Spring 1989

Andrological Methods of Predicting the Pregnancy Outcome of an *in vitro* Fertilization Program

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ANDROLOGICAL METHODS OF PREDICTING THE PREGNANCY OUTCOME OF AN IN VITRO FERTILIZATION PROGRAM

by

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M.S., JANUARY, 1983
San Francisco State University

A Dissertation Submitted to the Faculties of Old Dominion University and Eastern Virginia Medical School in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES
CLINICAL CHEMISTRY

OLD DOMINION UNIVERSITY
and
EASTERN VIRGINIA MEDICAL SCHOOL

(May 1989)

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ABSTRACT

ANDROLOGICAL METHODS OF PREDICTING THE PREGNANCY OUTCOME OF AN IN VITRO FERTILIZATION PROGRAM

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May, 1989

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Current advances in fertility treatment emphasize extracorporeal procedures, such as in vitro fertilization (IVF)/embryo transfer. IVF is now used to treat several types of infertility, including male factor and idiopathic infertility. However, IVF is time consuming, costly, unpredictable, and often yields a low pregnancy rate. Thus, it would be valuable to devise laboratory procedures for better prediction of IVF outcome.

The male infertility rate can be as high as 50%. Since sperm count and percent of motility alone do not appear to be strong predictors of IVF outcome especially when these parameters are not severely abnormal, a multivariable analysis of other parameters was made. In addition, because many IVF centers use

swim-up preparations of semen samples for insemination, whole semen and swim-up samples obtained for IVF were analyzed. Major sperm motion characteristics (such as sperm mean velocity and linearity), adenosine triphosphate (ATP), creatine kinase (CK) activity in whole semen and sperm, hypoosmotic swelling test (HOST), and sperm morphology were evaluated.

A total of 138 patients from Norfolk IVF series 27 and 30 were studied. To minimize bias, only semen samples with normal count ($\geq 20 \times 10^6$/ml) and normal percent of motility ($\geq$40%) were used in the study. Possible variation introduced by abnormal oocytes was reduced by including only patients with at least one mature (metaphase II) oocyte. Semen samples with low ($\leq$40 picomol/10^6sperm) swim-up ATP or with a poor (P) morphology pattern (<4% normal forms) produced no pregnancies. Samples with a normal (N) morphology pattern ($\geq$14% normal forms) or with normal (>40 picomol/10^6sperm) swim-up ATP had the highest pregnancy rate (41%). Samples with a good (G) morphology pattern ($\geq$4-<14% normal forms) produced a pregnancy rate of 25.8%.

Analysis of sperm motion characteristics in the group with the highest pregnancy rate indicated that sperm in swim-up samples of this group had significantly ($p<0.01$) lower mean linearity and slightly higher velocity than sperm in the group producing no pregnancies. This finding may indicate sperm hyperactivation in the samples from the group with the highest rate. By discriminant function analysis, it was found that ATP content of whole semen, ATP content of sperm in swim-up, the percent of normal morphology, sperm velocity in semen, sperm

linearity in swim-up, and the percent of motility in swim-up are useful in predicting pregnancy outcome in human IVF, and formulae to predict such outcome are presented.

I dedicate this work to my family,

Dr. Frank Bayliss of San Francisco State University, and to Silvina Bocca for her extraordinary sacrifice in helping me prepare this manuscript.

Without their help there would be no work to dedicate.

ACKNOWLEDGMENTS

Many people have helped me travel this long road and have touched upon my life during the course of this study. I would like to sincerely thank my advisor Dr. James Yuan for his contribution of expertise, time, patience and support. I would also like to take the opportunity to thank Drs. Anibal Acosta, Steven Ackerman, Patricia Pleban and Keith Carson for the hours they have spent reviewing my work, listening to my progress reports and reading my writings. My sincere gratitude goes to Dr. Acosta for his continuous support in providing patients and much needed clinical advice. I also sincerely thank Dr. Ackerman for the fine ideas that he provided me and for his continuous support and allocation of his valuable time. My very special gratitude goes to Dr. Pleban. During my course of study, as well as for this work, she has helped me tremendously. Thanks for her valuable advice and encouragement. I also thank Dr. Carson for his time reviewing my work.

I have learned much from the very fine people in the Andrology Lab of ODU/EVMS. However, they have been more than technical advisors to me. Many times Mrs. Rosita Acosta, Anne Cromwell and Kathryn Simmons shared my frustrations when ideas weren't working and my happiness when everything seemed to fall into place. I thank them all. It is also very appropriate to thank Drs. Swanson and Gordon who offered their support during

the course of my study.

My special thanks to Lucinda Veeck, Simona Simonetti, Lisa Brothman and Cathy Amundson of the Embryology Laboratory of the Jones Institute for providing very much needed samples. Without their help, this study could not have been completed.

I would like to extend a special note of gratitude to my other friends who have also touched upon my life. Always remembered are my very dear friends, Dr. William Bigler of San Francisco State University, Ann Nowell, Mrs. Margaret Broddus, Midge and Peter as well as other members of the Hutchison family, and Mrs. Kathy Chang (in her memory), whose help and encouragement cannot be forgotten. There have been many times when they have been both spiritually and physically uplifting. Thanks to them all.

A final note of sincere thanks to my very special friends Majid and Nahid Mahani, Badri Nayebi, Mohammad and Nahid Saberi, Ebrahim Sarmadi, Hassan and Christina Djafari, Siavosh Mirzaii, and Susan and Mike Powell for their valuable help and support.

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CHAPTER I

INTRODUCTION

Human reproduction is a cyclic process. Under normal conditions, in a sexually mature individual an oocyte is fertilized by a spermatozoon and a conceptus may be formed. The conceptus later implants in the uterus and development of an individual ensues. A child is born and grows to become sexually mature and may resume a new cycle of reproduction, leading to creation of another individual. Therefore, study of infertility requires a comprehensive knowledge of both male and female reproductive systems. Since the focus of this work is on the possible contribution of the male to infertility, it begins with a description of the anatomical and major functional characteristics of the human spermatozoon. A brief description of infertility and one of the treatment methods, in vitro fertilization (IVF) and embryo transfer, follows. Then comes a discussion of some of the laboratory assays for evaluating male partners of infertile couples, followed by a discussion of techniques to predict the pregnancy outcome of human IVF by evaluating results of various andrological assays performed on semen samples of the male IVF partners. Finally, the use of andrological assays to predict the pregnancy outcome of our IVF program is discussed. Formulae to predict such an outcome are presented.

A. The Spermatozoon

1. Testicular Spermatozoa

Testicular spermatozoa are not capable of forward movement and to date have not been shown to have fertilizing ability (1-3). They are, however, similar in biochemical composition and structure to spermatozoa in other regions of the reproductive tract and ejaculated spermatozoa. Compared to ejaculated spermatozoa, testicular spermatozoa contain more proteins, sodium, and calcium, a higher rate of glucose oxidation and lactic acid production, higher levels of neutral lipids and phospholipids, and lower levels of fatty acids (2, 4, 5, 9). Adenosine triphosphatase (ATPase) in testicular spermatozoa is less than that of ejaculated spermatozoa and is moderately activated by calcium and magnesium. Unlike ejaculated spermatozoa which are stimulated by sodium and potassium at acid pH, testicular spermatozoa require an alkaline pH for their stimulation. Glucose in the presence of phosphate stimulates oxygen uptake linearly in these cells. The regular uptake of oxygen without glucose is not affected by the presence or absence of phosphate. In ejaculated spermatozoa the glucose stimulation of oxygen uptake occurs in the absence of phosphate. Increased temperature inhibits the utilization of oxygen and glucose by testicular spermatozoa but appears to have no effect on the generation of lactate (5). Lipogenesis by testicular spermatozoa is not affected by heat. Compared with ejaculated spermatozoa, testicular spermatozoa

incorporate a small amount of labeled glucose into fatty acids, but higher amounts are incorporated into neutral lipids and phospholipids (9). Differences in lipid content and composition of testicular spermatozoa plasma membrane compared with that of ejaculated spermatozoa are the reason why ejaculated spermatozoa are more sensitive to cold shock than are testicular spermatozoa (2). Differences in the amount and composition of lipids in the plasma membrane of testicular spermatozoa may also account for the higher charge density at the phospholipid-water interface of spermatozoa and their higher membrane fluidity compared with epididymal and ejaculated spermatozoa.

2. Epididymal Spermatozoa

The luminal surface of the epididymal tubule is lined by cells that either do or do not possess stereocilia (microvilli). The testicular spermatozoa and fluids pass to the head (caput) of the epididymis, then through the middle (corpus) portion into the tail (cauda) of the epididymis (3). Transport of the spermatozoa from the corpus to the cauda requires from one to three weeks (5).

The epididymis, besides being a secretory organ, is a resorptive organ. It resorbs not only water but also certain biochemical components from the testicular fluid. Recent pregnancies, achieved using epididymal spermatozoa from different regions of the epididymis, have cast doubt on the notion that epididymal passage is required for sperm maturation

and fertilizing ability (77). It is known however, that spermatozoa undergo a series of biochemical changes as they are transported through the epididymis (5).

Only a few of the biochemical components of the epididymal fluid are primarily synthesized by the epididymis. The best studied are the lipids such as carnitine, acetyl carnitine, and glycerylphosphorylcholine (7, 9).

The net negative surface charge is greater on spermatozoa from the cauda than on those from the caput epididymis. An increase in sialic acid moieties may be responsible for the change in surface charge during epididymal passage (5).

The lipid content of whole sperm decreases during epididymal transport. The cholesterol concentration and cholesterol/phospholipid ratio are decreased. However, the amount of sulfo-conjugated sterols increases (9). These changes may make the epididymal, as well as the ejaculated, spermatozoa more sensitive to cold shock.

The sperm plasma membrane also undergoes major changes in protein composition in the epididymis. These changes occur by addition of new components to the sperm surface, by unmasking or modifying of pre-existing sperm surface moieties, or by loss of sperm surface components. Studies have found a 37,000-dalton glycoprotein present on sperm from the cauda but not on sperm from the corpus epididymis of the rat (9). Other studies have reported that the major change during rat sperm maturation is the increase in a 31,000-, 32,000-, 34,000- or 37,500-dalton surface glycoprotein.

Immunological approaches have also identified changes occurring in the surface composition of spermatozoa as they pass through the epididymis. Antisera raised against either spermatozoa or epididymal fluid have often reacted with both. It has been found that the epididymal fluid component binds to the sperm surface. Studies have shown that a 33,000-dalton acidic epididymal glycoprotein purified from the rat epididymis bound to spermatozoa as they left the initial segments of the caput epididymis. Acidic epididymal glycoprotein was found to have a slight stimulatory effect on sperm motility, but bovine serum albumin and rabbit serum were equally effective (5). In humans, an antiserum to ejaculated spermatozoa reacted specifically with epididymal fluid and with epididymal spermatozoa, but not with testicular spermatozoa, and apparently identified an epididymal secretory product that binds to sperm (5).

