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Aspects of the Regulation of Human Sperm Motility

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To all those who have helped me see this project through...

With sufficient thrust, pigs fly just fine. However, this is not necessarily a good idea. It is hard to be sure where they are going to land, and it could be dangerous sitting under them as they fly overhead.
–RFC 1925

Abstract

In this thesis I address some of the possible causes for oligozoospermia and low sperm motility, two factors that, singly or in combination, are often seen in cases of male subfertility.

In the first study we evaluated the effect on human spermatozoa of the toxicity of compounds present in diesel exhaust. The compounds tested were derivatives of 2-nitrofluorene, and some of them were found to drastically lower the motility of human spermatozoa. We concluded that human spermatozoa may be a working model system for testing potentially toxic compounds.

Sperm Activating Protein is a complex of immunoglobulin G4 and apolipoprotein A-I, previously shown to be a major extracellular factor that increases sperm motility. In the second study we determined the exact identity of the components. In addition we were able, using selective proteolytic digestion and Western blot, to demonstrate that the apolipoprotein A-I is bound in the Fab-portion of the immunoglobulin G4.

Oligozoospermia is an other common abnormality seen in male subfertile patients, and can be due to defects in cell division. In the third study we focused on the synaptonemal complex, which is a structure that takes part in the joining of homologous chromosomes during meiosis. The structure is almost universally conserved in eucaryotic species, and controls the number and distribution of cross-overs and converts these into chiasmata. In this work we found that the synaptonemal complex protein 1 is present in all testicular biopsies from patients with a partial or fully functional spermatogenesis.

List of original publications

I. P. K. G. Leijonhufvud, Å. Pousette, L. Möller & B. Fredricsson: *Derivatives of 2-Nitrofluorene Cause Changes of Human Sperm Motility*, Pharmacology & Toxicology, 1994 **75**:310-314.

II. P. K. G. Leijonhufvud, E. Åkerlöf & Å. Pousette: *Structure of Sperm Activating Protein*, Molecular Human Reproduction 1997 **3**(3):249-253.

III. Å. Pousette, P. K. G. Leijonhufvud, U. Kvist, S. Arver, J. Pelttiari & C. Höög: *Presence of Synaptonemal complex protein 1 transversal filament like protein in human primary spermatocytes*. Human Reproduction, 1997 **12**(11):2414-2417.

Contents

List of abbreviations

The units are indicated in parenthesis where applicable.

ALH	Amplitude of Lateral Head displacement (μm)
BSA	Bovine Serum Albumin
CASA	Computer Aided Sperm Analysis
EBSS	Earle's Balanced Salt Solution
FITC	Fluorescein Isothiocyanate
FPLC	Fast Protein Liquid Chromatography
ICSI	Intracytoplasmic Sperm Injection
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kD	Kilodalton
LIN	Linearity (VSL/VCL, given as %)
MW	Molecular weight
PBS	Phosphate Buffered Saline
ROSNI	Round Spermatid Nuclear Injection
SC	Synaptonemal Complex
SCP1	Synaptonemal Complex Protein 1
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis
SPAP	Sperm Activating Protein
STR	Straightness (VAP/VCL, given as %)
VAP	Velocity Along Path (μm/s)
VSL	Straight Line Velocity (μm/s)
VCL	Curvilinear Velocity (μm/s)

Introduction

Spermatogenesis and spermiogenesis.

Human spermatozoa develop at a constant rate during a 70 day period. The release from the testis and the transport through the epididymis take 2–3 weeks, but this can vary significantly in some cases.

Spermatogenesis

The germ cells in the tubuli develop into spermatogonia, thereby starting the first step in the process to that leads to a mature spermatozoon. During spermiogenesis the spermatogonia undergo mitotic division, giving rise to an increase in the number of daughter cells. Some of these will proceed into spermatogenesis, or degenerate. The three types of spermatogonia that exist in the human — dark type A (Ad), pale type A (Ap), and type B, or differentiating spermatogonium — are the stages through which a spermatogonium progress before they turn into primary spermatocytes.[4] At this point the first meiotic division occurs and secondary spermatocytes are formed.

Spermiogenesis

During this phase the spermatid matures into a spermatozoon. There are changes in the chromosome structure, and the flagellum develops into the characteristical 9 + 2 axoneme.

The synaptonemal complex

The synaptonemal complex participates in the meiotic pairing of homologous chromosomes. This complex is almost universally conserved in eucaryotic organisms.[6] Synaptonemal complexes control the number and distribution of cross-overs and convert these into chiasmata, thereby ensuring the proper disjunction of homologues.[7]

Synaptonemal complex protein 1

One part of the synaptonemal complex (SC) is the synaptonemal complex protein 1 (SCP1), that has been studied in detail and shown to be a major constituent of the transversal filament of the SC. A human SCP1 cDNA was recently isolated, and was found to correspond well with that of other mammalian species at the amino acid level. The data that are known indicate that it is a structural protein that is directly involved in the zippering process that brings the two homologous chromosomes together during meiosis.[8][9][10][11][12]

Sperm transport

Sperm progressive motility is essential for fertilization, since the spermatozoa must cover a significant distance between the testes and the oocyte. Apart from this also the sperm concentration is of importance for fertilization.

