European Society of Human Reproduction and Embryology

Andrology Pre-congress course

"Sperm Maturation in Men: Adluminal Compartment and Epididymis"

1 July 2001

Lausanne - Switzerland

PRE-CONGRESS COURSE EVALUATION FORM

Pre-congress course Andrology

"Sperm Maturation in Men: Adluminal Compartment and Epididymis"

DEMOGRAPHICS

1. Professional status (tick appropriate box) ❑ Resident ❑ Fellow ❑ Private practice (no teaching) ❑ Full-time faculty ❑ Private practice (clinical faculty) ❑ NurseResearch/Lab ❑ Other

2. Years since completion of training: ❑ less than 5 ❑ 5-9 ❑ 10-19 ❑ 20&over ❑ currently in training (tick appropriate box)

GENERAL INFORMATION (tick appropriate box)	Strongly Agree	Moderately Agree	Moderately Disagree	Strongly Disargee
1. The course objectives were clearly stated	❑	❑	❑	❑
2. The course objectives were clearly met	❑	❑	❑	❑
Faculty-participant interaction was satisfactory	❑	❑	❑	❑
The coruse was well-organised	❑	❑	❑	❑
Site accessibility was not a problem	❑	❑	❑	❑
Meeting facilities were adequate	❑	❑	❑	❑
Course co-ordinator conducted program well	❑	❑	❑	❑
Overall course grade (circle one)	❑	❑	❑	❑
Overall syllabus grade (circle one)	❑	❑	❑	❑

EDUCATIONAL VALUE (tick appropriate box)				
1. I learned new that was important	❑	❑	❑	❑
2. I verfied some important information	❑	❑	❑	❑
3. I plan to discuss this information with colleagues	❑	❑	❑	❑
4. I plan to seek more information on this topic	❑	❑	❑	❑
5. My attitude about this topic changed in some way	❑	❑	❑	❑
6. This info. is likely to have an impact on my practice	❑	❑	❑	❑

FACULTY EVALUATIONS

Directions: As a participant in this program, your assessment is very mportant. Please evaluate each faculty member on all 8 dimensions according to the scale

Strongly Agree **4**	Moderately Agree **3**	Moderately Disagree **2**	Strongly Disagree **1**	**HUSZAR**	**FRASER**	**BERNARDINI**	**DE JONG**	**YEUNG**	**MOORE**	**JEGOU**	**AITKEN**	
A. PRESENTATIONS												
1. Sufficient depth												
2. Concepts clearly explained												
3. Enhanced understanding of key matters												
4. Without bias												
5. Slides readable												
B. SYLLABUS CONTENT												
1. Learning objectives clear												
2. Helpful for future reference												
C. OVERALL GRADE - Grade A (high) through D (low)												

Additional Comments: _____

_____

_____

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Please submit completed Pre-congress Course Evaluation form to the Course Co-ordinator

Course Program

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09.00-09.30: Sperm Cellular Maturation during Spermiogenesis - G. Huszar (USA)

09.30-10.00: Peptide Modulators of Sperm Function - L. Fraser (UK)

10.00-10.30: Discussion

10.30-11.00: Coffee

Session B: DNA Structure - *Chair:* L. Fraser (UK)

11.00-11.30: Aneuploidy Frequency in Mature and Diminshed Maturity Sperm - L. Bernardini (I)

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12.00-12.30: Discussion

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15.00-15.20: Coffee

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15.20-15.50: Toxicants? Testicular Events? Epididymis - B. Jegou (F)

15.50-16.20: Reproductive Toxicity and the differentiation of a functional gamete - J. Aitken (AUS)

16.20-17.00: Discussion and Conclusions

Sperm Celluler Maturation During Spermiogenesis

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Learning Objectives

Within the scope of the relationship between the 70kDa HspA2 chaperone protein, fertilizing potential and chromosomal aneuploidies, we will discuss four issues that are related to human sperm cellular maturation during spermiogenesis.

(1) Cytoplasmic retention as an evidence of sperm immaturity, and the two waves of HspA2 chaperone protein expression during meiosis and terminal spermiogenesis.
(2) Cellular maturation, plasma membrane remodeling and fertilization function of human sperm;
(3) Relationship among sperm immaturity, diminished DNA integrity and increased frequencies of chromosomal aneuploidy.
(4) Selection of mature individual sperm for ICSI.

Cytoplasmic Retention and Other Biochemical Markers of Sperm Cellular Maturation

The primary interest of our laboratory has been the development of objective biochemical markers of human sperm maturity and function which would predict male fertility, independently from the traditional semen criteria of sperm concentration and motility. In measurements of sperm creatine-N-phosphotransferase or creatine kinase (CK), we found significantly higher sperm CK activities in men with diminished fertility [1]. We addressed the reasons underlying the sperm CK activity differences by labeling the enzymatic-active site of sperm CK with 14C-FDNB followed by autoradiography. In another approach, we visualized the CK in individual sperm with CK-immunocytochemistry. The autoradiographic and CK immunostaining patterns indicated that the high sperm CK activity was a direct consequence of increased cytoplasmic protein and CK concentrations in the spermatozoon. The combination of increased CK and protein concentrations, coupled with the diminished fertility suggested to us that we had identified a sperm developmental defect in the last phase of spermiogenesis when the cytoplasm (unnecessary for the mature sperm) normally is extruded and left in the adluminal area as “residual bodies" [2].

Upon electrophoretic analysis of human sperm extracts, in addition to the CK-B isoform, we found another ATP-containing protein, which was proportional to the incidence of mature sperm, characterized by low CK-activity and no cytoplasmic retention in semen samples. We have

recently identified this developmentally regulated protein as the 70 kDa testis expressed chaperone protein, which in man is called HspA2 [3,4]. The close inverse correlation between the proportions of sperm with cytoplasmic retention and low expression of HspA2 and those sperm with lack of cytoplasmic retention and increased expression of HspA2, indicated that cytoplasmic extrusion and the commencement of the HspA2 synthesis are related developmentally regulated spermiogenetic events (In three independent studies the correlation between HspA2 levels and CK-activity was r=-0.69, -0.71 and –0.76, P<0.001, N=159, 134, and 119).

HspA2 which, due to it's electrophoretic properties and ATP-content, we initially assumed to be an unusual form of sperm specific CK-M isoform (several properties have indicated that it was not a conventional CK-M, ref 4), Nevertheless, HspA2 is a most useful objective biochemical marker. We have shown that mature and immature sperm are different with respect to degree of cytoplasmic retention, HspA2 ratio, as expressed by the concentrations of sperm CK and HspA2 [%HspA2/(HspA2 + CK-B)], morphological and morphometrical attributes, zona pellucida-binding properties and fertility [5,6]. Furthermore, we have established that in spermiogenesis, simultaneously with cytoplasmic extrusion and the commencement of HspA2 synthesis, the sperm plasma membrane also undergoes a maturation-related remodeling. This remodeling step facilitates the formation of the sites and receptors for zona-binding and for hyaluronic acid binding in mature sperm [7]. We have also shown, along with another laboratory, that immature sperm have increased rates of lipid peroxidation [8,9]. Immature sperm also exhibit higher frequencies of chromosomal aneuploidies [10]. Finally, we established that all sperm maturational events, related to the decline of CK activity and increase in HspA2 expression, are completed by the time the sperm enter the caput epididymidis [11].

Cellular Maturation And Development of The Fertilization Function

The predictive value of CK-activity, representing cytoplasmic retention, was tested in couples with oligospermic husbands treated with intrauterine insemination. In spite of identical sperm concentration and motility parameters in husbands of those couples that have or have not achieved pregnancy, had four-fold difference in sperm CK activity (p<0.001, those with pregnancies had the lower CK-activity). Also, a logistic regression analysis indicated that sperm CK activity, but not sperm concentrations, contributed significantly to the predictive power [12]. The value of the HspA2 ratios in the assessment of male fertility was tested in two blinded studies of couples undergoing in vitro fertilization. In the first, we classified 84 husbands from two different IVF centers (without any information on their semen parameters or reproductive histories) based only on their sperm HspA2 ratios into “high likelihood” (>10% HspA2 ratio) and “low likelihood” (<10% HspA2 ratio) for fertility groups. All pregnancies occurred in the “high likelihood” group. No pregnancy occurred in the “low likelihood” group. In the “high likelihood” group, if at least one oocyte was fertilized, indicating the lack of oocyte defects in the wife, the predictive rate of HspA2 ratio for pregnancy was a very high 30.4% per cycle. An additional important utility of the HspA2 ratio became apparent: 9 of the 22“ low likelihood” men were normospermic but had diminished fertility. Thus, the HspA2 ratio provided, for the first time, a diagnostic tool for unexplained male infertility (infertile men with normal semen, ref. 6). More recently, we reexamined the utility of CK-M ratios in predicting IVF failure in 119

couples treated at Yale. Similar to the 1992 study, none of the 25 men with <10% CK-M ratios achieved pregnancy whether they had low or high sperm concentrations. The value of sperm CK studies has been confirmed by other laboratories.

To identify the steps of the fertilization process, at which the low HspA2 immature sperm are deficient, we explored human sperm-oocyte binding. With the study of sperm-hemizona complexes, we established that only the clear headed (low CK), mature sperm were able to bind to the zona [Figure 1, ref. 5]. Sperm with retained cytoplasm were deficient in the oocyte binding site. In a further study, we confirmed that a plasma membrane remodeling occurs in human sperm, simultaneously with cytoplasmic extrusion, during spermiogenetic maturation. This was demonstrated by the close correlation(r=0.8) between CK concentration or the HspA2 ratio and the density of the sperm plasma membrane-specific enzyme, b1,2,-galactosyltransferase in sperm fractions of various maturities [7]. This finding well explains two major characteristics of sperm with diminished maturity: cytoplasmic retention and deficiency in zona pellucida binding.
In general, chaperones facilitate the assembly and intracellular transport of proteins. Indeed, the expression of HspA2 is simultaneous with major sperm protein movements underlying cytoplasmic extrusion and remodeling of the human sperm plasma membrane. This in turn facilitates the development of the zona pellucida-binding site. We believe that retention of the cytoplasm, and the lack of zona-binding sites in immature sperm are likely related to the diminished expression of HspA2, and also to diminished DNA integrity, as a consequence of the impaired delivery of DNA repair enzymes during and following meiosis. In order to confirm our finding regarding the expression of HspA2 during terminal spermiogenesis, we also examined the expression pattern of HspA2 in human testicular tissue (Figure 2). Varying low levels of immunostaining was evident in spermatocytes and spermatids reflecting the presence of HspA2 in the synaptonemal complexes, but the staining was particularly striking in the cytoplasm of elongating spermatids and mature sperm about to be released from the adluminal compartment. This pattern is consistent with the biochemical data, which indicated that HspA2 is developmentally regulated during spermatogenesis and expressed simultaneously with cytoplasmic extrusion in late spermiogenesis [3,4].

From the perspective of male infertility, it is important that synthesis of the HSP70-2 family of proteins is developmentally regulated and that HSP70-2 appears during meiotic prophase as a component of the synaptonemal complexes. The testis expressed Hsp70-2 protein has been identified in the mouse, and it is expressed in pachytene spermatocytes during the meiotic phase of spermatogenesis and in spermatids and mature sperm [13]. The apparent functions of Hsp70-2 in mice are maintaining the synaptonemal complexes and assisting chromosome crossing over during meiosis and spermatocyte development. Accordingly, the targeted disruption of the hsp70-2 gene causes arrested sperm maturation and azoospermia. These events could be related to faulty meiotic recombination in spermatocytes, disruption of the meiotic cell cycle regulatory machinery, or perhaps to a more direct disruption of the apoptotic machinery in spermatocytes or even in spermatids or ejaculated immature sperm. Regarding human sperm, our laboratory was the first to demonstrate the expression pattern of the HspA2 protein in human testis and sperm and to correlate the expression level of HspA2 to sperm function. Because we had already identified maturational differences in cytoplasmic content, plasma membrane remodeling, DNA integrity and aneuploidy rates, we explored whether the plasma membrane structure differences and features specific for mature sperm could facilitate the selection of mature sperm for ICSI.

Relationship Between Sperm Immaturity, DNA integrity and Chromosomal Aneuploidies

Because HspA2 is a component of the synaptonemal complex in rodents, assuming that this is also the case in men, we hypothesized that the frequency of chromosomal aneuploidies will be higher in immature vs. mature sperm. We have examined this question in sperm arising from semen and from 80% Percoll pellets (enhanced in mature sperm) of the same ejaculate in 10 oligozoospermic men. Immature sperm with retained cytoplasm, which signifies spermiogenetic arrest, were identified by immunocytochemistry. We have evaluated with FISH approximately 7000 sperm nuclei in each of the 20 fractions (10 semen and 10 80% Percoll fractions, 142,086 sperm in all) using centromeric probes for the X, Y, and 17 chromosomes. The proportions of immature sperm were 45.4±3.4% vs. 26.6±2.2% in the two groups (medians:48.2% vs. 25%, $P<0.001$, N= 300 sperm per fraction, 6000 sperm in all). There was also a concomitant decline in total disomy, total diploidy and total aneuploidy frequencies in the 80% Percoll vs. semen fractions (0.17 vs. 0.54%, 0.14 vs. 0.26% and 0.31 vs. 0.81%, respectively, $P<0.001$ in all comparisons). The mean decline of aneuploidies was 2.7-fold. Regarding our hypothesis that aneuploidies are related to sperm immaturity, there was a close correlation between the incidence of immature sperm and disomies ($r=0.7$, $P<0.001$), indicating that disomies originate primarily in immature sperm. Thus, the idea that the common factor underlying sperm immaturity and aneuploidies is the diminished expression of the HspA2 appears to be valid [8]. The concepts regarding the central role of HspA2 and cytoplasmic extrusion/retention in aspects of spermiogenetic maturation are summarized in Figure 3.

Selection of Mature Sperm with High DNA Integrity and Low Frequency of Chromosomal Aneuploidies

We have also found earlier that mature, but not immature, spermatozoa in response to hyaluronic acid (HA) showed increased velocity and retention of long-term motility [14]. Based on the association between sperm maturation and plasma membrane remodeling, we have also expected that the HA receptor will be present in the mature, but not in immature, sperm and that an HA-coated surface will facilitate the selection of single mature sperm with high DNA integrity and low frequency of chromosomal aneuploidies for ICSI.

It was previously established that immature sperm with increased cytoplasmic retention show a higher rate of lipid peroxidation. Reactive oxygen species contribute to DNA degradation [8,9]. We hypothesized that the increased rates of DNA degradation, coupled with the DNA repair defects secondary to diminished HspA2 in immature sperm would cause an association between diminished sperm maturity and increased DNA degradation.

In order to test these ideas with respect to sperm with high DNA integrity, we exposed sperm suspensions to HA-surfaces. After 30 minutes the free sperm fraction was removed by washing, and the HA-bound sperm were released by 0.1% Triton. Both the free and bound/released sperm fractions were embedded in agarose-coated slides for the detection of DNA damage by the electrophoretic single cell DNA comet assay. The intact DNA in the sperm nucleus remains immobilized, forming the head of the comet, while the degraded DNA fragments extend as comet tails. After ethidium bromide staining, the %tail DNA area was estimated by the Komet image system. Data were analyzed by T-test and Fisher test (SigmaPlot).

