SPERM DNA DAMAGE: from Research to Clinic
11–13 March 2009, ROME (Italy)

Program

11 March 2009

15:30 – 17:00  Registration
17:00 – 18:00  Clinical round table
19:00 – 20:30 Welcome party

12 March 2009

I SESSION: Environment, genes and sperm DNA
Moderators: C. Krausz, Italy – R. S. Swerdloff, USA

8:30 Environmental hormones and male reproduction
(J. P. Bonde, Denmark)

9:00 Gene environmental interaction: the impact of persistent organohalogen pollutants on sperm characteristics and genital malformations
(Y. Giwercman, Sweden)

9:30 Cryopreservation of sperm DNA
(S. Lewis, Northern Ireland)

10:00 – 10:30  Coffee break

II SESSION: Epigenetics and DNA damage repair
Moderators: E. Baldi, Italy – S. Krawetz, USA

10:30 Epigenetic control in male germ cells: the chromatoid body as an RNA-processing center
(P. Sassone-Corsi, USA)

11:00 Effect of chemotherapy and folate pathway deficiencies on the sperm epigenome
(J. Trasler, Canada)

11:30 Our genome in the male germ line: is it safe?
(A. Grootegoed, The Netherlands)

12:00 Clinical significance of sperm DNA fragmentation assays
(M. Spanò, Italy)

12:30 – 14:15  Lunch and poster session
III SESSION: Chromosomes and mtDNA
Moderators: R. McLachan, Australia – G. Micara, Italy

14:15 Sperm aneuploidy and ART offspring
   (L. Gianaroli, Italy)

14:45 Y chromosome rearrangements: their cellular origin and clinical consequences
   (C. Krausz, Italy)

15:15 mtDNA and sperm function
   (J. St John, UK)

15:45–16:15 Coffee break

IV SESSION: Epigenetics and sperm DNA
Moderators: P. Sassone-Corsi, USA – M. Stefanini, Italy

16:15 Sperm chromatin packaging and DNA methylation: relevance to ART
   (D. Carrell, USA)

16:45 Defining sites susceptible to DNA damage within the sperm nucleus: the nuclear matrix connection
   (S. Krawetz, USA)

V SESSION: Selected oral presentations (I)
Moderators: F. Lanzafame, Italy – G. Balercia, Italy

17:15 Characteristic histone modifications and timing of histone to protamine switch in Drosophila sperm
   chromatin
   (S. Awe, Germany)

17:30 Persistence of DNA damage and its consequence for mutagenesis in male germ cells of OGG1−/− Big
   Blue® mice exposed to benzo(a)pyrene
   (A.-K. Olsen, Norway)

17:45 Cellular mechanism underlying the effects of paternal acrylamide-exposure on preimplantation development
   in mice
   (S. Shahzadi, Norway)

18:00 Epydidimal glutathione peroxidase 5 contributes to the maintenance of sperm DNA integrity and to
   embryo viability
   (J. Drevet, France)

VI SESSION: Selected oral presentations (II)
Moderators: G. Franco, Italy – G. Morrone, Italy

18:15 Male-to-female sex-ratio is potentially correlated to air pollution levels
   (J. Hallak, Brazil)

18:30 Analytical investigation on TUNEL/PI assay for the determination of sperm DNA fragmentation:
   pitfalls and possible solutions
   (M. Muratori, Italy)
18:45 Impact of environmental exposure to perfluorinated compounds on sperm DNA quality
(L. Governini, Italy)

19:00 Chromomycin A3 staining vs. TUNEL assay: different prognostic value on ART outcome
(M. Nadalini, Italy)

19:15 General discussion

19.15 Social dinner

13 March 2009

I SESSION: Selected oral presentations (III)
Moderators: E. Greco, Italy – G. Colpi, Italy

8:00 Apoptosis and sperm DNA fragmentation in infertile patients with Chlamydia and Mycoplasms
infection
(S. Alvarez, Mexico)

8:15 Inflammatory mediators induce apoptosis in ejaculated spermatozoa in in vitro conditions
(M. Fraczek, Poland)

II SESSION: ROS and DNA damage (I)
Moderators: M. Maggi, Italy – M. Costa, Italy

8:30 ROS induced damage and its clinical significance
(J. Aitken, Australia)

9:00 Diagnostic tests for monitoring scrotal hyperthermia
(A. Ledda, Italy)

9:30 Testicular hyperthermia and the pathways leading to DNA breakage
(C. Wang, USA)

10:00 – 10:30 Coffee break

III SESSION: ROS and DNA damage (II)
Moderators: M. Maggi, Italy – M. Costa, Italy

10:30 Pathways of ROS generation in male germ cells: insights generated by proteomics
(J. Aitken, Australia)

11:00 Genital tract inflammation and its consequences on sperm DNA
(A. Calogero, Italy)

11:30 Antioxidants and sperm DNA damage
(A. Zini, Canada)

12:00 General discussion

12:30 Closing remarks
INVITED ORAL PRESENTATIONS
Environmental Hormones and Male Reproduction

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The discovery in recent years that several widespread industrial and environmental chemicals have weak hormone disrupting properties through a number of different mechanistic pathways have greatly increased public and scientific interest into environmental effects on male reproductive function. Data indicating the increasing occurrence of male reproductive disorders in several countries have further fuelled hypotheses that environmental xenobiotics may cause reduced sperm counts, cryptorchidism, hypospadias and testicular cancer through disruption of endocrine homeostasis. Experimental studies provide remarkable evidence that chemicals may interfere with normal development of the male fetal gonad at rather low exposure levels in the range of environmental exposures in humans if exposure takes places during specific critical time periods of development. An early example was the demonstration of strongly reduced sperm counts in rodents that were exposed to dioxins at extremely low levels (64 ng/kg) once during pregnancy [Mably et al. 1992]. The mechanism, however, is not known and is not necessarily related to endocrine disruption. Phthalate esters are widely used in plastics and are, in spite of rapid metabolism and excretion, detectable in urine in the majority of people in affluent countries. Testicular effects of certain of these widely spread compounds have been known for many years but were not considered a major problem because effects were only seen at high exposure levels (g/kg/day). New studies of effects following in-utero exposure have changed this assessment [Foster 2006]. In-utero exposure to, in particular, di-butyl, di-(2-ethylhexyl) and butyl benzyl esters produce reproductive tract anomalies resembling disorders of testicular mal-development in humans including malformations of external genitalia, cryptorchidism and testicular injury [Skakkebaek 2003]. Critical to the induction of these effects is a substantial reduction in fetal testicular testosterone production, which is mediated by changes in gene expression of a number of enzymes in the fetal Leydig cell. Swan et al. [2005] provided the first evidence of an association of phthalate exposure of mothers during pregnancy and attenuation of androgenic action in their male babies. Reduction of AGD in rats occurs after dosing the pregnant mothers with phthalates at concentrations above 250 mg/kg/day [Mylchreest et al. 2000], while urine phthalate metabolite concentrations in humans are within the range of 3–40 microgram/l corresponding to in the range of three orders of magnitude lower concentrations [Marsee et al. 2006]. The question is, therefore, whether humans are much more susceptible to the action of phthalates than rodents [Sharpe 2005]. So far findings have not been independently replicated in epidemiological studies but anogenital distance was not reduced following high maternal exposure to the known anti-androgen p,p-DDE in a study performed in Mexico [Longnecker et al. 2007].

A review of more than 80 published studies addressing pregnancies with deviant hormonal profiles because of maternal obesity, twinning, low or high birth order, preeclampsia, severe nausea or treatment with hormonal active pharmaceuticals did – with the possible exception of testicular cancer – not provide evidence of links between these conditions and male reproductive tract anomalies in offspring [Storgaard et al. 2005]. For example, sperm count and other indicators of semen quality was not reduced in twin brothers in comparison with single-born brothers in spite of much higher levels of free estradiol during all weeks of gestation in twin pregnancies. It seems therefore unlikely that environmental xeno-estrogens are important factors in causing male reproductive tract abnormalities unless exposures are extremely high, dose-response relationships are non-monotonic (for instance U-shaped) or mixtures of xeno-estrogens have strong synergistic effects.

Few epidemiological studies that directly address male reproductive effects of endocrine disrupting chemicals are now emerging. A large European study addressing the impact of biopersistent organic pollutants (POPs) on
human reproductive function included interview and biological data from some 2300 women and their spouses in four regions spanning large contrasts in exposure to polychlorinated biphenyls (PCBs) and 1,1-dichloro-2,2-bis-(p-chlorophenyl)ethylene (p,p′-DDE). Indications were found that these compounds may interfere with male reproductive function, but apparently without major impact on biological fertility in these populations [Bonde et al. 2008]. For example, high PCB serum concentrations were related to decreased sperm motility and sperm chromatin integrity but not in any consistent way to time taken to conceive. Although findings in this large study are rather reassuring, effects of exposure during critical windows during development are not addressed.

Epidemiological studies that explicitly examine male reproductive tract anomalies following pre- or perinatal exposure to specified endocrine disrupting compounds are few. Follow-up studies of residents that were exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) after the Italian Seveso accident in 1976 have shown reduced sperm counts and sperm motility in adults that were 1–9 years of age when exposed while opposite effects were seen if exposure took place during puberty 10–17 years of age [Mocarelli et al. 2008]. Other persistent organochlorine chemicals as DDT and its metabolites have been associated with increased frequency of cryptorchidism and or hypospadias in few studies [Fernandez et al. 2007] while the majority of studies so far have provided little support of an association [McGlynn et al. 2006; Pierik et al. 2007; Longnecker et al. 2002; Bhatia et al. 2005]. Similarly, occupational exposure to endocrine disrupting chemicals including phthalates have not shown a relation to hypospadias or cryptorchidism [Vrijheid et al. 2003].

In conclusion, there is increasing but still limited evidence to indicate that environmental endocrine disrupting chemicals are contributing to the increase of male reproductive tract disorders. Studies with strong exposure contrasts linking documented levels of exposure in specified pre- and perinatal time windows with reproductive tract anomalies are highly warranted.

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Gene Environmental Interaction: the Impact of Persistent Organohalogen Pollutants on Sperm Characteristics and Genital Malformations

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Several observations suggest that male reproductive health has been declining in many countries. The incidence of testicular cancer and congenital malformations such as hypospadias and cryptorchidism has been increasing, whereas semen quality has been decreasing [Sharpe and Skakkebaek 2008]. Because these changes have occurred over a short time span, it could be argued that they only reflect adverse changes by exogenous agents in the environment that modulate endocrine signaling pathways by interfering with the synthesis, secretion, binding, action, or elimination of natural hormones. Agents of concern are primarily persistent organohalogen pollutants (POP) that activate or inhibit estrogen receptor and androgen receptor induced responses.

The androgen receptor (AR) mediates all androgen dependent processes. Mutations in the AR gene have occasionally been identified in subjects with infertility or severe hypospadias, but are not frequently found in these conditions. Consequently, decreased AR activity caused by milder variations in the AR gene, i.e. polymorphisms, are more likely to affect the phenotypic outcome. The AR gene contains two polymorphic sequences; a glutamine repeat and a glycine repeat commonly referred to as the CAG and the GGN repeat, respectively. Since both repeats are of importance for AR activity, it could be hypothesized that some individuals could be more susceptible or in contrary more resistant to adverse POP-effects based on variations in the CAG or GGN repeats, i.e. an interaction between genetic and environmental factors might be the underlying mechanism behind the disorders mentioned above.

In 2002–2006, an EU-financed project under the acronym INUENDO (www.inuendo.dk) was conducted. The study had an objective to study markers of reproductive function in European populations with varying POP exposure. The study cohort consisted of Swedish fishermen from the polluted east coast; pregnant women as well as their partners recruited among Greenland Inuit, with one of the highest body burdens of POP in the world; Ukrainians, the exposure being due to use of pesticides; and a Polish cohort, where an average European exposure level was expected. Exposure levels were assessed by measuring serum concentrations of CB-153 and \( p,p'-\text{DDE} \). Of specific interest was to investigate whether the polymorphisms in the androgen receptor gene modify the effect of POP exposure on human sperm characteristics i.e. volume, sperm concentration, total sperm count, proportion of progressively motile sperms, sperm DNA integrity and morphology in mentioned populations. The proportion of \( Y \)-chromosome bearing sperms was also assessed. Another aim was to investigate whether POP could also disrupt male sex differentiation.

A short CAG repeat (<20) in combination with high POP exposure was in the men enrolled in the INUENDO study associated with approximately 40% lower sperm concentration and total sperm counts compared to other CAG numbers [Giwercman et al. 2007]. Similar effects were seen in relation to sperm motility and DNA integrity as assessed by Sperm Chromatin Structure Assay, demonstrating that the AR CAG repeat length modifies the susceptibility of an individual to the adverse effects of POP exposure on semen quality. A statistically significant interaction was also found between \( p,p'-\text{DDE} \) and the CAG repeat in relation to the proportion of \( Y \)-bearing sperms [Tiido et al. 2007]. This interaction was even more pronounced in subjects
carrying a short CAG repeat in combination with a G-allele in their dioxin receptor repressor gene. The endocrine disrupting action of POP in relation to observed changes in sperm Y:X ratio may thus be modulated by genes involved in sex steroid and dioxin-mediated pathways.

Furthermore, by utilizing the birth register of Greenland, 11,076 live male births during 1982–2002 were identified [Giwercman et al. 2006]. Through a local register on congenital malformations all reported cases of hypospadias were traced. Only two cases of hypospadias were identified in Greenland during the actual period, corresponding to an incidence of 2:10,000, which is approximately 10 times lower than among Swedish boys, who have an incidence of 20:10,000 (www.icbd.org). Interestingly, we also found that 85% of the population in Greenland carried the androgen receptor GGN23 genotype. In vitro, the ability of the GGN23 variant to activate a reporter gene was superior compared to other lengths [Lundin et al. 2007], indicating that this variant could, in combination with other factors of relevance, be predisposing to the remarkably low incidence of genital malformations observed in the Inuit population. Another possibility is that GGN23 directly modifies the anti-androgenic effects of endocrine disrupters, as was shown for the CAG repeat on sperm production.

In summary, the AR CAG and GGN repeat length may modify the adverse effects of POP on male reproductive function. This gene-environment interaction may either be direct between the chemical and the AR or could also include other hormone regulating pathways.

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Cryopreservation and Sperm DNA

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Sperm cryopreservation is routinely used in a variety of circumstances including assisted reproduction, pre-radiation or chemotherapy treatment, as ‘fertility insurance’ for men undergoing vasectomy and for storage of donor semen until seronegativity for HIV and hepatitis is confirmed. It is also used for storage of sperm retrieved from azoospermic patients who have undergone testicular sperm extraction or percutaneous epididymal sperm aspiration to prevent the repetition of invasive biopsies. Cryostorage of testicular and/or epididymal sperm can, in theory, ensure sperm are available for multiple ICSI treatments from a single biopsy.

Studies investigating the effects of cryopreservation have largely been limited to conventional parameters. Reports include changes in sperm morphology including damage to mitochondria, the acrosome and flagellum. Unfortunately, therefore, the proportion of fully functional sperm that retain intact membranes, tail and mitochondrial activity after freeze-thawing is low. Sperm motility has also been shown to be particularly sensitive to such damage but, while it is generally accepted that sperm motility is reduced by cryopreservation, the mechanism by which this occurs is, as yet, unclear. However, since up to 40% of sperm are still motile post thaw, these sperm have been used clinically and little work has been performed to improve procedures. Despite many advances in animal cryobiology the success of protecting human sperm fertility potential has changed little, perhaps because studies determining cryoinjury and strategies for its limitation to more meaningful diagnostic parameters such as DNA quality are scarce.