Sperm transport in the male

The spermatozoa enter the seminiferous tubules, from which they later enter the epididymis by way of the vas efferentia. In the epididymis the sperm concentration increases 100-fold.[5] By the time they reach the vas deferens they have acquired the ability to move progressively. During ejaculation the spermatozoa, together with the fluid from the epididymis, and the other accessory sex glands are expelled from the penis.

Sperm Activating Protein

It has long been known that serum has a positive effect on human sperm motility when used as an additive *in vitro*. The basis for this effect has not been fully explored, and we therefore felt it was of importance to determine what parts of serum affects human sperm motility and how this effect is generated. When serum was fractionated with liquid chromatography it was found that the major part of the motility increasing effects were caused by a protein with a molecular weight of 180 kD. When this complex was further studied it was identified as a complex of immunoglobulin G4 and apolipoprotein A-I.[13][14][2]

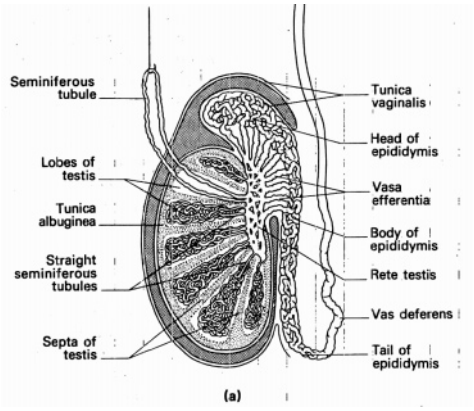

Figure 1: Section through adult human testis.[5]

Sperm transport in the female

In the human the semen is ejaculated in the upper part of the vagina, and most likely also partially directly into the cervix. There it coagulates into a gelatinous consistency. This coagulum is dissolved in less than one hour under normal circumstances.

Spermatozoa that reach the oocyte need to be capacitated for fertilization. In this process an outer layer of glycoproteins is stripped off. Once this has occurred the next step, *activation*, can occur. The double lipid membrane of the acrosome fuses in a number of points, and is removed, thus releasing the contents of the *acrosomal vesicle*. At this time the movement pattern also changes, with much more vigorous tail movements and less linear movement.

Infertility

Infertility is defined as a condition where the couple have unsuccessfully been trying to conceive for over one year.[15] One can divide most cases of infertility into four major categories: disorders of the female genital tract, disorders of ovulation, spontaneous

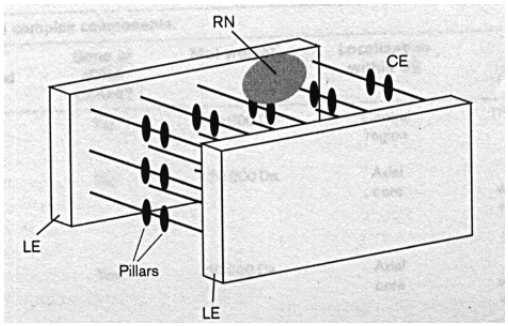

Figure 2: Segment of an SC, showing a schematic representation of its detailed three-dimensional structure.[7]

abortion and seminal inadequacy.[5]

Infertility and subfertility affect as many as 10–15% of all couples.[5] It is assumed that one third of these cases are due to causes of primarily female origin, another third due to those of primarily male origin, and the final third due to a combination of both.

Male infertility

Male infertility has several different possible causes, such as primary and secondary testicular failure, infection, and obstruction, but the most common diagnosis is idiopatic infertility, which accounts for 60–70% of the patients.[16] One definition states that "... idiopatic infertility designates diagnosis by exclusion. [...] Seminal parameters are frequently subnormal and may be associated with elevated serum follicle-stimulating hormone (FSH), indicating spermatogenic failure.".[15]

Diagnosis, evaluation and treatment

When evaluating and treating male sub- or infertility the first step is usually to perform a thorough semen analysis, as well as — when indicated — a full work-up of hormones, other diseases, drug use and possible environmental exposures.

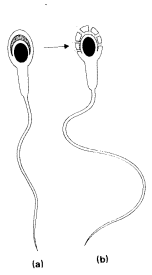

Figure 3: The acrosome reaction in a human spermatozoa.[5]

Sperm motility analysis

Sperm motility analysis methodology can roughly be divided into two categories: manual (i. e. based on direct visual observations) and automatic. The clinically most useful methods today are manual methods based on trained observers, and can give very reproducible measurements.[17][18] These methods evaluate physical characteristics of the sample (volume, consistency, pH, etc), as well as motility (both the fraction motile and grading the motility on a four step scale), sperm concentration, and the fraction abnormal spermatozoa[18].