The sperm surface galactosyltransferase may bind to N-acetylglycosamine of the zona pellucida as part of the fertilization process. Competitive substrates for the enzyme, α -lactalbumin, and enzyme substrate analogs inhibited sperm binding to the zona pellucida. Enzymatic removal or unmasking of terminal N-acetylglucosamine residue also inhibited or stimulated binding, respectively (9). Studies have shown that a) purified galactosyltransferase produced a dose-dependent inhibition of sperm binding to the zona pellucida and caused sperm bound to the zona pellucida to be released, and b) monospecific antiserum to the enzyme produced a dose-dependent

inhibition of sperm binding to the zona pellucida and concomitantly blocked sperm galactosyltransferase activity. The enzyme was localized on the plasma membrane over the dorsal surface of the mouse-sperm acrosome (5).

3. Ejaculated Spermatozoa

a. General description: An ejaculated spermatozoon with a small metabolizing intracellular volume of only 16-20 cubic micrometers (μm) is the end product of spermatogenesis and passage through the reproductive tract. It is about 60 μm long and has two main components, the head and the tail or flagellum. The head is somewhat flattened, ellipsoid, and about 5-6 μm long, 3.5-4 μm wide, and 0.3-0.5 μm thick. The tail measures about 50 μm in length, has a diameter of > 1 μm at its base (neck) and tapers progressively toward its tip. The head is crossed by a transverse constriction, which corresponds to the termination of the acrosomal cap approximately 2/3 along its length, making the posterior portion slightly but abruptly narrower than the anterior (3). After a junctional length of about 1 μm, termed the neck, the tail shows a section of a large spiral structure beneath the membrane, called the middle piece (midpiece), which contains the mitochondria. At the end of midpiece region, the surface becomes smooth and the diameter decreases abruptly. This portion, about 45 μm long, is the principal piece. Following this, after a further abrupt decrease in diameter, there is a

terminal region about 5 μm long called the end piece (Fig. 1). Although spermatozoa are uniform in size and shape in most species, in the human there is often variability in the size and shape of the head, even in fertile individuals (5).

b. The sperm head: The sperm head (Fig. 2) can be divided into two parts: the nucleus and the surrounding membrane structures. The nucleus contains a haploid number of chromosomes and the nuclear proteins. Chromosomes and nuclear proteins are so densely packed that individual chromosomes are not distinguishable even by electron microscopy. Many vacuoles, of sizes averaging 0.5 μm in diameter, are seen in the nucleus. A high concentration of potassium with unknown function is found around, but not in, the vacuoles (9, 12).

The membrane structures of the sperm head consist of 1) the plasma membrane that covers the entire surface of the sperm head, 2) the acrosome, a baglike structure that surrounds the anterior portion of the nucleus, 3) the postnuclear cap, a cytoplasmic sheath that covers the posterior part of the nucleus, and 4) the equatorial segment, which represents the area of overlap between the posterior cap and acrosome (Fig. 2). The acrosome consists of an inner acrosomal membrane that is in close contact with the nuclear membrane and an outer acrosomal membrane that is in proximity to the plasma membrane (5).

The sperm head also contains cytoskeletal components which lie in a narrow space between the inner acrosomal membrane and the nuclear membrane, and also just below the plasma membrane

Figure 1

Schematic drawing of a human spermatozoon

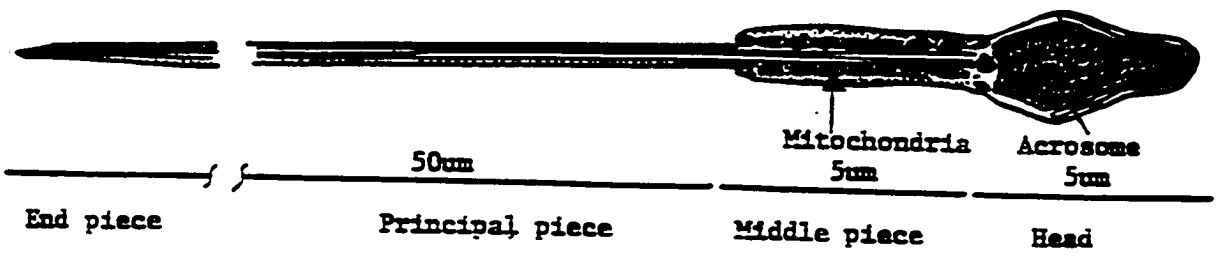
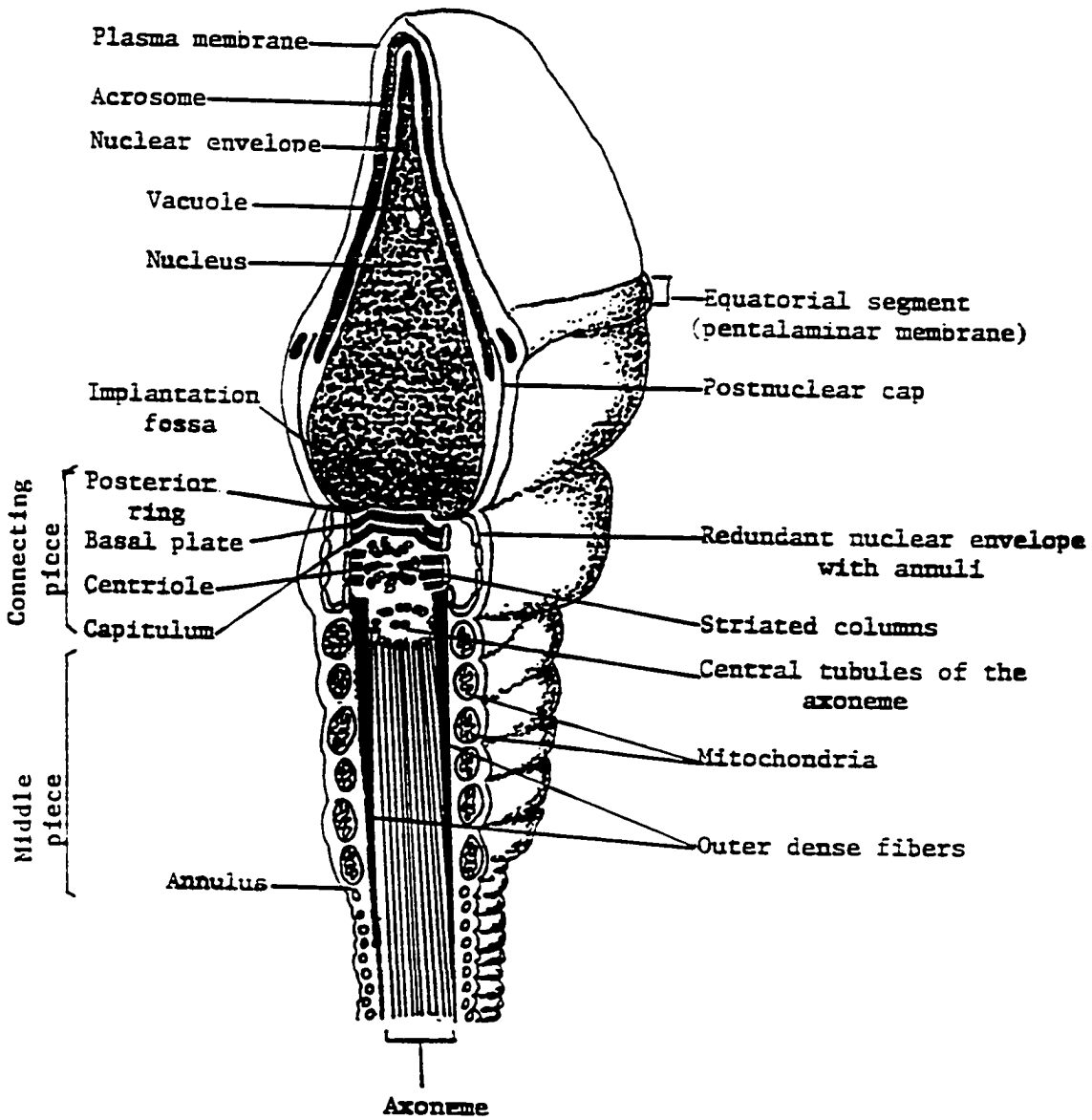


Figure 2.

Schematic drawing of the longitudinal section of the head, neck, midpiece, and portion of the principal piece of a human spermatozoon.



(9). The sperm head is connected to the tail by a narrow region called the neck, which is the most fragile portion of the spermatozoon. The neck contains a centriole, from which the tail fibers and microtubules originate (Fig. 2). The centriole serves as an organizing center for the formation of the axoneme and striated columns. However, its presence is not required for initiation or propagation of waves or bending along the tail (9).

The head has two important functions. It carries the genetic information, the haploid chromosomes, and it contains the acrosome, a region containing enzymes essential for penetration through the layers surrounding the oocyte. Contact with the oocyte induces biochemical changes in the sperm head along with the fusion of the plasma membrane and outer acrosomal membrane. In the fusion process, called the acrosome reaction, a series of enzymes is released. Hyaluronidase of the acrosome seems to be specific for the cumulus oophorus and acrosin, for the zona pellucida. These are the two major enzymes released during the acrosome reaction. Their release, as well as chemical changes occurring during sperm-oocyte contact, may be important for sperm-oocyte recognition and binding, penetration of the vitelline membrane, and sperm nuclear decondensation.

The proteinase that is responsible for decondensation of rabbit and bull sperm chromatin has properties similar to those of human acrosin (9). The nucleus usually occupies more than half of the mature sperm head, stains gram-positive, contains

K^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, phosphate, DNA, RNA, and nuclear proteins high in disulfide bonds and arginine (5). The highly condensed nuclear chromatin appears to be composed almost entirely of double helical DNA strands that aggregate into thick bundles, which in turn condense into tightly packed supercoiled structures. The DNA molecules form complexes with basic nuclear proteins (protamines), giving rise to a tertiary configuration, which confers a high degree of stability of the genetic materials against environmental changes and denaturation. The major nuclear proteins associated with sperm DNA are protamines, which are relatively small (5000-7000 molecular weight, 27-65 aminoacids), highly basic, and rich in arginine and cysteine. They contain a large amount of histidine and glutamic acid; unlike other mammals, they are associated with DNA in approximately 1:1 ratio by weight. The mRNA encoding for protamines is probably synthesized in spermatids, indicating that protamines are products of haploid genes (9).

The highly condensed protamine-DNA complex is stabilized by disulfide bonds between protamine molecules. A high degree of hydrogen bonding in the DNA molecule also aids stabilization. DNA binding to protamines and protamines to themselves is so tight that it makes transcription almost impossible. Most mammals have only one protamine type, but humans are reported to have two (9). Upon electrophoresis, human sperm protamines show two major bands: protamine 1, which resembles to that of other mammals; and protamine 2, rich

in lysine and histidine, specific for human sperm (9). There are two general models showing how protamines associate with DNA. One suggests that protamines are present in an extended configuration and lie in the major and minor grooves of the DNA double helix. They presumably cross-link the chromatin by forming covalent disulfide linkages with protamine on nearby DNA. The other model suggests that protamine is packaged into α-helical cylinders. These are thought to lie in the major or minor DNA grooves, to facilitate orderly DNA condensation, and subsequently to cross-link with neighboring cylinders to effect stabilization (9). However, it should be noted that these models are based on the presumption that sperm nuclei lack nucleosomes, although the presence of nucleosomes has been reported (10). Although the amount of DNA might be directly proportional to the size of the sperm nucleus, this is not true, since different sperm species undergo different degrees of condensation of the nuclear material during spermiogenesis. Typical properties of the nucleus include a high degree of stability and inertness, a high density, tight packing of DNA and nuclear proteins, supercoiling, replacement of somatic histones (lysine rich) by arginine-rich protamines, and a lack of DNA transcription. A high arginine content of sperm nuclear proteins not only may influence the supercoiling of DNA but also may increase the thermal stability of the DNA-nucleoprotein complex (10).

