidative damage.

is the control of sperm capacitation (Bize *et al.*, 1991) through the redox regulation of tyrosine phosphorylation (Aitken *et al.*, 1995b, 1998a). The stimulation of tyrosine kinase activity is mediated by a redox controlled increase in intracellular cAMP and the resultant stimulation of protein kinase A (Zhang and Zheng, 1996; Aitken *et al.*, 1998a). The ability of spermatozoa to regulate an intracellular cAMP–tyrosine phosphorylation axis by managing their redox status is a unique signal transduction cascade that has not, to date, been reported for any other cell type.

Oxidative stress and DNA damage

The excessive generation of ROS associated with defective sperm function does not only attack the fluidity and integrity of the sperm plasma membrane, but also attacks the DNA in the sperm nucleus. DNA fragmentation is commonly observed in the spermatozoa of infertile patients and there is strong evidence that this damage is free radical mediated and is induced by oxidative stress (Kodoma *et al.*, 1997; Ni *et al.*, 1997; Sun *et al.*, 1997). The amount of DNA damage observed in human spermatozoa is negatively associated with traditional criteria of semen quality (sperm count, motility and morphology) as well as *in vitro* fertilization rates (Sun *et al.*, 1997; Irvine *et al.*, in press). Fertilization failure in this context is presumably due to collateral peroxidative damage to the sperm plasma membrane, and ensures that spermatozoa with severely damaged genomes cannot participate in the normal process of fertilization. However, this biological safeguard is of limited effectiveness, since studies involving the exposure of spermatozoa to progressively increased oxidative stress have demonstrated that cells with significantly damaged DNA retain a residual capacity for fertilization (Aitken *et al.*, 1998a). Moreover, the use of intracytoplasmic sperm injection (ICSI) as a treatment for male infertility circumvents any peroxidative damage to the sperm plasma membrane. Even if severe oxidative DNA damage is experimentally induced in populations of human spermatozoa by co-incubation with activated leucocytes, exposure to hydrogen peroxide or the excessive stimulation of the spermatzoon's own free radical generating system with NADPH, successful fertilization can still be achieved with ICSI (Twigg *et al.*, 1998).

Consequences of fertilization involving DNA damaged spermatozoa

Cancer

The consequences of fertilizing the oocyte with spermatozoa exhibiting severe oxidative damage to the genome are not known. However, recent data from the analysis of smokers suggest a link between oxidative DNA damage in the spermatozoa and the appearance of neoplastic disease in the offspring. Thus, heavy smoking induces a state of oxidative stress that is associated with free radical-mediated damage to sperm DNA in concert with a decrease in the antioxidant capacity of seminal plasma (Fraga *et al.*, 1996). This DNA damage has consequences for the health of the offspring, who show a particularly high incidence of childhood cancer. In a recent analysis originating from China, paternal smoking was associated with an overall four-fold increased risk of developing childhood cancer (Ji *et al.*, 1997). Furthermore, an independent epidemiological study in the UK, based on records of the Oxford Survey of Childhood Cancers, concluded that 14% of all childhood cancers could be attributed directly to paternal smoking (Sorahan *et al.*, 1997).

Infertility

In addition to childhood cancer, another possible consequence of free radical-mediated damage to DNA in the male germ line is infertility in the offspring. This possibility relates specifically to forms of male infertility involving deletions on the long arm (q) of the Y chromosome. In this non-recombining area of the Y chromosome (NRY), three regions have been identified that contain genes of importance to spermatogenesis; these loci have been designated AZF (azoospermia factor) a, b and c. Deletions in these areas are associated with severe disruptions of spermatogenesis and infertility (reviewed by Roberts, 1998). According to Vogt *et al.* (1996) deletions in each of these areas produce a particular testicular phenotype: deletions in AZFa produce the Sertoli cell only syndrome, AZFb deletions are associated with germ cell arrest at the pachytene stage, and deletions in AZFc generate arrest at the spermatid stage of development. These deletions are not observed in fertile men or in a majority of the fathers of affected patients. Therefore, the Y chromosome deletions leading to male infertility must arise *de novo* in the germ line of the patients' fathers.