The automatic semen analysis is generally referred to as "Computer Aided Sperm Analysis" (CASA). These methods rely on a system for (1) recognizing a spermatozoa, and (2) analyzing the "tracks" that each spermatozoon makes while it is being observed (formed by interpolating a series of "snapshots"). The great advantages of the CASA systems are that they provide not only numerical data that simplifies statistical evaluation, but also information on detailed parameters of spermatozoal movements (e. g. VSL, AMP, VCL, ALH, LIN, STR, VAP) that are not normally measurable with manual methods.

Sperm separation techniques

Sperm separation is performed either diagnostically (typically as a swim-up), as a preparative step for artificial fertilization, or for research purposes. The plain human ejaculate contains, in addition to spermatozoa, a number of different components, some of which will tend to deteriorate the quality of the spermatozoa (e. g. cellular debris that tend to release reactive oxygen species, ROS). In order to obtain mainly motile spermatozoa in a standardized medium (with the potential for maintaining sperm

motility for an extended period) it is necessary to transfer the spermatozoa from the seminal plasma.

The methods used can roughly be divided into two categories: self migration, and centrifugation.

Self-migration methods

These methods are based on the ability of spermatozoa to swim in a pre-defined medium or environment. They include the classic swim-up, swim-over, and variously complex density gradient systems.

Centrifugation methods

These methods are based on the fact that the density of spermatozoa varies and is directly proportional to motility. Traditionally Percoll has been used for building the gradients necessary for these methods.

Assisted fertilization techniques

There are few conditions where natural male fertility presently can be restored. Primarily these are obstructive infertility (including absence of parts of the vas), hormonal imbalances, and infectious.[15]

A number of methods exist for achieving fertilization in cases where natural fertility does not exist or cannot be restored. These range from intrauterine insemination to direct injection of gametes into oocytes.

Mixing of gametes: insemination and IVF

In vitro fertilization (IVF) is presently the most common form of assisted fertilization. Oocytes are retrieved from the woman, generally after hormonal stimulation, and allowed to be fertilized *in vitro* by the mans spermatozoa, that has prior to this been separated from the rest of the ejaculate. The pre-embryo is then cultured for approximately two days, and then returned to the woman.

Direct injection of gametes: ICSI and ROSNI

Today there are a number of methods for treating infertility where the motility of the male gamete is of little or no importance. In Intracytoplasmic Sperm Injection (ICSI), or its "extensions" such as Round Spermatid Nuclear Injection (ROSNI)[19], it is possible to attain fertilization from totally immotile spermatozoa, or even from immature spermatids obtained by means of a testis biopsy.

Human sperm concentration and motility in perspective

The "Kamikaze sperm" hypothesis

The "Kamikaze sperm" hypothesis was first advanced by Baker & Bellis in 1988.[20] Briefly it states that the large proportion of defective sperm in the human is an adaptive trait in that these sperm aid in sperm competition. As examples they point to the known systems in e. g. some invertebrates of "anti-sperm spermatozoa", post-coital plugs, and claim that these things exist in the human, and are an effect of sperm competition. The theory has not received any measurable support in the scientific literature.[21]

Toxicological screening using spermatozoa

Rather little is known regarding the effect of endogenous and exogenous factors on sperm motility and concentration. It has been suspected that toxic effects can lead to sperm abnormalities. When performing toxicological screening of potentially toxic

substances the ideal system would test the toxicity to humans, rather than to some model animal or cell culture. But it is obviously not acceptable to test unknown substances on humans. For those reasons most toxicological screenings today are performed using animal models or cell cultures, generally on non-human cells.[22][23]

Is sperm concentration decreasing?

Another problem related to male infertility is the reported gradual lowering of sperm counts Carlsen and co-authors[24] attempted to perform a meta-analysis of previously published works on human sperm motility. They examined a total of 61 published works, spanning the period 1938–1991. The analysis found that there was a lowering in both sperm count and seminal volume.

While these data have only partially been validated by other workers [25][26], and the analysis has been contradicted by others[27][28] [29], it is clear that in at least some data sets [25] the decline appears to exist. One should also be aware that there has been criticism directed against the statistical interpretation of the material in the original work.[29] It has also been suggested that self-selected volunteers differ from the average population due to various bias factors.[30] So far all of these studies restrict themselves to European or American populations, with no studies at all for the Southern Hemisphere.

In addition the hypothesis that the decrease is caused by environmental contaminants is contradicted by the fact that veterinarians have seen no corresponding decrease in the semen of cattle.[31] A possible effect in humans would therefore have to be species specific.

The aims of this study

Low sperm motility and oligozoospermia are the two most common problems seen in cases of male factor infertility. Since the origins of male factor infertility are often unknown we have seen it to be of great interest to further study some aspects of each of these factors. We chose to investigate one endogenous and one exogenous motility-affecting compound, as well as one factor that can affect the production of spermatozoa.