In the free and HA-bound/released fractions we evaluated DNA fragmentation in 220 and 450 individual spermatozoa, respectively. We classified the sperm DNA as "intact" if the area of the comet tail extension was <10%, and "fragmented" if >20%. There were significant differences in DNA integrity between the free and the HA-bound fractions, sperm with fragmented DNA in the HA-bound vs. free fractions: 6.8±2% vs. 50.4±12%; sperm with intact DNA in the HA-bound vs. free fractions: 66.6±8% vs. 28.2±10% ($P<0.02$ in both, all data mean±SEM). The distribution of sperm with intact and fragmented DNA in the HA-bound (N=147 vs. 14, $P<0.001$) and free fractions (N= 70 vs.126, $P<0.001$) has further confirmed that mature sperm, with intact DNA, preferentially binds to HA, whereas immature sperm with diminished DNA integrity do not bind.

In another study, we further tested whether the exclusive binding of mature sperm by their receptors to HA, would also facilitate the selection of sperm with aneuploidy frequencies similar to that of normospermic fertile men [16]. Semen of 8 men with borderline oligospermia (conc: 22.5±2.4) were divided into aliquots. One aliquot was fixed on glass slides (control sperm, CSP). Another aliquot was placed on HA-coated glass slides (HASP). After 15 minutes of incubation at 37∞ C, the semen and unbound sperm were washed away and the HA-bound sperm (HASP) were fixed to the slide. The sperm were subjected to FISH with probes for the X, Y, and 10 chromosomes. Other CSP and HASP slides were treated with CK-imunocytochemistry in order to detect the retained cytoplasm in immature sperm. For ICSI we examined 120,000 sperm nuclei in the 8 men. For immunocytochemistry we evaluated 300 sperm from each of the 16 sperm fractions (8 HASP and 8 CSP samples).

The incidence of diminished maturity sperm with cytoplasmic staining was lower in HASP than in CSP (19.9±0.3% vs. 47.3±0.4%, $p<0.001$). The total incidence of aneuploidies was 4.5 times lower in HASP than in CSP (0.21±0.05 vs. 0.96±0.14, $p<0.001$, range 1.9-11). The incidence of X and Y aneuploidies was 4.7-times higher in CSP vs. HASP ($p<0.001$, range: 1.9-6.8), which is comparable to the over 4-times increased rates of sex chromosome aneuploidies reported in ICSI children. There was also a correlation between the incidence of total aneuploidies and proportion of immature sperm with cytoplasmic retention in the fractions ($r=0.65$ $p<0.01$, N=16), indicating that the aneuploidies primarily originate in immature sperm. Selection of mature sperm for ICSI by HA will likely reduce or eliminate the potentially adverse genetic effects of ICSI. Because HA is a physiological component of the cumulus, there are no concerns related to use of HA in assisted reproduction. This method is expected to address the concerns about the quality of sperm used for ICSI.

Summary

We identified the expression of a 70 kDa chaperone protein, HspA2 (formerly CK-M), in mature human sperm. We have established the central role of HspA2 as a measure of sperm cellular maturity, DNA integrity, aneuploidy frequency and function, including fertilizing potential. The presence of HspA2 in the synaptonemal complex likely provides the link between the lack of HspA2 expression and the increased frequency of aneuploidies in diminished maturity sperm. The spermiogenetic events of cytoplasmic extrusion and remodeling of the plasma membrane, which facilitates the formation of zona pellucida binding sites in human sperm, are related. Accordingly, in sperm with arrested maturation, which do not express HspA2, there is

cytoplasmic retention and a failure of binding to the zona pellucida. In extensive clinical studies we have demonstrated that low levels of HspA2 in human sperm predicts diminished fertility by natural conception or in vitro fertilization. These findings provide the biological rationale for the role of the human HspA2 as an objective biochemical marker of sperm cellular maturation, function and male fertility. Also, the localization of the HspA2 to the synaptonemal complex explains the higher level of aneuploidies in immature sperm with diminished HspA2 expression. Finally, the presence of the HA-receptor on the plasma membrane of sperm that completed cellular maturation, coupled with the HA coated surface method, will facilitate the selection of single mature sperm for ICSI.

Legends

Figure 1: CK-B-immunostained sperm-hemizona complex (from ref. 5)

Figure 2: Human testicular biopsy tissue immunostained with HspA2 antisera. The sections A,B, and C in the composite reporesent three difgfferent magnifications to show tubular structure and and the immunostaining pattern in the adluminal area. HspA2 expression begins in meiotic spermatocytes, and is prominent during terminal spermiogenesis.

Figure 3: A model of normal and diminished sperm maturation: In normal sperm maturation HspA2 expression commences in spermatocytes and facilitates normal meiosis. HspA2 may also be involved in late spermiogenesis: cytoplasmic extrusion as represented by the loss of residual body (RB), plasma membrane remodeling and the formation of the zona pellucida binding site (stubs). Diminished maturity sperm lacks HspA2 expression, which may lead to meiotic defects and a higher rate of chromosomal aneuploidies. Diminished spermiogenetic maturation would lead to cytoplasmic retention (increased activity of CK and other cytoplasmic enzymes), abnormal sperm morphology, deficiency in zona-binding sites, high levels of lipid peroxidation and consequential DNA fragmentation, and diminished fertility.

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FIGURE 1

FIGURE 2

FIGURE 3

Peptide Modulators of Sperm Function

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Learning Objectives

- Structure and function of fertilization promoting peptide (FPP) and related peptides
- TCP11 – putative receptor for FPP
- Mechanism of action of FPP – interaction with adenosine receptors; capacitation-dependent regulation of adenylyl cyclase(AC)/cAMP pathway
- Calcitonin and angiotensin II – effects used in isolation and in combination with FPP
- Importance of regulating AC/cAMP.

Introduction

It is well known that when mammalian sperm are released from the male reproductive tract, they are morphologically complete and able to express independent motility but they are non-fertilizing, even if added directly to unfertilized eggs in vitro. Given sufficient time and appropriate conditions, sperm will 'switch on' physiologically and acquire the capacity to fertilize eggs, i.e., they will become capacitated and able to undergo an acrosome reaction when they encounter an unfertilized oocyte. However, studies carried out in vitro indicate that once capacitation has been initiated, it will usually continue so that the cells may 'over-capacitate', culminating in sperm undergoing spontaneous acrosome reactions before they contact unfertilized oocytes. Biologically this is extremely undesirable since such acrosome-reacted cells, even if motile, are non-fertilizing (Yanagimachi, 1994). Regulation of capacitation is needed to prevent this. Recent in vitro studies have identified several small molecules, found in seminal plasma, that bind to specific receptors on mammalian sperm and appear to regulate the adenylyl cyclase (AC)/cAMP signal transduction pathway in biologically significant ways. These actions in vitro suggest that the molecules may also play a role in stimulating and then maintaining sperm fertilizing potential in vivo.

Structure and function of FPP

The first of these molecules, fertilization promoting peptide (FPP), is a tripeptide related to thyrotrophin releasing hormone (TRH). Structurally FPP is pGlu-Glu-ProNH2, while TRH is pGlu-His-ProNH2. FPP was isolated from prostate tissue and identified as being distinct from TRH by Cockle et al. in 1989. It has subsequently been identified in the prostate gland and/or semen of a number of other mammals including several rodents and man, where the mean concentration in seminal plasma is ~ 50 nM.

Initial physiological investigations of this peptide were undertaken using uncapacitated epididymal mouse sperm incubated in the presence of nanomolar concentrations of FPP. This approach was chosen because (1) epididymal sperm would not have yet come into contact with FPP and (2) there was already in place a well-characterized mouse sperm in vitro capacitation/ fertilization system that could be used to investigate responses to the peptide (Fraser, 1993). Sperm suspensions were incubated in the presence of FPP, then analyzed cytologically using chlortetracycline (CTC) to evaluate effects on capacitation and functionally using in vitro fertilization (IVF) (Green et al., 1994).

In the initial studies, FPP stimulated capacitation per se, but did not stimulate the acrosome reaction. This was unusual because, in general, treatments that stimulate capacitation also cause spontaneous acrosome loss. Using more conventional analytical technique, the failure of FPP to stimulate acrosome reactions would have led to the conclusion that the peptide had no effect and would probably have led to abandonment of the investigation. However, CTC results were encouraging and so responses to a range of FPP concentrations were evaluated. A minimum of 25 nM was required to obtain a significant effect, with the maximum response being obtained using 100 nM; no further differences (either positive or negative) were noted at higher concentrations. Given the presence of more capacitated, acrosome-intact cells following FPP treatment, it was hypothesized that FPP-treated sperm would be more fertile in vitro and this proved to be the case. Since sperm must express hyperactivated motility in order to fertilize intact eggs, the enhanced fertilizing ability of the treated suspensions indicated that this motility must have been stimulated. Subsequent evaluations of sperm motility using computer assisted sperm analysis (CASA) confirmed that FPP was able to stimulate hyperactivated motility in mouse sperm (Green et al., 1996c).

Following experiments on mouse sperm, responses of uncapacitated, ejaculated, Percoll prepared human sperm to FPP were investigated. As with mouse sperm, CTC analysis revealed that 25-200 nM FPP significantly stimulated capacitation in human sperm but had no detectable effect on acrosome loss. Using the zona-free hamster oocyte test, human sperm treated first with FPP and then progesterone (to trigger the acrosome reaction) were shown to be significantly more fertile than those in other treatment groups (untreated controls, FPP only, progesterone only), consistent with data obtained earlier in the mouse (Green et al, 1996a). More recently, FPP has also been shown to significantly stimulate capacitation in boar sperm over the same range shown to be effective with mouse and human sperm (Funahashi et al, 2000). This is interesting because boar seminal plasma contains only low picomolar concentrations of FPP, yet the cells clearly have FPP receptors.

Next, responses of capacitated mouse sperm to FPP were evaluated. Although it is relatively uncommon for a single molecule to have significant effects at both the beginning and the end of capacitation, FPP not only significantly stimulated capacitation in uncapacitated sperm but also significantly inhibited the spontaneous acrosome reaction in capacitated sperm. Furthermore, these cells could still undergo the acrosome reaction in response both to the normal trigger, the oocyte and its surrounding layers, and to progesterone, indicating that FPP did not interfere with sperm's ability to respond to biological stimulators of the acrosome reaction (Green et al., 1996b).

Interestingly, capacitation-dependent changes in sensitivity to FPP were observed. At least 25 nM FPP was required to stimulate capacitation, yet 6.25 nM FPP was as effective as 100 nM FPP in inhibiting the acrosome reaction (Fraser and Adeoya-Osiguwa, 1999). Recent studies have demonstrated that FPP also inhibits spontaneous acrosome loss in boar sperm, with significant responses being obtained using 6.25-200 nM FPP. Biologically this is extremely important, since it indicates that the sperm can be 'switched on' by FPP and then held in a potentially fertilizing state for some period of time. Given that FPP is a component of seminal plasma, it is plausible that FPP binds to its receptor on sperm at ejaculation and then elicits the same responses in vivo that have been observed in vitro. Thus FPP could help to maximize the chances of successful fertilization in vivo of the relatively few cells that reach the ampulla of the uterine tube, the usual site of fertilization in vivo.

Molecules related to FPP

Various peptides related to FPP were evaluated for biological activity. Deamidated FPP (pGlu-Glu-Pro) had no effect on uncapacitated mouse sperm and it failed to interfere with responses to FPP, suggesting that the amide group is necessary for FPP binding and that deamidated FPP does not bind to cells. TRH at 250 nM was required to elicit the same response obtained with 100 nM FPP, suggesting that FPP acts at a site other than the TRH receptor. Responses to pGlu-Phe-ProNH2 were similar to those obtained with TRH, while a methyl derivative [pGlu-Glu(OMe)-ProNH2] had no effect when used alone and did not interfere with responses to FPP, suggesting that the latter did not bind to cells.

The most interesting peptide was p-Glu-Gln-ProNH2 (Gln-FPP). On its own, Gln-FPP had no effect on uncapacitated mouse sperm but, used with FPP, it inhibited responses to FPP in a concentration-dependent manner; Gln-FPP also abolished FPP's effects on capacitated sperm. These results suggest that Gln-FPP binds to the FPP receptor but cannot elicit a response, thus acting as a competitive inhibitor of FPP (Fraser et al, 1997). Consistent with those observations in the mouse, Gln-FPP also inhibited boar sperm responses to FPP (Funahashi et al, 2000). These results suggested that FPP binds to a specific receptor, but there was no information about the identity of the FPP receptor. A plausible candidate for the FPP receptor emerged serendipitously from collaboration between two laboratories, one working on a gene in the mouse t-complex and the other working on physiological responses in sperm to FPP, a peptide with no known relationship to t-complex gene products.

TCP11, the putative FPP receptor

The mouse t-complex on chromosome 17 contains genes that influence male but not female fertility (Fraser and Dudley, 1999). Although male mice heterozygous for the chromosome 17 in the t-complex configuration produce equal numbers of sperm with the normal and the t-chromosome 17, these males can transmit the t-chromosome to as many as 95% of their offspring; this is referred to as transmission ratio distortion. The gene Tcp11 maps to the mouse t-complex and is expressed only in the testes. It is transcribed during late spermatogenesis and translated during spermiogenesis, consistent with its having a role somehow related to sperm function. The deduced amino acid sequence of TCP11 revealed that it has an RGD (arginine-

glycine-aspartic acid) motif and a leucine zipper, but details about its possible function were unknown until recently.

A. TCP11 has been localized to the acrosomal cap of acrosome-intact sperm, but not acrosome-reacted sperm, and to the flagellum. Fab fragments of polyclonal antibodies raised to bacterially expressed TCP11 protein were added to uncapacitated mouse sperm suspensions and the suspensions were sampled after 30 and 120 min. After 30 min in the presence of Fab, there were significantly more capacitated, potentially fertilizing cells and after 120 min there were significantly fewer acrosome-reacted sperm, compared with untreated controls at the same time point. IVF experiments revealed that after 30 min preincubation, the Fab-treated suspensions were significantly more fertile than controls and after 120 min preincubation, the Fab-treated suspensions and the controls were equally fertile (see Fraser and Dudley, 1999).

These responses were essentially the same as those obtained using FPP, leading us to hypothesize that TCP11 is the receptor for FPP. It is not uncommon that an antibody binding to its antigen can act as an agonist, and that would appear to be the case in this instance. If TCP11 is the FPP receptor, then Gln-FPP should competitively inhibit binding of Fab fragments to sperm. This proved to be the case. In the presence of a fixed concentration of Fab fragments, Gln-FPP inhibited responses to the antibodies in a concentration-dependent manner. Furthermore, both Gln-FPP and Fab fragments significantly inhibited binding of 3H-FPP to sperm membranes, suggesting that FPP, Gln-FPP and Fab fragments all compete for the same binding sites, consistent with TCP11 being the receptor for FPP.

Mechanism of action of FPP

The capacitation-dependent effects of FPP on mammalian sperm were reminiscent of earlier studies demonstrating that adenosine had capacitation-dependent effects on activity of adenylyl cyclase (AC) and consequent production of cAMP, with adenosine initially stimulating and then inhibiting enzyme activity. When adenosine was evaluated using the FPP protocols, the responses were the same as those obtained with FPP: adenosine stimulated capacitation in uncapacitated sperm and then inhibited spontaneous acrosome reactions in capacitated cells (in both mouse and boar sperm). Furthermore, combinations of FPP + adenosine were more effective than either used singly: combined high stimulatory concentrations resulted in an augmented stimulatory response, while combined low non-stimulatory (when used individually) concentrations resulted in a significant stimulation of capacitation. This suggested that FPP and adenosine acted via separate, specific receptors but somehow modulated the same signal transduction pathway; otherwise, the combination of two different effectors, each at non-stimulatory concentrations, would not have had a significant effect. A combination of a low, non-stimulatory concentration of Fab fragments of anti-TCP11 antibodies + adenosine also produced an augmented response, consistent with responses to FPP + adenosine.