Recently there has been much debate as to the usefulness of conventional semen analysis. It has been proven variable and lacking in prognostic value so, over the past decade, more useful molecular fertility biomarkers have been explored. Amongst the tests showing most promise in predicting the successful treatment of male infertility patients are those measuring sperm DNA quality. Sperm DNA damage has been demonstrated to have amongst the lowest variability of all semen parameters thus giving it an excellent credential as a diagnostic criterion in the clinical assessment of semen quality. It has also has been closely associated with numerous indicators of fertilization, embryo quality, implantation, spontaneous abortion and childhood diseases so it has great potential as a prognostic test for assisted conception when couples are presenting with male infertility. Sperm DNA is particularly susceptible to damage due to its high content of polyunsaturated fatty acids acting as substrates for reactive oxygen species (ROS) and also because of its lack of repair mechanisms. This also makes it a sensitive marker for cryoinjury and a potential area for cryo-improvement with addition of antioxidants.

In this session, a background to cryopreservation including its history will be given and the milestones in freezing sperm at different stages of maturity will be examined. Effects of cryopreservation on nuclear and mitochondrial DNA will be discussed. Success rates for assisted conception cycles for fresh and frozen testicular and ejaculated sperm will also be reviewed. Finally, the latest advances and challenges such as cryobanking testicular tissue for prepubertal boys and testicular stem cell transplantation will be explored.
Epigenetic Control in Male Germ Cells: the Chromatoid Body as an RNA-Processing Center

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The control of germ cell differentiation utilizes a complex and unique epigenetic program that involves specialized histone variants, the histone-to-protamine transition and key histone post-translational modifications. In addition, a highly specialized control of gene expression both at transcriptional and translational levels exist in spermatogenesis, ensuring the completion of the complex differentiation program that insures the production of haploid germ cells and thus the maintenance of the species [Kimmins and Sassone-Corsi 2005]. During the differentiation program of male germ cells there is a time lag of 4–5 days post-meiotically during which transcripts are stored and untranslated. The chromatoid body (CB) is a cytoplasmic structure of male germ cells that has attracted the attention of many researchers in the past 30 years, although its composition and function remained obscure [Kotaja et al. 2006a]. It is a cloud-like structure that moves rapidly in the perinuclear region of the cytoplasm and that resembles the nuage in Drosophila. Recent findings have highlighted the role of CB in storing and processing of haploid gene products by demonstrating that CBs are composed by elements of the microRNP complexes – including Dicer, Ago proteins and microRNAs – thus operating as intracellular platforms of the microRNA pathway [Kotaja et al. 2006b]. In addition, CBs have been shown to resemble somatic P-bodies that act as sites for both microRNA and RNA decay pathways. There is also new information about possible mechanisms governing the movements of the CB, and transport of RNA from the nucleus to the CB, as well as about the factors required for the CB formation [Kotaja and Sassone-Corsi 2007].

On the basis of the available information, we suggest a model in which pre-miRNAs transported to the cytoplasm are loaded through nuclear pores to the chromatoid body, where male germ cell specific RNA-binding proteins MIWI and MVH, as well as miRNP components, such as Ago proteins and Dicer, are localized. KIF17b, a germ cell specific kinesin [Kotaja et al. 2006b; Macho et al. 2002], functions as a shuttle transporting RNAs. Thus, the chromatoid body acts as a subcellular platform for components of the miRNA pathway, centralizing the miRNA post-transcriptional control system in the cytoplasm of haploid male germ cells. Since both miRNA and RNA decay pathways are located in the CB, it may function as a sorting center for mRNAs [Nagamori and Sassone-Corsi 2008].

These findings constitute a major stride towards the understanding of the phenomenon of spermatogenic translational repression and reveals, for the first time, the physiological role of the CB. On the basis of all the available information, we are able to propose a model of the CB function and involvement of miRNA pathway in the posttranscriptional regulation of gene expression in haploid male germ cells [Nagamori and Sassone-Corsi 2008].

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Effects of Chemotherapy and Folate Pathway Deficiencies on the Sperm Epigenome

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The term ‘epigenetics’ refers to heritable non-sequence based mechanisms that regulate gene activity. Three main types of mechanisms, including DNA methylation, small RNA species and histone modifications have been associated with the epigenetic silencing of genes. To date, the most well studied DNA modification associated with the modulation of gene activity is methylation of cytosine residues within CpG dinucleotides occurring at about 20–30 million sites throughout the mammalian genome. DNA methylation plays a role in regulating genes during development and has been implicated in gene regulation, genomic imprinting (variation in the expression of a gene according to its maternal or paternal origin), and X inactivation. Abnormalities in DNA methylation are associated with perturbations in growth, placental function and neurobehavioral processes, as well as carcinogenesis.

Methylation of DNA is initiated in the germline and catalyzed by DNA (cytosine-5)-methyltransferases (DNMTs). Gametic methylation patterns differ between oocytes and sperm and are further modified during early embryo development. In the male, the bulk of gamete methylation is initially acquired during germ cell development in the fetal testis; DNA methylation patterns in male germ cells continue to be remodeled postnatally during the transition from spermatogonia to pachytene spermatocytes. Recent results in mouse and human indicate that genome-wide methylation patterns are unique in sperm as compared to the patterns in somatic tissues. The expression of the DNA methylating enzymes, the DNMTs, is highly regulated during prenatal and postnatal male germ cell development. Gene-targeting experiments in mice have revealed critical roles for the DNMT enzymes, especially for DNMT3A and DNMT3L, in establishing methylation on imprinted genes, repeats and other sequences in the male germline as well as in meiosis.

In the field of male-mediated developmental toxicity, adverse effects on the offspring in animal studies often occur at levels too high to be accounted for by mutagenesis, leading to the suggestion that alternative mechanisms, including epigenetic processes, may be affected. Recent studies have reported that male-mediated effects on the progeny can be passed across generations and have implicated epigenetic mechanisms. In addition, a number of recent studies have linked the use of assisted reproductive technologies with growth and genomic imprinting disorders in children; epigenetic processes appear to be involved as the imprinting disorders found were associated with DNA methylation abnormalities. However, in the latter studies it was unclear whether the birth defects were related to the underlying infertility or the treatments (i.e. ICSI, superovulation, culture conditions) being used. A number of studies have reported DNA methylation abnormalities in the sperm of men with infertility. Since DNA methylation events and enzymes are well conserved across mammals, the rat and mouse have served as excellent models relevant to human studies. To address how alterations in DNA methylation affect spermatogenesis and subsequently embryogenesis, we and others have used pharmacological and genetic approaches. Treatment of male mice with the demethylating cytidine analogue 5-aza-2'-deoxycytidine, a drug used in anticancer regimens, resulted in decreases in fertility and sperm counts and increases in early embryo loss associated with decreases in the methylation of sperm DNA. In current studies we are examining the effects on the sperm epigenome of combination chemotherapy used to treat testicular cancer. In another model of methylation deficiency, mice deficient in the folate pathway enzyme methylenetetrahydrofolate reductase (MTHFR) had reduced germ cell numbers and fertility that were
ameliorated by supplementation with the methyl donor, betaine. The latter studies have important clinical implications since folate and multivitamins are used in the treatment of infertility in men and deficiencies/polymorphisms in folate pathway enzymes are common.

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Our Genome in the Male Germ Line: is it Safe?

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Our genome is being damaged in many ways, due to a variety of internal and external factors. DNA would not be able to serve as a trustworthy storage of genetic information, in the absence of mechanisms for DNA repair. Such mechanisms have developed early in evolution, and mouse and human make use of repair proteins that are very similar to orthologs encoded by bacterial and yeast genomes.

Most mistakes made by the replicative DNA polymerase, if not corrected by its proofreading activity, are repaired by mismatch repair (MMR). Repair mechanisms for other types of damage include nucleotide excision repair (NER), and repair of DNA double strand breaks (DSBs) by an error-prone end-joining (EJ) mechanism or a much more error-free homologous recombination (HR) machinery. Postreplication repair, also known as DNA damage bypass, allows cells to continue DNA replication over a damaged template, for later repair.

On the one hand, one would expect that DNA repair mechanisms act full speed to maintain germ line genome integrity, so that our gametes would contain a painstakingly accurate copy of each parent’s haploid genome. On the other hand, a 100% fidelity of parental DNA transmission to the offspring would limit genetic diversity among generations, which would not be optimal from an evolutionary point of view.

Spermatogenesis seems ill-equipped to safeguard our DNA. There is a high number of mitotic divisions of spermatogonial cells, and this number is also age dependent. When spermatogonia enter the meiotic prophase, they may already contain quite a lot of mutations which have escaped MMR. Then, in meiotic prophase, DSBs are generated by the enzyme SPO11. This active introduction of DSBs is a risky undertaking, but the error-prone EJ repair mechanism is suppressed, and DSBs are repaired by HR between non-sister chromatids of homologous chromosomes. This results in either gene conversion or crossovers. From the approximately 300–400 DSBs that are induced by SPO11, only around 25 per mouse spermatocyte nucleus are converted into crossovers, leading to meiotic recombination.

Meiotic recombination, linked to sexual reproduction, has evolved thanks to the fact that proteins acting in DNA repair pathways were successfully recruited to play a specific role in meiotic prophase. Proteins involved in HR and MMR in somatic cell types, are also engaged in meiotic recombination. Gametogenesis uses DNA repair proteins for germ line specific demands.

In male meiotic prophase, the highly heterologous X and Y chromosomes become heterochromatic and transcriptionally silenced. This meiotic sex chromosome inactivation (MSCI) is evoked by a mechanism for meiotic silencing of unsynapsed chromatin (MSUC). Being embedded in a special chromatin domain, X and Y may be prevented from triggering any checkpoints detecting unsynapsed chromatin, and from undertaking faulty recombinations. The XY body shows signs of the presence of DSBs and their repair by HR (for example presence of RAD51 foci). We also find accumulation of several proteins from DNA damage bypass mechanisms, including UBE2B and RAD18. These proteins act in protein ubiquitination, with UBE2B as a ubiquitin-conjugating enzyme and RAD18 as a ubiquitin ligase. Loss of UBE2B activity in a mouse Ube2b knockout results in an increased number of autosomal crossovers, and enhanced expression of several X-chromosomal genes in spermatids. This latter observation suggests a role of UBE2B in post-translational modifications of chromatin proteins related to the XY body. Such modifications may concern MSUC, or DNA repair, or both. In view of possible interactions between repair mechanisms, damage bypass proteins might promote HR repair of DSBs for the heterologous regions of the X and Y chromosomes.

After completion of the meiotic divisions, the haploid genome of spermatids undergoes the histone-to-protamine transition, which needs DNA topoisomerase II activity to eliminate DNA supercoils. If this would lead to an excess
of DSBs, these cannot be repaired by HR in an error-free manner, in the absence of a sister chromatid in the haploid spermatid nucleus. The histone-to-protamine chromatin reorganization process therefore would have to rely on very accurate functioning of the DNA topoisomerase II machinery, to make and seal transient DSBs.

Taken together, we are dealing with a trade-off: germ line specific advantages of the use of DNA repair proteins and mechanisms are intrinsically coupled with an increased chance for introduction of genetic and epigenetic defects. However, to some extent, we can count on repair of DNA damage contained in our gametes in the early embryo. If that fails, or if problems come up that cannot be repaired or reprogrammed, embryo lethality is a last line of defense before congenital defects will occur.
Clinical Significance of Sperm DNA Fragmentation Assays

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New assays for male factor idiopathic infertility would be clinically useful because the conventional semen analysis is not accurate enough to predict probability of conception. The most promising assays are those measuring in situ sperm DNA integrity. These assays are finding application to evaluate the individual fertility potential during assisted and natural conception or in those situations where the sperm DNA integrity is at stake because of iatrogenic, occupational, and environmental stressors. The integrity of the genetic material is a prerequisite for normal fertilization and transmission of paternal genetic information. Finely tuned differentiation steps of the male germ cell line, during its active lifetime, ensure this goal and any derailment is thought to be crucial, especially during spermiogenesis when repair systems fade leaving DNA more vulnerable. There is a large body of literature on paternally transmitted genetic damage in rodents associated with pregnancy loss, developmental and morphological defects, infant mortality, infertility, and genetic diseases in the offspring, including cancer.

The dominant lethal assay, widely used in toxicological testing, relies on the principle that paternal exposure to genotoxic agents can overwhelm the oocyte repair capabilities impacting on the viability of the embryo and fetus. Epidemiological evidence also links paternal exposure to occupational or environmental agents with an increased risk of abnormal reproductive outcomes. Causes of sperm DNA damage are numerous, and not all mechanisms are known. Multiple (testicular and extra-testicular) sources have been proposed including abortive apoptosis, abnormal chromatin packaging during the transition from round to elongated spermatids, and oxidative stress. Among the types of genetic defects that may be transmitted through sperm (whole- and segmental-chromosomal aneuploidies, mutations, trinucleotide repeat-length variations, defects in the imprinting profiles) we will restrict the analysis to unspecific DNA breaks.

Sperm DNA damage detection has been addressed by using different approaches, encompassing direct tests that work on the site of strand breaks (i.e., the TUNEL – Terminal deoxunucleotidyl transferase mediated dUTP Nick End Labeling – and the single cell gel electrophoresis Comet assays) and indirect tests that exploit nucleic acid denaturability associated with DNA strand discontinuity (i.e., SCSA – Sperm Chromatin Structure Assay – and SCD – Sperm Chromatin Dispersion – tests). Notably, these tests can be performed directly on a semen sample, once routine measures have been obtained, thus complementing the information extracted from standard semen quality analysis. A few studies compared alternative assays, suggesting only a moderate level of correlation possibly reflecting the differences in protocols and in the parameters supposedly measured by each assay. Their application has shown that sperm DNA damage can be associated with reduced rates of fertilization in vivo, by natural conception or intrauterine insemination. Less unequivocal information exists regarding the link between DNA strand breaks and in vitro fertility. Unexplained recurrent pregnancy loss has also been suggested to be associated with a variety of DNA anomalies including sperm DNA fragmentation and the abortion rate was found to be higher in apparently fertile couples where the partner’s sperm had poor chromatin quality. So far, the SCSA has demonstrated to be the most clinically relevant assay, as a threshold for impaired fertility has been consistently shown at $\geq 30\%$ DFI (DNA Fragmentation Index) for both spontaneous
pregnancies and assisted conceptions \textit{in vivo}. ICSI is advised when DFI is above cut-off levels. More variable thresholds have been derived by using the TUNEL and COMET assays, probably related to different protocols used. In spite of these insights, the inclusion of sperm DNA integrity tests into the andrological practice is under scrutiny. Criticism claims that current data are only sufficient to suggest rather than demonstrate an association between sperm DNA damage and ART (Assisted Reproductive Technologies) outcomes and there are not enough data to prove the clinical value of DNA integrity analysis in infertility assessment. If tests become more standardized, accessible, and reliable, possibly after large multinational trials, the prospect of their routine use in clinical practice will become more likely. Obviously, the predictive value of each DNA fragmentation test can be affected by several factors associated with the DNA damage itself, which need to be elucidated. These include the discrimination between single strand breaks vs. the most dangerous double strand breaks, the level of DNA fragmentation per spermatozoon, whether DNA damage affects coding or regulatory sequences, and the ability of the oocyte and of the embryo to repair sperm DNA damage. Resolving the nature of sperm DNA lesions will be an important step toward understanding the aetiology of this pathology and rational strategies could be developed for the clinical management of this condition.
Sperm Aneuploidy and Art Offspring

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According to the data derived from the analysis of abortuses in the general population, aneuploid pregnancies are maternal in origin in 90% of cases. Besides the hypothesis stating that trisomies of paternal origin could be less viable compared to those carried by the oocyte, the possibility that the situation could be different in cases of severe male factor infertility, including azoospermia, whose treatment by ICSI completely bypasses any mechanism of natural sperm selection should be considered.

In the attempt of answering this query, studies on numerical and structural chromosomal abnormalities on sperm were performed by multicolor fluorescence in situ hybridization (FISH) with the aim of verifying whether male factor sperm samples could be exposed to an increased incidence of aneuploidy compared to the normospermic population.