Materials & methods

Model for sperm motility test

Two different methods were used for these studies. In one method we separated spermatozoa on a discontinuous Percoll gradient, and then removed the Percoll by means of a pump-filter arrangement. In the other we used a standard swim-up procedure.

Percoll gradient In some of the studies we used self-migration on a discontinuous Percoll gradient. The gradient had 7 layers, 80-32% Percoll (80, 72, 64, 56, 48, 40 and 32%, highest in the bottom), using RPMI-1640 medium with 10% human donor serum. One ml of semen was placed on top, and the gradient was left for 3 h in an incubator (37 °C). The bottom 3 ml were kept. The Percoll was removed from the sperm samples with a pump-filter system (0.2 μm nitro-cellulose filters).[32]

Swim-up separation (in EBSS, Earle's balanced salt solution, with 13% donor serum).

Concentration was adjusted by either centrifuging at 600×g for 10 minutes or diluting with RPMI-1640 until a concentration of 20–60 $\times 10^6$ spermatozoa/ml was attained.

Derivatives of 2-nitrofluorene cause changes in human sperm motility (I)

This paper deals with the effect of exogenous substances on human sperm motility. The experiments were performed using a CASA system for analyzing the motility of human spermatozoa when influenced by various compounds that can be found in automobile exhaust. The purpose of the study was partially to determine more precisely in what ways the various components of diesel exhaust (in particular nitrofluorenes[33]) affect human spermatozoa[34], but also to further evaluate the potential of human spermatozoa for toxicological screening.[23]

Only semen samples that were normal according to WHO criteria (>20 $\times$ 10^6 cells, > 50% motile)[18] were used.

Procedure

The compounds tested were 2-nitrofluorene (fig. 5) and its derivatives (see table 1 on page 16, figure 4, and table 1 in article I for more information on the substances tested). All the compounds were dissolved in acetone, and pure acetone was used as a control. The test substance solutions was then added to the swim-up prepared sperm solution. All incubations were performed at room temperature in the dark. Analysis was performed initially (< 5 minutes) and after 24 hours. All experiments were performed in duplicate.

Sperm motion analysis was performed using the Cellsoft system (Cryo Resources, New York, USA), and the motility and other parameters were recorded.

Structure of Sperm Activating Protein (II)

It has long been known that serum has a positive effect on human sperm motility when used as an additive *in vitro*. The basis for this effect has not been fully explored, and this project is the continuation of a project aiming at determining what components in serum mediates these effects.[14] In earlier studies a protein complex had been purified from donor serum that proved to have strong effects on the motility of Percoll separated sperm in RMPI-1640. The Sperm Activating Protein (SPAP) complex was shown to consist of apolipoprotein A I and immunoglobulin G4.[13][14][2]

Studies on the structure of Sperm Activating Protein

Purification of Sperm Activating Protein

Sperm Activating Protein was purified by a four step procedure, as has been described earlier.[13]

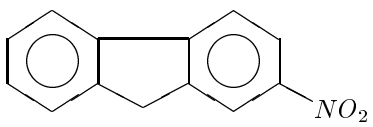

Figure 5: The 2-nitrofluorene molecule. This is the base structure for the nitrofluorenes studied. See figure 1, in article I for information on the structure of the substances tested

Ion-exchange chromatography Human donor serum was fractioned on a DEAE-Sepharose column, using phosphate buffer and an 0–0.25 M NaCl gradient.

Chromatofocusing The SPAP was eluted in the pH-range 5.0–5.3.

Gel filtration using FPLC The SPAP was eluted with Fast Protein Liquid Chromatography (FPLC) as a peak at 250 kD.

Blue Sepharose Chromatography In order to remove loosely bound albumin the sample was purified with a Blue Sepharose column.

Purification of F(ab′)$_2$ from Sperm Activating Protein

Purified SPAP was digested with pepsin to cleave off the intact F(ab′)$_2$ from SPAP. The F(ab′)$_2$ was then separated from the partially digested Fc-fragments with a protein A Sepharose column.

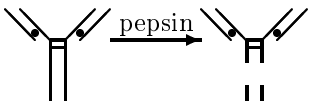

Figure 6: The IgG4 portion of SPAP was digested with pepsin to F(ab′)$_2$ and Fc-fragments.

Antibodies

Polyclonal antibodies against Sperm Activating Protein were generated in the rabbit.

Bacterial expression products C23 and ZZ-T

The bacterial expression products C_{23} and ZZ-T were provided by Dr. Roland Andersson (Karolinska Institutet, Stockholm). They have been shown to bind to specific epitopes on immunoglobulins.[35] Both the C_{23} and ZZ-T proteins were labelled with ^{125}I for use in dot-blots.[36][37]

Anti-SPAP binding to spermatozoa

Swim-up separated spermatozoa were allowed to dry on slides overnight. The slides were then incubated with anti-SPAP serum (diluted 1:100). After washing the slides were incubated with Fluorescein Isothiocyanate-labeled (FITC) anti-rabbit IgG serum. The slides were then examined in a UV microscope at 100–250× magnification.