After penetration of sperm into the oocyte, decondensation of its nuclear chromatin into DNA and protamines takes place.

However, the artificial dissolution of DNA and nucleoproteins in vitro is very difficult because of the tight binding of the DNA with the basic nuclear proteins and the large number of disulfide bonds in the proteins. Reducing agents such as mercaptoethanol, alkaline thioglycolate, 2,3-dimercaptopropane, and dithiothreitol, alone or in combination with other reducing agents such as cysteine, urea, guanidine HCl, or sodium dodecyl sulfate (SDS), are effective in extracting the nuclear contents from spermatozoa. Once the nuclear material is extracted, the proteins are hydrolyzed by treatment with trypsin, thermolysin, or proteinase K before further purification of DNA. The DNA content of a single human spermatozoon is approximately 1.36 picograms (9).

Although fertile men have a fairly constant DNA content per sperm nucleus, subfertile men show discrepancies and a variable amount of DNA in their spermatozoa. In an extensive study of subfertile men, large abnormalities in the DNA content were noted in most spermatozoa with approximately normal morphology, size of the sperm nuclei, and motility (10). By contrast, no significant differences were observed in the DNA content of the spermatogonia from fertile and subfertile men. The changes in DNA may occur during spermatogenesis (9, 10).

The amount of RNA in mature spermatozoa is extremely low. One group of investigators claims that the mature spermatozoa are capable of transcription and translation but that these activities are associated with the mitochondria of the sperm rather than the nucleus. This remains to be confirmed (10).

Recently, several reports have shown that sperm nuclei are capable of a very low level of nucleic acid synthesis and contain high-molecular-weight RNA polymerase and DNA-dependent DNA polymerase associated with the particulate fractions of the head. This DNA polymerase, in association with the particulate complex, can synthesize DNA even in the absence of an exogenous template such as polynucleotides. In the absence of the complex, polynucleotides are needed (9).

c. The sperm flagellum (tail): The flagellum of the sperm consists of four distinct segments: the connecting piece (neck), the middle piece (midpiece), the principal piece, and the end piece (Figs. 1 and 2). The major structural features of the flagellum consist of a central part, the axoneme, the mitochondrial sheath, the outer dense (coarse) fibers mainly characteristic of mammalian spermatozoa, and the fibrous sheath.

The main structural components of the connecting piece are the capitulum, striated (segmented) columns, basal plate and posterior ring, along with the centriole (Fig. 2). Fine filaments traversing the narrow region between the capitulum and basal plate presumably are responsible for attaching the capitulum of the flagellum to the basal plate of the head. This region between the capitulum and basal plate is the site of the cleavage of heads from tails following trypsin treatment (11). Decapacitation of sperm with primary amines or sodium dodecyl sulfate usually results in cleavage between inner and outer nuclear membranes, next to the basal plate. Heads and

tails can also be separated by sonication, but the cleavage site is not predictable. The basal plate and capitulum are composed of proteins that are soluble in ionic detergents containing a disulfide reducing agent such as dithiothreitol (9). The midpiece section contains the mitochondrial sheath, while the principal piece contains the fibrous sheath (Fig. 3). The mitochondria underlie the plasma membrane in the midpiece with the fibrous sheath underlying the plasma membrane in the principal piece. The nine dense (coarse) fibers are found in the midpiece. They extend from the neck into the posterior part of the principal piece, where they reduce to seven, and terminate in the proximal half. In connection with the two missing dense fibers in the principal piece, the fibrous sheath thickens in that region, forming two longitudinal columns along the principal piece (Fig. 3, bottom). Dense fibers are situated between the mitochondria and the axoneme in the midpiece and between the fibrous sheath and the axoneme in the principal piece (11).

Zinc, which is localized in the dense fibers (9), is required for spermatogenesis and is incorporated into the flagellum in late spermatids (11). A correlation between the radius of beat curvature in human sperm and the size of the dense fibers has been reported (9). This suggests that the dense fibers might influence the form of the beat by determining the elastic properties of the sperm tail. At the center of the flagellum is the axoneme with the characteristic 9 + 2 microtubular structure which extends the full length of

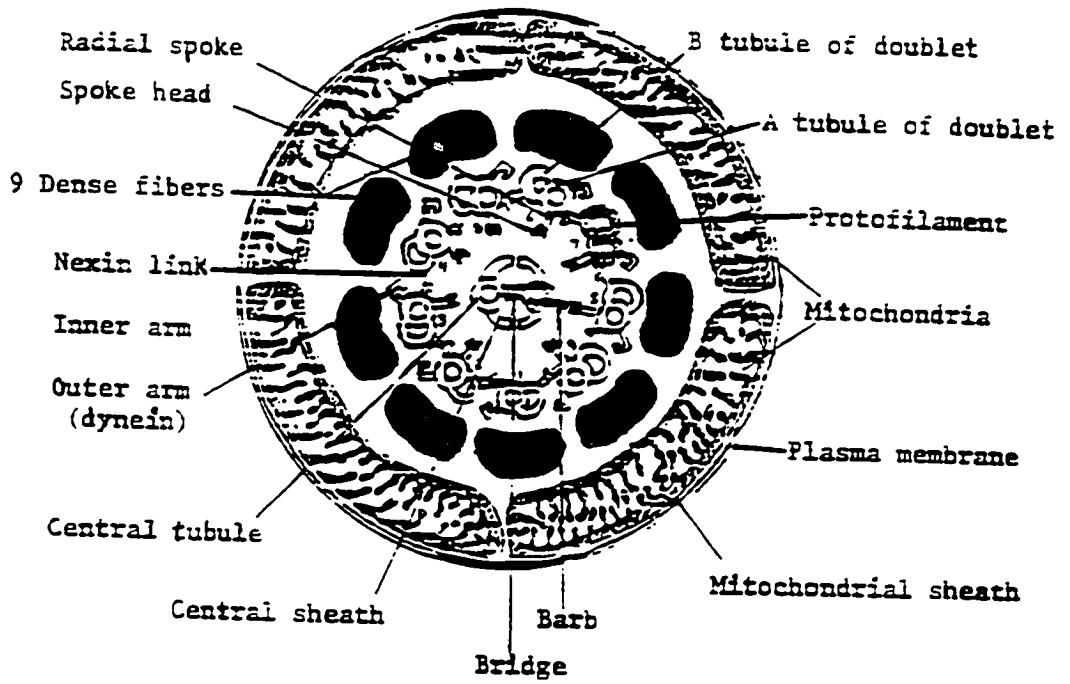
the flagellum. This structure two central microtubules surrounded by nine microtubule doublets (Fig. 3). Each doublet consists of a complete A microtubule, onto which is attached an incomplete (C-shaped) B microtubule. Two arms extend from the A microtubule toward the B microtubule of the adjacent doublet. When the axoneme is viewed from base (neck) to tip, the arms project clockwise. The doublets are numbered clockwise 1-9 with 1 being the doublet on a plane perpendicular to that bisecting the central microtubule pair.

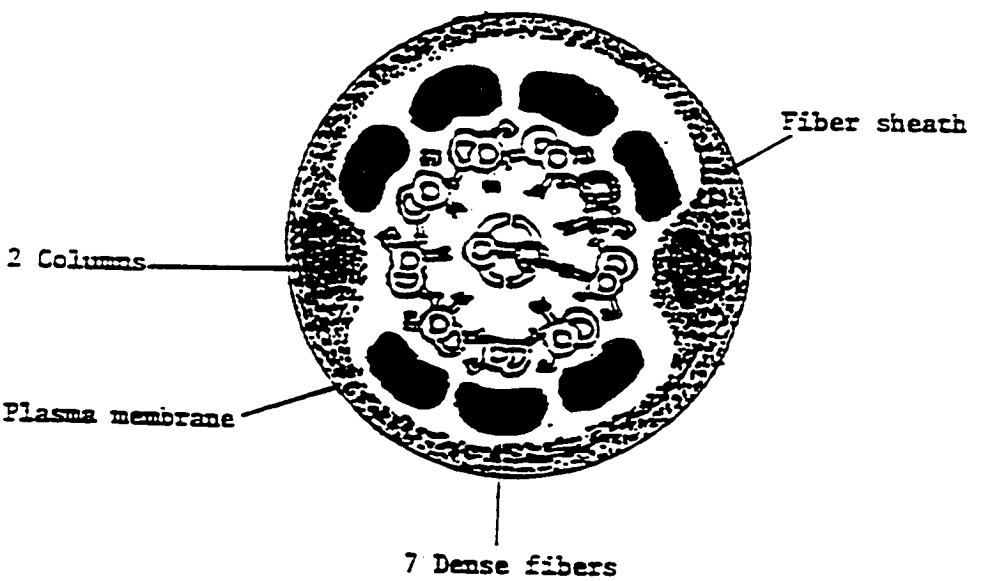
The microtubules are composed of α-tubulin and β-tubulin proteins. Kinesin is another interesting protein associated with microtubules (9); it is complex, composed of a 110,000- and a 65,000- to 70,000-dalton subunit, which interact with the surface of microtubules to effect movement. Isolated microtubules move over the surface of a microscope slide coated with kinesin, and latex beads are moved along microtubules in the presence of this protein. It requires ATP but differs from dynein in its structural and enzymatic characteristics. It will be interesting to learn whether kinesin is present in the human sperm and whether it has a role in generating or propagating the flagellar wave.

The last segment of the flagellum, the endpiece, lacks any structural detail other than the axoneme. The flagellum provides the motile force for the sperm to reach the oocyte and achieve fertilization by gaining entry through the outer investments of the oocyte. Different elements of the flagellum are involved a) in generating and shaping the waves of bending

Figure 3.

Schematic drawing of the human sperm axoneme in the midpiece region (top) and in the principal piece (bottom).





that produce this force and b) in propagating the waves from the base (neck) to the tip of the flagellum. The motile force (motility) may serve another function in allowing the sperm to circulate rapidly within the fluid of the reproductive tract and thus avoiding depletion of nutrients and oxygen in the surrounding microscopic environment.

The mitochondria found in the midpiece provide most of the chemical energy used by the spermatozoon under aerobic conditions. The enzymes of the glycolytic pathway, the major pathway providing energy for the spermatozoon, are thought to exist within the midpiece, although their exact location has not been determined (5). Hexokinase, for example, is also thought to be bound to structural components of spermatozoa, such as the axonemal complex or the plasma membrane. There is ample evidence that the plasma membrane may be associated with aldolase and glyceraldehyde-3-phosphate dehydrogenase (5). It is possible that all glycolytic enzymes exist in free solution in the limited volume of the cytosol of the flagellum. The role of mitochondria and ATP in providing energy for different spermatozoal functions is discussed in more detail below.