Adenosine was already known to modulate AC/cAMP in sperm, suggesting that this is the signal transduction pathway involved in responses to FPP as well. Subsequent experiments confirmed that both FPP and Fab fragments of anti-TCP11 antibodies significantly stimulated cAMP production in uncapacitated sperm. Adenosine, like FPP, is present in seminal plasma and so

sperm would come into contact with both molecules at the time of ejaculation, making it plausible that both could affect sperm in vivo as well as in vitro.

Subsequent experiments provided evidence for the involvement of both stimulatory and inhibitory adenosine receptors in responses to adenosine. The use of specific agonists for adenosine receptors provided evidence that stimulatory A2a adenosine receptors function only in uncapacitated cells, while inhibitory A1 adenosine receptors function only in capacitated cells. Furthermore, specific antagonists for adenosine receptors inhibited responses to FPP as well as to adenosine, providing evidence that FPP receptors and adenosine receptors somehow interact. Because adenosine receptors in somatic cells modulate AC activity via G proteins, possible G protein involvement in both uncapacitated and capacitated sperm was investigated. Cholera toxin, which activates stimulatory Gas or related a subunits was shown to stimulate both capacitation (assessed using CTC) and cAMP production, to the same extent and within the same time frame as FPP (Fraser and Adeoya-Osiguwa, 1999). Pertussis toxin inhibits G proteins containing inhibitory Gai/o subunits and the use of pertussis toxin + FPP on capacitated suspensions abolished responses to FPP: the incidence of spontaneous acrosome loss was as high as that observed in untreated control suspensions and FPP's inhibition of cAMP production was abolished (Fraser and Adeoya-Osiguwa, 1999).

Since these results suggested the presence of both stimulatory and inhibitory Ga subunits, sperm membrane preparations were subjected to electrophoresis and Western blotting, using commercial antibodies. Positive identification of Gas, Gai2, Gai3 and Gao was made (Fraser and Adeoya-Osiguwa, 1999). These results were particularly interesting and important because earlier studies on mammalian sperm, using a variety of techniques, had identified various inhibitory Ga subunits, but none had identified stimulatory Gas subunits. Despite those unsuccessful earlier attempts, we were able to identify a total of 3 isoforms of Gas; the most abundant was ~48 kDa, followed by a 45 kDa form and the least abundant was ~52 kDa. Isoforms were seen with two different antibody preparations, one commercially available and the other one prepared 'in house' and given to us by Dr JS Lymn (St Mary's Hospital, London). As controls, a human cell line was used and while both the 45 and 48 kDa isoforms were seen, the relative abundance was opposite that seen with sperm membranes, i.e., in the somatic cells the 45 kDa isoform predominated.

Very recently we have demonstrated that the stimulatory responses to FPP and adenosine detected in uncapacitated mouse sperm correlated with enhanced tyrosine phosphorylation of phosphoproteins within the range of ~30-140 kDa. Phosphorylation was stimulated by FPP, adenosine, and cholera toxin. In contrast, the inhibitory responses to FPP in capacitated cells correlated well with reduced tyrosine phosphorylation of an array of phosphoproteins in FPP-treated capacitated sperm. The inclusion of pertussis toxin, which abolished FPP's inhibition of cAMP production, resulted in phosphorylation patterns very similar to those seen in the untreated control capacitated samples (Adeoya-Osiguwa and Fraser, 2000). Some of the phosphoproteins identified in uncapacitated sperm treated with FPP were also involved in capacitated sperm responses to FPP, but a few were unique to either the uncapacitated or capacitated state.

Calcitonin and angiotensin II

In addition to FPP and adenosine, both calcitonin and angiotensin II are found in seminal plasma. While the latter small peptides are primarily known for their significant effects on various somatic tissues and systems, recent investigations have revealed that they also elicit physiologically important responses in mammalian sperm. Earlier studies had provided evidence for both calcitonin and angiotensin II receptors on mammalian sperm, but there was relatively little unequivocal evidence that they might have important effects on sperm function.
New studies have demonstrated that both calcitonin and angiotensin II, used individually on mouse sperm, are able to significantly stimulate capacitation in uncapacitated cells as shown first by CTC analysis and then by demonstration of enhanced fertilizing ability in vitro (Fraser et al., 2001). When used in combination, augmented responses were obtained in the presence of both high, stimulatory and low, non-stimulatory (when used individually) concentrations of the peptides. These results suggested that both peptides were acting on the same, but unidentified, pathway; if they modulated different pathways, then it is unlikely that the low concentrations used in combination would have had the stimulatory effect observed.

The similarity of these responses to those obtained with FPP led to experiments on uncapacitated sperm using calcitonin and angiotensin II in combination with FPP. Combinations of low, non-stimulatory concentrations of FPP plus either calcitonin or angiotensin II produced a significant stimulatory response, again suggesting activation of a common pathway. Because FPP is known to act via the AC/cAMP signal transduction pathway, we have hypothesized that calcitonin and angiotensin II are also acting in some way on this signal transduction pathway (Fraser et al., 2001). We have preliminary (unpublished) evidence that calcitonin does stimulate cAMP.
Although similar results were obtained when uncapacitated mouse sperm were treated with calcitonin and angiotensin II, results obtained with capacitated cells revealed important differences. Whereas both FPP and calcitonin were able to inhibit spontaneous acrosome reactions in capacitated cells, angiotensin II had no detectable effect on this parameter. Furthermore, the inclusion of pertussis toxin along with FPP or calcitonin totally abolished inhibition of spontaneous acrosome reactions, suggesting the direct involvement of inhibitory Ga subunit-containing G proteins in responses to these ligands. Interestingly, a combination of calcitonin, angiotensin II and FPP inhibited the spontaneous acrosome reaction, indicating that angiotensin II did not interfere with the inhibitory responses elicited by calcitonin and FPP.
Importance of regulating AC/cAMP: It has been known for some considerable time that cAMP plays an important role in mammalian sperm physiology, but most studies have focused on factors that might stimulate cAMP production. Our recent studies have provided evidence that unregulated production of cAMP may lead to 'over-capacitation' and consequent spontaneous acrosome loss. Given that acrosome-reacted sperm are non-fertilizing (Yanagimachi, 1994), this is biologically undesirable. Both FPP and adenosine have been shown to act as ‘first messengers’, binding to specific external receptors, regulating the activity of AC and therefore regulating availability of the ‘second messenger’, cAMP. In so doing, FPP and adenosine initially stimulate capacitation but then arrest it, inhibiting spontaneous acrosome reactions and maintaining fertilizing potential. Evidence indicates that there is interaction between FPP receptors and adenosine receptors in the regulation of this signal transduction pathway, leading to alterations in the availability of cAMP and consequent effects on protein tyrosine phosphorylation and sperm function. The more recent results obtained with calcitonin suggest

that it too can act as a first messenger to regulate cAMP production, although how this integrates with responses to FPP and adenosine has yet to be determined. Angiotensin II can at least stimulate capacitation and the fact that a combination of low, non-stimulatory concentrations of angiotensin II and FPP significantly stimulates capacitation suggests that both are activating the same pathway, namely AC/cAMP. At present, however, there is no firm evidence for the mechanism whereby angiotensin II might achieve this.

This recent evidence that FPP, adenosine, calcitonin and angiotensin II may all affect AC/cAMP in some way suggests that there is redundancy in mechanisms that modulate this signal transduction pathway and emphasize the importance of AC/cAMP in regulating sperm function.

Conclusions

FPP, adenosine, calcitonin and angiotensin II are found in seminal plasma of many mammals and so mammalian sperm would contact all of them at ejaculation. Sperm have specific receptors for all four of these molecules and respond to them in vitro in biologically significant ways, suggesting that similar responses may also occur in vivo. Adenosine, calcitonin and angiotensin II are found in many body fluids and so would probably be present in female reproductive tract fluids; this is also possible for FPP.

Of the two responses elicited by FPP, adenosine and calcitonin, inhibition of the spontaneous acrosome reaction is probably the more biologically important. It is therefore striking to note that much lower concentrations of FPP and adenosine are needed to inhibit the spontaneous acrosome reaction than to stimulate capacitation, strongly suggesting that inhibition of early acrosome loss plays a particularly important role in sperm function.

In mammals, sperm are deposited in either the vagina or the uterine horn(s) of the female reproductive tract, but fertilization usually occurs in the upper region of the uterine tubes known as the ampulla. Especially for species such as the human, where sperm deposition is in the vagina and sperm must first penetrate cervical mucus before entering the main lumen of the uterus, only a very small proportion of the cells ejaculated into the female tract will reach the ampulla. Therefore, to maximize the fertilizing potential of these few sperm, one would wish the cells to be capacitated but still acrosome-intact and so ready to interact with any egg that might be contacted. This is exactly the effect elicited by FPP and the other molecules.

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Table I. Relationship between peptide structure and ability to stimulate capacitation in uncapacitated mouse spermatozoa

Peptide name	Peptide structure	Charge	Bioactivity*
FPP	pGlu-Glu-ProNH_2	negative	+++**
deamidated FPP	pGlu-Glu-Pro	negative	—
Gln-FPP	pGlu-Gln-ProNH_2	neutral	—
MeO-FPP	pGlu-Glu-(O Me)ProNH_2	neutral	—
Phe-FPP	pGlu-Phe-ProNH_2	neutral	+ - ++
TRH	pGlu-His-ProNH_2	neutral	+ - ++

*Data from Fraser *et al.*, (1997), Green *et al.* (1994; 1996a,b). **Bioactivity: denotes responsiveness to 50 - 100 nM peptide in uncapacitated spermatozoa, relative to stimulation elicited by 100 nM FPP.

Table II. Comparison of the abilities of FPP, adenosine, calcitonin and angiotensin II to elicit responses in uncapacitated and capacitated spermatozoa.

Molecule	Uncapacitated cells*	Capacitated cells**
FPP	+	+
Adenosine	+	+
Calcitonin	+	+
Angiotensin II	+	—

* A '+' indicates a stimulation of capacitation assessed by chlortetracycline fluorescence and in vitro fertilization.

** A '+' indicates an inhibition of spontaneous acrosome reactions assessed by chlortetracycline fluorescence; a '–' indicates that the molecule has no detectable effect. Based on data in Green *et al.* (1994, 1996b), Fraser *et al.* (2001).

Aneuploidy frequency in mature and diminished maturity sperm

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Learning objectives

Literature data on sperm aneuploidy frequencies are here reviewed with the objective to provide the most accurate reproductive counseling for couples undergoing infertility treatment. To this end, data on sperm aneuploidy rates will be presented taking into consideration 4 different biological conditions:

1. Mature sperm in ejaculate from healthy donors.
2 Mature sperm in ejaculate from infertile patients:
a) normal karyotype b) abnormal karyotype
3. Immature germ cells in ejaculated semen.
4. Mature and immature sperm cells from testicular tissue.

Mechanisms of aneuploidy origin and clinical implications will be discussed.

Lecture summary

Application of in situ hybridization techniques (fluorescent or not: FISH or ISH) have allowed in recent years to analyze all types of cytogenetic constitutions present in human gametes and embryos. As compared to conventional karyotyping the use of FISH on gametes and embryos has greatly improved time for cytogenetic analysis and number of cells analyzed. Recently, our knowledge about sperm ñchromosomes meiotic anomalies occurring in a variety of different conditions has been enormously increasing. This lecture will focus on numerical chromosome abnormalities in human male germ cells (sperm aneuploidy) found in 4 main different biological and clinical situations:

1. Mature sperm in ejaculate from healthy donors:

Most of the information today available on the baseline frequency of sperm disomy and diploidy derives from FISH-studies where healthy donors or fertile men were compared to patients with poor semen quality. At least 200 normal men have been so far studied though a limited number of different probe-set was employed in each study. Although almost all human sperm chromosomes have been studied most part of data produced relate only to some of them (1, 8, 13, 17, 18, 21, X, Y). Approximately, it has been estimated that in normal healthy men the per-chromosome

average rate of sperm disomy is around 0.05% with a total baseline rate of sperm disomy equal to 1.15% (0.05 x 23). The frequency of sperm diploidy would be 0.12% and the frequency of other chromosomal aberrations (structural) close to 1.4-% (1). It must be clearly noted however that a certain degree of variability exists in terms of baseline frequency of sperm aneuploidy from study to study due to multiple factors including inter-individual variability, type of probes used, number of sperm counted, scoring criteria and sperm head-decondensation method used. Perhaps other confounding variables occur which have been in part object of independent analysis:

Impact of paternal age: current evidence suggests that aging is associated with a reduced semen quality and increased sperm aneuploidy in humans (particularly > 60 years) and mice.

Impact of smoking: cigarette smoking in teenagers has been found to increase sperm disomy and reduce the quality of semen. Other life style factors as drinking alcohol or caffeine are probably involved but are more difficult to be evaluated separately (2).

Impact of drugs: this is an area of continuous investigation and many different compounds are under study. Type, time and dose of exposure are critical factors as well as time free interval passed by. For instance, preliminary data suggest that diazepam acts during chronic exposure as an aneugen during meiosis in human male spermatogenesis while some chemotherapy agents (NOVP) only temporally affect sperm disomy in Hodgkin disease patients.

Impact of constitutional factors: FISH studies have also been made to ascertain whether fathers of children with chromosomal anomalies would show increased frequencies of specific chromosome disomies (fathers of children with Down or Klinefelter or Turner syndromes) or higher frequency of sex chromosomes and autosomal aneuploidy are present in semen from men partners of women with habitual abortion. No more than 50 patients from few studies have been investigated in this sense and a preliminary evidence supports a mild association between recurrent pregnancy loss and increased rates of sperm aneuploidy and diploidy. The association becomes stronger whenever the semen parameters decrease in quality. This represents a confounding factor because male factor infertility carries per se intrinsic risk for higher sperm chromosome abnormalities.

2. a. Mature sperm in ejaculate from infertile patients with normal blood karyotype:

More than 300 infertile men with poor semen quality have been studied so far in terms of sperm aneuploidy by at least 20 separate investigators. Overall, most of these studies demonstrate statistically significantly higher sperm disomy and diploidy rates in patients presenting disturbances of the semen parameters in respect with their internal controls. Only 3 out of 20 studies disagree with this conclusion. It is here stressed that there is a general consensus on a strong association between severe oligospermia (<5 millions/ml) and higher sperm aneuploidy and diploidy. It is more debated whether also a decrease in motility and morphology might affect sperm disomy and diploidy as well. On this regard some authors emphasize the impact of one over the other though often it is difficult to separate data because of the concomitant alteration of multiple factors (oligo-astheno-teratozoospermia). For this reason some investigators prefer to

score the aneuploidy rates on the bases of the overall total normal motile sperm count (TNMC) (3). Our personal experience indicates that the poor the semen quality the higher is the chance to find, in morphologically normal spermatozoa, higher sperm aneuploidy rates (in particular for sex chromosomes). In addition, clear data show that certain specific types of morphological sperm aberrations (macrocephalic heads, double tails sperm) with no doubt carry very high sperm aneuploidy and diploidy rates. Unfortunately after FISH it is often difficult to recognize the original sperm morphology and information on aneuploidy rates present in sperm with other morphological defects is still lacking. Another issue object of interest has been the detection of chromosomal abnormalities in immotile viable spermatozoa present in some ejaculates. FISH analysis after hypo-osmotic swelling test have shown that morphologically normal immotile but viable spermatozoa have an aneuploidy rate similar to that of normal motile spermatozoa (4).

b. Mature sperm in ejaculate from infertile men with abnormal blood karyotype:

All the data available on FISH performed on semen samples of infertile men with different types of sex chromosomal abnormalities or carriers of Robertsonian or reciprocal translocation indicate an interchromosomal effect whose entity varies with the type of individual karyotype and intensity of mosaicism. A review of the literature data will be presented. Aneuploidy for sex chromosomes occurs in approximately 15% of the sperm cells of the XYY males and in 3-20% of the XXY males depending on the mosaicism. In the ejaculates of these men there is a low percentage of sperm cells (70%). The other cells are represented by immature germ cells (IGC) like spermatogonia and spermatocytes arrested at various stages of spermatogenesis. Very likely, in XYY or XXY men these IGC are shed into the semen to an increased extent as compared to normal fertile men. The sex chromosome constitution of these IGC is heterogeneous. The finding that the majority of sperm in semen of XYY and XXY males carries a single sex chromosome suggests that a 46,XY germ cell line must be there with a proliferative advantage over the 47,XYY or 47,XXY cells.