Presence of SCP1 transversal filament-like protein in biopsies from human testicles (III)

This study was intended to further evaluate the potential basis for male subfertility, by linking the presence or absence of a vital synaptonemal complex portion (synaptonemal protein complex 1, SCP1) to errors in the spermatogenesis.

Testicular biopsies

Testicular biopsies were taken from men explored for infertility (N=18). The material was divided into two pieces, one of which was placed in 4% formaldehyde (in phosphate buffer, pH 7), and the other placed in a cryotube and frozen (-70 °C).

Evaluation of spermatogenesis

The formaldehyde-fixed biopsies were embedded in methylacrylate-resin, and sections stained with Giemsa technique. The spermatogenesis was evaluated by light-field microscopy (×60 under oil), and grouped into five categories:

A. Normal spermatogenesis
B. Sertoli Cell Only
C. Meiotic disturbances
D. Spermiogenetic (i. e. differentiation) disturbances
E. other, combined disturbances

Immunoflourescence microscopy

The frozen (-70 °C) biopsies were sectioned (7 μm) at -25 °C. Each section was attached to an immunoflour object glass and fixed for 10 minutes with acetone. The sections were then air-dried, and stored at -25 °C until use.

Blocking and primary antibody The sections were transferred to a moisture chamber and to each a blocking solution of 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) was added. After 30 minutes of incubation the blocking solution was decanted and 75 μl of primary antibody solution was added (rabbit anti-mouse SCP1, diluted 1:10–1:100 in PBS with 3% BSA). Incubation was run at room temperature for 1–2 h. The sections were then rinsed 3 × 5 minutes in PBS.

Secondary antibody The secondary antibody was swine anti-rabbit IgG, conjugated with FITC (diluted 1:50 in PBS with 3% BSA). Each section was incubated with 75 μl of this in darkness for 45-60 minutes at room temperature in a moisture chamber. The sections were then rinsed 3 × 5 minutes in PBS.

Mounting The slides were mounted using a medium made by dissolving 0.5 g Diazabiicyclo-octane in a mixture of 0.5 ml PBS and 4.5 ml glycerol, and pH set to 8.5–8.9. These slides were also counter-stained using Hoechst 33258 to label the nuclei. The slides were analyzed using a fluorescence microscope (Zeiss) and photographed. As controls incubations were performed without primary antibody.

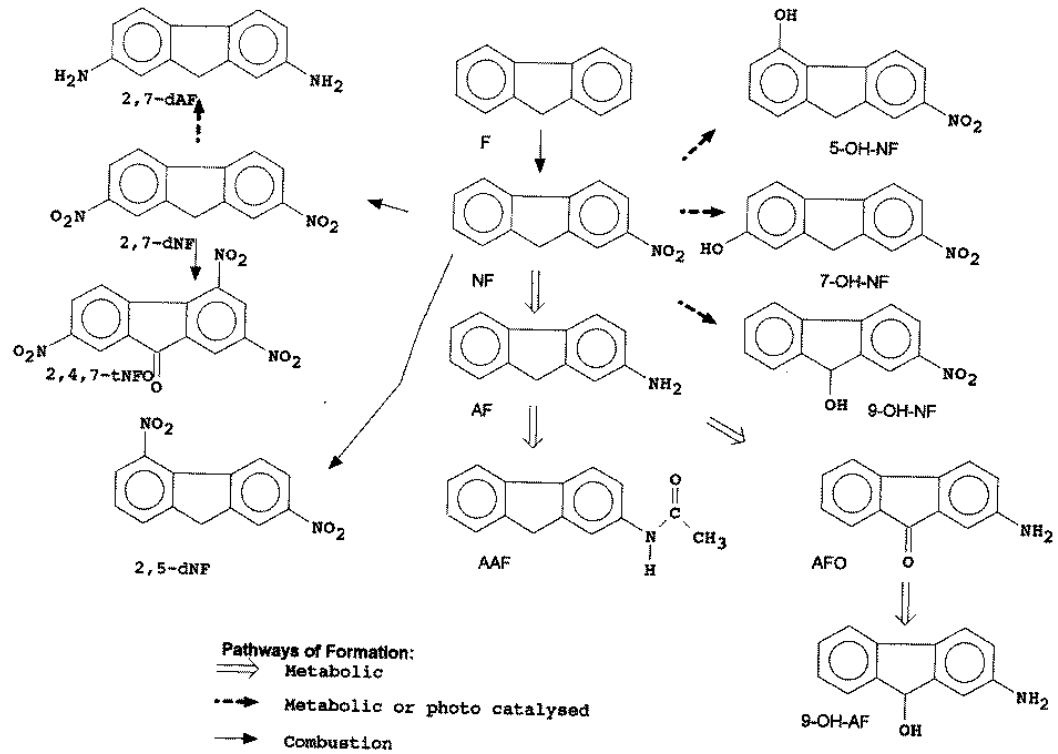

Fig. 1. 2-Nitrofluorene and its derivatives, formed in combustion or during *in vitro* metabolism.