According to the data obtained from the study of 672 samples from infertile patients which underwent a FISH test for nine chromosomes (XY, 13, 15, 16, 17, 18, 21 and 22), an inverse correlation between sperm indices and the incidence of numerical chromosome abnormalities was reported. The highest frequency of aneuploid cells was detected in samples belonging to severe OAT (3.3%) and TESE (4.1%) compared to MESA (2.3%), moderate OAT patients (1.7%), and normospermic samples (1.2%). Nevertheless, when compared with female gametes, the proportion of aneuploid sperm cells was generally low even in pathological samples, where it reached the highest figure in testicular spermatozoa (17.7%) followed by severe OAT (6.1%). The analysis of aneuploidy for the tested chromosomes, showed different frequency of variation with the highest values for the gonosomes followed by the chromosomes 21, 22, 17 and 13. Most samples with a significantly higher incidence of aneuploidy presented aneuploidy events involving four chromosomes suggesting that aneuploidy is not randomly distributed. These findings suggest that aneuploidy derives from alterations of the meiotic spindle, in which each chromosome is allocated to a settled place.

Whether the high incidence of aneuploidy in sperm cells from severe male factor patients could have implications in the clinical outcome of the generated pregnancies is still to be determined. However, independent data suggest that this could be the case. First of all, an increased incidence of de-novo chromosomal abnormalities in the children born after ICSI has been reported with a notable rise in sex chromosome aneuploidy (1.6% vs. 0.45% in the normal population). Secondly, the chromosomal analysis on blastomeres biopsied from preimplantation embryos are in agreement with the data from FISH on spermatozoa and shows that the incidence of chromosomal abnormalities increases proportionally to the severity of the male factor condition. Therefore, the possibility exists that the introduction of ICSI could lead to recalculating the contribution of spermatozoa to the generation of aneuploid embryos.

For this reason, having information on the chromosomal status of the injected spermatozoon could be of great importance in determining the viability of the resulting embryo. As the majority of the techniques used for this purpose are highly invasive and cannot be used to select spermatozoa for ICSI, specific strategies have been investigated that could assist in the identification of the most viable spermatozoa without affecting their viability. In view of these considerations and to evaluate the organelle organization in both compartments of the sperm head, nucleus and acrosome, polarization microscopy has been applied to the ICSI technique as a novel tool for sperm selection based on the properties of birefringence in sperm cells. This strategy improved clinical outcome after ICSI for extremely severe male factor infertility and azoospermia for which testicular spermatozoa were used. The take-home baby rate was especially relevant when using birefringent cells
(29.5% per pick-up) and this was due to an increased implantation rate (24.3%) in concomitance with a low abortion rate (10%).

In order to better investigate the reasons for the resulting clinical outcome, sperm samples were both analyzed at the polarizing microscope for the proportion of head birefringent spermatozoa and tested for the aneuploidy of the 9 chromosomes by FISH. According to the results, the proportion of birefringent spermatozoa varied proportionally to sample concentration, vitality and motility, while there was an inverse correlation between the frequency of aneuploidy and either the proportion of total birefringent spermatozoa ($R \pm 0.56$) or the proportion of motile birefringent spermatozoa ($R = -0.59$). In other words, the quality of sperm samples is i) directly correlated with the incidence of abnormalities in their protoplasmic compartment, as measured by the proportion of birefringent spermatozoa, and ii) inversely correlated with the predisposition to chromosomal errors that increase proportionally with the severity of the male factor condition. It can be concluded that the selection of birefringent spermatozoa increases the chances of identifying a vital sperm cell having a normal chromosomal complement.
Structural polymorphism is increasingly recognized as a major form of human genome variation, and is particularly relevant on the Y chromosome. The structure of the Y chromosome predisposes it to deletions, segmental duplications and to copy number variations. Three regions on the Y chromosome, called AZF (Azoospermia Factor) regions (AZFa, AZFb, AZFc) contain genes involved in spermatogenesis [Skaletsky et al. 2003]. Their complete removal following deletions causes impairment of spermatogenesis and therefore they are considered clear cut causes of spermatogenic failure [Krausz and Degl’Innocentini 2006]. Two other structural variations are known on the Y chromosome with potential effect on spermatogenesis: i) partial AZFc deletions/duplications, and ii) the copy number variation of the TSPY cluster.

Among partial AZFc deletions the gr/gr deletion has been reported as a genetic risk factor for impaired sperm production [Repping et al. 2003]. In the largest study population (n = 1,061) analyzed to date, we found an OR = 7.9 (95% CI 1.8–33.8) [Giachini et al. 2008]. Given that the number of genes removed by the gr/gr deletion is half that of the classical AZFc deletion its effect on spermatogenesis seems to be milder, and thus we should consider it as a co-factor for spermatogenic impairment with variable penetrance. On the other hand its pathogenic effect may also be related to distinct Y-linked or non-Y genetic factors. We have carried out a multicenter study to investigate the contribution of Y-chromosomal factors to the extensive and puzzling phenotypic variation exhibited by gr/gr deletion carriers, which ranges from normal spermatogenesis to azoospermia [Krausz et al. 2008]. The factors examined included both the known AZFc structural variants associated with this deletion – removal of different DAZ and CDY1 gene copies, deletion followed by duplication – and the more general Y chromosome background. We found significant geographic differences in the distribution of deletion subtypes which may affect the outcome of case control association studies in different geographic areas. However, the phenotypic variation of gr/gr carriers in men of European origin seems to be largely independent of the Y-chromosomal background.

Homologous recombination between AZFc amplicons can generate, other than partial AZFc deletions, also partial AZFc duplications, which may occur among different amplicons. In a recent study on a Taiwanese population, Yen and her group found an association between AZFc partial duplications and male infertility [Lin et al. 2007]. The authors suggested that some AZFc gene would be dosage sensitive and their increased expression may interfere with normal spermatogenesis. This finding was not confirmed in a large study population of Italian origin [Giachini et al. 2008].

The testis-specific protein Y-encoded (TSPY) gene is the putative gene for the gonadoblastoma locus on the Y chromosome (GBY) that predisposes dysgenetic gonads of intersex patients to gonadoblastoma development. An overexpression of the TSPY protein has been reported also in seminoma. The TSPY protein is involved in germ cell proliferation and/or differentiation and its role in spermatogenesis is plausible. It is expressed in early gonocytes in prenatal and postnatal testes and spermatogonia and, to a certain extent, round spermatids of adult testis. The TSPY gene is a tandemly repeated gene on the short arm of the human Y chromosome and is organized in a so called TSPY array which contains a variable number of gene copies. We addressed the role of copy number variation in male infertility. Using a validated method against the gold standard method and avoiding selection biases (matching for Y chromosome haplogroup distribution in cases versus controls) our data
show that the mean copy number of infertile men is significantly lower than that found in controls. The origin of copy number contraction/expansion in relationship with Y hgrs is currently under study.

The large majority of ‘classical’ AZF deletions are de novo i.e. the father of the carrier shows intact Y chromosome in his genomic DNA. It has been proposed that deletions may originate either in the testis of the father or during embryogenesis. In the testis, deletions may occur during meiosis through non allelic homologous recombination or may be caused by chromosome breakage induced by free radicals during later phases [Aitken and Krausz 2001]. In order to define the presence of spermatozoa carrying a deleted Y chromosome, we developed a deletion junction fragment PCR for the detection of classical AZFa deletion in DNA originated from sperm suspension. Our preliminary data in normozoospermic men indicates that a proportion of Y chromosomes originating from sperm DNA are deleted. This observation supports a potential testicular origin for deletion formation. The comparison of deletion frequencies between subjects who generated sons with Y deletions and those who had sons with intact Y chromosome, will provide further insights into the origin of deletion formations.

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The role of mitochondria and the extranuclear genome, mitochondrial DNA (mtDNA), in gametogenesis, fertilization and embryogenesis has been controversial. Human mtDNA is a circular genome that is 16.6 kb in size. It encodes 13 polypeptides of the electron transfer chain (ETC) that are vital for the generation of ATP through the process of oxidative phosphorylation (OXPHOS). It also encodes 22 tRNAs and 2 rRNAs and consequently transcription, replication and translation are highly dependent on chromosomally-encoded factors being translocated to the mitochondrion. The importance of this symbiotic relationship between the cell, the mitochondrion and the mitochondrial genome is further demonstrated by those mtDNA genomes possessing certain lethal or severely debilitating mutations which will affect offspring survival and quality of life due to constituent cell function being severely impaired.

Early investigations indicated that ATP for sperm motility was primarily generated by glycolysis. However, analysis of pathogenic mutations and deletions to the mtDNA genome and different mtDNA haplotypes has provided evidence to suggest that OXPHOS has a key role to play in sperm motility, function and morphology, especially in the context of men presenting with varying degrees of male subfertility. Whether OXPHOS is required for all stages of the fertilization process, i.e. from the point of ejaculation to syngamy, remains to be determined. However, due to the few mitochondria present in human sperm, approximately 22, it is likely that they may have a specialized role. In somatic cells, mitochondria can switch from dormant to active roles suggesting specialized roles. Indeed, recently, it has been demonstrated that sperm mitoribosomes might be key factors for protein synthesis during capacitation.

Another area of controversy is the association between CAG repeats in the mtDNA-specific DNA Polymerase, Polymerase Gamma (PolG), and semen quality. There appear to be regional differences and trends, which suggest that in some, but not all, cases there may be associations. Interestingly though, good quality sperm samples have more sperm expressing PolG and mitochondrial transcription factor A (TFAM), the mtDNA-specific transcription factor also involved in mtDNA replication, than poorer quality samples, as defined by World Health Organization criteria. There is also a strong association between semen quality and mtDNA copy number in sperm, where men exhibiting good quality semen parameters have few copies of the mtDNA genome per sperm. Typically, the sperm from those men with poor semen characteristics, such as those categorized as oligoasthenoteratozoospermics, have increased numbers of sperm mtDNA and these sperm exhibit poor motility, morphology and function. This suggests that mtDNA copy number could be a useful marker for the efficient completion of spermatogenesis, as sperm mtDNA copy number decreases 10-fold during the transition from spermatogenesis to spermiogenesis.

Throughout evolution, the number of genes present in the mitochondrial genome has decreased as some genes have translocated to the nucleus, cf yeast versus human mtDNA genomes. Equally so, the transmission of mtDNA is biparental in mussels and some fruit flies while it is maternal only in other strains of fruit fly and mammals. In mammals, it has been proposed that sperm mitochondria are eliminated through a process of oocyte-mediated ubiquitination. In pigs and cattle, there appears to be a large-scale loss in mtDNA throughout preimplantation development, suggesting that not only is sperm mtDNA targeted for destruction but so is oocyte mtDNA. Whatever the process, it appears not to be foolproof as experiments involving interspecific crosses of strains or breeds in mammals (mice through to monkeys) have shown low levels of sperm mtDNA
transmission to the offspring; although this appears to only persist for one generation. In abnormal human embryos (3 pronuclei embryos), sperm mtDNA can persist up to the blastocyst stage but the frequency is low. However, there is one reported case of sperm mtDNA transmission in a male patient suffering from a mitochondrial myopathy. In this instance, subsequent analysis demonstrated that the sperm mtDNA genome had fused and thus recombined with the oocyte-derived genome resulting in hybrid molecules.

The introduction of more sophisticated reproductive technologies opens the door for the transmission of more than one population of mtDNA, creating a situation known as heteroplasmy. A clear example in recent clinical practise was the use of cytoplasmic transfer where cytoplasm, containing mitochondria, from younger oocyte donors was transferred into oocytes from women with repeated embryonic arrest. Blastomere nuclear transfer in rhesus macaques has also been shown to result in sperm mtDNA transmission, i.e. from the sperm mtDNA resident in the interspecific donor blastomere. As there are subtle differences in the coding sequences for mtDNA genomes from various haplotypes within a species, a certain amount of caution is required when planning more sophisticated assisted reproduction protocols, where it might be more appropriate to match (sperm and oocyte) donors for mtDNA compatibility.
Sperm Chromatin Packaging and DNA Methylation: Relevance to ART

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Severe male factor infertility is a common cause of infertility. The introduction of assisted reproductive therapies (ART) has allowed the treatment of many patients with severe defects of spermatogenesis. However, live birth rates for couples with severe male factor infertility undergoing intracytoplasmic sperm injection (ICSI) are generally lower than other etiologies treated by ART. Numerous pathologies possibly contributing to the diminished ART pregnancy rate associated with a subset of these patients include elevated sperm DNA fragmentation, abnormal sperm chromatin packaging, highly correlated with protamine abnormality, and an elevated risk of methylation perturbations at imprinted genes. Such observations have stimulated an interest in our laboratory to study sperm DNA packaging and the affect of abnormal sperm packaging on DNA methylation.

During spermiogenesis, sperm undergo profound morphological changes, including extensive remodeling of the sperm chromatin. Protamines replace somatic histones in a stepwise manner [Dixon et al. 1986]. Somatic histones are initially replaced by testis-specific histone variants, that are subsequently largely replaced by transition proteins (TP1 and TP2). The transition proteins are lastly replaced by protamines. Protamines are positively charged molecules that facilitate a higher order of DNA packaging in sperm than found in somatic cells, which is thought to protect the genetic integrity of the paternal genome during transport through the female reproductive tract, and may also be involved in silencing the paternal genome [Brewer et al. 2002; Balhorn et al. 1999; Gatewood et al. 1987]. In humans, approximately 85% of the histones are replaced by protamines [Hecht 1989, 1990; Dadoune 1995; Steger 1999; Oliva and Dixon 1990].

Humans express two protamines, protamine 1 (P1) and protamine 2 (P2), both of which are expressed in roughly equal quantities [Balhorn, et al. 1999; Corzett et al. 2002]. Previous studies by our lab, and others, have shown that the mean P1/P2 ratio in human sperm is approximately 1.0, and that abnormal ratios are associated with severe male factor infertility, including diminished counts, fertilizing ability, and reduced IVF outcome. In addition, patients with abnormal protamine levels also have increased DNA damage, as measured by the TUNEL or SCSA assays, and altered sperm packaging [Aoki et al. 2005].

To better understand the role of histone and protamines in chromatin packaging and early embryogenesis we have localized histones genome-wide. Retention of canonical histones in sperm chromatin is likely not a result of ‘inefficient protamine replacement’, but rather a unique and important mechanism used for epigenetic poising of the sperm genome for early embryogenesis. These findings elicit concern of whether the reduced fertility potential observed in abnormal protamine patients may be due to a reduction or increase in histone levels retained.

Recent speculations have been made on whether sperm chromatin structure (determined by sperm nuclear protein content) may be critical for the establishment and maintenance of proper epigenetic patterns during gametogenesis [Paldi 2003]. To address the question of whether abnormal packaging is associated with changes in DNA methylation, Aoki et al. [2006] evaluated global DNA methylation levels using 5-methyl cytosine antibody. In this study, no differences were observed in the mean global DNA methylation levels of fertile and abnormal protamine patients, however, significant variation in the 5-methyl cytosine intensity was observed in a sperm population, explaining sample heterogeneity and variable success rate following ART.
[Aoki et al. 2006]. The use of immunoflorescence to examine methylation status is limiting and requires more sensitive methodologies for evaluating subtle changes in methylation at developmental and imprinted genes involved in embryonic processes or disease onset.

Recently, a few reports have suggested that epigenetic changes are present in the mature sperm of oligozoospermic patients [Kobayashi et al. 2007; Marques et al. 2004]. However, CpG methylation changes at imprinted genes of abnormal protamine patients have not been reported. A number of imprinted genes are currently being evaluated in both oligozoospermic and abnormal protamine patients. Consistent to what has been reported we do note that significant change in DNA methylation is observed in both oligozoospermic and abnormal protamine patients when compared to fertile donors at many imprinted sites, but whether methylation aberrations extend beyond imprinted genes is unknown.