3. Immature germ cells in ejaculate semen:

from initial FISH studies on ejaculated semen of men with normal peripheral karyotype we found increased rates of abnormal forms and immature germ cells (large, roundish, brightly stained by Diff-Quik) whenever the quality of semen was very poor as compared to controls (Fig. 1). In the immature germ cells the rate of aneuploidy and diploidy was very high and based on the cytogenetic finding we scored these as spermatocytes (diploid) or spermatids (disomic) (Fig 2). The conclusion of our studies was that in case of severe oligoasthenoteratozoospermia something happens at the early stages of spermatogenesis that is translated in abnormal function of meiosis, abnormal spermiogenesis and shedding of abnormal products (abnormal sperm and immature cells) in the ejaculate. All this was correlated with increased rates of sperm aneuploidy and diploidy found in morphologically normal sperm. This observation is similar to that made in ejaculate of patients with abnormal blood karyotype (XXY, XYY males).

Other more sophisticated studies are aimed to identify morphometric changes in spermatozoa correlated with sperm maturation and spermiogenesis process (5). These studies suggest that significant differences exist in the mid-piece and tail between mature and diminished mature sperm present in the ejaculate and prove that biochemical markers of sperm maturity (heat shock

protein HspA2) and changes in sperm morphology involve common spermiogenic events. The same authors also showed that higher sperm aneuploidy rates occur in these immature sperm and studies are ongoing to detect and select mature spermatozoa with fluorochrome-coupled biochemical probes for clinical use. Similarly, other studies (6) indicate that zona-bound spermatozoa have lower frequency of aneuploidy than swim-up or Percoll motile fractions. This may represents another way to select for human spermatozoa with normal maturity and functional competency.

4. Mature and immature sperm cells from testicular tissue:

firstly, a distinction must be done between patients with obstructive azoospermia and patients with non-obstructive azoospermia. Secondly between different types of immature cells generally seen in wet preparations after MESA and TESE. Morphological analysis of epididymal sperm retrieved in patients with obstructive azoospermia shows no particular difference with mature sperm present in ejaculate. FISH analysis reported from previous studies are limited and suggest that in epididymal sperm the aneuploidy rate is only moderately higher than that found in normal ejaculate spermatozoa but significantly lower than that found in ejaculate sperm from OAT patients. It is known that a clear correlation between the incidence of meiotic anomalies and decreasing numbers of motile sperm in the ejaculate as well as with increased levels of serum FSH exists (7). FISH studies on testicular spermatozoa of men with non-obstructive azoospermia are as yet quite limited and control studies on baseline frequency of testicular sperm aneuploidy in normal fertile men almost absent. Despite the fact that probably many studies are ongoing, no more than 20 patients with non-obstructive azoospermia have been studied so far and a very limited number of sperm could be scored each time (30-3000 cells per patient). The number of sperm cells found varies as a function of the severity of the spermatogenetic process impairment (maturation arrest, hypospermatogenesis). All data suggest increased rates of sperm aneuploidy and diploidy mainly due to abnormalities in sex chromosomes disjunction process. Scoring FISH signals on testicular sperm is affected by the impossibility to accurately recognize type of cell under analysis (morphologically abnormal spermatozoon or morphologically normal immature germ cell). Traditional criteria of sperm morphology evaluation do not fit when testicular sperm are analyzed particularly when this derives from infertile patients. Anomalies of meiosis have been studied in primary spermatocytes by different approaches including synaptonemal complexes spreads, metaphases preparations, and FISH studies. All the studies agree in the conclusion that in men with different degrees of spermatocyte arrest there is a failure in bivalent formation, which correlates with the chance to find mature spermatids in wet preparation. Sex chromosome bivalent is found in a lower percent than the autosomal bivalent. Separate consideration must be made for spermatids, the youngest post-meiotic haploid stage of the male germ cell, which can be also utilized for ICSI. Baseline frequency of aneuploidy found in spermatids from men with obstructive azoospermia (controls) resulted to be around 7% (8). Others and we have found very high aneuploidy and diploidy rates in round spermatids from small groups of men with non-obstructive azoospermia. Clinical implications of such data will be discussed.

Conclusions

Evidence is growing to support an association between sperm chromosomal aneuploidy and severe oligo-astheno-teratozoospermia, between sperm chromosomal aberration and sperm heads defects, between sperm chromosomal aneuploidy and spermiogenesis defects (function, motility, maturity), between sperm chromosomal aneuploidy and spermatogeneis defects (non-obstructive azoospermia) and ultimately between sperm chromosomal aneuploidy and failure to obtain a successful pregnancy.

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Uniqueness and Predictive Power of the Sperm Chromatin Structure Assay (SCSA) in the Infertility Clinic

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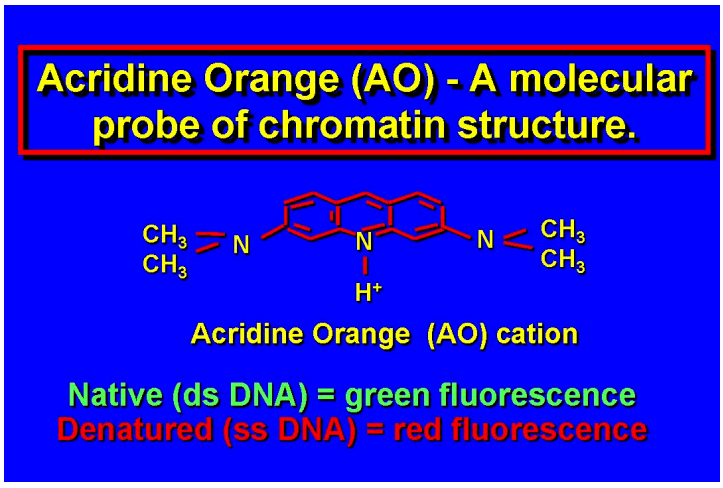
Learning Objectives

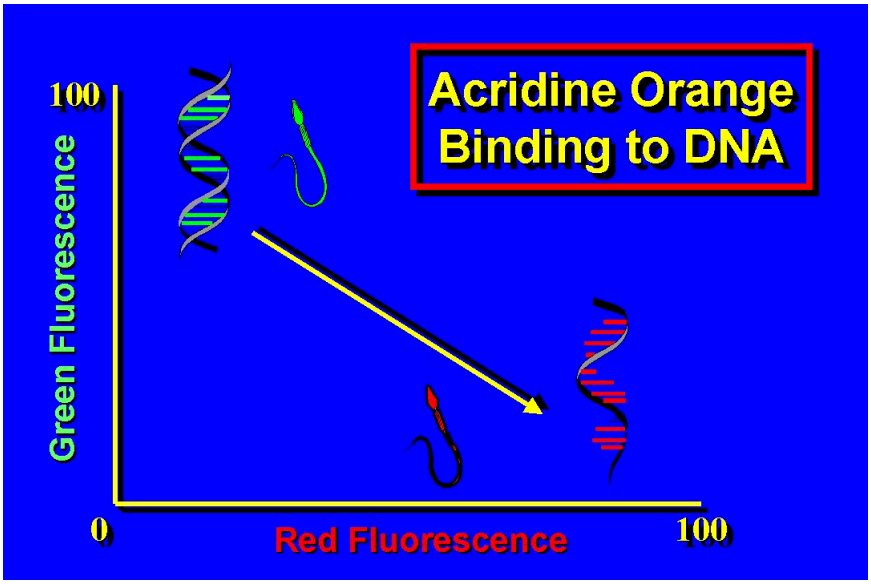
The overall objective is to increase understanding of what the Sperm Chromatin Structure Assay (SCSA) is and what it can do by addressing the following questions:

I. What is the SCSA?
II. What does the SCSA measure?
III. How does the SCSA compare to other semen quality tests?
IV. What is the role of the SCSA in the human infertility clinic?
V. What are the advantages and disadvantages of the SCSA test for ART clinics?

I. What is the SCSA?

The SCSA is a unique, rapid, objective and statistically robust flow cytometric protocol for measuring sperm nuclear DNA/chromatin integrity in fresh or frozen/thawed semen.





History of the SCSA

Pioneer publication: Evenson DP, Darzynkiewicz Z, Melamed MR. Science. 1980; 240:1131-1133. 4

✓ sperm DNA denaturation induced by 100∞C temperature for 5 min.
✓ stained with AO

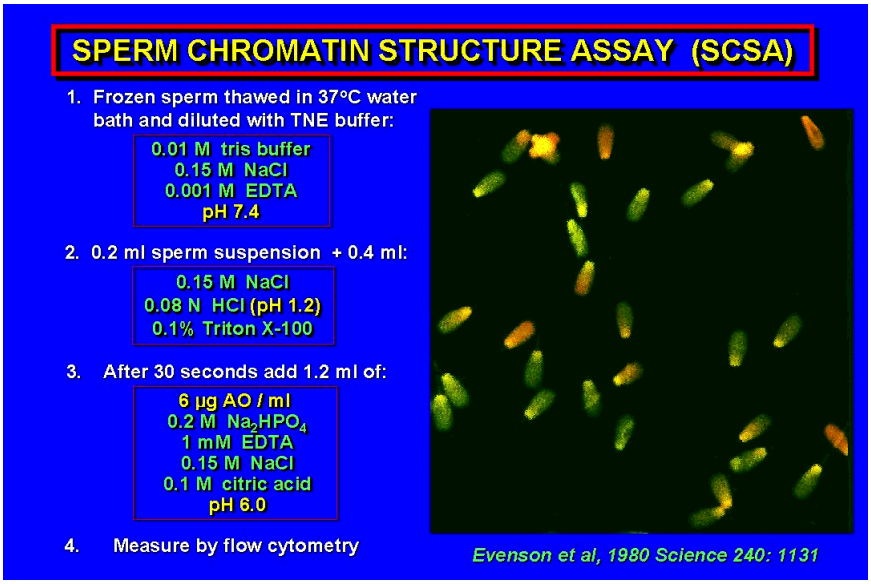
Observed that FERTILE MEN had significantly lower levels of DNA denaturation than infertile men (and bulls).

Concluded “We expect this assay to have application in many research areas, including animal husbandry, human infertility, and environmental and public health.”

Refinement of the SCSA

Evenson DP, Higgins PH, Grueneberg D, Ballachey B. Cytometry 1985; 6:238-2535.

✓ sperm DNA denaturation induced by pH 1.2 buffer for 30 sec.
✓ no cell loss due to sperm sticking to test tubes during heating, much simpler and easier, less time



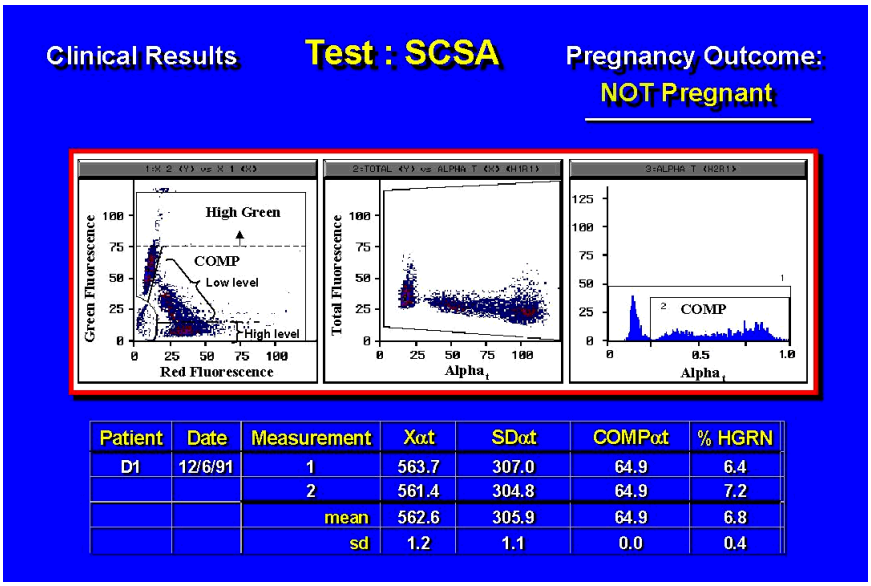
II. What does the SCSA Measure?

A. The percent of up to four major populations of sperm that may be present in semen samples:

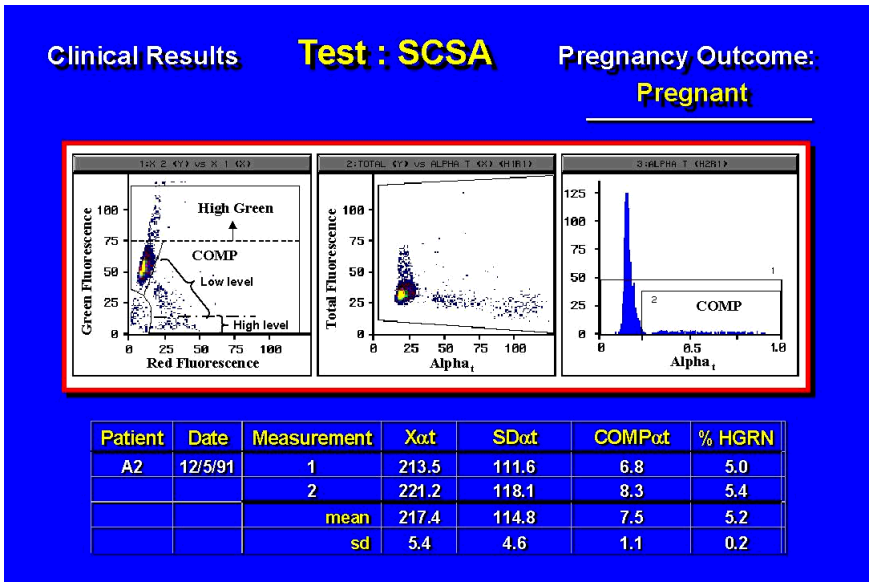
1. Sperm with "normal" chromatin structure defined as the "Main Population"

   Sperm with increased susceptibility to DNA denaturation, those sperm Cells that fall Outside the Main Population of sperm (COMPat), representing sperm with denatured (fragmented) DNA.