Figure 4: The tested compounds and their formation from 2-nitrofluorene.[1]

Results

Derivatives of 2-nitroflourene cause changes of human sperm motility (I)

Four of the substances (2,4,7-trinitrofluorene, 2,5-diaminofluorene, 7-hydroxy-2-nitrofluorene, and 2,7-diaminofluorene) caused a strong decline in motility after 24 h (a strong decline is here defined as <50% of the original motility) at a final concentration of 1000 μM, while two of the substances (2,4,7-trinitrofluorene and 2,7-diaminofluorene) also caused significant effects at 100 μM. Since these substances in some cases totally removed all motility it was sometimes impossible to measure motility parameters.

Most of the substances were tested at 100 and 1000 μM. There were three substances (2,5-dinitrofluorene, 2,7-dinitrofluorene, and 9-hydroxy-2-aminofluorene) which could not be dissolved at sufficient concentrations to enable testing at the higher concentration, and therefore lower concentrations were used in those cases, as per table 1.

It was found that 2,7-diaminofluorene (100 μM) and 7-hydroxy-2-nitrofluorene (100 and 1000 μM) decreased the linearity and velocity.

Weak effects inhibiting motility were found using 2-nitrofluorene (100 and 1000 μM) and 2,5-dinitrofluorene (100 μM). A stimulatory effect was found with 2-acetoaminofluorene (1000 μM) and 2,7-dinitrofluorene (50 μM).

IUPAC Name	(μM)
2,4,7-trinitrofluorene	1000, 100
2,5-dinitrofluorene	100, 10
2,7-diaminofluorene	1000, 100
2,7-dinitrofluorene	50, 10
5-hydroxy-2-nitrofluorene	1000, 100
7-hydroxy-2-nitrofluorene	1000, 100
9-hydroxy-2-nitrofluorene	1000, 100
2-acetoaminofluorene	1000, 100
2-aminofluorene	1000, 100
2-aminofluorene-9-one	1000, 100
9-hydroxy-2-aminofluorene	200, 20
2-nitrofluorene	1000, 100

Table 1: Substances and concentrations used in nitrofluorene studies.

Conclusion

We found that it was possible to determine which of the tested substances affected sperm motility, and that the methodology therefore may be useful for toxicological screening.

The structure of Sperm Activating Protein (II)

Purification of Sperm Activating Protein

Sperm Activating Protein was purified to homogenity using the four step process described earlier

(p. 12). Starting with 100 ml human donor serum (containing about 5 g protein) we obtained about 50 μg pure SPAP (10–250 μg, N=15). All preparations increased sperm motility in the SPM test[38], and contained a band at 180 kD when analyzed with SDS-PAGE under non-reducing conditions.

Studies on the structure of Sperm Activating Protein

Sperm Activating Protein contains IgG4 kappa and occluded apo A-I

Purified Sperm Activating Protein reacted with antibodies against IgG (but not IgA, IgD, IgE or IgM). Similarly, it was shown that only the antibodies against IgG4 (as opposed to IgG1, IgG2 or IgG3) reacted with SPAP.

As regards the apo A-I it was shown that only some polyclonal antibodies to apo A-I reacted with unreduced SPAP. None of the 14 monoclonal antibodies against apo A-I that were tested reacted with SPAP. These did, however, all react with reduced SPAP (i. e. SPAP that had been exposed to SDS and therefore had its disulfide bonds broken).

Production and analysis of F(ab′)$_2$

Proteolysis of Sperm Activating Protein with pepsin yielded a F(ab′)$_2$ product that had a molecular weight (MW) slightly higher than that of IgG4. The successful proteolysis also indicated that the hinge region is, at least in part, unblocked.

Western blot analysis[39] of the F(ab′)$_2$ showed that it contained apo A-I.

C_{23} and ZZ-T bound freely to Sperm Activating Protein

Binding assays were performed where ^{125}I-labeled C_{23} and ZZ-T were allowed to bind to both SPAP and IgG4. Using increasing concentrations we obtained binding curves with a high degree of parallelism, indicating that the C_{23} bound the same way to both SPAP and IgG4 (Fig. 7).

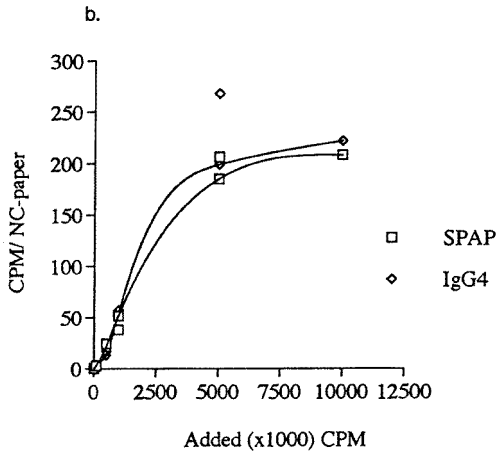

Figure 7: Binding of radio-labelled C_{23} to SPAP and IgG4.[2]

Sperm Activating Protein does not form spontaneously in vitro

Under none of the conditions tested was it possible to form SPAP spontaneously.