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Defining Sites Susceptible to DNA Damage Within the Sperm Nucleus: the Nuclear Matrix Connection

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The sperm genome is compacted to \(\sim 1/14\) the size of the oocyte that it will fertilize. In both mouse and man, this level of condensation is brought about by the replacement of the majority of the histone complement with protamines. Interestingly, in mouse, replacement is essentially complete with 98% of its spermatozoal genome packaged by protamines. In contrast, 15% of the human spermatozoal genome remains histone bound. While the protamine containing regions are dispersed throughout the genome, the histone bound regions that include the telomeres are localized towards the nuclear annulus.

Both the mouse and human sperm genomes are structurally segmented resolving as a series of toroids. It has been suggested that the base of each toroid is attached to the nuclear matrix arranging the sperm genome into a series of 50 kb loops. In turn, it has been proposed that the toroids are stacked to form a radial coil structure, each connected at its base to the nuclear matrix. While this is an interesting model, our preliminary data show that the distribution of matrix attachment sites is more complex, with the loops exhibiting a biphasic distribution. These varied sites of attachment may be reflective of the wide range of functions specified by the nuclear matrix that are integral to both replication and transcription while acting as a sink to a diverse population of spermatozoal RNAs. Together this suggests that segments of the genome may be regionally localized in association with other nuclear structures to functionally compartmentalize specific chromosomal segments. Even though the sperm genome is transcriptionally inert, and is tightly packaged into this unique structure, segments remain in a comparatively open potentiated state. This can be revealed as a function of their sensitivity to exogenous nuclease and are likely the primary candidates for DNA damage. Accordingly, one must consider these regions and the mechanism by which they are formed as both a pathway to destruction that is responding to differentiation that has gone awry as well as an opportunity for differentiation.

To address these issues we have begun to examine the mechanism and pathway that leads to the production of a mature spermatozoon. Using the protamine cluster as a model and by comparing both the mouse and human systems, we have examined how chromatin domains open during spermatogenesis. The dynamic nature of this mechanism, which can be likened to a ‘tug-of-war,’ has been resolved. Specification, that is opening, appears to be initiated through a series of relaxases. This includes Topoisomerase II that, by interacting with the nuclear matrix, marks segments and initiates chromatin opening. Upon receiving this signal, the loop domains are formed then consolidated as part of a transcription factory. Interestingly, these domains can remain in a comparatively open confirmation even in mature spermatozoa. We have begun to characterize these sites as markers of segments that are susceptible to DNA damage. Sites of nuclear matrix attachment and histone/protamine association throughout a series of human chromosomes have been mapped by competitive genomic hybridization as well as 454 and G2A sequencing. This has revealed a unique set of targets and structural properties that may render these regions susceptible to modification.

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Poor semen quality is a characteristic feature of the human condition. Not only is the motility, morphology and fertilizing potential of human spermatozoa frequently flawed but the integrity of DNA in the male germ line is also commonly compromised. Moreover, this DNA damage has purported links to poor rates of conception, impaired embryonic development, an increased incidence of miscarriage and the appearance of various kinds of morbidity in the offspring, including childhood cancer. Difficulties in interpretation arise however, because these associations are not consistently observed across all data sets. Such inconsistency reflects the inherent complexity of the reproductive process, large variations in sample size, differences in patient selection, inadequate study design as well as inter-individual differences in the type of DNA damage being detected and the effectiveness of repair mechanisms in the oocyte. Analysis of oxidative base formation in human spermatozoa shows a very high correlation with DNA damage as measured with the TUNEL assay. Such data suggest that most of the DNA damage observed in human spermatozoa involves the creation of oxidative stress. The significance of oxidative stress in this context is supported by an increasing number of studies demonstrating the beneficial effects of antioxidant therapy in the treatment of patients possessing high levels of DNA damage in their germ line.
Diagnostic Tests for Monitoring Scrotal Hyperthermia

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Human scrotal/testicular thermoregulation is a complex process that maintains the temperature of the testes at levels compatible with a normal spermatogenesis. Various techniques have been used in the evaluation of the effects of heat on the testes and to measure cutaneous blood flow, cutaneous scrotal temperature, thermal inertia and sympathetic skin response. In the last decade the high-resolution infrared telethermography has been widely used in the study of testicular thermal stress. Such an approach to thermal medical imaging requires both new methodologies and tools, like diagnostic paradigms, appropriate software for data analysis and a complete new way to look at data processing. In conclusion scrotal telethermography can be considered a very interesting method for a better understanding of the physiopathological modifications of testes at temperature elevation.
Testicular Hyperthermia and the Pathways Leading to DNA Breakage

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Testicular Hyperthermia and Apoptosis in Rodents: Our laboratory has demonstrated that mild testicular hyperthermia induces accelerated germ cell apoptosis predominantly via the mitochondrial-dependent death pathway [Sinha Hikim et al. 2003a]. The DNA breakage leading to germ cell apoptosis occurs predominantly at early (I–IV) and late (XII–XIV) stages. Spermatocytes, including pachytenes at stages I–IV and IX–XII, diplotene and dividing spermatocytes at stages XIII–XIV, and early (steps 1–4) spermatids were most susceptible to heat [Lue et al. 1999, 2000]. The effect is mediated principally through translocation of Bax (a pro-apoptotic protein) from cytosol to the paranuclear areas of susceptible germ cells [Yamamoto et al. 2000]. Relocation of Bax is accompanied by organelle (mitochondria, ER and Golgi) sequestration into paranuclear areas, cytosolic translocation of cytochrome c; activation of initiator caspase 9, executioner caspases 3, 6 and 7, and cleavage of PARP (hallmark of apoptosis) [Sinha Hikim et al. 2003a, b]. Additional studies, using the gld and lpr cgh mice, which harbor loss-of-function mutations in Fas L and Fas, respectively, showed that heat-induced germ cell apoptosis is not blocked, thus providing evidence that the Fas signaling system is not required for heat-induced germ cell apoptosis in the testes [Sinha Hikim et al. 2003a, b]. Our more recent studies showed that heat induced apoptosis and DNA breakage are initiated by p38 MAP Kinase. Heat stress resulted in increased in stage-and cell-specific activation of both p38 MAPK and ERK but not JNK. Inhibition of p38 MAPK but not inhibition of ERK resulted in a decrease in male germ cell apoptosis [Vera et al. 2006]. The action of p38 MAPK is mediated by phosphorylation and thus loss of function of the pro-survival protein BCL-2 promoting the mitochondrial intrinsic signaling pathway and activation of the apoptosis cascade [Jia et al. submitted].

Testicular Hyperthermia and Germ Cell Death in Monkeys and Men: Heat applied to the testis at 43°C for 30 min for two days to cynomolgus monkeys resulted in transient decrease in sperm counts and marked acceleration of germ cell apoptosis [Lue et al. 2006]. Heat stress induced activation of both ERK and p38 MAPK, increases in BCL-2 levels, inactivation of BCL-2 through phosphorylation at serine 70, release of cytochrome c and DIABLO from mitochondria, induction of the caspase cascade, germ cell DNA breakage and cell death [Jia et al. 2007]. We then studied the role of p38 MAPK in inducing apoptosis in human seminiferous tubules in vitro, induced germ cell apoptosis which can be partially inhibited by co-incubation with a selective p38 MAPK inhibitor. This suggests a role of p38 MAPK in inducing germ cell apoptosis in man [Vera et al. 2006]. When men were exposed to transient testicular hyperthermia at 43°C for 30 min for 6 consecutive days, sperm concentration decreased progressively for 6 weeks and by 9 weeks sperm concentration began to recover. Testicular germ cell apoptosis occurred by 2 weeks after treatment and at 9 weeks the testis biopsies were similar to the pre-treatment biopsies. Thus testicular hyperthermia causes a transient increase in germ cell death with rapid recovery after the cessation of the intervention [Wang et al. 2007].

In summary, studies in rodents, monkeys and men showed that transient testicular hyperthermia induced DNA fragmentation resulting in germ cell apoptosis. The heat stress is mediated by the stress kinases such as p38 MAPK leading to phosphorylation and inactivation of the pro-survival protein BCL-2, cytochrome c and DIABLO release from the mitochondria, induction of the caspase cascade leading to DNA breakage and germ cell death. Cessation of heat treatment results in rapid recovery of spermatogenesis.
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Pathways of ROS Generation in Male Germ Cells:
Insights Generated by Proteomics

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The application of advanced proteomics techniques to the study of human spermatozoa has shed new light on the molecular mechanisms that regulate sperm function. The proteomic profile of these cells reflects their vulnerability to oxidative stress since these cells contain a range of antioxidant enzymes including glutathione peroxidases, glutathione transferase, several forms of thioredoxin-dependent peroxiredoxins, thioredoxin reductase and superoxide dismutases type 1 and 2. Intriguingly, catalase does not appear to feature significantly in the defence of these cells, despite their susceptibility to hydrogen peroxide attack. Oxidative stress is known to be associated with a loss of sperm function, particularly motility, as well as the induction of DNA damage to the nuclear and mitochondrial genomes. Given the risk of oxidative damage to these cells, it is surprising that they generate reactive oxygen species and that these molecules play a physiological role in the promotion of sperm capacitation. ROS are generated spontaneously on dilution of murine spermatozoa in culture medium via mechanisms that involve electron leakage from the mitochondria.

Human spermatozoa also generate ROS from their mitochondria although, in this case, the spontaneous production is low, but becomes elevated in cases of defective sperm function. The factors responsible for elevating mitochondrial ROS generation in human spermatozoa in vivo are unknown although free unsaturated fatty acids, certain retinoids and radiofrequency electromagnetic radiation are known to stimulate mitochondrial ROS generation in vitro. Whether there are other sources of ROS generation in human spermatozoa is still uncertain. Proteomic analysis has indicated the presence of a calcium sensitive NADPH oxidase in these cells, known as DUOX, as well as certain components of the NOX 2 system seen in phagocytic leukocytes. Further studies are needed to determine the significance of these redox systems to the oxidative stress experienced by defective human spermatozoa.
Genital Tract Inflammation and its Consequences on Sperm DNA

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Male accessory gland infections (MAGI) are of worldwide distribution in both industrialized and developing countries. Except the uncommonly acute, symptomatic conditions, MAGI have an underhand beginning, and a chronic, symptomless or rather paucisymptomatic course. Clinically, MAGI include: i) uncomplicated MAGI represented by prostatitis; and ii) complicated MAGI: prostatovesiculitis (PV) or prostatovesiculo-epididymitis (PVE), when the inflammation involves more than one gland.

Prostatitis is the most common prostate disease (5–14% in Europe). It is today more frequently referred to as ‘prostatitis syndrome.’ According to the National Institutes of Health (USA), it may be classified in four categories:

I Acute bacterial prostatitis
II Chronic bacterial prostatitis
III Chronic abacterial prostatitis/chronic pelvic pain syndrome which may be subdivided into:
   IIIA. Inflammatory chronic pelvic pain syndrome (significant leukocytes in: semen, expressed prostatic secretion, third voided urine specimen)
   IIIB. Non-Inflammatory chronic pelvic pain syndrome (non-significant leukocytes in: semen, expressed prostatic secretion, third voided urine specimen)
IV Asymptomatic (histological) inflammation of prostate

Chronic prostatovesiculitis (PV) and prostatovesiculo-epididymitis (PVE) are due to an ascending spread of urethral pathogens via the ejaculatory duct into the vas deferens up to the epididymes. PV or PVE epidemiological data are scanty.

The incidence of epididymitis is approximately 600,000 cases/year with the highest prevalence in young men (19–35 years). In young men, it is usually associated with a past or concurrent history of sexually transmitted urethritis. In older men, it is more often associated with bacterial prostatitis or PV. It is responsible of scrotal, testicular or epididymal discomfort or pain of at least 3 month duration.

MAGI have been reported with a wide frequency (1.6–39.1%) in infertile patients. It contributes to infertility to an extent which depends upon the site and the extension of the inflammation as well as on the intensity of the inflammatory response (leukocytospermia, production of reactive oxygen species, ROS, and/or cytokines). It has been shown that patients with PVE have worse sperm parameters compared with patients with prostatitis alone or PV.

Several lines of evidence suggest that MAGI may be responsible for male infertility not only by altering conventional sperm parameters but also by damaging sperm DNA. This may be achieved by: i) direct effects: caused by the germ per se and ii) indirect effects: caused by germ molecular components, and over-production of ROS and/or cytokines (IL-6, IL-8, TNFα, MIF, etc.).

We have shown that the experimental infection of normal motile spermatozoa with Candida albicans or Chlamydia (C.) trachomatis increase the number of spermatozoa with sperm DNA fragmentation in a concentration- and time-dependent manner. More insight studies have shown that the chlamydial lipopolysaccharide (LPS) induces sperm apoptosis. A greater degree of apoptosis was seen with LPS extracted from C. trachomatis serovar E than from serovar LGV. The pro-apoptotic LPS-induced apoptosis can be blocked by
pan-caspase or selective caspase-3 inhibitors. Subsequent studies showed that the lipid A, a toxic component of LPS, and 3-deoxy-D-manno-octulosonic acid (Kdo), an additional component of the *C. trachomatis* LPS, cause an apoptotic-like caspase-dependent effect in spermatozoa. A vast literature has established a tight relationship between sperm DNA fragmentation and ROS over-production.

We and others have shown that *in vitro* exposure to TNFα increases the percentage of spermatozoa with DNA fragmentation in a concentration- and time-dependent manner in normozoospermic volunteers. More recently, a significant correlation between polymorphonuclear elastase and TNFα concentration in the seminal plasma and apoptotic spermatozoa has been reported in patients with signs of chronic genital tract inflammation. These patients also had a significantly higher percentage of apoptotic spermatozoa compared to patients without MAGI.

MIF, a ubiquitous cytokine that functions in reproduction and plays an important role in sperm maturation and motility, has also been shown to induce sperm apoptosis. Interestingly, its seminal fluid concentrations showed a biphasic profile in patients with altered sperm parameters being high in patients with low sperm count. In these patients, MIF may, therefore, contribute to the increased sperm apoptosis rate.

The interplay between cytokine and germ on sperm apoptosis has been further highlighted by a recent study showing an elevated production of IL-1α, IL-6 and IL-8 by immortalized normal human urethral epithelial cells and immortalized normal adult human prostate epithelial infected with *C. trachomatis*. These findings support the hypothesis that male-derived cell lines initiate a proinflammatory response to infection.

In conclusion, these observations suggest that MAGI may damage sperm DNA with multiple mechanisms. A thorough diagnostic approach of the infertile patient aimed at establishing the presence of MAGI, the identification of the microbial agent responsible (when possible), the extension of the inflammatory process, and the therapeutic strategy is mandatory to reduce the number of spermatozoa with damaged DNA.
Antioxidants and Sperm DNA Damage

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There is evidence to show that infertile men possess substantially more sperm DNA damage than do fertile men and that human sperm DNA damage may adversely affect reproductive outcomes. This is particularly relevant in an era where advanced forms of assisted reproductive technologies (ARTs) are commonly utilized (these technologies often bypass the barriers to natural selection), as there is some uncertainty regarding the safety of utilizing DNA-damaged spermatozoa in this setting. Therefore, it is important to identify strategies that may reduce sperm DNA damage. At present, there is some evidence to suggest that antioxidants may be useful in this regard. The etiology of sperm DNA damage is multi-factorial and may be due to primary testicular or secondary (e.g. environmental) factors. Sperm DNA damage is believed to be the result of aberrant protamine expression, excessive ROS (reactive oxygen species) generation and abortive apoptosis during spermatogenesis.