The plasma membrane undoubtedly plays a special role in the initiation and control of motility by virtue of its function in nutrient and ion transport. It lies tightly adjacent to the mitochondria and perhaps in contact with them. Freeze-fracture studies of guinea pig spermatozoa by Friend have shown rows of particles in the plasma membrane of the region where it is in contact with the mitochondria. The

particles appear to change in their distribution with a change in motility or after disruption of mitochondrial function by poisons such as cyanide. There is evidence that a glycoprotein-polysaccharide layer, or glycocalyx, is associated with the plasma membrane of spermatozoa. Recent studies implicate the glycocalyx and peripheral surface glycoproteins in the control of ion transport and motility (5).

d. Metabolism in the spermatozoon: The major metabolic reactions occurring in the spermatozoa involve pathways for the utilization of simple sugars to form chemical energy to drive the motile machinery and to maintain cellular osmotic balance. Very little, if any, sugar is converted to glycogen. Hexose catabolism involves the conversion of hexose (e.g. glucose) to pyruvate or lactate by the Embden-Myerhoff (glycolytic) pathway and by oxidation of either or both of these products within the mitochondria (Fig. 4). Glycolysis proceeds at almost the same rate, both anaerobically and aerobically. Potent mitochondrial poisons such as azide or dinitrophenol have, in contrast to their effects on most other cell types, only a small effect on glycolysis in spermatozoa, and these cells maintain high motility in the presence of these compounds or in the absence of air. Oxygen consumption, although small, can support motility in the absence of glycolysis (5).

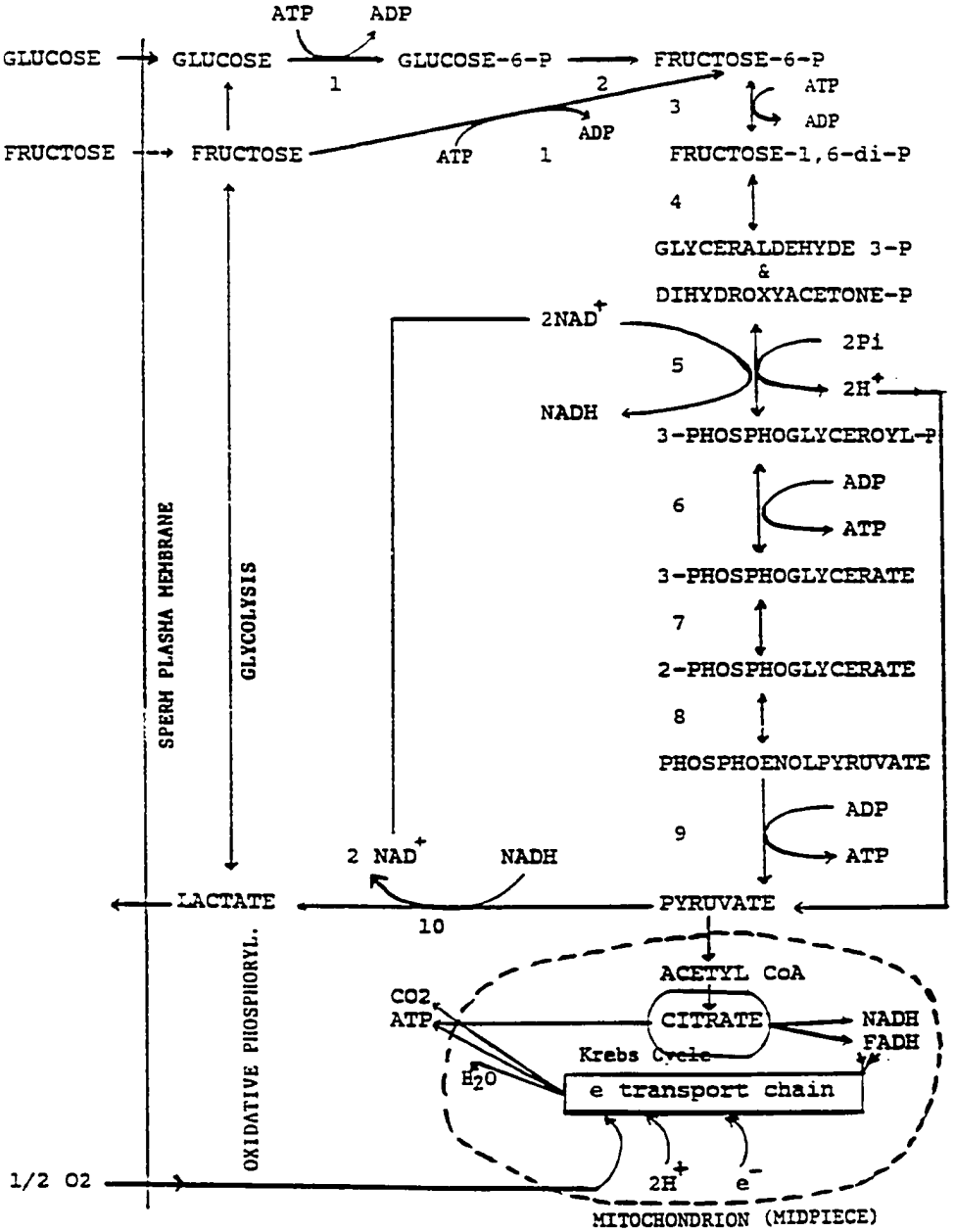
The high rate of aerobic glycolysis in spermatozoa is similar to that of many tumor cells. This rate reflects, in part, an absence of significant feedback on the glycolytic

Figure 4.

Pathways of energy metabolism (glycolysis and mitochondrial oxidative phosphorylation) in human spermatozoa.

Enzymes catalyzing numbered reactions are:

Reaction 1: Hexokinase
Reaction 2: Phosphoglucoisomerase
Reaction 3: Phosphofructokinase
Reaction 4: Aldolase
Reaction 5: Glyceraldehyde 3-phosphate dehydrogenase
Reaction 6: Phosphoglycerate kinase
Reaction 7: Phosphoglyceromutase
Reaction 8: Enolase
Reaction 9: Pyruvate kinase
Reaction 10: Lactate dehydrogenase (LDH-X, LDH-4)



pathway by the metabolites of mitochondrial metabolism (the Pasteur effect). Although the reason for this has not been adequately explained, it does not appear to be due to any unusual properties of key glycolytic enzymes, which respond to the allosteric effects of mitochondrial metabolites much as do these enzymes in other cells. One explanation is that a significant portion of the energy generated by the mitochondrial electron transport system is converted into the energy needed to carry out non-ATP dependent ion transport, which may be very active in motile cells (5).

Glucose and fructose serve as sources of metabolic fuel in spermatozoa, although mannose and possibly galactose may also be used. Fructose, found in ample concentration in seminal plasma, is formed by a nonphosphorylative pathway in which glucose is enzymatically reduced to sorbitol by the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), and then oxidized to fructose by nicotinamide adenine dinucleotide (NAD). The spermatozoa uptake mechanism shows a considerably higher affinity for glucose than for fructose. Since seminal plasma does not pass through the cervix, glucose (which is found in ample concentration in the female reproductive tract) is probably the major energy source for spermatozoa in the female reproductive tract.

Transport of glucose and fructose into the spermatozoon appears to have independent mechanisms. Low concentrations of the antibiotic cytochalasin B and other specific inhibitors of the hexose transport inhibit transport of glucose but not

fructose into the spermatozoon. Glucose transport is probably not a limiting factor in glucose utilization by the spermatozoon because the initial rate of glucose uptake exceeds the rate of lactate production at physiological glucose concentrations.

The glycolytic pathway seems to be the only significant pathway for glucose degradation in spermatozoa. The relatively large contribution of glycolysis to ATP production by spermatozoa is probably an adaptation in sperm transport to the sites of fertilization, since the oxygen content may be quite low in the female genital tract. The enzymes of the hexose monophosphate shunt have been found in mature spermatozoa, but it is likely that the enzymes are associated with residual bodies rather than within the spermatozoa themselves. The comparative concentrations of the glycolytic enzymes found in spermatozoa, as well as the steady-state concentrations of metabolites, have indicated the probable sites of glycolytic control (5). These include the enzymes phosphofructokinase and D-glyceraldehyde phosphate dehydrogenase.

In one study of the control sites of glycolysis in spermatozoa, it was postulated that glycolytic rates operate at near capacity and are controlled primarily by the energy needs of the cell (5). The investigator argued that when motility is increased by elevation of intracellular cAMP (he believes that cAMP stimulates motility), the glycolytic rate in spermatozoa increases but at the expense of a decrease in the ATP/ADP ratio in the cell. This can be explained by the inability of

glycolysis to increase the rate of ATP production sufficiently when the energy demands of increased motility exceed a certain limit. A lower steady-state of ATP/ADP is established under these conditions of near-maximum glycolysis (5).

Measuring the changes in the steady-state concentration of the substrates and products of each glycolytic enzyme before and after an induced change in the glycolytic rate, has made possible the identification of those spermatozoal enzymes primarily involved in controlling glycolytic rates. Phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase play key roles in the regulation on glycolysis. Due to allosteric effects, the activities of these enzymes are inhibited by ATP; therefore the rate of glycolysis in spermatozoa decreases when energy reserves in the form of ATP are high.

That energy metabolism in spermatozoa operates at near capacity is perhaps not surprising, in view of the short life span of these cells and the absence of significant biosynthesis that would cause tighter regulation of high energy metabolites, such as that occurring in the more complex metabolic pathway of the somatic cells.

Mitochondrial oxidative metabolism is capable of maintaining spermatozoal motility in the absence of exogenous substrates, indicating that endogenous oxidizable reserves, presumably lipids, are present in spermatozoa. It is probable that fatty acids and triacylglycerols are synthesized in spermatozoa (5). Human spermatozoa are not able to oxidize

pyruvate, lactate, or α-glycerophosphate rapidly.

Lactate dehydrogenase (LDH), particularly the X isoenzyme (LDH-X), appears to play an important role in mitochondrial metabolism in spermatozoa. The enzyme, which is located in the cytosol of most other cells, is present almost exclusively in the mitochondria of spermatozoa. Lactate dehydrogenase competes effectively for reducing equivalents (NADH, FADH) against the electron transport chain enzymes and permits the direct oxidation of the lactic acid produced by glycolysis. LDH-X may participate in shuttling these reducing equivalents from the cytosol to the mitochondria, where the energy yields are higher (5).

An important role in the mitochondrial metabolism for L-carnitine and L-carnitine acetyl transferase has been recently demonstrated (5, 9). These compounds are present in high concentration in epididymal spermatozoa, and active acetate in the form of acetylcarnitine may provide fuel for spermatozoa during epididymal transit. Acetylcarnitine may function in carbohydrate metabolism and in the utilization of pyruvate. To ensure a steady flow of intermediates into the Krebs cycle, pyruvate and lactate may be converted to acetyl carnitine. This intermediate can serve as a reserve supply of acetate needed regularly for energy production. These conversions and reserve sources seem particularly suited for the maximum utilization of glucose and pyruvate by spermatozoa under aerobic conditions, particularly since these compounds have been implicated as major sources of energy during capacitation.

It is difficult to determine the relative contribution of glycolysis and respiration to the overall energy demands of the spermatozoon. In one study with bull sperm, which is very similar to human spermatozoa, it was estimated that approximately 25% of the free energy of metabolism was generated by fructolysis. Of the free energy generated by mitochondrial metabolism, only 30% to 40% was available to directly support motility (ATP generation). The remaining free energy was presumably used to drive ion transport, which suggests the importance of this process in motility.