Anti-SPAP binding to spermatozoa

When spermatozoa were incubated with anti-SPAP and a FITC-labeled secondary antibody, the spermatozoa displayed a distinct band around the lower part of the sperm head. In the control experiments (i. e. no anti-SPAP added) no such fluorescence was detected (see figure 3 in II).

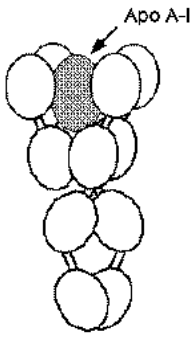

Figure 8: Proposed structure of the Sperm Activating complex. The immunoglobulin G4 is shown in white, with the apolipoprotein A-I in grey.

Conclusion

Sperm Activating Protein complex, one of the major sperm motility enhancing factors in serum, consists of one apo A-I and one IgG4 molecule, and the apo A-I is located in the $F(ab)_2$ region of the IgG4, most likely nested in between the arms.

Presence of SCP1 transversal filament-like protein in biopsies from human testicles (III)

Evaluation of spermatogenesis

The evaluation of testicular morphology and assessment of the 19 biopsies categorized them as

3 with normal spermatogenesis
4 with Sertoli cell only syndrome
3 with meiotic disturbances
4 with spermiogenetic (i. e. differentiation) disturbances
4 with other, combined disturbances.

Immunocytochemistry

The ability of the 2-aminofluorene anti-rodent SCP1 antibody to stain the synaptonemal complex (SC) in meiotic cells in mice and man was initially analyzed. We found that the SCP1 antibody strongly stained the meiotic SC structure in both organisms. This shows that the SCP1 protein is conserved and that it is part of the SC also in man. The SCP1 antibody was therefore used as a marker to analyze the spermatogeneic differentiation process in testis biopsies taken from men with different spermatogeneic disturbances. In this way it should be possible to divide the patients into different categories based on their SCP1 labeling pattern. In all three men with normal spermatogeneis the primary antibody distinctly stained the synaptonemal complexes of primary spermatocytes, whereas no specific staining was seen in spermatogonia, spermatids or Sertoli cells.

We also analyzed the SCP1 labeling pattern in patients having spermatogenetic disturbances. In the groups categorized as meiotic disturbances, spermatogenetic (i. e. differentiation) disturbances and other, combined disturbances, all showed the same SCP1 staining pattern, a pattern similar to what was seen in men with normal spermatogenesis. No staining was seen in controls (i. e. sections stained without the primary antibody).

Conclusion

Synaptonemal complex protein 1 can be detected in testis biopsies and it was present in the patients with some known spermatogenic abnormalities, indicating that when spermatogenesis takes place the SCP1 is present.

Discussion

Since both endogenous and exogenous factors affect sperm motility, I chose in this thesis to examine one model system from each category: SPAP as an endogenous factor, and 2-nitrofluorene as an exogenous. Another aspect of subfertile men is low sperm counts, and we have therefore studied SCP1, since spermatogenic failure can affect sperm concentration.

Human sperm for toxicity screening

This work showed that it may be possible to use the motility of human sperm as an indicator for cytotoxicity. This would allow the use of a human cell culture system in determining toxic effects. The marker we chose to use was a decrease in motility, but there are a number of possible factors that could be analyzed in a further development of the method. We chose to initially concentrate on motility since it is easy to determine, and motility problems are often seen in subfertile males.

In many cases there exists a need for determining a screening procedure, to test if a given substance is toxic to human cells or not. In those cases it is valuable that the cell culture used for the test is of human origin, since this ensures that one is measuring toxicity to humans, without having to extrapolate from an animal model. One such possible model is human spermatozoa.

Human spermatozoa do pose several problems when used on a larger scale, since a donor program needs to be maintained to ensure a continuous supply. It is possible to freeze sperm for future use, but this is associated with lowered motility as well as other less well known effects. It is also well known that there is a significant variation between samples, something that needs to be controlled for, perhaps by pooling samples. Many of the "purification" methods used with sperm allows one to select motile sperm, but these populations are still not homogenous in all respects. If one has a need for testing with spermatozoal cells it ought to be possible to use e. g. bull spermatozoa in at least the initial screening phases, since these are much more homogenous, in addition to being available commercially.[22][23] These advantages of bovine materials should, however, be weighed against the advantages that a human system gives.

Using human spermatozoa for toxicological screening

When screening potentially toxic substances there are several factors that need to be taken into consideration, such as convenience of the model system, the appropriateness of the system, etc. Spermatozoa have been evaluated for use in these contexts by a few groups, both using bull and human spermatozoa.[22][23][1][40][34] The advantages of using human spermatozoa is that you are actually testing on human cells, while the bull system allows you to work with a more readily available source of material, as well as more homogenous populations.