The association between sperm DNA damage and ROS is the basis for the use of antioxidants in the treatment of sperm DNA damage. High levels of ROS have been detected in the semen of 25% of infertile men and have been associated with sperm DNA damage. While the generation of low levels of ROS is necessary for normal sperm function, high levels of ROS are generated by defective spermatozoa and by activated semen leukocytes, resulting in sperm dysfunction. The susceptibility of human spermatozoa to oxidative stress stems primarily from the abundance of unsaturated fatty acids in the sperm plasma membrane. These unsaturated fatty acids provide fluidity that is necessary for membrane fusion events (e.g. the acrosome reaction and sperm-egg interaction) and for sperm motility. However, the unsaturated nature of these molecules predisposes them to free radical attack and ongoing lipid peroxidation throughout the sperm plasma membrane. Once this process has been initiated, accumulation of lipid peroxides occurs on the sperm surface [Alvarez et al. 1987] and oxidative damage to DNA can ensue [Fraga et al. 1991; Twigg et al. 1998]. The association between sperm DNA damage and sperm-derived ROS suggests that DNA damage may be due to a defect in spermiogenesis, whereas the association between sperm DNA damage and leukocyte-derived ROS suggests that the DNA damage may be due to a post-testicular defect.

Seminal fluid is an important source of ROS scavengers (antioxidants) and is probably important in protecting sperm from oxidative injury due to ROS. The endogenous free radical scavenging enzymes in the male reproductive tract include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). In semen, the antioxidant defense is largely conferred by the seminal plasma since spermatozoa have little cytoplasmic fluid (antioxidant enzymes are generally intracellular) and no capacity for protein synthesis. Indeed, a high level of antioxidant activity has been detected in semen and much of this activity is non-enzymatic [Zini et al. 1993]. A small number of clinical studies have evaluated the relationship between semen antioxidants and sperm DNA damage [Song et al. 2006]. These studies have generally shown that a deficiency in semen antioxidants is associated with sperm DNA damage.

Repetitive washing of sperm, which removes seminal fluid, can increase ROS production dramatically. This is clinically relevant as sperm washing is performed prior to ARTs (assisted reproductive technologies such as intrauterine insemination and in vitro fertilization) and the process may result in oxidative injury to the sperm DNA. This is particularly true of semen samples with significant ROS levels. The addition of antioxidants to sperm preparations can protect spermatozoa from oxidative stress. Vitamins C and E, hypotaurine and reduced glutathione have been shown to reduce oxidative sperm DNA damage in vitro [Hughes et al. 1998; Lopes et al. 1998]. However, under certain conditions, some antioxidants may have a pro-oxidant (and detrimental) effect on sperm DNA when used in vitro [Hughes et al. 1998].
There are few reports on the effects of dietary vitamin and antioxidant supplementation on sperm DNA integrity with conflicting results. In 1991, Fraga et al. demonstrated that dietary vitamin C has a beneficial effect on the integrity of sperm DNA (DNA oxidation) in male smokers. More recently, Greco et al. [2005] have shown that oral vitamins (C and E) can improve sperm DNA integrity. Menezo et al. [2007] have shown that although vitamins (vitamins C, E, zinc, selenium and ß-carotene) can reduce sperm DNA fragmentation, they may increase sperm head decondensation (increase DNA stainability). In contrast, Silver et al. [2005] and Piomboni et al. [2008] have shown that vitamin intake is not associated with improved sperm DNA integrity.

In summary, the data suggest that ROS appears to play a significant role in the generation of sperm DNA damage. Although in vitro studies have demonstrated a beneficial effect of antioxidant supplements in protecting sperm from oxidative DNA injury, the beneficial effect of dietary antioxidants on sperm DNA integrity has not been clearly demonstrated.

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SELECTED ORAL PRESENTATIONS
Characteristic Histone Modifications and Timing of Histone to Protamine Switch in Drosophila Sperm Chromatin

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The nuclear chromatin of maturing spermatids in mammals as well as in Drosophila becomes highly compacted by replacing histones with small, basic protamines on the DNA in post-meiotic stages [Jayaramaiah Raja and Renkawitz-Pohl 2005]. Various histone modifications preceding this chromatin reconfiguration have been analyzed in our group [Rathke et al. 2007]. Among these, histone H3 K9 and K27 methylation have been observed from pre-meiotic stages until histone removal in post-meiotic stages. Since, in Drosophila, transcription ceases almost completely after meiosis, it is worthwhile to note that H3K9me and H3K27me are known to indicate a repressive chromatin state [Lachner et al. 2003]. Additionally, as known from mammals, in Drosophila histone H4 hyper-acetylation directly precedes the histone to protamine switch, while DNA breaks coincide with the following 'transitional' stages [Rathke et al. 2007].

In Drosophila this switch in chromatin configuration appears to be a global process, since somatic core-histones could not be detected in late spermatids by immunohistological assays [Rathke et al. 2007]. The time required for completion of this process was, so far, unknown in either mammals or Drosophila. We were able to narrow down the time-frame of the switch to around five hours in Drosophila using live-cell imaging of spermatids expressing both H2AvD-RFP and ProtamineB-eGFP.

The molecular mechanism behind this apparently comprehensive exchange of the major chromatin comprising proteins is still unknown. In ongoing experiments we test the relevance of H4 acetylation and other characteristics for the molecular mechanism.

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Persistence of DNA Damage and its Consequence for Mutagenesis in Male Germ Cells of OGG1−/− Big Blue R Mice Exposed to Benzo(a)pyrene

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The paternal genome is important for fertility and the health of coming generations. Male germ cells are targets for DNA-lesions, especially oxidated DNA-lesions that are strongly associated with poor semen quality and impaired embryo development. We have previously reported distinct DNA-repair differences between human and rodent male germ cells, with poor repair of bulky DNA–adducts in both species whereas oxidized DNA-lesions are poorly repaired in humans as opposed to rodents. This raises the question: Do unrepaired DNA-lesions persist in male germ cells to the stage of mature spermatozoa or are they transformed into mutations prior to fertilization? A mouse line with deficient repair of oxidized purines (Ogg1−/−-mice) was proposed as a model for human male germ cells, in a background that allows mutation analyses (Big Blue®). Mice were exposed to Benzo(a)pyrene (B(a)P), inducing both bulky DNA adducts and oxidized lesions, and sacrificed at time points, to attain specific information on the susceptibility of different stages of spermatogenesis. Preliminary results indicate that both B(a)P-DNA adducts and oxidative lesions are present in caput sperm, and that there is a trend towards increased mutation rates with a statistically significant increase for exposed stem cell spermatogonia. This suggests that DNA-damage is induced – and not repaired – during later stages of spermatogenesis. Such lesions will therefore be transmitted to the oocyte upon fertilization. The data corroborate the poor repair earlier reported in male germ cells, and suggest that persisting DNA-lesions from the sperm may lead to death or de novo mutations in the zygote.
Cellular Mechanisms Underlying the Effects of Paternal Acrylamide-Exposure on Preimplantation Development in Mice

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Sperm with excessive DNA damage retain the ability to fertilize oocytes, but normal development of the early embryo is often compromised, resulting in reduced fertility or developmental toxicity. We have studied effects of acrylamide, a toxicant that humans are exposed to via heat-treated starch-rich food. Acrylamide is a germ cell mutagen inducing clastogenic effects in cells of the male germline. Mating acrylamide-exposed males with unexposed females has been shown to result in reduced fertility and to induce pre- and post-implantation loss.

We present measurements of acrylamide-induced DNA damage in individual sperm and the induction of stress responses in the early embryo. This study is part of a larger project the primary objective of which is to clarify mechanisms underlying negative effects of environmentally induced paternal DNA modifications on early embryo development. Epididymal sperm was isolated at different time points following acrylamide exposure for analysis of DNA lesions by the Comet assay. The results indicate that the highest amount of damage in cauda sperm results from an exposure 7 days earlier, a time point which has been associated with induction of preimplantation loss. The Comet assay showed increased damage levels in the majority of exposed cauda sperm and not in a sub-population. In contrast to testicular cells, no fpg-sensitive DNA lesions (representing oxidated purines) were observed in sperm following in vivo acrylamide exposure. Paternal acrylamide exposure influenced the early embryo cleavage rate and induced the expression of DNA damage response proteins like γH2AX and p53.

Acknowledgments: We thank the Norwegian Research Council for financial support.
Epididymal Glutathione Peroxidase 5 Contributes to the Maintenance of Sperm DNA Integrity and to Embryo Viability

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Epididymal sperm maturation is considered an essential step for the transformation of immature testicular gametes to mature spermatozoa capable of fertilization. Reactive oxygen species (ROS) have clearly been shown to be key actors in this maturation process. However, spermatozoa are extremely sensitive to oxidative stress that is correlated with lipid peroxidation, DNA damage and a decline in sperm motility. GPx5, a glutathione peroxidase highly expressed in the epididymis was proposed to be a key element in the sperm protection against ROS. To find out what could be the real impact of this protein in the mouse epididymis and also in sperm physiology, GPx5-null mice were generated. Histological analyses of the GPx5−/− epididymides followed by cytological sperm examination did not show any obvious signs of morphological alterations. The fertility parameters of GPx5−/− sexually mature male mice were not affected. However, sporadic developmental defects were noticed with aged GPx5 deficient males (over one year old) compared to wild type males. Cytometry analysis has shown a significant decrease in sperm DNA compaction in cauda epididymides from GPx5 null mice. In addition, sperm DNA oxidation was noticed in GPx5−/− mice using 8-oxodG as a marker. Nuclear peroxidative attacks were observed in cauda epididymidis epithelium. Quantitative PCR analyses have confirmed that the cauda epididymidis of the GPx5−/− mice is engaged in an antioxidant response. Overall, the data collected show that the lack of GPx5 protein leads to oxidative stress within the epididymis resulting in sperm nucleic alterations.

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Male-to-Female Sex-Ratio is Potentially Correlated to Air Pollution Levels

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Sex ratio, the ratio of male to total live births, has been declining in industrial countries. Trends in the sex ratio of live births in the city of São Paulo have not been investigated. Data on male and female live births in nine cities of the Metropolitan Region of São Paulo from 1999 to 2003 were obtained. Daily records of PM_{10} concentrations for the Metropolitan Region were obtained from the monitoring stations and concentrations averaged in annual terms. Stations were classified into three main categories of PM_{10} levels (least polluted, medium and most polluted -40.7 µg/m³, 50.8 µg/m³ and 70.9 µg/m³, respectively). The districts and cities were correlated to a given monitoring station if they were in the wind route and at no more than 2 km away from the station. The male proportion was determined by dividing the number of live born males by the number of live births and analyzed by 3D Scatter Plotter.

For the year 2000, the sex ratio in the least polluted area was 51.3%, decreasing to 49.6% (p < 0.05). However, the sex ratio showed a significant increase, from 51.1% in 1999 to 52.0% in 2002, in the five-year period, in agreement to a reduction of PM_{10} levels measured by all the stations (Fig. 1).

We found a significant negative association between sex ratio and levels of air pollution measured in PM_{10} when comparing the three different areas. In the most polluted area in the year 2000, a corresponding loss of 1.7% in male births which represented a cumulative loss of 1,996 male births was noted. Also, we found a relative increase of male births over the five years studied reflecting the actual improvement in air quality control policy implemented. The annual PM_{10} concentration has been declining, from 50.5 µg/m³ in 1999 to 45.6 µg/m³ in 2003, a corresponding reduction of 9.7%. Sex-ratio can be recovered if government policies of improving air quality are conducted.

![Figure 1](image-url)
Analytical Investigation on TUNEL/PI Assay for the Determination of Sperm DNA Fragmentation: Pitfalls and Possible Solutions

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In the past decades, a bulk of studies were conducted to determine the impact of sperm DNA fragmentation (sDF) on ART outcome. However, data are controversial. One crucial reason is represented by the techniques used to reveal sDF, that are different and often lack standardization. Our group studied TUNEL coupled to flow cytometry (FC) and identified some pitfalls of such technique. These are:

i) The presence of M540 bodies in semen of subfertile patients. Such bodies have variable sizes including those similar to sperm heads. This feature localizes M540 bodies in the same FSC/SSC region of sperm and, if not excluded from the sperm analysis, yield a variable underestimation of measures of sDF. To overcome this bias, we modified Tunel assay by simultaneous staining with the nuclear probe propidium iodide (PI). The new version of the technique (Tunel/PI) allows the exclusion of M540 bodies because of their lack of nuclei.

ii) The lack of standardization in performing Tunel assay and in elaborating flow cytometric data on sDF. We found that when different aliquots of the same samples are processed with Tunel/PI in the same day, the resulting measures have very good CVs (intra-assay Cv = 6.9 ± 4.7, n = 13). However, when different aliquots of the same samples are processed in different days and in the meanwhile they are kept in the widespread used fixative buffer (3.7% paraformaldehyde, pH = 7.4), the resulting measures yield poor CVs (inter-assay CVs = 55.0 ± 16.0, n = 9). Hence, to evaluate sDF by Tunel/PI a time standardization following sample collection is needed. Another issue is the method to elaborate flow cytometric data of sDF. The two main methods are the threshold and the subtraction method. We have found that the two methods yield different results (TM, 51.0 ± 18.6%; SM, 66.0 ± 16.2%, p < 0.0001). In addition, whereas the measure of sDF obtained with TM correlates to poor semen parameters, the association with semen quality is lost when the measures are obtained with SM. This finding suggests that a clinical meaning of sDF is masked if SM is used.

iii) There is a discrepancy when measures obtained by FC are compared with those obtained by fluorescence microscopy (FM) in semen samples processed with the same technical procedure. Even if the two types of measures show a good correlation, the results of FC are much greater than those of FM [Muratori et al. 2008].

In conclusion, several procedures need to be followed in order to obtain reliable measures of sDF by TUNEL technique.

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Impact of Environmental Exposure to Perfluorinated Compounds on Sperm DNA Quality

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Recent studies have suggested that exposure to certain compounds is associated with reproductive toxicity, including structural and functional alterations of human sperm and DNA damage in particular. An association has been postulated between the global decline in semen quality and increased exposure to environmental chemicals that act as endocrine disruptors. Among these a new class, the Perfluorinated Compounds (PFCs), is suspected to have adverse effects on human fertility.

The objective of this study was to evaluate PFC contamination in whole blood and seminal plasma from subfertile men (n = 59) and to investigate the main semen parameters (sperm concentration, motility, morphology) and the quality of sperm DNA. PFC contamination was present in 42.40% of subjects; semen alterations were detected in 77.97%. Sperm DNA fragmentation was evaluated by flow cytometry and double staining with TUNEL and propidium iodide (PI). This method makes it possible to exclude M540 bodies from the final evaluation of DNA fragmentation and to distinguish two sperm populations, stained with low (PI$^{\text{dim}}$) and high (PI$^{\text{br}}$) avidity for PI.