Increased levels of intracellular cAMP have marked effects on the motility and metabolism of spermatozoa. Garbers et al. (13) showed that the increased levels of cAMP induced by caffeine and papaverine, both potent cAMP phosphodiesterase inhibitors, increased fructolysis, respiration, and motility of bovine spermatozoa. The opposite effects occurs in the presence of imidazole, an agent that stimulates phosphodiesterase. Later it was found that protein kinases are also involved in the process (5). Both the plasma membrane and axonemal proteins have been implicated as the sites of action of cAMP-activated protein kinases. Adenylate cyclase activity is concentrated in plasma membranes of the spermatozoa, as it is in most cell types. A portion of adenylate cyclase may also be located along the axonemal complex, but more confirmatory studies are needed to prove that. The role of cAMP in motility may be due to an alteration in the rate of ion flow across the plasma membrane. The association of the change in

ion flow across the plasma membrane and the change in motility in human sperm has been observed (5). However, too little is known about the sperm motility apparatus to permit anything but guesses on the effects of phosphorylation of proteins associated with the apparatus.

e. The sperm plasma membrane, ion transport, and motility: Little is known about the composition of the plasma membrane of the flagellum. Regional differences in the charge density of the surface membrane have been noted, and certain surface antigens appear to be restricted to discrete regions of the plasma membrane. Differences between the proteins of the head and flagellar plasma membranes are not marked, however, and appear as differences in the concentration of specific proteins, as well as the presence or absence of certain proteins which remain constant throughout the entire plasma membrane.

Studies using membrane-active drugs have indicated that the plasma membrane plays an active role in the control of metabolism and motility. Ouabain, which specifically interacts with the membrane-bound Na^+ -K^+ -activated ATPase, inhibits metabolism and motility in spermatozoa (5). However, testosterone, which stimulates motility, could block the ouabain action and vice versa. This suggests that both ouabain and testosterone act on the same enzyme. Although these effects could be due to changes in intracellular ATP levels induced by inhibition or stimulation of enzyme activity, a direct effect of a change in ion flow on motility can also be

proposed as an alternative mechanism. Indeed, there is substantial evidence that motility is highly sensitive to the ionic composition of the supporting medium. For example, very high (6 millimolar) or very low concentrations of K^{+}, low concentrations of Cl^{-} or Na^{+} ions, and Ca^{2+} concentration exceeding 1 mM are known to depress sperm motility (5).

Ion fluxes, perhaps by their effects on the surface or by intracellular-propagated action potentials, play key roles in developing progressive forward flagellar motion. Recent studies on the role of ions in motility have focused on Ca^{2+} because this ion is involved in acrosome reaction and there is some evidence that Ca^{2+} may increase motility during capacitation. Reports on the effects of Ca^{2+} on spermatozoa motility vary considerably and may reflect species differences, some of which seem clearly related to effects on metabolism. The rapid uptake of Ca into the mitochondria of spermatozoa that are highly dependent on oxidative metabolism may lead to an uncoupling of phosphorylation and cell death. Recent reports regarding an increase in cAMP levels in capacitated guinea pig spermatozoa in the presence of Ca^{2+} suggest that activation of adenylate cyclase by Ca^{2+} may play a role in this change of motility (5).

f. Other mechanisms of motility control: A neuronal theory for the control of motility has been suggested (5). This idea arises from the effects of cholinergic drugs on spermatozoal motility. After establishing the presence of a cholinesterase in spermatozoa, further studies showed that

inhibitors of cholinesterase, such as eserine, stimulated motility. Acetylcholine also stimulated motility; provided that the spermatozoal membrane was made permeable to allow access to intracellular binding sites, anticholinergic compounds inhibited motility. The existence of a cholinergic receptor oriented in the plasma membrane so that its active site is located on the cytoplasmic surface has been proposed (5). Activation of the receptor by binding to acetylcholine or its analogs induces changes in ion transport that increases motility. Calcium may play a key role in these changes as a stimulus for an excitation wave that is responsible for the propagation of contractile events.

g. Drug effects on sperm motility and metabolism:

A normal man can ejaculate about 300 million spermatozoa every 2-3 days (14). The germinal epithelium of the testis is one of the most active tissues in the body and therefore potentially susceptible to the effects of pharmacological agents (Table 1). Drugs may affect spermatozoa by altering their production or fertilizing ability, or, theoretically at least, by causing abnormalities of the offspring. This subject is important because male infertility can be caused inadvertently by drugs or toxic chemicals and the effects may not always be identifiable. Drugs, particularly hormones, may also be used to treat infertile male individuals. (The effects of certain drugs and other chemicals on spermatozoa have been summarized in Table 1). As far as the effects of drugs and some other chemicals on sperm motility are concerned, the following are

important to note. In contrast to cholinergic agents that can stimulate motility, certain pharmacologic agents have been shown to inhibit motility. Their actions may be due to their interactions with plasma membrane. Agents such as propranolol (a beta blocker), lidocaine, and diphenhydramine depolarize the membrane of spermatozoa. The effects of these agents (especially that of propranolol) become even more pronounced if the female is taking these medications. These agents tend to concentrate more in cervical mucus than in seminal fluid. Some of these agents such as chlorpromazine and benztropin mesylate are particularly potent and inhibit sperm motility at concentrations as low as 50 μM (15). An initial membrane depolarization is induced by these agents, and can, in part, be reversed by the potassium specific ionophore, Valinomycin. However, it cannot reverse the second and slower depolarization induced by these agents. The effects of these agents are therefore, partially related to changes in plasma membrane ion flow . Propranolol and certain local anesthetic-like compounds displace divalent cations from plasma membranes in some somatic cells.

The plasma membrane also interacts directly with the glycolytic apparatus. Glycolysis (or some glycolytic enzymes) is inhibited by propranolol in intact spermatozoa. It causes accumulation of fructose 1,6-diphosphate and glyceraldehyde-3-phosphate by inhibiting enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase, respectively (5).

A particularly interesting agent is tetraphenylboran (TPB)

TABLE 1

EFFECT OF DRUGS AND OTHER CHEMICALS ON SPERM PRODUCTION

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1. AGENTS WHICH MAY IMPAIR SPERM MATURATION
   a. Antiandrogens (e.g. cyprotrone acetate)
   b. α-chlorohydrin, chlorinated sugars
   c. Sulphasalazine (salazosulfapyridine)
   d. Gossypol
2. AGENTS WHICH MAY IMPAIR SPERM MOTILITY
   a. Acting before ejaculation: nicotine
   b. Acting after ejaculation: propranolol, vaginal spermicides (e.g. nonyxnol-9)
3. AGENTS WHICH DIRECTLY AFFECT SPERMATOGENESIS
   a. Heavy metals (Cd, Hg,Pb): damage blood-testis barrier
   b. Chemotherapeutic agents, dibromochloropropane, gossypol: affect germinal epithelium
4. AGENTS WHICH MAY REDUCE SPERM COUNT
   a. Antibiotics (e.g. trimethoprim, nitrofurantoin, gentamicin, oxytetracycline, antimalarials)
   b. Cimetidine, marijuana
5. AGENTS WHICH ACT ON ENDOCRINE SYSTEM
   a. GnRH agonists, androgens, estrogens, progestins, reserpine, valium ?, chlorpromazine: inhibit gonadotropin secretion, decrease sperm production
   b. Spironolactone: inhibit androgen biosynthesis
   c. GnRH, Bromo-criptine, antiestrogens such as clomiphene & Tamoxifen: may stimulate gonadotropin release

TABLE 1 CONT.

DRUG	EC 50 (mmol/L)	PHARMACOLOGICAL CLASSIFICATION
Acebutolol	11.0	Beta-blocker
Amitriptyline	0.17	Antidepressant
Arachidonic acid	3.5	Fatty acid
Chlorpromazine	0.22	Neuroleptic
Clonidine	11.0	Antihypertensive
D-Propranolol	1.3	Beta-blocker
Delta-9-tetra-hydrocannabinol	1.2	Cannabinol (MJ)
Diltiazem	1.4	Calcium blocker
Doxorubicin	0.4	Chemoterapeutic
Docoxahexanoic acid	2.0	Fatty acid
Flecainide	1.15	Antiarrythmic
ICI-118551	0.3	Beta-blocker
Imipramine	0.16	Antidepressant
Indoramin	4.0	Alpha-blocker
Labetalol	2.2	Antihypertensive
Lysophosphatidylcholine	0.8	Phospholipid
Metoprolol	6.2	Beta-blocker
Mexiletine	2.8	Antiarrhythmic
Norpropoxyphene	0.8	Analgesic
Oxprenolol	4.2	Beta-blocker
Procaine	18	Local anesthetic
Propranolol	0.8	Beta-blocker
Quinidine	0.5	Antiarrhythmic
Verapamil	0.8	Calcium blocker

The effect of these drugs on human sperm motility was assessed using the transmembrane migration method, which measures the proportion of sperm that moves across the 5 μm pores of a nucleopore membrane in 2 hours of incubation at 37°C. Water soluble drugs are first dissolved in phosphate buffer saline, pH 7.3, and then mixed with fresh semen. Water insoluble drugs are pipetted into glass tubes, dissolved in chloroform, and heated on a sand plate to allow evaporation of chloroform before semen is added. The effective concentration 50 (EC 50) decreases sperm motility by 50% of that of the control sample.

which, unlike many other active drugs, reversibly inhibits spermatozoal motility during short periods of contact. This lipophilic organic anion binds to the exoplasmic, positively charged membrane phospholipids. It can cause a decline in sperm cAMP. The enzyme adenylate cyclase in isolated membranes is not affected by the agent if the membranes are treated with a detergent such as Triton X-100. This suggests that membrane phospholipids may play an important role in adenylate cyclase activity in the intact spermatozoon. The effects of tetraphenylboran can be reversed by albumin or K ions which insolubilize the drug, and motility can be reinitiated by these antagonists (5).

h. The motility apparatus: The sliding microtubule model originally proposed by Satir (16) for cilia and flagella satisfies many conditions for movement. Electron micrographs established that neighboring outer microtubule doublets of the axonemal complex slide past each other without contracting (Fig. 3). This sliding, which induces a localized bending of the flagellum, is apparently due to the cyclic attachment and detachment of the outer arms of the A tubule (complete,13-tubulin unit) with an adjacent B tubule (Fig. 3). These arms contain the ATPase dynein, thought to be involved in these cyclic reactions. It is postulated that the sliding of the outer doublets and the constraints imposed on sliding by the nexin fibers and the two radial spokes that connect the sliding doublet to the central sheath give rise to characteristic bending patterns. The length of the tubules does not change

during bending. Experiments by Gibbons (17) on isolated demembranated axonemes of sea urchin spermatozoa clearly showed that ATP induces sliding between the tubules. Using trypsin to selectively destroy supporting structures (nexin and radial spokes), he showed that in the presence of ATP, the outer tubules rapidly slide past one another; as one tubule of the doublet became exposed, it was destroyed by the trypsin in the medium. He also showed that removal of the dynein arms by salt extraction reduced the motility by a factor of two (17). In the absence of ATP, demembranated sea urchin spermatozoa showed a specific rigorous wave characteristic (flagella frozen in a given oscillatory state), which continued until ATP was provided. Dynein is a Mg-activated ATPase similar to the Ca - activated ATPase of muscle (myosin).

i. Propagation of wave motion: Although the sliding of microtubule doublets and the constraints imposed on sliding by the central doublets, radial spokes, and nexin links explain flagellar bending, they do not explain propagation. Since wave motion occurs in demembranated spermatozoa, wave propagation is apparently an intrinsic property of the axoneme. Portions of the axoneme that are not so actively sliding may resist the sliding of neighboring doublets (5). This may activate the sliding mechanism in that adjacent section and thus propagate bending along the flagellum. This mechanism requires that the ATPase involved in active sliding exist along the entire axoneme.