Nitrofluorene toxicity to human spermatozoa

The 2-nitrofluorene itself probably has low effects on human spermatozoa, but it is clear that some of its metabolites do have a highly significant effect on human spermatozoa. Since we do not have any definitive data on the concentration of these substances in exposed individuals, it is difficult based on that to give any opinion regarding the toxic potential of *in vivo* 2-nitrofluorene exposure. It is, however, known that metabolites from some of these pathways are distributed through the body after exposure, and 2-nitrofluorene is a well known marker substance for exposure to nitro-polycyclic aromatic hydrocarbons.[33]

Nitro-polycyclic aromatic hydrocarbons such as 2-nitrofluorene enter the body from a number of sources, such as internal combustion engines, tobacco smoke and food processing.[33]

The structure of Sperm Activating Protein

The Sperm Activating Protein complex was originally discovered in an attempt to fractionate human donor serum in order to determine what portions of it provides the effects that it has on human sperm motility.[41] It was determined that the majority of the positive effects came from a complex consisting of one apolipoprotein and one immunoglobulin.[13][14] In article II we determined the exact identity as well as the position of both components.

It was found that the structure that was most consistent with the available data is one where the apo A-I is enfolded by the Fab-arms of the IgG4-molecule. It was demonstrated that the immunoglobulin part of SPAP is made up of IgG4, most likely with a predominance of the kappa light chain, but the lambda has also been shown to be present.

The ZZ-T and C_{23} bound to SPAP and IgG4 in the same manner, making it clear that the epitopes of their binding are unobstructed by the bound apo A-I.

The fact that it was impossible to detect the apo A-I in intact SPAP when using most of the antibodies against apo A-I that we tested, and that those that did detect the apo A-I only did so weakly, would tend to indicate that the apo A-I is somehow either hidden or modified structurally when it is a part of the SPAP complex. This view is supported by the fact that when Western blots of reduced $F(ab')_2$ are analyzed with anti-apo A-I antibodies it is found that apo A-I is present in these fragments.

Based on the structure of the IgG4 molecule there are only two places where the apo A-I could conceivably be hidden to any extent: in the opening between the Fc-arms, and between the Fab-arms.

The significance of the individual components

The immunoglobulin component, previously identified as IgG by Åkerlöf *et al*[13], has now been narrowed down to IgG4 kappa by immunological studies. This is interesting, since only approximately 7% of all serum immunoglobulin is IgG4.[42][43][44][45][46] While this makes speculation tempting, we hesitate to speculate further on this item, but rather leave it open.

Mechanism and possible biological role

The fluorescence experiments (see p. 13) showed that Sperm Activating Protein binds to the lower part of the sperm head. This indicates that SPAP is bound directly to the head of the spermatozoon,

and therefore presumably has a more or less direct effect. The nature of this effect is unknown, but we speculate that it might be either a receptor mediated or enzyme linked effect. This is supported by the fact that only a small amount of SPAP is required for improving the motility of the spermatozoa. One possible role is to increase the levels of intra-cellular calcium (Ca^{2+}), probably by means of a membrane effect

Presence of SCP1 transversal filament-like protein in biopsies from human testicles.

The SCP1 has been shown to be a major component of the structures that take part in the meiotic pairing process. It is therefore of interest that we have now shown that SCP1 exists also in men with severe meiotic disturbances, which indicates that the absence of SCP1 is not responsible for these disturbances.

Of more interest is perhaps to note that in all cases where there was any meiosis the SCP1 was also present. This indicates that an intact synaptonemal complex is essential for meiosis to take place.

The synaptonemal complex in the rat and other animals has been shown to be a meiosis-specific structure essential for synapses of homologous chromosomes. The synaptonemal complex protein 1 (SCP1) is a major constituent of the transversal filament, a fibrous structure that connects the central element of the synaptonemal complex with the two lateral elements. The SCP1 protein form filamentous dimers with the two molecules having the same polarity, the C-termini being anchored in the lateral elements and the N-termini reaching into the central element, possibly acting as a molecular zipper during the meiotic pairing process.

In the present study we demonstrate that antibodies specific to the mouse SCP1 specifically identified the synaptonemal complexes of human primary spermatocytes. The characterization of this complex using immunofluorescense techniques gives a result that is very similar to what earlier has been described in other sexually reproducing eucaryotic organisms. This strongly indicates that a SCP1-related protein is conserved and present also in man.

The antibodies identified SCP1 protein also in cases with meiotic disturbances. This means that the meiotic disturbances in these cases were not caused by an absence of the SCP1 protein, but we can not exclude that a nonfunctional SCP1 protein had contributed to these disturbances. Future studies may reveal to what extent the absence or mutation in the SCP1 protein contributes to meiotic disturbances in man.

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