The DNA fragmentation index (PI$^{\text{br}}$ + PI$^{\text{dim}}$) results were higher (29.94% ± 3.82) in patients with poorer sperm morphology and reduced motility, in comparison to normospermic subjects (20.13% ± 7.44). No significant difference was found when the percentage of DNA fragmentation in PFC ‘positive’ men (27.07% ± 3.88) was compared to that detected in PFC ‘negative’ subjects (31.42% ± 7.35). Nevertheless, the percentage of PI$^{\text{dim}}$ populations was significantly higher in PFC ‘positive’ than in PFC ‘negative’ males (72.44% vs. 56.94% p < 0.05). Our results suggest a negative interference by PFCs on sperm DNA quality.
Chromomycin A3 Staining vs. TUNEL Assay: Different Prognostic Value on ART Outcome

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The aim of this study was to evaluate the impact of abnormal protamination on sperm parameters, sperm DNA fragmentation and ART outcome and the link between protamine deficiency and seminal plasma’s antioxidant ability. Sperm protamine deficiency and DNA damage were analyzed employing chromomycin A3 staining and TUNEL assay, respectively, in 132 patients (82 IVF, 50 ICSI). In 10 men we analyzed seminal plasma’s antioxidant ability, using the Total Oxidant Scavenging Capacity assay. A significant negative correlation was found between abnormal protamination and sperm parameters, including sperm DNA integrity ($P < 0.001$). We found a close relationship between sperm protamination and fertilization and pregnancy only in IVF ($P = 0.004$ and $P < 0.04$, respectively); in ICSI there was a correlation just between DNA integrity and pregnancy ($P = 0.031$). Finally, we found a negative correlation between chromatin underprotamination and seminal plasma’s antioxidant ability ($P < 0.01$). Results of this study underline that, despite sperm abnormal protamination and DNA fragmentation being positively correlated, they affect the reproductive outcome in different manners: in particular we stressed the good prognostic value of CMA3 analysis only in IVF, whereas DNA fragmentation analysis is prognostic only for ICSI outcome. We also provided data supporting the idea of a relationship between a defective antioxidant system activity and impairment of chromatin packaging.
Apoptosis and Sperm DNA Fragmentation in Infertile Patients with Chlamydia and Mycoplasms Infection

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The conjugal infertility is a problem of increasing magnitude; in many cases the cause is not identified. The association of germs commonly observed in genital infections include *Chlamydia trachomatis* (Ct) and Mycoplasms (Mg).

The apoptotic process associated with elevated levels of reactive oxygen species (ROS) and the damage in the spermatic DNA have been associated with the presence of infectious agents and may have a crucial influence on the process of fertilization. A total of 37 patients were grouped according to the microbiological results Mg(+)/Ct(−), Mg(−)/Ct(+) and Mg(+)/Ct(+). We also studied a control group of 11 normozoospermic healthy men. We determined sperm apoptosis using phosphatydilserine translocation (APOAC, Sigma) and spermatic DNA fragmentation (SCD; INDAS). Fragmented DNA in the group Mg(+)/Ct(−) was 14.5 ± 6.3%; in the group Mg(−)/Ct(+) was 23.1 ± 10.4% and the group Mg(+)/Ct(+) presented an average of 27.4 ± 11.1%, the latter two groups had a statistically significant difference (*p* < 0.05). The average of apoptotic cells in the group Mg(+)/Ct(−) was 25.0 ± 2.8%; in the group of Mg(−)/Ct(+) it was 20.0 ± 3.5%, while in group Mg(+)/Ct(+) it was 22.5 ± 5.6% and in the three groups it presented a statistically significant difference (*p* < 0.05). In the group studied, high levels of DNA breaks were associated with the presence of Ct and Mg that in combination had a synergistic effect as inducers of damage to the structure of DNA. There is an association between high levels of apoptosis/DNA sperm fragmentation and seminal infection by *Chlamydia trachomatis* and Mycoplasms.
Inflammatory Mediators Induce Apoptosis in Ejaculated Spermatozoa in In Vitro Conditions

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We have evaluated an in vitro induction of apoptosis in three sperm subpopulations exposed to mediators participating in the inflammatory process such as white blood cells (WBC), combinations of proinflammatory cytokines (interleukin (IL)-6 + IL-8, IL-12 + IL-18), and selected bacterial strains (Escherichia coli, Bacteroides ureolyticus). Semen samples of normozoospermic volunteers were differentiated by swim-up (swim-up fraction) and Percoll gradient procedures (90% and 47% Percoll fractions). WBC were isolated from the whole heparinized blood using density gradient centrifugation. DNA fragmentation in sperm fractions was evaluated using the Comet assay and flow cytometry with TUNEL labeling. Among all the inflammatory factors tested, only bacteria significantly increased the percentage of TUNEL-positive spermatozoa compared to untreated cells (p < 0.05). In the case of B. ureolyticus, these changes were accompanied by a significant increase in the mean fluorescence intensity (MFI) levels of TUNEL-positive spermatozoa from the 90% Percoll fraction. Moreover, this apoptotic index was the highest among all the inflammatory factors applied. The incubation of sperm subpopulations with proinflammatory cytokines significantly increased MFI levels only in the swim-up and the 90% Percoll sperm fraction, respectively for combinations of IL-6 + IL-8 or IL-12 + IL-18 (P < 0.01 and P < 0.05 when compared to sperm incubated alone). The results indicate that bacteria or their toxins may directly induce apoptosis in ejaculated spermatozoa in in vitro conditions. Proinflammatory cytokines may therefore additionally influence DNA of spermatozoa possibly with already initiated apoptosis. The selection of spermatozoa by gradient procedures seems to increase the risk of using apoptotic sperm for assisted reproduction.
SELECTED POSTER SUBMISSIONS
P 01: Association between Sperm DNA Fragmentation and Alterations of Sperm Nuclear Matrix

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Disturbances in the organization of the genomic material in sperm nuclei can have a serious impact on the growth of the offspring, therefore a stable nuclear matrix is crucial for participation in embryonic development. It has been shown that the sites at which DNA associates with the sperm nuclear matrix contain chromatin structures that are linked with specific functions. Recent data also suggest that the sperm nuclear matrix plays an essential role in the paternal pronucleus of the fertilized oocyte.

Moreover, the phenomenon of DNA fragmentation in sperm cells is a potential new parameter of semen quality. It has been suggested that altered nuclear chromatin structure or damaged DNA in spermatozoa is implicated as a possible cause of infertility in males. However, the condition of the nuclear protein in the sperm cells with fragmented DNA has never been considered.

In this work 20 human semen samples, including samples from fertile (n = 4) and infertile males (5 normozoospermic patients and 11 with abnormal semen parameters), were processed for determination of DNA fragmentation using an in situ diffusion assay, so that spermatozoa containing fragmented DNA showed remarkably large halos of disseminated DNA fragments after a lysis treatment in an agarose microgel. Specific protein staining revealed that nuclear matrix proteins do not remain in the core from sperm nucleoids without DNA fragmentation, whereas spermatozoa with fragmented DNA tended to retain residual nucleoskeletal protein in a collapsed and condensed state. This result suggest that a modified nuclear protein matrix associates with fragmented sperm DNA.

P 02: Polymorphisms in the Protein C Inhibitor Gene in In Vitro Fertilization Failure

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Many of the couples undergoing IVF experience unexplained total fertilization failure. In mice the protein C inhibitor (PCI) gene has been found to be an absolute requirement for fertilization. In experiments where the PCI gene was knocked-out, male mice produced normal amounts of sperm, but these were unable to penetrate oocytes. We used a genetic approach to test whether total fertilization failure in human IVF could at least partly be explained by changes in the PCI gene.

Leukocyte DNA was extracted from peripheral blood in 46 men involved in IVF where no fertilization occurred. Fifty-one men undergoing IVF with normal fertilization were used as controls. Patients and controls were screened for mutations in the PCI gene by direct sequencing. The difference in distribution of genetic variants among patients and controls was evaluated using Fishers exact test. Among 15 different single nucleotide polymorphisms, an A/G transition at position 1389 in exon 6 was significantly more common in patients involved in IVF fertilization failure compared to controls (10.9% vs. 0%, p = 0.02). Two other novel mutations were also detected. However, these were as frequent in patients as in controls. The results suggest that total fertilization failure after IVF in a subgroup of cases may be caused by polymorphisms in the PCI gene.
P 03: TSPY Array and its Role in Male Fertility

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The human TSPY (testis-specific protein, Y-linked) gene family (30–60 copies) is situated in the MSY (male-specific) region of the Y chromosome. The expression pattern of TSPY indicates that the gene may play a role in gonadoblastoma, seminoma and spermatogenesis. To define the role of the TSPY gene in male infertility we analyzed 90 idiopathic infertile men (29 azoospermic and 61 oligozoospermic men) and 90 normozoospermic controls. Syber Green real time PCR was applied to evaluate the number of TSPY copies. Differences in threshold cycle (ΔCt) was calculated for each individual by subtracting mean PMP22 (diploid control gene) Ct from mean TSPY Ct. The method was validated against gold standard method (pulsed-field gel analysis).

The copy number of the TSPY gene was significantly higher in controls in respect to patients (mean 32.9 versus 28.0; \( p < 0.002 \)). Subjects with >33 copies have a significantly lower probability to have an impairment of spermatogenesis with an OR = 0.51; 95% CI 0.3–0.8 (\( p < 0.01 \)).

Our result is in contrast with a previous study. The discordance may derive from the small sample size, lack of matching of cases versus controls and the use of a non validated method in the previous study. Matching of cases versus controls is particularly relevant since TSPY gene copy number may vary according to different Y haplogroups. In our study the Y hgr distribution was similar among patients and controls indicating that the difference in TSPY copy number reflects a genuine variation of TSPY copy number and thus it directly influences the efficiency of spermatogenesis.

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P 04: gr/gr Deletions: their Clinical Significance and Relationship with Y Chromosome Background and AZFc Rearrangements

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The role of the gr/gr deletion in spermatogenic impairment is still debated, especially because the associated semen phenotype ranges from normo- to oligo/azoospermia. The basis of this variation is unknown but with differences in the genes removed, the occurrence of subsequent duplications have been suggested as possible causal factors. The aims of this study were to evaluate: i) the clinical significance of gr/gr deletions and ii) if Y-linked factors are involved in the heterogeneous phenotype.

A total of 634 infertile patients and 487 normozoospermic controls from Italy were screened for gr/gr deletions with a combined method based on STS +/− followed by CDY1-DAZ gene dosage and copy analysis; 169 gr/gr deletion carriers from six centers in Europe and one in Australia, with known phenotypes, were characterized for: a) DAZ-CDY1 copy dosage, b) the presence of secondary rearrangements and c) the Y-chromosome haplogroups.

The first part of the study showed a significantly higher gr/gr deletion frequency in patients versus controls (3.2% versus 0.4%; \( p < 0.001 \)), with an OR = 7.9. In the multicenter study we observed: i) no differences between patients and controls concerning the distribution of Y haplogroups and secondary rearrangements and ii) a significant geographical difference in the frequency of subtypes of gr/gr deletions with potential relevance for studies in admixed populations.

Our study on the Italian population – the largest in the literature – provides strong evidence that gr/gr deletion is a risk factor for spermatogenic failure. The phenotypic variation of gr/gr carriers in men of European origin is largely independent from the Y-chromosomal background.

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Brazil has a successful ethanol program which reduces automotive pollution. Part of sugar cane harvesting is preceded by burning the plantation, since harvest is done manually. In this study we explored the possible reproductive adverse effects due to biomass burning using sex ratio as the end point. Such information is not hitherto explored in the literature. Data on male and female live births obtained from the São Paulo state of Brazil from 2000, 2001 and 2002, on more than 204,000 births were obtained from the annual publication of the databank of the Brazilian Ministry of Health.

The annual male proportion was obtained by dividing the number of live born males by the total number of live births. In cities with over 100 million/ton/cane/year, information on the extension of sugar cane plantations, number of foci of fires on daily basis, by satellite monitoring, was obtained. We separated the agricultural cities from each region in five categories according with the agricultural area in relation to total area of the city – [1] 0–15%, [2] 16–30%, [3] 31–45%, [4] 46–60%, [5] > 60%.

Sex ratio was aggregated for the 3 years of study for each region and compared with the mean number of plantation fires, also expressed as the mean of the 3 years. The significance of the correlation between sex ratio and the two estimators of exposure – relative proportion of sugar cane plantation area and number of foci of fires, was assessed. A graphical representation of sex ratio as a function of mean number of plantation fires is depicted in Figure 1. Negative significant correlations between sex ratio of exposure were obtained ($p = 0.01$ for both estimators). A significant reduction of the proportion of male births was observed across two estimators of sugar cane production (0.3%). Fewer male births may implicate that oocytes are more likely to be fertilized by sperm carrying X chromosome and that Y chromosome sperm may be more susceptible to the effects of toxicants.
Trends in the sex ratio of live births in Brazil have never been investigated. The objective of our study was to determine the sex ratio data on live births of this country from 1994 to 2002, comparing different regions of the country, from the Amazon to polluted and industrialized regions in the south. Data was collected on male and female live births from all states of Brazil from 1994 to 2002. We calculated the annual male proportion by dividing the number of live born males by the total number of live births [(males)/(males + females)] for each studied year. These proportions were calculated for Brazil as a whole and for 5 main regions: North (Amazon-forest region); Mid-West (intermediate development); Northeast (Atlantic Coastal areas); Southeast (industrialized areas); and South. The scatter plot of the results showed a trend in male proportion from 1994 to 2002 (Fig. 1), that appears to be relatively stable ($p = 0.003$) for the studied years. The number of live births in Brazil from 1994 to 2002 was 27,042,051.

When analyzed separately by regions, however, the sex ratio in the North and Northeast regions was 51.41% and 51.37%, respectively and the sex ratio in the Southeast and South regions was 51.16% and 51.22%, respectively ($p = 0.05$). However, the statistical significance was correlated between Northeast vs. Southeast regions ($p = 0.002$) and North vs. Northeast regions ($p = 0.041$).

A statistical difference in the male-to-female sex-ratio was clearly observed to be higher in the most polluted southern areas of Brazil, whereas the Amazon region preserves the male-female proportion seen in mammals. The decrease suggested a north-south gradient ($p = 0.01$) (Fig. 2). This finding has not been previously reported.
P 07: Positive Association between Air Pollution and Low Birth Weight in the City of São Paulo, Brazil

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Epidemiological studies in São Paulo have confirmed a positive correlation between air pollution and mortality and respiratory morbidity. São Paulo has a fleet of 8.5 million vehicles that emit 68 million tons/year of PM10. The objective was to examine specifically the association between air pollution exposure and Low Birth Weight (LBW), by examining the live birth data of different polluted areas from 2004. The ecological study analyzed data from districts of São Paulo. We obtained singleton certificates in the São Paulo city between January and December 2004 from the State of São Paulo official demographic Institution. We considered as having gestational age between 37 and 41 weeks and separated the weight in two categories – [1] < 2499 g (500–999 g, 1000–1499 g, 1500–1999 g, 2000–2499 g) and [2] ≥ 2500 g (2500–2999 g, and 3000–3499 g, 3500–3999 g). Live-birth infants with birth weights < 500 g or ≥ 4000 g were excluded from all analyses. Annual Low Birth Weight proportion was calculated by dividing the category < 2499 g by < 2499 g + ≥ 2500 g categories ([1]/[1 + 2]). Air pollution data from Environmental Agency of State regarding concentration of PM10 was obtained from monitoring stations and for each birth, measurements obtained from monitors within 4 km of the mother’s residence. A total of 12,920 singleton live births among residents of the study area were identified. The annual mean exposure to PM10 was 51.5 μg/m³ in the most polluted districts and the annual mean exposure to PM10 was 33.5 μg/m³ in the least polluted districts. At the study period the LBW proportion in the least polluted districts was 2.7%, increasing to 5.1% in the most polluted districts.

We have demonstrated a consistent effect of PM10 on birth weight that were born between 37–41 weeks of gestation. A positive association has been observed between the risks for term Low Birth Weight (LBW) and exposure to CO, PM10, SO2 and TSP. There is evidence to suggest that air pollutant exposure during pregnancy has an adverse impact on birth weight.