2. Low level COMPat (lessor level of red fluorescence)
3. Higher level COMPat (greater level of red fluorescence)
4. Sperm with immature chromatin (lack of condensation that allows a higher degree of DNA stainability; High Green)

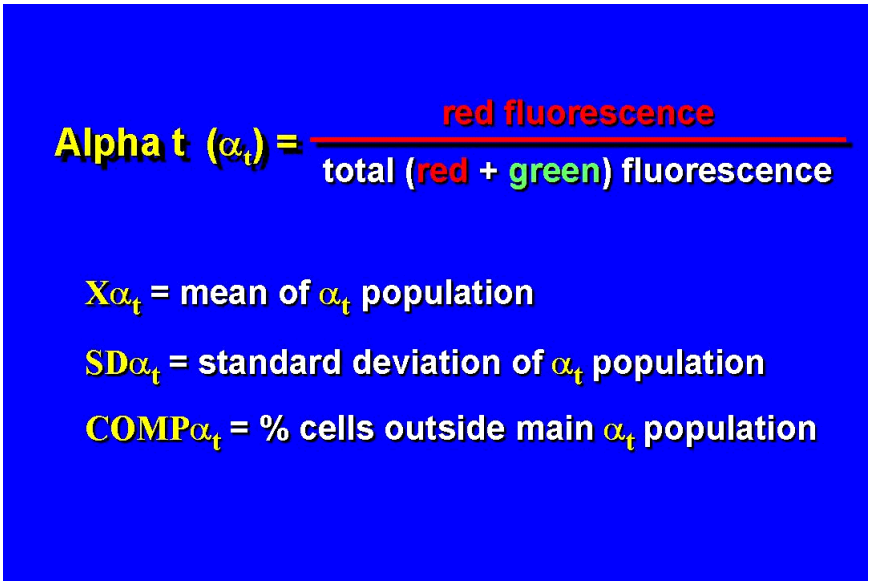


Patient	Date	Measurement	Xαt	SDαt	COMPαt	% HGRN
D1	12/6/91	1	563.7	307.0	64.9	6.4
		2	561.4	304.8	64.9	7.2
		mean	562.6	305.9	64.9	6.8
		sd	1.2	1.1	0.0	0.4



Patient	Date	Measurement	Xαt	SDαt	COMPαt	% HGRN
A2	12/5/91	1	213.5	111.6	6.8	5.0
		2	221.2	118.1	8.3	5.4
		mean	217.4	114.8	7.5	5.2
		sd	5.4	4.6	1.1	0.2

B. The degree of normality / abnormality of the COMPat population(s) (groups 2 & 3) is determined by the computer calculation of alpha t (at = red / (red + green) fluorescence). Note that flow cytometers measure 1024 channels (shades) of green and red fluorescence compared to light microscope measurements of red, yellow, and green.



C. In addition, SCSA data show the presence of very immature sperm cells (round spermatids and prior) and seminal contamination with bacteria and somatic cells including leukocytes and cellular debris.

D. The SCSA does not measure:

1. Mutations and translocations, unless they affect DNA / chromatin integrity
2. Aneuploidy, unless it represents a significant amount of total DNA content

What is the Rationale for Using the SCSA in the ART Laboratory?

Incorporating the SCSA into conventional semen analysis will likely enhance the predictive power of male fertility potential.

III. How Does the SCSA Compare to Other Semen Quality Tests?

A. The SCSA has a huge advantage6 over existing clinical assays of sperm quality because

1. it measures 5000 (or more) individual sperm in seconds of time,
2. it randomly measures all cell types in semen as opposed to evaluation of only washed samples,
3. data are from objective, machine-defined criteria rather than from biased human eye measurements,
4. data have a higher level of repeatability than those of any other currently used semen parameter,
5. data are significantly correlated with male factor infertility with reasonable sensitivity and precision, and
6. data provide both a diagnostic and prognostic evaluation of the man's potential for sub/ infertility.

B. NO other semen quality test matches all of these criteria.
C. In addition, easily prepared frozen samples can be sent by air courier to a diagnostic laboratory for SCSA evaluation.

Some conclusions about the SCSA

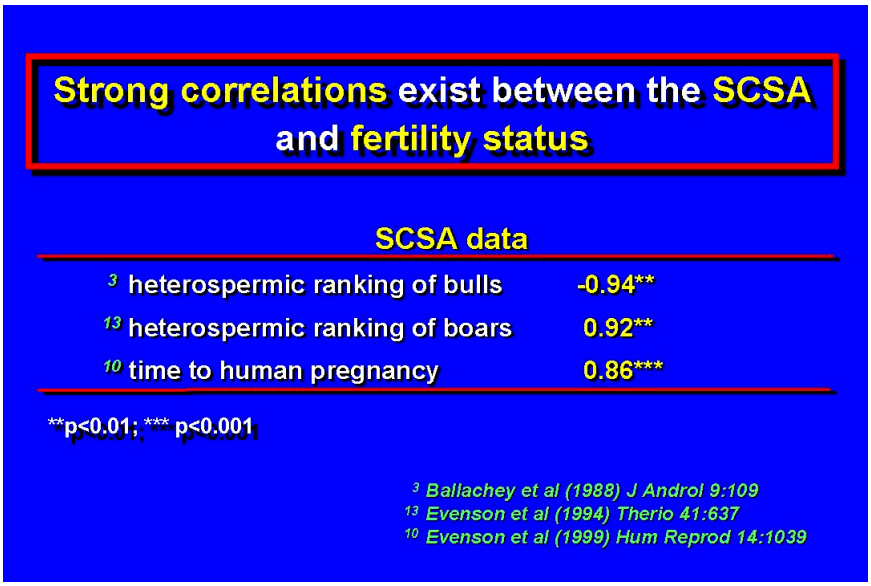
1. The SCSA is an independent assessment of sperm quality because SCSA data are weakly correlated with other WHO semen quality parameters.

Of importance - SCSA values were not well correlated with any of the classical semen quality measures, e.g.

- **% live (-.22)**
- **counts (-.16)**
- **% normal morphology (.30)**
- **% motile (.43)**

Suggests that the SCSA is an independent parameter

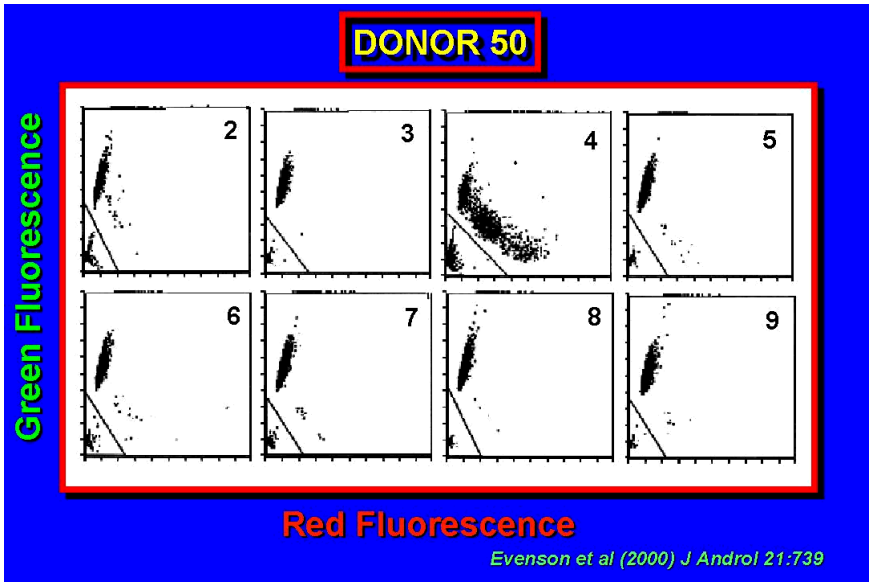
2. Both animal and human studies show a strong correlation between SCSA data and fertility status.



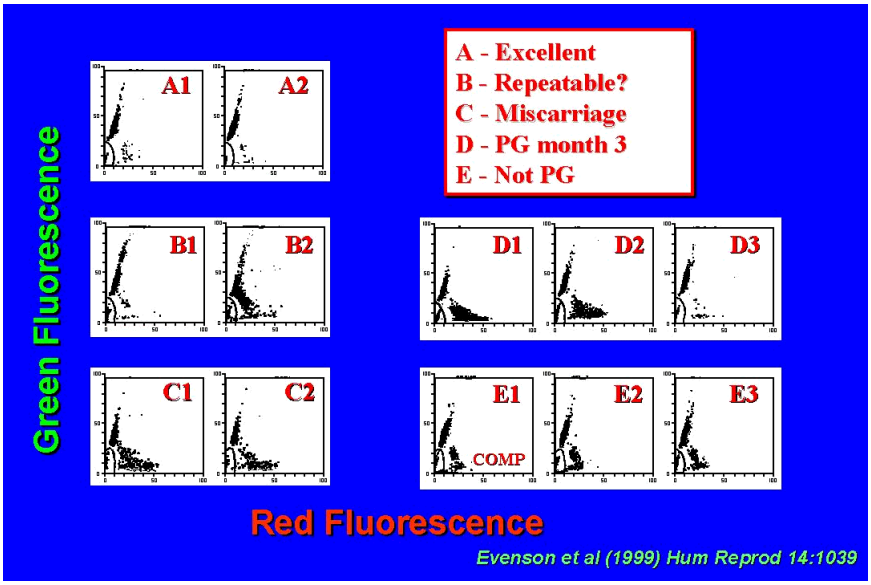
Characteristics of sperm chromatin revealed by the SCSA

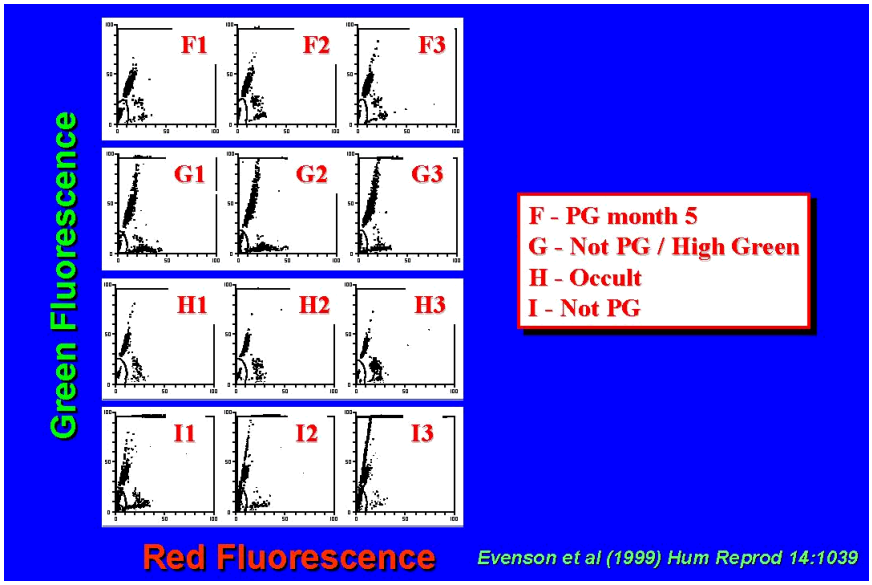
1. The SCSA is the most stable measure over time as compared to classical semen parameters7.

Example: Consecutive monthly semen samples from the same donor.



2. Can it change with time? YES!10

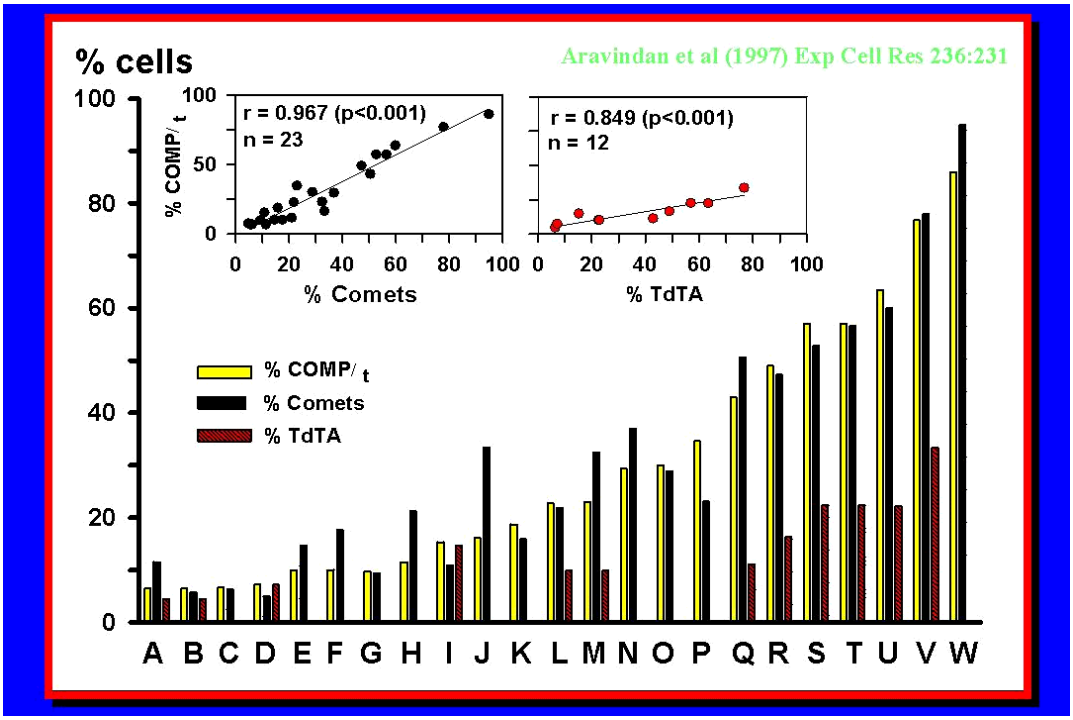




The nature of COMPat sperm

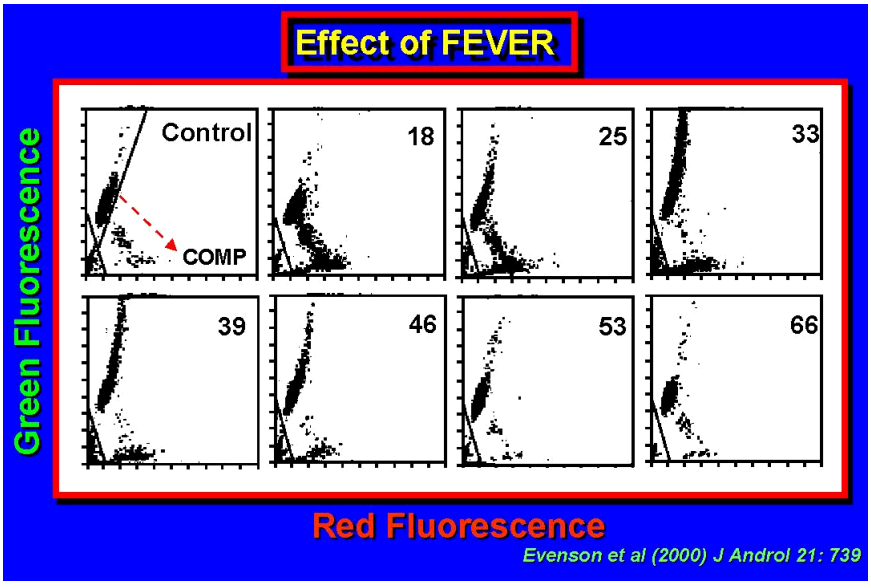
1. COMPat sperm have extensive DNA strand breaks that give rise to the DNA denaturation.