The mechanism by which the doublets undergo coordinated

sliding to provide the various types of wave motion observed in flagella, including variations in amplitude and direction, remains to be resolved. Also unresolved are the factors that control beat frequency and the induction of spontaneous beating. Protein phosphorylation mediated by cAMP appears to play a role in the control of beat frequency in mammalian spermatozoa, and perhaps the cholinergic mechanism described above also plays a role. The responsiveness of the flagellum to external stimuli and the direct coordinated motion induced by surface glycoproteins also indicate the importance of the plasma membrane in motility.

j. Excretory genital ducts, male accessory sex organs and formation of seminal fluid: The ducts that transport spermatozoa produced in the testis toward the upper portion of the male reproductive system are the ductuli efferentes, the epididymis, the ductus deferens (vas deferens), and finally the urethra when spermatozoa are ejaculated. Epithelial cells of the ductuli efferentes are ciliated. The rapid movement of the cilia impels the spermatozoa, which lack fertilizing ability and progressive motility, toward the epididymis (7, 3).

The epididymis, about 4-7 meters long, serves many purposes for the newly arrived spermatozoa. It is a major site of sperm storage, it concentrates testicular plasma by absorption (90%) of the fluid, and, under hormonal control, it serves as a site for developmental (maturational) changes observed in spermatozoa as they progress from the head (caput)

to tail (cauda). The latter function of the epididymis has recently been disputed (77). The epididymis also removes dead, dying, or otherwise defective spermatozoa primarily by phagocytic activity of the macrophages and neutrophils, and by the action of lysosomal enzymes such as proteases, deoxyribonuclease, and acid phosphatase. "Normal" spermatozoa are protected from these activities by their mucoprotein coat and also by the presence of enzyme inhibitors. All of these degradative activities contribute to the cellular debris observed in ejaculated semen. It has been estimated that the epididymal transit of spermatozoa requires 4-12 days (78).

Upon leaving the cauda of the epididymis, spermatozoa (semen) enter the vas deferens, a pair of straight tubes with thick, muscular walls measuring 25-45 centimeters (cm) each. The sperm continue toward the dilated section of the vas deferens (ampulla) and to the prostatic urethra, if ejaculation occurs (7).

The peristalsis of the distal epididymis and vas deferens at the time of ejaculation is mediated by the sympathetic nervous system. The vas deferens is richly innervated with adrenergic fibers and contains large quantities of catecholamines along with alpha receptors. The failure of ejaculation following retroperitoneal surgeries (or even vasovasostomy) may be due to damage to the sympathetic innervation of the epididymis and vas deferens (7).

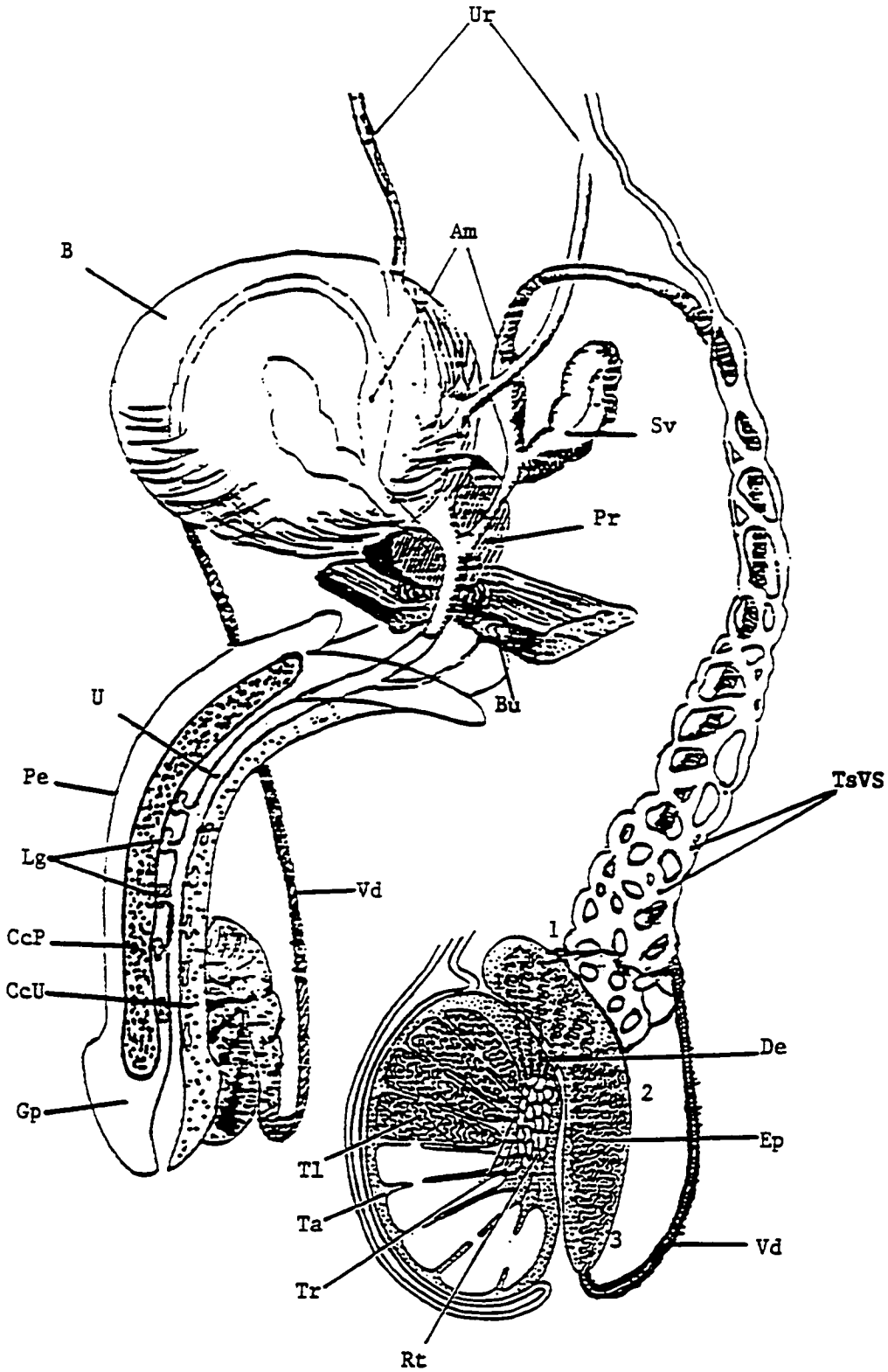
During embryonic development, the terminal portion of each vas deferens becomes dilated to form ampullary glands. At 13

weeks of embryonic development, a diverticulum forms on each ampulla which develops into the seminal vesicle (79). At most, secretions of the ampullary glands and vas deferens form 10% of the ejaculate volume. The ampulla does, however, store a significant amount of spermatozoa (semen). With a single ejaculation, the spermatozoa come primarily from the ampulla, vas deferens, and cauda epididymis (7).

Seminal vesicles and the prostate (Fig. 5) are the main parts of the male accessory sex organ system. The sole physiological function of these parts is to contribute to the production of seminal plasma. Seminal vesicles, so named from the early misconception that they stored sperm, are two distinct lobulated structures, each 10-15 cm long. The bulk of the seminal plasma constituents produced at ejaculation are secreted by the seminal vesicles. Major human seminal components assimilated by the seminal vesicles include fructose, prostaglandins, inositol, ascorbic acid, sorbitol, glucose, and enzymes associated with semen coagulation (Table 2). The degree of activity of secretory processes is highly androgen-dependent. Fructose, a major secretory product of seminal vesicles, is formed from blood glucose via an enzymatic pathway (described below). Since both the seminal vesicles and the vas deferens are of mesonephric duct origin, congenital absence of the vas deferens may accompany a lack of the seminal vesicles as well (congenital bilateral aplasia of the vas deferens and seminal vesicles). The affected patients will be azoospermic. The cause(s) of azoospermia can be verified by

Figure 5.

The male reproductive system: Tl, testicular lobules (seminiferous tubules, interstitial tissue); Ta, tunica albuginea; Tr, tubuli recti; Rt, rete testis; De, ductuli efferentes; Ep, epididymis (1, the head or caput; 2, the body or corpus; 3, the tail or cauda); Vd, vas deferens; Ts V S, testicular venous system (spermatic veins which may dilate to cause varicocele) along with sympathetic nerves; Ur, Ureters; Am, ampulla; Sv, seminal vesicle(s); Bl, bladder; Pr, prostate; Bu, bulbourethral (Cowper's) gland; U, urethra; Pe, penis; Lg, Littre's (urethral) glands; Ccp, corpus cavernosum of the penis; Ccu, corpus cavernosum of the urethra; Gp, glans penis.



testing for fructose in the seminal plasma. The point at which each seminal vesicle stems from its respective ampullary gland gives rise to the contralateral ejaculatory ducts which converge in the prostate and open into the prostatic urethra.

The prostate is probably the best known of the male accessory organs because of its propensity for causing problems in later life. It is the largest of the accessory glands and is of endodermal origin. Contrary to the seminal vesicles development of which is under the control of testosterone, prostatic endoderm does not begin development until testosterone is converted to dihydrotestosterone. The prostate gland contributes about 15-20% of seminal plasma volume. Its major secretions are acid phosphatase, spermine, zinc, calcium, citric acid enzymes involved in semen liquefaction and a host of other compounds (Table 2).

Ejaculation is a sequential event with the various organs contributing their contents to the ejaculatory stream (usually) in a specific order. The urethral (Littre's) and bulbourethral (Cowper's) glands in the distal portion of the urethra release their mucoid secretions during the excitatory phase and may be emitted (in part) as a pre-ejaculatory secretion. These scant secretions may make up only 3-5% of the total volume. Prostatic secretions constitute the majority of the first portion of the ejaculatory stream, comprising 15-20% of the total volume. The initial portion of the ejaculate is also high in spermatozoa. Secretions of epididymal and ampullary origin constitute 7-10% of the total volume, which may be

expelled with the first part of the ejaculate. The seminal vesicle secretions are emitted last and make up the majority of the total seminal plasma volume, approximately 70-80%. Table 2 lists some of the common semen components in association with their probable gland of origin (8, 78).