P 08: DNA Adducts and Mutations in Male Germ Cells from Mice Exposed to Benzo(a)pyrene

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The importance of the paternal genome for fertility and health of the offspring has become increasingly evident. DNA lesions in sperm are associated with poor semen quality and negative birth outcome after in vitro fertilization (IVF). We have previously shown that male germ cells exhibit low or non-functional repair of some types of DNA lesions. Benzo(a)pyrene (B(a)P), a component of cigarette smoke, forms BPDE-DNA adducts, and Zenzes and coworkers showed that such adducts are detectable both in spermatozoa and in preimplantation embryos of smoking fathers. These observations should be investigated further since they suggest persistence of paternal DNA lesions to the stage of the early embryo. Using Big Blue® mice given 3 doses of 50 mg/kg bodyweight of B(a)P (i.p.) we observed that BPDE-DNA adducts that were introduced at the stage of primary spermatocytes or later during spermatogenesis persisted to the stage of caput sperm. No BPDE-DNA adducts were detectable in caput sperm from progenitor cells exposed at earlier stages of spermatogenesis. There was a trend towards increased mutation frequencies that were statistically significant for stem cell spermatogonia. We are currently conducting mice IVF, using sperm from males exposed to B(a)P, to study the paternal transmission and removal of BPDE-DNA adducts.
P 09: DNA Fragmentation in Spermatozoa of Mammalian Species: a Dynamic Process

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Spermatozoal haplotypic DNA is prone to damage, leading to male fertility problems. In this investigation we determined the differential dynamic increase of sperm DNA fragmentation (SDF) in different mammalian species after exposure to a temperature excursion episode (chilled/frozen) and 37°C temperature rewarming to simulate insemination temperature recovery under biological conditions. Frozen sperm samples of human, boar, bull, ram, goat, stallion, donkey, rabbit and koala, were thawed and incubated at 37°C. Sperm DNA fragmentation was assessed using the Sperm Chromatin Dispersion test (Halosperm for humans and Halomax for animals) at different times (from T=0 up to various days). When spermatozoa experience a severe (frozen) or mild (cooled) change in the biological temperature, SDF is induced and causes the subsequent decline of sperm quality. However, the period of time for SDF triggering varies from one species to another and could be detected just at the onset of the biological temperature recovery (human, ram, goat, stallion, rabbit, donkey), could be delayed for a period of 72 h of incubation (bull), or several days (boar). The general pattern of SDF timing was not totally strict within the same species and differences were observed when different individuals were compared. This feature could be a useful measure for pre-screening males before selection for artificial insemination, especially when oocytes are limited or expensive.

P 10: Dynamics of Sperm DNA Fragmentation in Fresh and Frozen Human Semen Samples

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Sperm DNA fragmentation (SDF) must be considered as a dynamic process, i.e. SDF may vary through time after ejaculation. To analyze the incidence of this phenomenon on sperm quality, the dynamics of SDF was assessed in semen samples before and after freezing. Fifteen donors were included in the analysis and from the same ejaculate three aliquots were obtained. One of them was kept fresh in the seminal plasma and two were frozen-thawed. After thawing one aliquot was maintained in the freezing media and the other capacitated. For analyzing SDF all samples were processed with Halosperm. Sperm from different aliquots were incubated during a period of 72 h at 37°C and the rate of SDF at different incubation times (from 0 to 72 h) were scored. Results show that the dynamic behavior of the SDF before and after freezing are different and, in general, capacitation is selecting a specific sperm subpopulation which show less variance in the distribution of SDF values than in no capacitated samples. These results indicate that both the management and capacitation of human sperm for use in assisted reproduction techniques must be completed quickly in order to minimize the DNA damage. Theoretically, this practice should result in an improvement in pregnancy rates.
P 11: Influence of Semen Processing Technique on Human Sperm DNA Fragmentation
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The aim of this study was to determine the efficiency of the density gradient centrifugation to eliminate spermatozoa with nuclear DNA damage particularly with DNA fragmentation. Semen samples \( n = 22 \) were obtained from severely teratozoospermic men (atypical forms: 80–100%) presenting for infertility evaluation. None of the semen samples had significant leukocytospermia or necrozoospermia as per WHO guidelines. Individual samples were divided into two parts: one part of the semen was washed and spread on a slide and the remainder was prepared using density gradient centrifugation. Sperm DNA fragmentation was evaluated with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) assay and a minimum of 300 spermatozoa were counted per patient.

The patients with severe teratozoospermia (85.86 ± 6.11%) showed a significantly higher percentage of DNA fragmentation index (DFI) particularly in whole semen, the DFI had exceeded the value of 30%, in all cases. The percentage of spermatozoa with fragmented DNA was reduced significantly in Pure sperm selected spermatozoa compared with whole semen (19.27 ± 8.68% and 37.63 ± 8.63%, respectively, \( r = 0.802, p < 0.01 \)). In conclusion, our study demonstrated that semen processing by density gradient centrifugation is useful in selection of sperm with high underlying DNA fragmentation.

P 12: Evaluation of Nuclear DNA Damage in Ejaculated Human Spermatozoa
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The aim of our study was to detect DNA fragmentation in human spermatozoa, in order to investigate a correlation between DNA fragmentation index (DFI) and semen parameters. A total of 60 patients were divided into two groups according to their semen parameters, 30 patients with asthenozoospermia (group B) and 30 patients with teratozoospermia (group C). The percentage of sperm DNA fragmentation was evaluated by the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUDP nick-end Labelling) assay. Results were compared with those of spermatozoa sampled from 30 healthy men with normal semen profiles (group A).

The difference was not significant between the percentage of DFI in patients with asthenozoospermia and the normozoospermic men (9.46% ± 8.68 and 8.19% ± 6.84, \( p \) not significant). In addition, no significant correlation was found between the motility of spermatozoa and the DFI in the same group. The patients with teratozoospermia showed a significantly higher percentage of DNA fragmentation than the control group (21.37 ± 17.26% and 8.19% ± 6.84, respectively, \( P < 0.001 \)). There was a positive correlation between abnormal sperm forms and the DFI \( (r = 0.44, P < 0.01) \) in the same group (C). The DFI was particularly higher in patients with 91–100% teratozoospermia (group C2) than the other patients with 80–90% teratozoospermia (group C1) (29.55 ± 20.55 versus 14.3 ± 11.01, respectively). The highest percentage of DFI was found among the patients having a high percentage of microcephalic spermatozoa and acrosome anomalies.

Our study noted that impaired sperm parameters were associated with an increase of DNA fragmentation, this association was strictly related to atypical forms. This finding suggests that teratozoospermia may be the critical sperm parameter associated with hypofertility and when it exceeds the proportion of 90% it would be a prudent sign for analysis of sperm DNA fragmentation.
P 13: Increased DNA Fragmentation and Aneuploidy Rate in Sperm of Meiotic Abnormalities Carriers

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The objective of this study was to correlate the presence of testicular meiotic abnormalities with the percentage of DNA fragmentation and chromosome aneuploidies in ejaculated sperm. A testicular biopsy for meiotic analysis was performed in 36 males. Meiotic analyses were based on the evaluation of the different meiotic stages, chromosome synopsis and chiasmata count.

Sperm DNA fragmentation analysis was assessed with TUNEL (terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labelling) and FISH (Fluorescent in situ hybridization) analysis was done including chromosomes 13, 18, 21, X and Y. Patient’s characteristics are illustrated in Table 1.

**TABLE 1**

|                          | Normal Meiosis | Abnormal Meiosis | P-value |
|--------------------------|----------------|------------------|---------|
| Patient’s n (%)          | 11 (30.6)      | 25 (69.4)        |         |
| Age (Mean ± SD)          | 36.7 ± 4.8     | 37.4 ± 5.7       | NS      |
| Years of infertility (Mean ± SD) | 3.4 ± 1.7     | 4.9 ± 3.5        | NS      |
| Previous miscarriages (Mean ± SD) | 0.6 ± 0.8     | 1.0 ± 1.2        | NS      |
| Total progressive sperm count (Mean ± SD) | 80.0 ± 139.6 | 57.4 ± 63.8     | NS      |

Percentage of chromosome abnormalities in sperm tended to be higher when an abnormal meiotic pattern was observed (56.0% vs. 27.3%; \( p = 0.11 \)). There was 61.11% of coincidence between both tests. Sperm DNA fragmentation was increased in carriers of meiotic abnormalities (27.8% ± 12.0SD) with respect to the ones with a normal meiotic pattern (20.4% ± 13.1SD) \( p = 0.10 \). When meiosis and FISH were abnormal, sperm DNA fragmentation was higher (30.0% ± 12.5) than when both results were normal (19.8 ± 13.1) \( p = 0.09 \). Our study suggests that meiotic anomalies could lead to chromosome abnormalities in germ cells, and these seem to be related to an increase in the percentage of ejaculated sperm with fragmented DNA.
Since June 2007 TESE-ICSI is allowed in the Netherlands. With this technique, spermatozoa are retrieved directly from the testis by taking a tissue biopsy, and isolated spermatozoa are subsequently injected into oocytes. TESE-ICSI is used to treat infertile patients with non-obstructive azoospermia. However, there are uncertainties about the cell biological status of spermatozoa and preceding spermatogenic stages from these patients, in comparison with spontaneous reproduction and about the long-term consequences for the offspring.

Remnant material from sperm retrieval is used for nuclear spreading and sperm nuclear decondensation. Using immunofluorescence, synapsis and progression of recombination intermediates are followed as is sex body formation and sex chromatin inactivation. These observations are related to karyogenesis in round and elongating spermatids. The data thus recovered are further matched against reproductive success. As in the Netherlands, up to three artificial reproduction cycles are covered by health insurance, biopsies that result into a pregnancy in the first cycle are compared with biopsies that were followed by three failed attempts. To reduce the negative age-related female factor in the pregnancy chance, only biopsies are included from a couple in which the woman is 35 or below. Preliminary examples from the analysis will be shown, illustrating the usefulness of this approach.

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P 15: Evidence of Lower DNA Damage in Testicular Compared to Ejaculated Spermatozoa

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Men with elevated sperm DNA damage (>30%) often exhibit reduced fertility. Oral antioxidant therapy and the retrieval of testicular spermatozoa have been suggested as treatment options for these patients. The purpose of our study was to compare DNA damage in ejaculated and testicular spermatozoa collected on the same day in patients with previously unsuccessful oral antioxidant treatment. Following IREB approval, patients with DNA damage >30% as measured by flow cytometry using the DNA fragmentation index (DFI) were placed on a three month course of oral antioxidant treatment. In 12 patients with persistently high sperm DNA damage (430%), DNA damage in spermatozoa from testicular biopsies was compared to that in ejaculated spermatozoa. Both samples were collected on the same day immediately prior to ICSI. Since testicular tissue is unsuitable for flow cytometry assessment, slide based TUNEL was chosen for assessment of DNA damage in both types of samples. The Wilcoxon signed ranks test was used to evaluate differences between DNA damage in ejaculated and testicular spermatozoa. Results are expressed as mean ± SD. Ejaculated spermatozoa showed three-fold higher DNA damage when compared to testicular samples (39.7% ± 14.8, vs. 13.3% ± 7.3; P < 0.001). Our results indicate that retrieved testicular spermatozoa have significantly lower DNA damage than ejaculated sperm in patients with persistently high DFI following unsuccessful oral antioxidant treatment. A randomized controlled study is currently underway to compare pregnancy outcomes after ICSI with testicular vs. ejaculated spermatozoa in patients with persistently high sperm DNA damage.

P 16: Comparison of Methods to Detect DNA Damage in Sperm from Different Mammalian Species

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Sperm DNA integrity is pivotal for normal fertilization and transmission of paternal genetic information. Methods have been developed to assess the nuclear integrity of spermatozoa, including the sperm chromatin structure assay (SCSA), the terminal TdT-mediated d-UTP-nick-end-labeling (TUNEL) and the comet assay. These methods evaluate DNA integrity from different and complementary perspectives, and could offer a new class of biomarkers of the male reproductive function and of its possible impairment after environmental exposure, but their usefulness needs to be validated. The chromatin in sperm is extremely condensed and its structural remodeling is species-specific. These differences in chromatin structure may hide differences in DNA damage susceptibility to a given stressor. In this study the capacity of SCSA, TUNEL and comet assay to detect DNA/chromatin integrity has been evaluated in human, mouse and bull spermatozoa. In vitro treatments with DNAsel were performed considering that the activity of this enzyme depends on its accessibility to DNA thus reflecting the chromatin packaging characteristics of sperm. Results suggest a different sensitivity between the species with human spermatozoa being the most sensitive. Furthermore, data obtained show a good correlation among the three tests in revealing sperm with DNA strand breaks induced by DNAsel. These results can be useful to standardize the protocols used to detect DNA damage in sperm, and to determine how the results generated by the different assays correlate with each other among different species having in mind the problem of risk extrapolation in reproductive toxicology.
Improved Prediction of Male Fertility \textit{In Vivo} by Use of the Sperm Chromatin Structure Assay: a Case-Control Study

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Standard sperm parameters were shown to have a limited power in prediction of chance of natural conception. Recent studies have indicated the Sperm Chromatin Structure Assay (SCSA) DNA Fragmentation Index (DFI) is associated with fertility \textit{in vivo}. We wished to evaluate the value of this parameter for prediction of infertility. A total of 142 men from infertile couples with no known female factor were studied. As controls were 135 proven fertile men. Semen analysis was done as recommended by WHO. DFI was assessed using SCSA. Logistic binary regression was used for calculating the odds ratios (OR) for infertility. DFI was an independent predictor of infertility with OR of 2.5 (95% confidence interval [CI] 1.0; 6.1) for DFI between 10% and 20% and 8.4 (95% CI: 3.0; 23) for DFI \geq 20%. In men with normal standard semen parameters, the increase in the OR of infertility was seen for DFI \geq 20% (5.1, 95% CI: 1.2; 23) whereas if one of the standard parameters was abnormal, high OR for infertility was already seen at DFI above 10% (16, 95% CI: 4.2; 60). DFI is an independent predictor of chance of natural conception and the cut off level of DFI for increase in the risk of infertility depends on whether the standard sperm parameters are normal or not.

DNA Fragmentation and Adducts and their Relationship with Semen Analysis Parameters in Sperm of Infertile Men

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Conventional semen analysis for male infertility diagnosis depends on the microscopic assessment of sperm concentration, motility and morphology. However recent studies question their clinical value. Sperm DNA testing is increasingly recognized as a useful indicator of male fertility potential. The alkaline Comet assay is a rapid and sensitive method to detect DNA damage in individual sperm. To include the sensitive detection of potential DNA damage present in ejaculated sperm in the form of DNA adducts, we used formamidopyrimidine-DNA glycosylase (fpg) an enzyme which recognizes and converts oxidized purines into DNA strand breaks which can then be measured by the Comet assay. Semen and sperm prepared by density centrifugation from 87 men attending a Regional Fertility Centre, andrology lab were included in the study. No correlations were observed between semen analysis and DNA fragmentation specifically; DNA fragmentation and semen volume ($p = 0.759$), concentration ($p = 0.370$), progressive motility ($p = 0.982$), non-motile sperm ($p = 0.352$), morphology ($p = 0.729$) or total motile count ($p = 0.531$). However, DNA damage was significantly correlated with non-progressive motility ($p = 0.007$). Sperm DNA fragmentation in native semen was $43.3 \pm 3.7\%$ increasing to $51.2 \pm 3.9\%$ ($p < 0.001$) when DNA adducts were converted to strand breaks by fpg. Similarly, DNA damage in prepared samples was $32.3 \pm 3.1\%$ increasing to $39.7 \pm 3.5\%$ ($p < 0.001$) after incubation with fpg. The additional damage observed after fpg treatment indicates the impact of oxidative stress on sperm DNA. Adding potential and actual sperm DNA damage together may provide the basis for a useful prognostic test for male infertility.

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P 19: Effects of the Degradation of the Spermatozoa DNA on the Ovocyte Fertilization

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In the daily practice we often find variations of behavior of the seminal fluid, with parameters in normality, on the fertilizing ability. The objective of this work was to estimate if several degrees of DNA degradation of the spermatozoa influence the ovocyte fertilizing ability.

In order to estimate the integrity of spermatozoa DNA, the Acridine Orange, a specific dye of DNA, has been used. The formation of embryos by F.I.V.E.T. technique at 4–6 cell stage has been considered for the ovocyte fertilization. The 215 patients who have participated in this work were aged between 20 and 35 with an infertility history which goes from a minimum of two to a maximum of five years with a count of spermatozoa per the whole ejaculate of over 25 million, motility degree 3—/3+.