SCSA data correlate significantly with COMET ($p<0.001$) and TUNEL ($p<0.001$) data.2,12,15,22

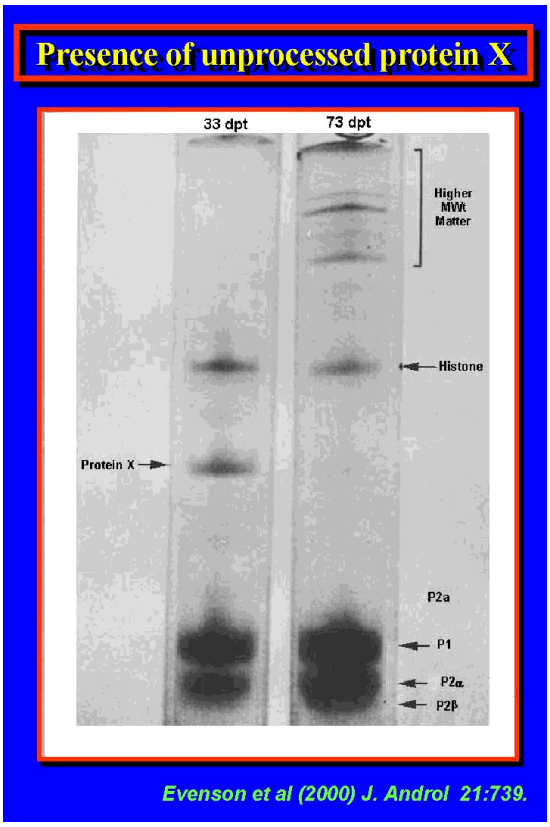


The nature of High Green DNA staining sperm[8]

1. Suggested that High DNA stainable sperm have abnormally condensed chromatin.
2. Day 33 sperm have unprocessed P2 protamine.



3. The presence of unprocessed protamine P2 (protein X) was shown.



IV. What is the Role of the SCSA in the Human Infertility Clinic?

Sperm nuclear DNA integrity is of paramount importance for competent expression and integration of the male genome with the female (oocyte) genome. Integrating a rapid, objective and highly reproducible DNA quality assessment (SCSA) into conventional semen analyses will be a valuable tool for enhancing the diagnosis of and treatment for male infertility. Note that sperm with DNA strand breaks can fertilize oocytes with the same efficiency as normal sperm1.

Criteria for whether or not a test is clinically useful

In order for a test to be clinically valuable, it must have a threshold above and/or below which it will provide discriminatory and predictive capabilities, with little overlap between unaffected and affected groups, e.g., fertile and infertile men. Conventional semen parameters often do not meet these standards. For example, a “normal” sample has a concentration of >20 million sperm/ml23; however, this was derived from a study18 where the median sperm concentrations in the fertile and infertile groups were 90 and 74 million, respectively. Obviously, sperm concentration, like other conventional parameters, has little power to identify potentially infertile men.

SCSA predictive status from a major in vivo fertility study10

The purpose of evaluating the male partner of a suspected infertile couple is to gather information about his fertility potential through history, physical and diagnostic testing. To achieve this, diagnostic tests should provide thresholds that discriminate between fertile and infertile men, to help predict fertility potential. SCSA data on hundreds of semen samples strongly predict a negative outcome for both in vivo and in vitro fertility and sustained pregnancy for patients with COMPat of >30%.

From 200 couples in the comprehensive "Georgetown male factor infertility study", a current threshold of >30% COMPat (% sperm with damaged DNA) was statistically derived for 'significant lack' of fertility potential, 15-30% for 'reasonable' and <15% COMPat for 'high' fertility status. These data show that the SCSA has reasonable sensitivity.

LSMeans of Sperm Chromatin Structure Assay (SCSA) Data on Georgetown Fertility Study

Fertility Status	n	$X\alpha_t$	$SD\alpha_t$	$COMP\alpha_t$	% High Green
PG in 3 months	78	239.6	141.9	12.3	9.0
PG 4-12 months	40	254.7 *	158.0 *	15.4 *	8.7
No Pregnancy	31	270.9 ***	174.1 ***	17.5 ***	15.0 ***

* $p < 0.05$
*** $p < 0.001$

1. SCSA data from those couples demonstrating pregnancy during the first 3 months of the study were significantly different ($P < 0.01$) from SCSA data on the 40 couples achieving pregnancy in months 4-12 and from the 31 couples not achieving pregnancy ($P< 0.001$).
2. 84% of the male partners of couples that conceived in the first three months (n=73) had a COMPat of <15%.
3. A sharp cut off in values was found between fertile and subfertile men ($p < 0.01$) and infertile men (1 year failure to achieve pregnancy; $p < 0.001$).
4. Specifically, the proportion of "normal" individuals predicted to have a fertility problem and who actually experienced a fertility problem was 52% (27/52).
5. If the SCSA data had been based on a single measurement taken within several days of attempted conception, rather than on a mean of several monthly samples, the predictive power likely would have increased.
6. Based on logistic regression, the percentage of sperm with denatured DNA was the best predictor for whether a couple would not achieve pregnancy.

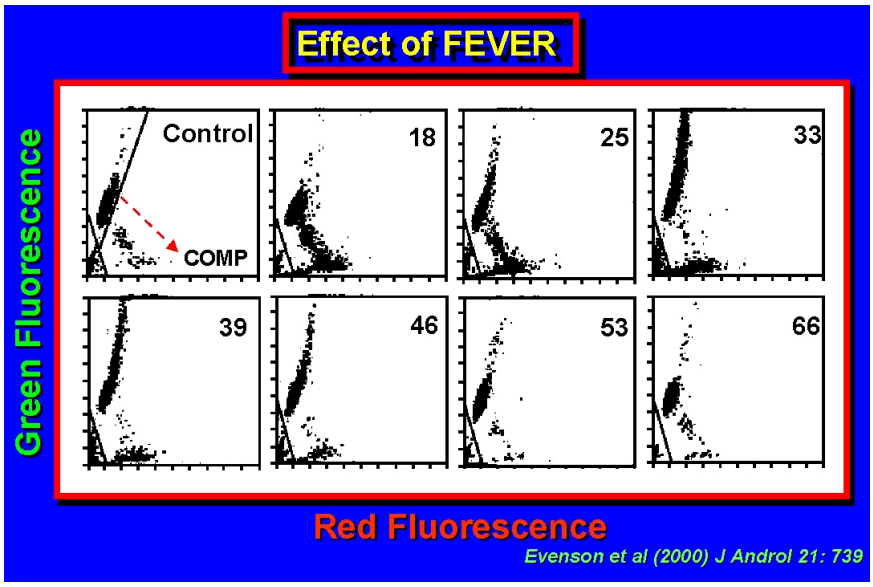
7. Using selected cut-off values for chromatin integrity, including COMPat (denatured sperm) AND those with abnormally high levels of stainability (high green fluorescence, HIGRN), the SCSA data predicted seven of 18 miscarriages (39%). This may be close to the expected male partner contribution.

SCSA predictions for in vitro and ICSI fertilizations17

A statistically established threshold was determined from an in vitro fertilization study for which no sustained pregnancies were observed in 25 cases of Intracytoplasmic Sperm Injection (ICSI) if the raw semen sample had > 27 % of sperm with denatured DNA. Currently, this threshold appears to quantify the functional competence of the sperm nucleus in providing genetic information for normal embryonic growth after fertilization17. Some of these ICSI failures are very likely due to defective DNA integrity, which the SCSA apparently detects.

What is the etiology of abnormal SCSA results?

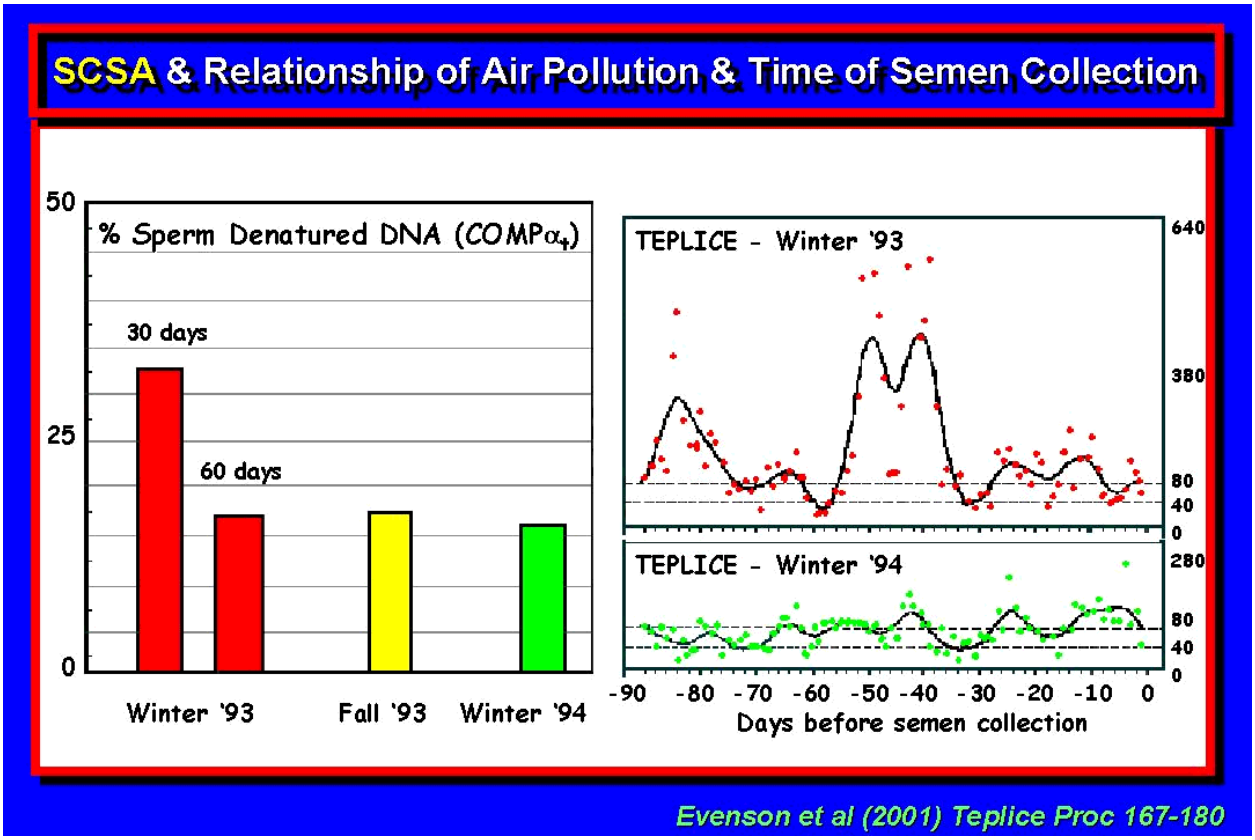
1. Fever, illness, drugs, chemicals, pollution, radiation, heat, leukocytospermia, trauma to testis.
2. Although the SCSA is generally stable within individual men over time, results may vary due to various stressors including infections involving leukocytospermia[20].
3. Febrile illness[7].
4. Testicular cancer[11,14,16] and other as yet undefined conditions
5. The direct correlation of leukocyte concentration to DNA fragmentation in immature and mature sperm indicates that the presence of contaminating leukocytes during periods of infection may alter the regulation of spermatogenesis and compromise male fertility[19].
6. High fever (39.9∞C) was also shown to be associated with both DNA fragmentation as well as abnormal DNA packaging (high DNA stainability, HIGRN) in a case study of a man with influenza[8]



Effects of environmental pollution[9]

The sensitivity of the SCSA has been utilized to measure the effects of high levels of environmental pollution on male fertility potential in men living in or near polluted areas.

1. Young Czech men living in a region with high levels of air pollution produced sperm with high levels of DNA fragmentation.
2. This level of fragmentation may explain the relationship between air pollution and the increased incidence of miscarriage and congenital abnormalities in children conceived by men from this region.
3. Therefore, the SCSA may be used as a biomarker of genotoxicant exposure that may identify men not only with reduced fertility potential but also with the increased risk of conceiving children predisposed to malformations, cancers and genetic diseases[21].



V. What are the Advantages and Disadvantages of the SCSA test for ART Clinics?

A. Advantages of the SCSA

1. The easiest clinical test for preparing samples for analysis at a later time or another place.
2. The semen should be frozen as soon as possible, but may wait up to 4 hrs (motility measurements must be done in under one hour).
3. Samples can be collected at home (or a field study) and brought to the lab.

4. A raw aliquot of semen can be frozen and sent to a diagnostic lab by overnight courier for results in < 24 hours. This is the best solution when dealing with a limited number of patients.

B. Disadvantages of the SCSA

1. cost - a new commercial benchtop (non-sorting) flow cytometer ~US $100,000. (annual service contract ~10% of purchase price).
2. Need a technician trained in flow cytometry.
3. The SCSA has some specialized requirements not typically utilized or recognized by core flow cytometry operators (experience indicates that core FCM facilities tend NOT to be reliable for SCSA data).

Biomedical Diagnostics, Inc.
P.O. Box 853
Brookings, SD 57006
Phone/FAX: (605) 692–5938
Email: bdigloba@itctel.com

Biomedical Diagnostics, Inc.

DATE: __/__/__

CLINICAL REPORT
Sperm Chromatin Structure Assay

PATIENT : _____ **MEDICAL RECORD NUMBER :** _____
DOB : __/__/__ **COLLECTION DATE :** __/__/__

Background

COMPα_t (% sperm cells containing denatured DNA) is the most important factor in determining fertility potential as measured by the SCSA and is defined in the Georgetown study[1] as:

a. <15% COMPα_t = high fertility potential
b. >15 to 29% COMPα_t = good to fair fertility potential that decreases as it approaches 30%
c. > 30% COMPα_t = In published studies, NO sustained pregnancies have been observed from samples measured the month of potential conception. Spontaneous abortions have occurred.

% High Green Fluorescence (Sperm with higher DNA stainability or immature chromatin)[1]:
a. < 15% immature sperm = normal
b. > 15% immature sperm = high

A combination of these two parameters plus any other factors significant to this particular sample goes into an **estimate** of overall fertility potential checked below.

[1]Evenson DP, *et al*. (1999) Utility of the sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. Human Reproduction 14(4):1039-1049.

SCSA Results

% COMPα_t = 32.5 %
% HIGREEN (immature chromatin) = 6.5 %

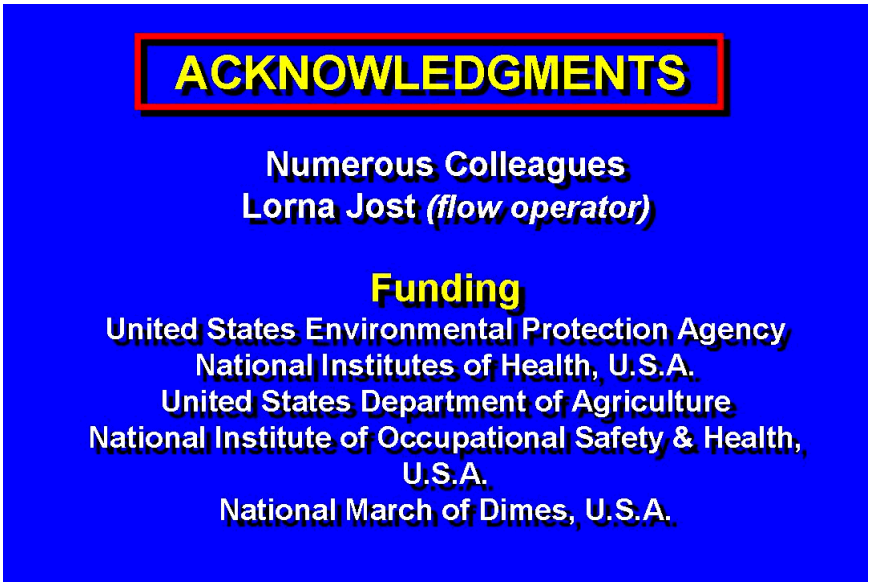
Other Comments:

***Estimate* of Overall Fertility Potential (based on SCSA data only)**

❒ Excellent Fertility Potential
❒ Good Fertility Potential
❒ Fair Fertility Potential
❒ Poor Fertility Potential

Conclusion

The Sperm Chromatin Structure Assay (SCSA) predicts significantly reduced male fertility potential based on the level of DNA fragmentation in ejaculated spermatozoa[10].