TABLE 2

SOME PHYSICOCHEMICAL PROPERTIES OF HUMAN SEMINAL PLASMA

PARAMETER	MEAN VALUE (RANGE VALUE)	PRIMARY SOURCE
Volume (ml)	3.5 (1.5-6.0)	
Freezing-point Depression (°C)	(-0.55 to -0.58)	
Protein (gm/100 ml)	4.5 (3.3-6.8)	
Water (gm/100 ml)	91.8 (89.1-99.4)	
Specific gravity	1.035 (1.031-1.039)	
Osmolarity (mOsmol)	(300-500)	
Acetylcarnitine	(0.006-0.28)[n]	epididymis
B-N-acetyl-galactosaminidase	11[a]	?
β-N-acetyl-glucosaminidase	7288[b]	?
Acid-phosphatase	66 (49-72)[c]	?
Adenosine triphosphate (μg/ml)	1.1	?
Adrenalin (μg/ml)	(1.0-2.1)	?
Alaline	29.1[k]	?
Alkaline phosphatase	(1.3-12)[c]	?
Aminoacides (Total)	1258[k]	?
B-Aminobutyric acid	31·7[k]	?
Ammonia	2[k]	?
α-Amylase	9 (3-25)[m]	prostate
Arginine	70-90[k]	?
Ascorbic acid	(2.6-14.4)	?
Aspartic acid	99.8[k]	?
Bicarbonate	54 (43-74)[k]	?
Calcium	30 (14-62)[k]	prostate
Cardiolipin	0.3[f]	?
Carnitine	(11.5-53.5)[k]	epididymis
Chloride	155 (100-203)[k]	?
Cholesterol	47 (16-94)[k]	prostate?
Choline-plasmalogen	0.31[f]	?
Citric acid	376 (96-1430)[k]	prostate
Copper	(0.006-0.0024)[k]	?
Creatine	20[k]	?
Cystine	3.4[k]	?

TABLE 2 cont.

Deamine oxidase (nmol/ml/30ml)	208		prostate
Dehydroepi-androsterone	0.0032[k]		?
DNAse I (units x 10^4/ml)	248		?
DNAse II (units x 10^4/ml)	376		?
Ergothionine	trace		?
Ethanolamine	?		?
Plasmalogen	4.8[f]		?
Fatty acids (volatile)(mg/l)	0.73	(0.5-0.95)	?
Fructose	235	(40-640)[k]	seminal vesicles
α-L-Fucosidase	4.3[g]		?
β-D-Fucosidase	0.2[g]		?
α-Galactosidase	0.2[g]		?
β-Galactosidase	68[h]		?
Glucose	0.4[n]		?
α-Glucosidase	82[h]		?
β-Glucosidase	60[h]		?
β-Glucuronidase	232[j]		?
Glutamic acid	6.5[n]		?
Glutathione	30[k]		?
Glyceryl-phosphoryl-choline	70	(74-90)[k]	epididymis (sem. vesicles)
Glyceryl-phosphoryl-inositol	3[n]		?
Glycine	58.9[k]		?
Glycogen	trace		?
Histamine (μg/ml)	2		?
Histidine (mg/100ml)	109.1		?
Hyaluronidase	1.8[g]		?
Inositol		(3-3.5)[n]	prostate
Isoleucine	62.4[k]		?
Lactate dehydrogenase	1.87	(0.68-4.75)[l]	prostate
Lactic acid	35	(20-50)[k]	?
Leucine	96.7[k]		?
Lysine	152.1[k]		?

TABLE 2 cont.

Magnesium	14[k]		?
x-Mannosidase	218[h]		?
B-Mannosidase	10[g]		?
Methionine	96.7[k]		?
Phenylalanine	28.2[k]		?
Phosphatidyl-choline (lecitin)	3.8[f]		?
Phosphatidyl-ethanolamine (cephalin)	3.3[f]		prostate
Phosphatidyl-inositol	0.7[f]		?
Phosphatidyl-serine	4.4[f]		?
Phospholipid	84	(48-133)[k]	?
Phosphorus			
Total	112	(90-120)[k]	?
Inorganic	11[k]		?
Potassium	89	(56-107)[k]	seminal vesicles
Proline	26.9[k]		?
Prostaglandins:			seminal vesicles
A+B	5[k]		
E	5.3[k]		?
F	0.8[k]		?
19-OH A+B	20[k]		?
Putrescine (nmol/ml)	230		prostate
Pyruvic acid	3.4[n]		?
Seminin (fibrinolysin) (ug% trypsin equivalent)	30	(20-50)	prostate
Serine	11[1].6[K]		?
Serotonine (ng/ml)	150		?
SGOT (AST)	14	(7-24)[i]	?
SGPT (ALT)	2.5	(1-4)[i]	?
Sialic acid	124	(64-219)[k]	?
Sodium	281	(240-319)[k]	seminal vesicles
Sorbitol	10[k]		?
Spermidine	trace		prostate
Spermine		(22-250)[k]	prostate
Sphingomyelin	17.16[f]		?
Sulfur	3 % of ash		?
Testosterone	0.068[k]		?
Threonine	47.7[k]		?
Total lipid	185	(166-206)[k]	?
Tyrosine	51.4[k]		?

TABLE 2 cont.

Urea	72[k]		?
Uric acid		(2-6)[k]	?
Valine	49.8[k]		?
Zinc	14	(5-23)[k]	prostate

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from references 1, 7, 78 and 85

a: µmoles aglycone/N-acetylglucoseamine liberated/hour at 37° C

b: µmoles phenol liberated/hour at 37° C
c: Sigma units
f: µg atoms lipid P/100ml
g: µmoles aglycone/N-acetylglucoseamine liberated/hour at 37° C
h: ug nitrophenol
i: King units
j: µg phenolphtalein liberated/hour/37° C
k: mg/100 ml
l: unit/ml
m: Street-Close Unit/ml/15 minutes at 37° C
n: mM

CHAPTER II

INFERTILITY

A. Definition

The normal events that take place during puberty in male individuals that lead to formation of spermatozoa have been described, together with factors that influence the development of male gametes. It must be noted, however, that these events may not always take place under normal conditions, and the outcome may not always lead to formation of healthy gametes or normal reproductive systems. Any deviation from the norm may cause infertility.

Infertility has been defined as failure to conceive during one year of unprotected coitus. This definition is based on a demonstrated monthly conception rate of 20% to 25% in fertile couples attempting to achieve a pregnancy (18). Given a monthly conception rate of 20%, approximately 95% of fertile couples should conceive within 13 months. The conception rate is somewhat lower in the first months after oral contraceptive medications are stopped. Age may also play a role. therefore, the definition of infertility might logically be extended to 15-24 months for these couples (18).

We discuss male infertility, IVF, and semen assays we found to be significant in couples who participated in series 27 and 30 of our IVF program.

B. Size of the Problem

The prevalence of infertility has climbed from 15% to 20% in the past decade (20). A different lifestyle has contributed to the increased number of infertility patients. Delayed childbearing in pursuit of a career and excellence, often accompanied with stress, obsession with being "thin and beautiful," and an epidemic of sexually transmitted diseases have been contributing factors.

Estimation of the prevalence of infertility in different communities has been plagued with numerous difficulties in data collection and interpretation, as well as varying definitions of infertility. In most Western societies the figure is about 10%, in some urban areas of Colombia is as high as 13-23%, and 42.5% in some districts of Sudan and other areas of Africa. In England the prevalence is 8%; in the USA it is about 17% (20).

Data from the past 20 years reveal that in approximately 33% of cases, significant pathology is found in the man alone; in another 20% both the man and woman are abnormal. Thus, in roughly 50% of infertile couples the male factor is at least partly responsible for the failure to conceive (6). Adding to the problem is idiopathic infertility. In one study at a male fertility clinic 25.4% of infertility cases were idiopathic (6).

C. IVF as a Technique to Aid Infertile Males

During the past decade several new and promising techniques have been developed to combat an ever increasing

number of reported cases of infertility. The new era began with the birth of the first baby to a woman without functional Fallopian tubes through a procedure now known as in vitro fertilization (IVF). This technique, first applied to patients with uncorrectable tubal disease, is now applied to treat many other causes of infertility, including male and idiopathic infertilities. IVF involves 1) deliberate hyperstimulation of ovaries to produce more than one follicle (oocyte), 2) induced follicle maturation and release at a specified time, 3) retrieval of oocytes and their insemination with spermatozoa outside the body (in vitro), and 4) transfer of the developed conceptus (concepti) into the uterine cavity.

Fertilizing ability of the mammalian oocyte is achieved only after a prolonged period of development and differentiation. In most mammals, fertilization occurs at metaphase II. Oocytes that have not reached this stage usually cannot be penetrated by spermatozoa or, if penetration does occur, the sperm nucleus is not transformed into a sperm pronucleus (20).

In the human, the meiotic process, which is required for the eventual formation of an oocyte with a haploid (22+X) number of chromosomes, is initiated during fetal life. After a series of mitotic divisions, the oocyte enters its first meiotic division and is arrested at the diplotene stage of prophase before or near birth. During fertile life, due to the effects of hypothalamic gonadotropin-releasing hormone (GnRH), a pulsatile pattern of FSH and LH secretion is established.

During each cycle, FSH and LH levels gradually increase, with a peak at midcycle. This increase and the subsequent peak result in three effects: 1) it may (directly or indirectly) cause termination of the first meiotic arrest, 2) it may affect the process of selection of a follicle destined to ovulate in the coming cycle, and 3) it affects development and eventual release of the dominant follicle.

Usually one follicle develops and ovulates in each natural cycle. The mechanism is a complex one whereby a single follicle, among the many that are recruited in the early follicular phase, attains and maintains dominance over the others which fail to develop and become atretic. The initial attainment of dominance appears to result mainly from local intra-ovarian regulation (paracrine function) by one of the many peptides in the ovary, or the dominant follicle itself which may interfere with proper selection, recruitment, and development of other follicles (94). Inhibin suppression of FSH may also play a role. Later in the follicular phase, a large amount of E2 secreted by the dominant follicle maintains its dominance by inhibiting gonadotropins (FSH, LH) and secreting E2 until the preovulatory surge of FSH and LH. Some early efforts at IVF, including the birth of the first child by IVF, used oocytes retrieved from ovaries in a natural, unstimulated cycle. Most regimens now involve hyperstimulation with exogenous gonadotropins to induce superovulation and retrieval of several oocytes. This hyperstimulation usually begins early in the follicular phase of the cycle to allow the

recruitment of several follicles before the selection of the dominant follicle. Three basic protocols are used to induce multiple follicular development: 1) human menopausal gonadotropin (hMG) administration followed by human chorionic gonadotropin (hCG), 2) a combination of pure FSH and hMG on days 3 and 4 of the cycle, followed by hMG and hCG, and 3) pure FSH followed by hCG (94). Synthetic compounds such as clomiphene citrate (Clomid) which stimulate endogenous secretion of gonadotropins have also been used but natural gonadotropins seem less likely to cause adverse effects on oocytes or the endometrium (94).

The chances of conception are increased if more than one fertilized oocyte is replaced, though whether this is due to a "local helper" effect of multiple blastocysts or simply to increased chances of implanting a fertilized oocyte of appropriate maturity is unknown.

Currently in Norfolk, hyperstimulation of the ovaries begins on day 3 of the menstrual cycle with intramuscular injection of gonadotropin(s). During the course of injections, daily (morning) determinations of serum E2 are carried out. Usually beginning on day 6 of the cycle, daily ultrasound examination is carried out to assess the number and size of the follicle(s). A pelvic examination is also performed daily for assessment of the quality of cervical mucus, percentage of lateral wall pyknotic cells, and dilation of cervical os. Administration of gonadotropin(s) is terminated when 1) concentration of serum E2 reaches the proper level (according

to the patient's response), 2) quality of cervical mucus (clear, volume >0.2 ml, spinnbarkeit >10 cm, 4+ ferning) appears to be good, 3) the cervical os is dilated, and 4) at least one follicle of size >12 mm is observed. Administration of hCG is usually around 50 hours after the last gonadotropin injection. Oocytes are retrieved 34-36 hours later via laparoscopy, and their stages of maturity are determined. Mature oocytes (metaphase II) are inseminated with swim-up samples within 2 hours of retrieval. Each oocyte is inseminated with 150,000 sperm/3 ml of Ham's F10 and 7.5% fetal cord serum (HF10-FCS). Immature oocytes, such as those in metaphase I or prophase stages, are incubated for several more hours to become mature and ready for insemination. After 16-18 hours of incubation at 37° C in 5% CO_2 in air, fertilized oocytes are transferred into the growth medium (HF10- 20% FCS) and eventually are transferred (up to 4 concepti) into the uterine cavity on the second day after oocyte retrieval.