The incidence of Y chromosome deletions is around 15% of patients with azoospermia or severe oligozoospermia and 10% in men with idiopathic infertility (Roberts, 1998). Although this is not a particularly high frequency, it should be recognized that more than 90% of the human genome is non-coding and would not produce a phenotype on deletion. Moreover, for most of the genome, homologous recombination could provide a theoretical mechanism for repairing double-stranded DNA deletions on autosomes or on the X chromosome (Liang *et al.*, 1998). However, since the Y chromosome possesses no homologue, this repair mechanism cannot be invoked and deletions on the non-recombining region of this chromosome will persist. Thus for Y chromosome deletions to occur at the frequency observed, there must be an extremely high spontaneous rate of DNA fragmentation in the male germ line, most of which is either undetected or is repaired. However, deletions on the AZF loci of the NRY cannot be repaired and produce an extremely obvious phenotype.

Even if homologous recombination is used to repair double-strand breaks on the autosomes, the allele carried by the gamete will be lost permanently and recombination repair using the allele donated by the oocyte will automatically lead to a loss of heterozygosity (Zhu *et al.*, 1992). Since the latter is known to be a key feature of the mutagenic changes that lead to neoplastic disease, it is possible that activation of the recombination repair pathway after fertilization is at the centre of the association between DNA fragmentation in the spermatozoa and the increased incidence of childhood cancer in the progeny (Fraga *et al.*, 1996; Ji *et al.*, 1997).

Concluding hypothesis

In this brief review a number of lines of evidence have been presented indicating that oxidative stress plays a key role in the aetiology of poor semen quality and male factor infertility. The male germ cell certainly has a capacity for oxygen radical generation that has been traced back as far as the pachytene spermatocyte stage of spermatogenesis (Fisher and Aitken, 1997). In the mature gamete, this system plays an important physiological role in the redox regulation of sperm capacitation via a cAMP-dependent mechanism. A free radical-generating capacity may also be important at earlier stages of sperm differentiation, influencing such events as the thiol-oxidation stages of DNA compaction (Godeus *et al.*, 1997) and regulation of the number of germ cells through the induction of apoptosis or the stimulation of cell division in spermatogonial stem cells. It is even possible that free radical generation by the male germ cell contributes to mutations that are of fundamental biological importance in creating the genetic diversity through which evolution acts (Agulnik *et al.*, 1997).

In contrast to such biologically useful roles for ROS generation by male germ cells, oxidative stress can arise as a consequence of the excessive production of reactive oxygen metabolites and an impaired antioxidant defence system. It is proposed that such oxidative stress precipitates the range of pathologies that are currently thought to afflict the male reproductive system. DNA damage resulting from increased ROS generation could accelerate the process of germ cell apoptosis leading to the decline in sperm counts associated with male infertility and the apparent deterioration of semen quality observed over the past four to five decades. ROS-mediated peroxidative damage to the sperm plasma membrane would also account for the defective sperm function observed in a high proportion of infertility patients. Enhanced oxidative stress in male germ cells could explain the sudden recent increase in the incidence of testicular cancer, considering the important role played by ROS in mediating the mutagenic events that lead to neoplasia.

Moreover, an impact of excess free radical generation on the integrity of the germ cell genome is supported by the oxidative base damage seen in the DNA of spermatozoa from infertile patients, which is inversely correlated with semen quality (Kodoma *et al.*, 1997; Ni *et al.*, 1997). ROS-induced DNA fragmentation could also be an important factor in the aetiology of infertility involving *de novo* deletions in the AZF regions of the NRY. Furthermore, free radical-mediated damage to the sperm DNA is known to be associated with significantly increased risks of various kinds of childhood cancer in the offspring. Such an association is a particular source of concern considering that defective spermatozoa with oxidatively damaged DNA are inevitably being used to fertilize human oocytes during the treatment of infertile patients with ICSI.

If oxidative stress in the male germ line is responsible for infertility and the induction of mutations associated with neoplastic disease, the mechanisms responsible for this pathology need to be elucidated. Excessive free radical generation by the germ cells could be induced by the redox cycling of xenobiotics, increased testicular temperatures, excessive NADPH oxidase activity or the increased availability of transition metals. Oxidative stress could also be associated with impaired antioxidant protection, possibly related to dietary deficiencies, age or genetic factors. Whatever the mechanism, a free radical hypothesis for impaired testicular function is an eminently testable proposal with wide ranging implications for the health and fertility of the human species.

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