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C-Ros Receptor Tyrosine Kinase Knockout Mouse as a Model of Epididymal Involvement in Sperm Maturation

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Introduction

Sperm produced in the testis pass through one of the several efferent ducts into the epididymis. In man, this takes 2 to 11 days (1), covering a tubule length of about 6 m (2). Maturational changes occur during this sojourn, as revealed by studies of the functional and structural differences between sperm obtained from different regions along the length of the epididymis. These involve changes in the potential of motility and kinematics, head morphology and chromatin staining, the ability to undergo the acrosome reaction, bind to the oolema and penetrate oocyte (see 3). With the successful and wide application of assisted reproductive techniques which bypass most of the natural events resulting in fertilisation, post testicular events regulating sperm function may have been neglected by some. On the other hand, precise functional entities of the epididymis in its influence on specific sperm maturational processes remain undefined, despite increasing number of specific epididymal secretions being identified (see 4, 5). In the c-ros transgenic mice, healthy homozygous males exhibit sterility by natural mating despite normal copulation and sperm output. The study of epididymal abnormality and sperm dysfunction in this animal model would help to clarify the influence of the epididymis on male fertility.

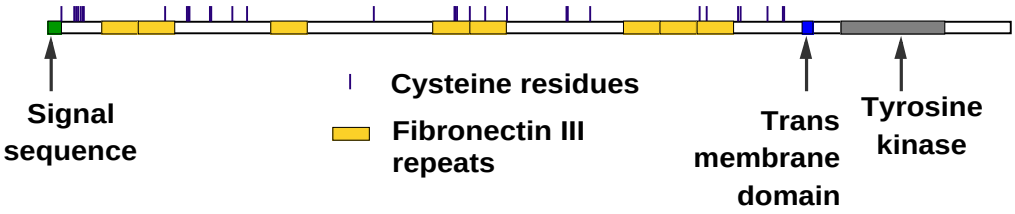
C-Ros and Male Fertility

Gene Expression and Function

The proto-oncogene c-ros was first cloned from a human mammary tumour cell line (6). The gene product is a receptor protein tyrosine kinase belonging to the insulin receptor subfamily in its molecular phylogeny (7). It has a MW of 260 kD, with a large extracellular domain.

Transcript of *c-ros* receptor protein tyrosine kinase

8 kb (2340 amino acids; 260 kD)



The c-ros transcript is expressed in embryonic epithelia of the kidney, lung, intestine and the Wolffian duct which develops into the male genital tract (8, 9). Expression disappears from all organs during postnatal development except in the proximal epididymis where it is up-regulated at the time of the prepubertal differentiation of the initial segment. Transcript in the testis is largely truncated and non-functional (10).

The study of the role of c-ros has been hampered by the unknown identities of both the receptor ligand and the substrates of its kinase activity upon activation. Studies using in vitro heterologous expression of chimeric receptors indicate phosphorylation of early signalling proteins (phospholipase Cg, MAP kinase, phospho-inositide-3 kinase, insulin receptor substrate1, SH2 domain of c-Ab1 and SHP1), as in the activation of many growth factor receptors (see 11, 12).

Infertility of Knock-out Male Mice

Unlike most knockout animals where a proto-oncogene is targeted, the c-ros knockout mice develop into healthy adults, indicating a replaceable role in the morphogenesis of the vital organs. However, there is no differentiation of the initial segment of the epididymis at puberty, and adult males are completely infertile despite deposition of normal numbers of sperm in the female tract. There is a complete failure of the ejaculated sperm in their migration from the uterus into the oviduct, caused by the angulation of the sperm tail into a hair-pin shape (13). Sperm released from the cauda epididymidis of the knockout mice into a culture medium of 310 mmol/kg exhibit various extents of tail angulation, which is an indication of cell swelling. Percentage motility is normal and kinematics is only slightly compromised (14).

Sperm Volume Regulation

Physiological Significance

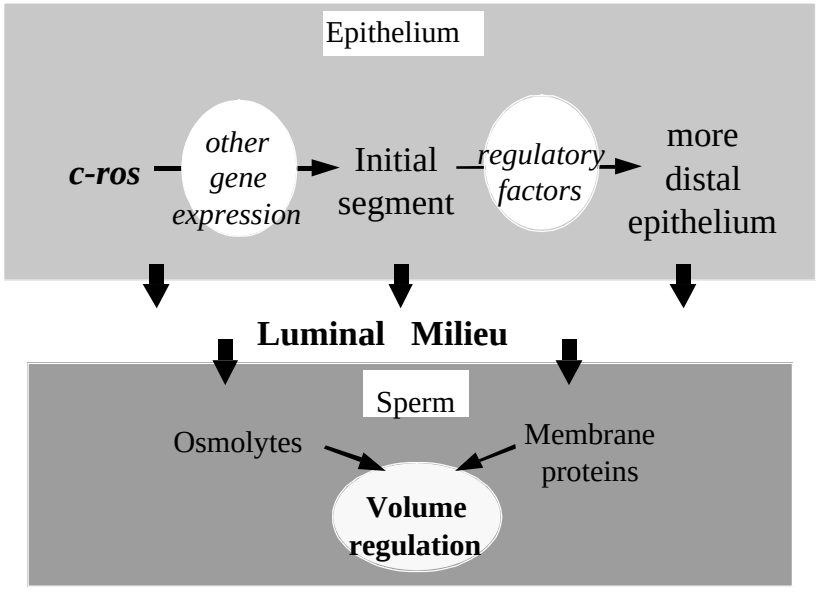
Spermatozoa leaving the testis (ca. 300 mmol/kg in the rete) encounter fluids of varying osmolarities before they reach the site of fertilisation, experiencing hypertonicity in the epididymis during maturation and then relative hypotonicity along the female tract. Data for human epididymal fluid are not available but the vas deferens contains fluid of 342 mmol/kg

(15) and epididymal fluid from many mammalian species is of high osmolality. After ejaculation into the female tract, spermatozoa experience a decrease in extracellular osmolality as they leave the seminal plasma (around 340 mmol/kg) to around 290 mmol/kg as they enter cervical mucus and are eventually bathed in follicular fluid (16, 17). Therefore, regulatory volume decrease (RVD) is required to counteract cell swelling. There is an indication that sperm acquire the ability for RVD on maturation in the epididymis, which is defective in the homozygous c-ros transgenic mice (14). Mouse sperm experience an osmolality of 430 mmol/kg in the cauda epididymidal fluid, and a drop to around 330 mmol/kg in the uterine cavity. Subsequently, sperm with defective RVD would swell, causing angulation of the tail and hindering migration into the oviduct. The documented association of infertility in domestic animals with swollen sperm tails, which are apparently of epididymal origin, may be manifestation of a similar defect.

Mechanism of Regulatory Volume Decrease (RVD)

In somatic cells, RVD involves effluxes of K+, Cl- and organic osmolytes through separate K+-channels and anion channels (see 18, 19). Various K+-channels have been demonstrated to be volume sensitive, depending on the cell type (see Wehner, 1998). These include the Ca2+-dependent, large conductance maxi-K+ channels; the mostly Ca2+-independent, low conductance K+-channels; the small molecular size, Ca2+- and voltage-dependent minK+-channels; and the voltage-sensitive shaker family members Kv1.3 and Kv1.5. Quinine is a wide spectrum channel blocker affecting voltage-sensitive K+-channels as well as Ca2+-activated ones (see 20). Besides K+-channels, quinine can also affect the volume-sensitive organic anion channels (VSOAC) (21, 22). These can be voltage-gated chloride channels (ClC-2), large conductance channels or outwardly rectifying intermediate conductance channels that permit passage of organic osmolytes (23, 24). Quinine and some other channel blockers for RVD induce swelling of the wild type mouse sperm, which is manifested in tail angulation at the cytoplasmic droplet of the swollen sperm, mimicking the defect of infertile sperm from the knockout males (14).

Implication of Epididymal Malfunction in Defects of Sperm Volume Regulation



Since the c-ros protein in the wild type animals is expressed only in the proximal epididymis and not in the testis, and spermatozoa carrying the mutant allele have normal fertility potential, as demonstrated in the genotype distribution of the offspring of chimeric mice, the underlying cause of sterility is believed to be epididymal malfunction (10, 11). The initial segment of the epididymis is characterised histologically by tall columnar epithelial cells bearing long stereocilia extending into the small tubule lumen. This type of epithelium is also found in the proximal epididymis of man, although not extensive enough to form a gross anatomical segment (25). The epididymal epithelium is very active in the transport of inorganic electrolytes and organic osmolytes, creating a unique environment for sperm maturation and storage (see 26, 27). It is known in many species that both transport and secretory function of the epididymal epithelium is regionalised, resulting in a unique milieu bathing the maturing sperm in the tubal lumen which varies in organic and inorganic composition along the entire length of the epididymis. Non-differentiation of the initial segment due to c-ros deletion could lead to an abnormal epididymal milieu, either locally or distally via regulatory factors, for the maturing sperm (see figure). This could directly, or indirectly via modulation of functional sperm membrane proteins (such as ion channels and transporters), deprive the sperm cells of the essential osmolytes for their volume regulation. Whereas the osmolytes are yet to be identified, likely candidates include substances present in high concentrations in various part of the epididymis such as K+, glutamate, taurine, myo-inositol, carnitine and glycerophosphocholine. Most of these are known osmolytes in somatic cells.

RVD in Human Sperm and its Possible Relevance in Sperm Transport in the Female Tract
In contrast to rodents, where sperm transport in the female tract demands vigorous forward progressive motility for penetrating the tightly folded utero-tubal junction, the main obstacle to sperm transport in the human female tract is less clear. Limited studies in few subjects, including fertile women, have indicated the presence of barriers since less than one in a million sperm deposited in the vagina can be recovered from the oviduct (see 28). Both the morphology and motility characteristics of sperm are known to be important factors for the penetration of cervical mucus (29-31). Despite a suggestion that sperm transport from the cervix into and through the uterus may be effected by uterine peristalsis, another significance of mucus penetration, such as in the creation of sperm reservoirs in the cervical crypts, cannot be ruled out anyway (32). There is also a correlation between sperm velocity measured in vitro and IUI success rates (33). Washed human spermatozoa change cell volume and shape in response to low concentrations of the ion-channel blocker quinine, as detected by flow cytometry and light microscopy respectively. The increase in sperm volume is accompanied by reduced straight-line velocity and linearity of the swim-path but increased lateral head displacement and curvilinear velocity, with percentage motility unaffected. Such decreases by quinine in the efficiency of forward progression are also observed in semen and in artificial cervical mucus, resulting in a marked reduction of mucus penetration and migration. Experiments have been performed, using ion-channel openers and blockers, to characterise the channels involved in human sperm RVD, and K+-channels have been implicated.

Characterisation of the epididymal abnormality in the c-ros knockout infertile mice model would reveal the epididymal factors involved in the normal maturation of sperm with respect to volume regulation. On the other hand, understanding the physiological significance and the mechanism

of sperm volume regulation would improve diagnosis of male infertility and provide a new lead to male contraception.

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Relationship Between Adluminal and Epididymal Sperm Maturation

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Learning Objectives

- ✓ The epididymis is involved in promoting sperm maturation and maintaining sperm viability
- ✓ Sperm maturation involves constitutive changes to epididymal sperm and changes to sperm which depend on the epididymal milieu
- ✓ Sperm maturation results in acquisition of sperm motility and fertilizing potential
- ✓ There is an intimate association between sperm and the epididymal epithelium (as demonstrated by culture experiments)
- ✓ The epididymal epithelium may regulate phosphorylation of sperm proteins involved in maturation processes
- ✓ Factors of epididymal origin most likely act in concert with constitutional changes to sperm which together permit full sperm function.

Introduction

In fertile men, the majority of epididymal sperm(atozoa) acquire the potential to fertilize (assessed with sperm function assays) on passage into the corpus and cauda regions of the epididymis. However, when the epididymal tubule is blocked or absent, a small proportion of sperm can acquire fertilizing potential in the more proximal region of the excurrent duct. This suggests that maturation and storage of human sperm may not be as closely dependent on passage through the caput and proximal corpus regions of the epididymis as in laboratory species. Although secretions of the epididymal epithelium are clearly important for sperm maturation and survival the exact nature of many of these factors has yet to be determined. The main interactions between the epididymal epithelium and sperm are presented in figure 1.

Factors Affecting Epididymal Sperm Viability

Sperm must be maintained in a viable condition throughout epididymal transit and during storage in the distal cauda epididymidis and vas deferens. Examination of human semen quality in relation to the interval between ejaculations has not indicated any consistent decline in semen quality after 10 days abstinence, but the precise conditions that sperm require for survival in the epididymis also remain unclear. In animals, it has been estimated that sperm viability is retained in the cauda epididymidis for 2-3 weeks or longer.

Sperm storage in the cauda region

Men are endowed with a poorly differentiated cauda epididymidis and have a relatively small sperm storage capacity in comparison to most animals. The normal storage capacity for human sperm within the cauda epididymidis is estimated to be only 3-4 days while the entire transit time for sperm from the testis to ejaculation is about 12 days.

Androgen-dependent factors

Changes in androgen levels can alter the capacity of the epididymis to store sperm by affecting ion and protein profiles of fluid from the cauda epididymal lumen.

Temperature-dependent factors

Temperature-dependent secretory factors from the epithelium may stabilize membranes to maintain sperm survival. In this connection, human sperm may be relatively insensitive to temperatures which in other species would compromise cell survival Underwear and many activities (i.e. prolonged sitting) cause elevated scrotal temperatures so that sperm are often stored at near body temperature, but despite this they still can maintain fertilizing potential.

Anatomical factors

Sperm are usually in a quiescent state within the epididymal luminal microenvironment but it unknown whether particular ions or proteins act to specifically prolong sperm viability. For obvious ethical reasons it is not possible to undertake the experiments carried out in animals of ligating the cauda epididymidis to isolate sperm and thereafter testing for their viability and fertilizing ability. But a number of indirect observations indicate that men might only be able to store sperm in a viable condition for a short period. Anatomically, the human cauda epididymidis is poorly differentiated and since, the tubule is not very convoluted and the lumen is relatively narrow it has less capacity to store sperm than that of the rodent or domestic animal

Major Functional Changes to Sperm During Epididymal Passage

During epididymal transit, mammalian sperm undergo numerous changes. These include morphological alterations, the stabilisation of nuclear chromatin and dense fibres by disulphide bonds, changes to the composition of membranes and many other biochemical modifications but only the major functional changes related to human sperm will be discussed here.

Sperm motility

Perhaps the most striking alteration is the increase in the potential for sustained forward motility of epididymal sperm when placed in the appropriate culture medium. Investigating epididymal samples retrieved from volunteers undergoing vasectomy under general anaesthetic has made an assessment of this aspect of sperm maturation. Most sperm recovered from the caput region remained immotile or exhibited a weak twitching motion in modified Tyrode's medium but a few

(~3%) did show progressive motility. In contrast, more than 60% of sperm from the cauda epididymidis displayed straight-line forward motility. Similar results for the proportion of motile sperm have been obtained for other patients with intact ducts. Since, the in vitro and in vivo fertilizing potential of an ejaculate is correlated with the proportion of sperm with a velocity above 25 mm/sec, this induction of straight-line motion in epididymal sperm is a significant development. Sperm motility changes are associated with phosphorylation of proteins in the flagellum.