The groups have been subdivided on the basis of DNA degradation: group A 58 patients - low; group B 87 patients – moderate and group C 70 patients – high. For our work we availed ourselves to retrospective studies on F.I.V.E.T. effected between 1998 and 2003. Fertilized ovocytes: group A 516 out of 813 (63.5%); group B 528 out of 907 (58.2) and group C 306 out of 774 (39.5%).

A high degradation of spermatozoa DNA is associated with a poor quality and a low amount of ovocyte fertilization. As completion of this work we also find a high degradation of DNA with low pregnancy rate.

P 20: Therapeutic Effects of FSHr and/or Tocopherol on Ovocyte Fertilization in Subjects with Grave Degradation of the Spermatozoa DNA

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The objective of this work is to estimate if a pathological degradation of spermatozoa DNA is recoverable by a medical therapy. In order to estimate the spermatozoa DNA integrity, the Acridine Orange, a specific dye of DNA, has been used. The formation of embryos by the F.I.V.E.T. technique at 4–6 cell stage, has been considered for the ovocyte fertilization. A total of 1,805 oocytes were used for this work altogether. The 148 patients participating in this work are aged between 20 and 37 with an infertility history which goes from a minimum of two to a maximum of six years with a count of spermatozoa per the whole ejaculate over 20 million, motility degree 3—/3+.

The groups have been subdivided in: group A 48 patients no therapy; group B 36 patients treated with FSHr 450UI/weekly/3 months; group C 41 patients treated with Tocopherol 600 mg/daily/3 months and group D 23 patients treated with FSHr + Tocopherol. For our work we have availed ourselves of retrospective studies on F.I.V.E.T. and therapeutic programs between 1996 and 2003. Fertilized oocytes: group A 229 out of 571 (40.1%); group B 254 out of 504 (50.4%); group C 271 out of 512 (52.9%) and group D 119 out of 218 (54.6%).

On the basis of these preliminary results, these drugs have proved to be useful in those pathophysiological conditions of male infertility. We are also convinced that further studies and higher number of patients are necessary to confirm.
Oxidative stress (OS), which is a result of the imbalance between reactive oxygen species (ROS) and antioxidants, can lead to sperm damage and eventually male infertility. Recently, the use of Hydroethidine (HE) and H2DCFDA probes has been revealed as a novel method for the study of intracellular ROS production by flow cytometry in semen samples. HE is a cell-permeant compound that can be used to measure intracellular oxidation by superoxide, whereas H2DCFDA is a cell-permeant indicator for reactive oxygen species that is nonfluorescent until removal of the acetate groups by intracellular esterases and oxidation occurs within the cell. The aim of this study was to determine if the HE and H2DCFDA analysis on human semen samples by flow cytometry is a useful assay to determine the intracellular ROS production in human spermatozoa.

Human semen samples from 40 patients and 51 unselected fertile donors were obtained by masturbation after 2 to 3 days of sexual abstinence. After liquefaction, semen analysis was performed following WHO criteria. Then, spermatozoa were processed on discontinuous two-step pure sperm density gradients (45% and 90%), resuspended on 0.5 ml HTF medium and reanalyzed for concentration, motility and morphology parameters. Fresh and selected spermatozoa were then washed twice (1,000 x rpm for 10 min) and adjusted to 0.5 x 10^6 cells/ml with HTF medium. For the assay, HE (3 μM) and H2DCFDA (10 μM) were diluted in PBS buffer and added to 0.5 x 10^6 fresh or selected spermatozoa in a final volume of 500 μl. The cells were then incubated in the dark at 37°C for 30 min (for HE) or 60 min (for H2DCFDA), washed twice (2,000 x rpm, 5 min) and the resultant red (HE) and green (H2DCFDA) fluorescence was analyzed by flow cytometry using a FACScan analyzer. Statistical analyses were performed using SPSS software. A p value <0.05 was considered statistically significant.

Comparisons of patient and fertile donor neat semen samples with respect to sperm variables showed a significant negative correlation between motile sperms and DHE or H2DCFDA expression. However, no correlation was found between concentration and ROS positive cells. The study of neat semen samples also showed that the percentage of both DHE and H2DCFDA positive cells were significantly lower in fertile donors (31.8% ± 6.8 and 58.3% ± 8.2, respectively) compared with patients (35.2% ± 7.2 and 62.8% ± 12.2, respectively). After sperm selection, the percentage of both DHE and H2DCFDA positive cells was diminished, showing no significant differences between the two groups (15.6% ± 10.3 vs. 15.2% ± 8.2, for DHE positive donor and patient cells, respectively, and 6.5% ± 5.7 vs 7.5% ± 6.1, for H2DCFDA positive donor and patient cells, respectively). Intracellular ROS determination is an independent marker of concentration but not motility parameters in neat semen samples. Sperm selection by density gradient significantly diminished the percentage of ROS positive cells at similar levels in both fertile donors and patients.
Sperm DNA integrity can be related with male infertility even in those cases of patients with moderate or no alterations of seminal parameters. In this study we have analyzed the relationship between the grade of sperm DNA damage in the first and the second day of the treatment (IUI) and the pregnancy rate.

Semen samples from 278 men and 31 fertile donors were obtained by masturbation after 3 to 5 days of sexual abstinence. After liquefaction, semen samples were evaluated according to WHO. An aliquot of the neat semen of each patient was reserved to analyze the sperm DNA fragmentation. The TUNEL assay was performed using the commercial kit ‘In Situ Cell Death Detection Kit Fluorescein.’ Semen samples were fixed in 4% PFA and permeabilized with 0.1% Triton 100 X. The TdT enzyme was added to each sample and incubated in a humidified chamber at 37°C for 45 min. The samples were analyzed by flow cytometry using FACScan. The remaining semen sample was capacitated, using the swim-up technique and an aliquot was taken just before giving the artificial insemination to the gynecologist in order to compare the sperm DNA fragmentation between the first and the second day of the treatment. Statistical analysis was performed with SPSS for Windows software package version 13.0. Statistical differences were considered significant at P < 0.05.

The results obtained did not show a significant difference in IUI by husband (P = 0.659) or IUI by donor (P = 0.502) when we analyzed the sperm DNA fragmentation in the first day with respect to the second day of the treatment. Similarly the results did not show a significant difference between sperm DNA fragmentation in the first day with respect to the second day of treatment in patients who obtained a pregnancy and those who did not (P = 0.504 and P = 0.838, respectively).

The results suggest that the sperm DNA fragmentation percentage does not change having a short or moderate sexual abstinence and so sperm DNA damage is similar in both days of the treatment. Finally there is no correlation between a high sperm DNA fragmentation grade and the absence of pregnancies.
The aim of this study is to investigate if novel sperm parameters like DNA fragmentation, chromatin packaging or HA binding are correlated with standard WHO sperm parameters like motility, concentration and morphology.

Spermatozoa were collected from 205 men undergoing a first IVF/ICSI treatment. Motility, concentration and morphology were analyzed according to WHO criteria. Sperm chromatin structure assay (SCSA) determined the DNA fragmentation index (%DFI) and High DNA stainability (%HDS). Poor chromatin packaging was visualized by chromomycin A3 (CMA3) staining. HA binding was studied using the Sperm-Hyaluronan Binding Assay. Statistics analysis included the Pearson Correlation coefficient evaluation.

Mean age of the patients was 35.6 years (SD ± 4.8). A mean concentration of 32.8 × 10^6 spermatozoa/ml (SD ± 32.8) was noted, 5.4% of normal forms (SD ± 2.9) and 21.2% and 30.4% Grade A and B motility respectively (SD ± 13.4; 11.5, respectively). An inverse correlation was found between %DFI (mean of 22.1%) and concentration (r = −0.27), Grade A (r = −0.32) Grade B motility (r = −0.30) and morphology (r = −0.30). A positive correlation was found with Grade D (immotile) motility (r = 0.40). %HDS (mean of 11.98%) was negatively correlated with concentration (r = −0.28), grade A motility (r = −0.20) and morphology (r = −0.24). Chromatin packaging (mean 19.21%) was inversely correlated with sperm concentration (r = −0.25) and positively with Grade C motility (r = 0.15). HA binding (mean of 71%) was significantly correlated with morphology (r = 0.4191). Analysis of HA binding, chromatin packaging and DNA fragmentation could give a better understanding of the overall quality of a semen sample.
P 24: Chronic Inhalation of Crack Cocaine Severely Affects Spermatogenesis in Young Mice

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Chronic inhalation of crack (57–66% of pure cocaine) on the spermatogenesis of young and mature mice was investigated. Balb/c mice of two different ages, pubertal and adult (n = 20/age), were exposed to the smoke of 5 g of crack cocaine in an inhalation chamber, 5 days/week/2 months. Control animals (n = 10/age) were kept in animal house during experimentation. Samples of transversal sections of testis were fixed in Bouin’s solution and paraffin embedded. Histological testis sections (4 μm) were HE stained for normal cells quantification or treated with caspase-3 for apoptosis immunolabelling score. Blood samples were collected for cocaine serum concentration determination. Morphometric quantitative analyses of normal cells in testis were made within a reference area (grid) and apoptosis were scored in 3 histological sections/testis/animal, optical microscope. Cocaine serum concentration: 212.5 ng/ml. Young intoxicated animals presented significant reductions of tubular sections undergoing phase VII (p = 0.023); reduction in Sertoli cells and elongated spermatids (p < 0.001 and p = 0.005, respectively); and increased number of round spermatids (p = 0.001). Leydig cells in testis of adult exposed animals presented lower population density (p = 0.023) and higher number of apoptotic cells (p = 0.002). Inhalation of crack-cocaine smoke induced spermatogenesis disruption of chronic exposed mice. Crack toxicity was more severe in pubertal mice when sexual gonad undergoes maturation. This finding is a public health issue since the crack-cocaine is an affordable illicit drug mainly abused by teenagers and kids, reaching alarming epidemic proportions.

P 25: PHGPx and Sperm Nuclear Matrix

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Normal sperm production in mammals is strictly dependent upon selenium, an element that is incorporated in the testis at the level of the selenoprotein Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx). Mouse germ cells express three isoforms of this enzyme, having multiple functions and representing the pivotal link between selenium, sperm quality and male fertility. It was proposed that the nuclear variant nPHGPx is involved in the stabilization of sperm condensed chromatin. In agreement with this, sperm of Se-deficient mice display abnormal heads. To gain more insight into the role of nPHGPx, we have studied the subnuclear localization and association with other nuclear components of this enzyme by both in vitro and in vivo experiments. Morphological and biochemical analyses of transfected cells showed that the exogenous nPHGPx was restricted to nucleus, being mostly localized at the level of the nuclear matrix. In line with this evidence, nPHGPx did not co-immunoprecipitate with either HP1β, or Histone H3. In addition, confocal microscopy analysis showed that nPHGPx did not co-localize with HP1β. Nuclear subfractionation protocol and western blot analysis performed on isolated germ cells at different steps of maturation demonstrated that nPHGPx was present in the nuclear matrix fraction of round spermatids, whereas it was distributed in both nuclear matrix and chromatin fractions of epididymal spermatozoa. The localization of nPHGPx has further been demonstrated by exploiting an in situ nuclear matrix preparation of spermatozoa heads. In conclusion, the shift of nPHGPx to different subnuclear regions during sperm maturation suggests more than a single role for this selenoprotein.
P 26: Impact of Metabolic Disorders on Sperm DNA Fragmentation

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The deleterious influence of diabetes and obesity on fertility is receiving increasing medical attention since prevalence and incidence of both disorders are growing steadily worldwide and the age at first diagnosis is continuously declining. As a consequence, diabetes and obesity are expected to affect many more individuals during their reproductive years. The aim of our study was to investigate the impact of metabolic disorders on sperm DNA integrity.

Semen specimen of 19 healthy donors, 21 overweight or obese males, 13 men with type I diabetes and 14 men with type II diabetes were prepared by density gradient centrifugation. Besides standard sperm parameters, serum hormone levels, serum lipids and HbA1c, body mass index and abdominal girth were analyzed. DNA fragmentation was measured by FACS using TUNEL assay.

Compared to healthy donors (TUNEL $^+$, Mean $\pm$ SEM: 8.2 $\pm$ 2.3%), semen samples from males with metabolic disorders showed significantly higher levels of DNA fragmentation (overweight/obese: 22.4 $\pm$ 3.2%, type I diabetes: 22.5 $\pm$ 2.3%, type II diabetes: 28.9 $\pm$ 4.9%, $p < 0.05$). High DNA fragmentation rates were significantly positive correlated to age, body mass index, abdominal girth and HbA1c serum levels. Serum hormone levels (Testosterone, LH, FSH) were not directly associated with the DNA integrity ($p > 0.05$). Serum lipid analysis revealed a significant correlation of TUNEL $^+$ sperm to the triglyceride levels, but not to HDL and LDL levels. Increased DNA fragmentation might be one factor for subfertility observed in patients with metabolic disorders.

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Epimutations may account for an increased incidence of certain congenital diseases in children conceived via assisted reproduction techniques (ART) and have been reported as a potential cause for male infertility. Thus we analyzed the paternal imprint H19 in a large cohort of idiopathic infertile men.

From 103 oligozoospermic and 77 normozoospermic men genomic DNA was isolated from swim-up purified spermatozoa. The methylation status of a 321bp fragment in a differentially methylated region of H19 was determined by Dye-Terminator sequencing that allows rapid methylation analysis at single CpG-site resolution. Mean methylation values were correlated with total sperm count, sperm motility and morphology.

Men with a total sperm count of < 40 million showed a significant decrease of H19 methylation (below 80%) compared to men with > 40 million spermatozoa (p=0.016). Moreover, 33% of severe oligozoospermic men (0.1–20 million total) showed hypomethylation of H19 while only 15% of normozoospermic men with high sperm count (100–1600 million total) displayed this defect (p=0.008). We also found a strong association of hypomethylation with sperm motility. Men with less than 50% progressive sperm motility displayed more severe and more frequent hypomethylation than men above 50% progressive motility. No association of H19 methylation with sperm morphology was detectable.

Our data show that epimutations are strongly associated with impaired spermatogenesis and sperm motility. We hypothesize that spermatogenic impairment does not only affect sperm function, but also has impact on epigenetic processes such as paternal imprinting.

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Aim of the present study was to determine ONOO\(^-\) production in semen and its correlation with kinetic features in spermatozoa, together with protein tyrosine nitration in the same samples.

Spermatozoa themselves generate small amounts of \(O_2^-\) and NO\(^-\). Under physiological conditions these compounds exist at very low concentration; however their local concentrations become significant close to the production sites and the formation of peroxynitrite (ONOO\(^-\)) appears likely. Peroxynitrite reacts rapidly with proteins, lipids and DNA. The nitration (addition of an NO\(_2\) group) of protein residues gives rise to 3-nitrotyrosine which represents a protein modification specific for ONOO\(^-\) formation \textit{in vivo}. Semen samples from 25 normal fertile donors (controls) and 40 infertile patients affected by idiopathic asthenozoospermia were analyzed according to WHO criteria. After liquefaction, one aliquot of semen was diluted with PBS and was stored at \(-80^\circ\text{C}\) until determinations. Peroxynitrite concentration was measured through the fluorescence of the DCFDA probe. Protein tyrosine nitration was determined by Western Immuno Blot with an appropriate antibody. Kinetic parameters of sperm cells were determined using CASA system.

Controls exhibited ONOO\(^-\) production significantly lower than asthenozoospermic patients \((p < 0.001)\); furthermore, ONOO\(^-\) exhibited a significant inverse correlation with the motility parameters. Moreover, in western immuno blot there was an increase in the nitration of the tyrosine residues in the asthenozoospermic samples compared to controls. The present data show a critical negative effect of peroxynitrite on sperm motility when spermatozoa concentration is normal, thus suggesting a possible pathogenic role in infertile men.
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