Potential for sperm-egg binding

Equally as important as changes in motility, epididymal sperm also acquire the ability to recognize and bind to oocytes and then to fuse with the oolemma during epididymal transit. Because of the practical and ethical difficulties of experimenting with human oocytes, the first in vitro investigations were performed with zona-free hamster oocytes. Sperm retrieved from the initial segment or caput region of proven fertile men or patients with a normal epididymis failed to bind or penetrate these oocytes while those from the cauda region were successful. These results provided an indirect measure of sperm fertilizing capacity but more recent investigations with human oocytes have confirmed the general conclusion. Although the ability of sperm to interact with the oocyte may reflect direct alterations in it's membranes during maturation most of the experimental results can be explained also by an enhanced ability of the cell to undergo the acrosome reaction. Both secondary binding of sperm to the zona and sperm-olemma fusion require an acrosome reaction to be completed.

Is there a direct relationship between epididymal epithelium and sperm maturation?
As mentioned above care must be taken in how experiments on sperm maturation are interpreted with respect to the acquisition of specific receptor molecules. Certainly, it has been shown that during maturation, the negative surface charge of human sperm increases due to a changing distribution and density of moieties over their plasma membrane. Moreover, some of these alterations undoubtedly result from the incorporation into sperm membrane of protein, sugar and lipid determinants of epididymal origin. Many studies have tried to establish whether such acquire epididymal determinants might engage with complementary molecules on the egg. While there is evidence that this might occur, a direct and definite relationship between the expression of a molecule of epididymal origin on sperm and the acquisition of a fertilizing function has yet to be demonstrated conclusively either in the human or in an animal model. This reflects the complexity of the process of sperm maturation. Many epididymal epithelial secretory proteins may undergo additional processing at the sperm surface, so that following the fate of a particular determinant, by for example immuno-localisation, can be difficult. For example, there may be membrane exchange of GPI anchored moieties between epididymal epithelium and sperm. Additionally, factors of epididymal origin most likely act in concert with constitutional changes to sperm which together permit full sperm function.

Epididymal epithelial cultures to study sperm-epithelial interactions

Plaques of epididymal epithelium can be generated from hamster or human tissue that evert overnight in culture and form contiguous spheres of epithelium with the apical surface facing

outwards. These tissue balls eventually attach and plate out on the bottom of the Petri dish after 3-5 days in culture and form nodes of epithelium. Even after 42 days in culture human epididymal principal cells displayed good polarity with apical microvilli and lateral tight junctional complexes. Similar procedures have been used to culture epididymal epithelia in the mouse and dog. Epididymal tubule plaques produced in this way retain initially their original epithelial architecture and cellular composition but will incorporate also the basal lamina and some of the underlying myoid, fibroblast, and endothelial cells that are closely associated with the tubule. In this regard, fibroblast cells of the myoid layer have been shown to maintain epithelial activity in culture. Interestingly, fibroblast overgrowth in these cultures is seldom a major concern and there is a reasonable proportion of cytokeratin positive epithelial cells maintained in culture for 2-3 weeks. This may indicate that the usual proliferative activity of fibroblasts is inhibited in these preparations. Principal cells of the epithelium may remain polarized and continue to secrete proteins for many days and sometimes weeks in the presence of androgens. In canine epididymal cultures, it was demonstrated epididymal-specific gene transcripts were maintained for up to 7 days with the expression of some genes being more stable than others

Co-incubation of epididymal epithelial cultures with sperm

The co-incubation of sperm with epididymal epithelial cultures provides further clues as to how sperm maturation may occur. However, co-incubation experiments alone, cannot as yet bring about all the changes to sperm that are required for acquisition of full fertility. Clearly, sperm in situ undergo sequential modifications as they migrate along the epididymis and this is difficult to reproduce in vitro. Human cauda epididymal or washed ejaculated sperm maintained good viability for 8 days (50% with progressive motility < 20 mm/sec) provided some medium was replenished every other day. A small proportion of these sperm was still viable after 17 days of co-culture and retained intact acrosomal membranes. This finding supports the contention that human sperm may be resilient to body temperature under the appropriate conditions.

Direct interaction between sperm and apical epithelial surface may be important for sperm viability

Extended cell viability was associated with the close attachment of sperm to the apical surface of principal cells in culture. In contrast, the co-incubation of human caput sperm with epididymal epithelial cultures only enhanced sperm motility for about 24 hours although over this period the ability of sperm to bind to salt-stored human zona increased significantly. All these changes were promoted by androgen-dependent factors since cultures maintained in the absence of testosterone or dihydrotestosterone failed to maintain sperm viability or induce maturation. Co-incubation of sperm with cultures other than of epididymal origin have been unsuccessful in promoting sperm maturation although in some cases they may enhance sperm motility and viability. Human oviductal cells in culture have a beneficial effect on washed ejaculated human sperm in terms of viability, synergistic induction of capacitation and induction of hyperactivated. These effects seem to be more specific than with other epithelial cell lines.

Sperm maturation is associated with changing patterns of protein tyrosine phorphorylation, which may be regulated by the epididymis

As sperm undergo maturation there is a change in the phosphorylation of major proteins in the sperm head and tail. These changes are associated with acquisition of progressive sperm motility, the ability of sperm to be capacitated and the acrosome reaction. Recent evidence suggests that particular phosphorylation patterns are associated with specific regions of the epididymis.

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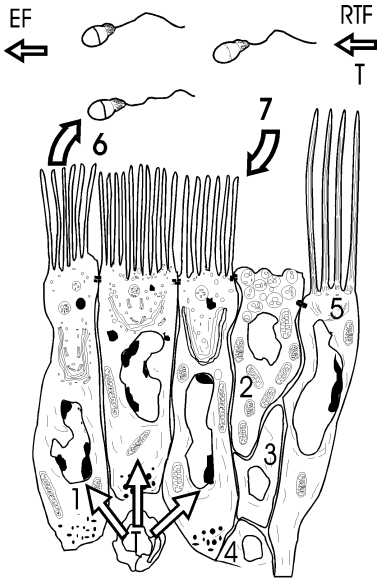

Figure 1. Function of the epididymal epithelium is dependent on androgens (mainly T) from the systemic circulation or directly from Rete testis fluid (RTF) and DHT formed by reduction of T by 5 a-reductase in the epithelium. The majority of the epithelium consists of principal cells (1). These are involved in both absorption and secretion of ions and proteins, lipids etc and regulate the composition of the epididymal fluid (EF). Tight junctions between adjacent epithelial cells form a blood-epididymal barrier restricting the access of epididymal spermatozoa to systemic macromolecules. A small proportion (5%) of mitochondrial rich clear (apical) cells (2) may be involved in acid regulation of the EF and the removal of particulate matter from sperm degeneration. Intra-epithelial lymphocytes (3, immune regulation?) and basal cells (4, stem cells?) are also present. In the efferent ducts there are true ciliated cells (5) while the luminal surface of principal cells display microvilli. As spermatozoa pass along the epididymis and interact with the epithelium (6) their surfaces are altered either by the enzymatic transfer (e.g. sugar transferases) of determinants; direct covalent coating (masking); or by the processing or removal of existing membrane macromolecules (endoproteolytic cleavage). A variety of epithelial secretory proteins (e.g. protease inhibitors) may serve to regulate this process and protect spermatozoa (e.g. antioxidants). Metabolites from spermatozoa are also removed (7). In vitro incubation of spermatozoa with epithelial can promote sperm maturation changes and prolong sperm survival.

Reproductive toxicology and the differentiation of a functional gamete

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Learning Objectives

As a consequence of this lecture the attendees will be able to:

✓ Critically evaluate the evidence suggesting that environmental toxicants have a causative role in the aetiology of male reproductive pathology

✓ Understand the need to identify the nature of these toxicants and the mechanisms by which they exert their toxic effects

✓ Define the wide range of biological processes that are known to be influenced by reproductive toxicants

✓ Give examples of reproductive toxicants that work via different mechanisms: testicular, post testicular and through disruption of DNA integrity in the male germ line

✓ Identify the directions that future research in this area will take and its relevance to clinical practice.

Introduction

The evidence suggesting that there has been a sudden deterioration in male reproductive health since the 1950's will be reviewed. An apparent decline in sperm counts over the past 40-50 years has stimulated intense interest in this area and has been taken to indicate an environmental impact on male reproduction in the wake of the sudden growth the chemicals industry since World War II. This evidence is controversial largely because:

✓ Sperm count is not a very robust measure of male reproductive competence. It is confounded by factors such as ejaculation frequency and may be profoundly influenced by the techniques used in performing these assessments.

✓ Considerable difficulty has been experienced in identifying an unbiased population of men for sampling purposes.

- ✓ There is a significant geographical impact on sperm counts due to factors that have not yet been identified. Such regional variation may, in countries such as the USA, be further confounded by differences in the racial composition of the populations sampled.

- ✓ There is only a weak link between sperm counts and fertility and the threshold values for normality have been progressively revised with time.

Such difficulties aside, detailed analyses of single-site data bases in Europe have provided suggestive evidence for a post war decline in sperm numbers. Moreover it remains a fact the greatest single cause of human infertility at the present time is defective sperm function. There has also been an incontrovertible global rise in the incidence of cancers of the male reproductive tract (prostate and testicular) over the past 40-50 years while cancers of the female tract (cervix, ovary, uterus) have remained unchanged (1).

Targets for Male Reproductive Toxicity

There are two major ways in which male reproductive toxicity can present itself:

- ✓ Male infertility. In this case the target of a given toxicant could be any of the major cell types within the testes (Sertoli, Leydig- and germ cells), or post testicular targets such as the epididymis and mature spermatozoa. In certain cases a testicular toxicant can have an effect on sperm differentiation that does not manifest itself until the mature spermatozoon is released. For example certain toxicants can lead to the retention of excess residual cytoplasm during spermiogenesis. This has no effect on sperm number but does impact upon the functional competence of the spermatozoa following ejaculation as a consequence of oxidative damage (2,3).

  It should also be recognized that the functional competence of mature spermatozoa can be compromised in many different ways. Some agents may effect sperm motility while others may disrupt the ability of these cells to capacitate, acrosome react or fuse with the vitelline membrane of the oocyte (4).

  It is also possible for reproductive toxicants to interfere with male reproductive function through centrally mediated effects. This is particularly true of the environmental estrogens. These compounds include phenols such as nonylphenol or 4-tert-octylphenol that are thought to disrupt spermatogenesis through the suppression of gonadotrophin production by the pituitary gland. It has been proposed that one of the consequences of this effect during foetal life is to limit Sertoli cell number as a result of inhibited FSH secretion. Since the number of germ cells the testes can support is strictly defined by the availability of Sertoli cells, prenatal exposure to environmental estrogens might be expected to drive down sperm counts in the adult (5,6,7).

  It should also be appreciated that environmental toxicants might be instrumental in the aetiology of male infertility even if the latter has a genetic basis, such as the deletion of key genes on the long arm of the Y chromosome (8). It such cases we might simply postulate that a xenobiotic factor has caused mutagenic (or promutagenic changes) in the germ line of the

father. According to this hypothesis redox cycling compounds might induce DNA fragmentation in the father's spermatozoa, that ultimately leads to the permanent deletion of genes on the Y–chromosome of male offspring. The Y-chromosome is particularly susceptible to double stranded DNA breaks and deletions because most of the genes on this chromosome cannot participate in recombination repair.

✓ Integrity of DNA in the male germ line. Reagents that induce DNA damage in the male germ line might not have a dramatic effect on sperm function, however they can seriously impair the ability of the spermatozoon to trigger the development of a normal human embryo. Such effects may interfere with the progress of pregnancy leading to early embryonic loss or may have long term effects on the well-being of the offspring such as childhood cancer or infertility (1).

Examples of Male Reproductive Toxicants

Examples of toxicants that affect testicular function include:

✓ Sertoli cell toxicants. Phthalate esters [eg mono-(2-ethylhexyl) phthalate] and 2,5-hexanedione (2,5-HD) are classic examples of Sertoli cell toxicants that induce high rates of apoptosis in the germ line via Fas ligand dependent mechanisms (9,10)

✓ Leydig cell toxicants. Ethane 1,2-dimethanesulfonate is a Leydig cell toxicant that suppresses intratesticular testosterone levels and induces germ cell apoptosis, again via Fas ligand dependent mechanisms (11).

✓ Germ cell toxicant. Clinically, the most important germ cell toxicants are chemotherapy drugs such as cisplatin or cyclophosphamide. These reagents that can induce DNA damage in the germ line via complex mechanisms that involve either the induction or suppression of apoptosis in germ cells (12, 13). Other potential germ cell specific toxicants include carbendazim (14), molinate (15).

Examples of testicular toxicants that affect DNA integrity in the germ line include:

1. Cigarette smoke. High levels of cigarette smoking induce a state of oxidative stress in the body characterized by reduced levels of antioxidants (vitamin E and C) in blood and semen. One of the consequences of this oxidative stress is the presence of high rates of DNA fragmentation in the male germ line. It is possible that this fragmentation is oxidatively induced and a reflection of the oxidative stress the testes are under in such patients. It is also possible that xenobiotics present in cigarette smoke, such as polycyclic aromatic hydrocarbons, induce DNA fragmentation as a consequence of the initiation of an apoptotic cascade (16).

✓ Metals. Certain metals such as iron, copper, lead, cadmium and selenium are either known or suspected inducers of DNA damage in the male germ line. In many instances redox mechanisms have been implicated.

✓ Environmental estrogens. There is at least one report of estrogenic compounds directly inducing DNA damage in human spermatozoa (17).

✓ Cytotoxic drugs. As indicated above, chemotherapeutic drugs such as cisplatin and cyclophosphamide can have a profound effect on DNA integrity in male germ cells.

These observations are important because DNA damage in the male germ line has, particularly in the case of smoking, been linked with the etiology of childhood cancer and may also play a role in the genetic defects that lead to male infertility (1,18).

Future Research Directions

The data reviewed in this presentation emphasise the powerful impact that environmental factors can have on male reproductive function. Such factors might induce male infertility via effects on a variety of disparate cell types in the male reproductive tract. They might also cause DNA fragmentation in spermatozoa and thereby compromise the ability of these cells to support normal embryonic development. Key tasks that remain to be engaged include:

✓ Characterize those xenobiotic structures that can compromise the functional integrity of male germ cells at different stages of differentiation.

✓ Elucidate the importance of such factors in the aetiology of spontaneous male infertility.

✓ Determine the mechanisms by which these compounds achieve their toxic effects.

✓ Resolve whether any of the toxic effects observed could be further exploited as the basis for male contraception.

✓ Characterize those environmental factors, including xenobiotics, that can induce DNA damage in the male germ line.

✓ Determine the mechanism by which such damage is induced.

✓ Elucidate the protective measures employed by germ cells and the early embryo to prevent DNA damage, including the nature of the DNA repair strategies employed by these cells.

✓ Determine the role played by such DNA damage in the aetiology of human disease.

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Recommended Reading

1. Aitken, R. J. (1999) The human spermatozoon: a cell in crisis? The Amoroso Lecture. J. Reprod. Fertil., 115, 1-7.
A review of our understanding of the defects that occur in human spermatozoa and their role in etiology of infertility and genetic disease.

2. Sawyer, D.E. and Aitken, R.J. (2001) Male mediated developmental defects and childhood disease. Reproductive Medicine Review 8, 107-126.
A recent review of the impact of occupational factors in the etiology of DNA damage in the male germ line

3. Turner, K.J. and Sharpe, R.M. (1997) Environmental oestrogens--present understanding. Rev. Reprod. 1997 2, 69-73.
A brief review of our current understanding of the impact of environmental estrogens on male fertility.