D. Tests to Evaluate Subfertile Males

Although many of our ancestors grieved for their failure to overcome infertility problems, we enjoy recently gained success. During the past 25 years possibilities of more precise localization of the problem(s) responsible for infertility have provided an impetus for transforming the largely empirical approach of treatment to a more practical one. In the past 5 years we have witnessed an increasing interest in the male factor and a concurrent appreciation of the subtle abnormalities of gonadal and spermatozoal dysfunction that can contribute to male infertility. New diagnostic assays have been developed to delineate previously unrecognized male factors. Once these male abnormalities are identified correctly, treatment regimens can be introduced. Etiological interpretation of causes of infertility related to the male and diagnostic procedures to evaluate the male partners of infertile couples have expanded tremendously and become less empirical. This indicates a modern trend in evaluation of male infertility. However, we are far from our goal of developing the "magic test(s)" that can accurately pinpoint the problem(s) related to the male infertility.

For years, conventional semen analysis has been used to assess male fertilizing potential. However, the traditional characterization of semen by sperm concentration, motility, and morphology has frequently yielded results that are unexpected or compromised. This has made the prognosis for fertility uncertain. Several investigators (28, 31, 32), as well as the

World Health Organization (WHO), have defined parameters for a "normal" semen sample with regard to sperm count, motility, and morphology. Nevertheless, pregnancy rates of about 30% in partners of men with semen characteristics below normal levels have been reported (34). Basic semen parameters, as they have been determined traditionally, are not sensitive monitors of sperm fertilizing capacity, although they may reveal the presence of a severe disturbance in the mechanism responsible for spermatogenesis and sperm maturation.

Several newer techniques to determine the fertilizing capacity of sperm have also been disappointing. Many usual biochemical and immunological procedures have not correlated with the functional competence of the sperm but have been useful only in understanding the factors involved in the pathogenesis of infertility.

In response to the urgent need for more definite results, newer approaches for determining the fertilizing ability of the sperm have been developed. Examples include biologically related and clinically valuable functional assays such as an estimation of the migration capacity of sperm and in vitro penetration to heterospecific and autospecific oocytes.

Reports on the ability of the cervical mucus penetration assay or migration test to discriminate between fertile and infertile sperm cells have been contradictory. Evaluation of cervical mucus from 132 infertile couples showed no penetration in 24%. However, upon retesting mucus, obtained from the same patients on other occasions, was penetrable by the husband's

sperm (35). When this test was used to evaluate sperm abnormality, only severe motility defects adversely and significantly influenced migration of sperm into the human mucus. In that study, 132 infertile patients were compared with 63 couples who entered IVF programs and became pregnant. Cervical mucus penetration results obtained from these two groups showed no differences in penetration.

Heterospecific fertilization of hamster oocytes by human sperm has been actively investigated: 1) to evaluate the influence of seminal plasma on fertilization, 2) to assess the chromosomal contribution of human sperm (36), 3) to determine the effects of steroids in the penetrating efficiency of sperm and, 4) to correlate the results obtained with the fertilizing ability of the sperm.

There are several reports (37, 38) of a close association between male infertility and the success of the hamster zona-free oocyte sperm penetration assay (SPA). Despite these findings, objective quantitation of the SPA as a predictor of fertility is still not available. The normal value for the test is uncertain, and the conditions for the assay have not been standardized. The widespread use of the test has also been hampered by major practical difficulties. Zausner-Guelman et al. (39) noted a wide range in the percent of penetration in males of proven fertility. Studying 54 patients undergoing IVF, Ausmanas et al. (40) could not find a definite lower limit of the SPA to define absolute male infertility. They found that a poor SPA was not always indicative of the

inability of sperm to fertilize human oocytes. Perreault and Rogers (41) suggested a 10% SPA as a cut-off point to delineate infertile from fertile males. Martin and Taylor (42) found that in 22 men with proven fertility, one showed no SPA and the other 21 had penetrations > 15%. Margolioth et al. (43) found a good correlation between a failed SPA and a failed IVF attempt. Using the same ejaculate for SPA and IVF, Wolf et al. (44) found that 11% (2 of 18) of the samples which fertilized human oocytes (IVF) had false negative SPA results.

Because of these problems, there is a great need for more critical and discriminating assessment of the quantitative semen parameters and the development of new methods to determine cause(s) of decreased fertility. In addressing this need, attention has recently been focused on the use of adenosine triphosphate (ATP) and creatine kinase (CK) determinations as possible markers of human fertility potential. The choices seem appropriate since many sperm functions are energy-requiring processes.

1. ATP and CK and their significance in infertility studies

Sperm cells do not undergo cell division and do not have major biosynthetic pathways. As a result, ATP is mainly used to initiate catalytic processes such as glycolysis, maintaining motility, and supporting miscellaneous processes such as maintenance of osmotic balance (30). Exogenous glycolyzable substrates such as glucose and fructose are transported into the cell and are directed toward the Embden-Myerhof pathway to

lactate because sperm cells do not possess a pentose phosphate shunt or enzymes for glycogen metabolism. The lactate thus formed is either excreted into the medium or oxidized to CO_2 and acetate by mitochondrial oxidation. Appropriate alternate exogenous substrates include lactate (oxidation by mitochondrial lactate dehydrogenase, LDH-X; conversion to acetyl CO A, and metabolism via the Krebs cycle), pyruvate (via mitochondria and the Krebs cycle), and β-hydroxybutyrate (oxidation by β-hydroxybutyrate dehydrogenase, conversion to acetyl CO A, and metabolism via the Krebs cycle).

Motility consumes about 70% of the spermatozoal ATP at 37 C (30). Sperm motility, both the percent of motile sperm in an ejaculate and the quality of movement, must be considered one of the most important parameters in evaluating the fertilizing competency of sperm. The flagellar activity of the sperm is needed for normal progressive transport through the female genital tract and for penetration of the cumulus layer and zona pellucida of the oocytes (45). The type of movement will also influence the capacity of the sperm. Sperm cells moving in very tight circles or without beating the tail vigorously cannot readily penetrate and fertilize the oocyte (46). The sperm tail beats approximately 10 times/second (46), and the form of the beat is asymmetric. The sperm tail propagates an undulating wave as it moves from the base of the flagellum to its tip, with the tip moving clockwise in approximately a semicircular pattern which drives the sperm head forward (17). The amplitude of bending increases progressively along the

flagellum toward the distal end. The movement in the proximal region is planar, whereas that of the distal region contains a significant three-dimensional component.

The mitochondrial sheath located in the midpiece region of the sperm is the power house for the mammalian sperm and has been likened to a combustion engine (46). ATP produced in mitochondria diffuses toward the distal end of the tail and is used to maintain motility and other active processes (47). No specific alterations of the sperm mitochondrial ultrastructure can be demonstrated in most men with decreased sperm motility. However, in rare cases of severe asthenospermia or necrospermia, defects have been observed in the internal structure of mitochondria (46).

The axoneme, the central core of the sperm tail, originates within the mitochondrial sheath and extends the length of the sperm tail. It consists of nine hollow doublet microtubules surrounding a central pair of single microtubules. Doublets, structurally made of protein tubulin subunits, contain A and B tubules. A tubule has both inner and outer arms, as well as the nexin links connecting them to the adjacent B tubules and the radial spokes connecting them to the central sheath (17).

Gibbons (17) was the first to characterize the major protein components of cilia and flagella. Two major proteins found in the flagella were the ATPase protein of the outer arms of A tubules and tubulin,the principle structural protein of microtubules, which account for about 15% and 70% of the total

axonemal proteins, respectively. The ATPase protein, dynein, is activated by Mg^{2+}, Mn^{2+}, small concentrations of Ca^{2+}, Co^{2+}, Ni^{2+}, and relatively high concentrations of K^{+}, which increase internal pH of the sperm. It is inhibited by Zn^{2+}, Cd^{2+}, Hg^{2+}, and sulfhydryl groups (17).

In 1955, Gibbons discovered that grasshopper sperm flagella, in which the selective permeability of the membrane had been destroyed by treatment with 50% glycerol, could be reactivated by addition of exogenous ATP and that beat frequency increased with ATP concentration up to about 1 mM (17). He also noted that Mg, a cation now known to be essential for the activity of ATPase and possibly other enzymes such as creatine kinase, was needed for motility. The presence of Ca-Mg ATPase in the plasma membrane of sperm may have a function in this regard (32). It may also serve to reduce intraflagellar Ca^{2+} concentration, which is essential for maintenance of sperm motility (48).

It is now generally accepted that two largely independent mechanisms are responsible for regulating the beat frequency and the wave form of the flagellum. The mechanism regulating beat frequency is related to the dynein arms (ATPase), whereas the mechanisms regulating wave form appear more sensitive to the mechanical resistance at the flagellar base and to the properties of the radial spokes and the nexin links. Cyclic detachment and reattachment of radial spokes from the central sheath in the flagellum may also be an active process, driven by ATP (17).

Besides dynein arms, other active processes may also be involved in the regulation of sperm motility. The sperm flagella of mammals, gastropods, and many insects are surrounded by an additional set of nine coarse fibers which end in the principal piece (17). Gordon et al. (48), in their study of the flagella of guinea pig sperm, found Ca^{2+}-ATPase and calmodulin sites on the inner surface of the fibers. These authors suggested that the presence together of calmodulin, a Ca^{2+} binding regulatory protein, and the Ca^{2+}-ATPase close to the axonemal complex may control the concentration of Ca^{2+}, thus regulating dynein ATPase activity and sperm motility.

Cyclic nucleotides, one of which is formed from ATP, have also been implicated in the regulation of sperm motility (45, 50, 51). Wasco and Orr (48) studied demembranated rat caudal epididymal sperm cells and found a calmodulin-dependent cyclic nucleotide phosphodiesterase associated with their tails and heads. They confirmed findings of other investigators (52, 53) that the regulation of flagellar movement by cyclic adenosine monophosphate (cAMP) and by Ca^{2+} probably involves phosphorylation and dephosphorylation of specific axonemal proteins. This finding has been further confirmed (54) by the discovery of a human flagellar protein, axokinin, which is positively correlated with an increase in sperm motility. One possible mechanism whereby cAMP and Ca^{2+} could regulate sperm motility has been described by Wasco and Orr (48). An increase in Ca^{2+} concentration in the vicinity of the tail activates a calmodulin-dependent phosphorylation leading to an increase in

cAMP degradation. This degradation results in a decrease of protein phosphorylation in the flagellum due to the reassociation of the catalytic and regulatory subunits of flagellar cAMP-dependent protein kinase.

In order to fertilize an oocyte, spermatozoa must be separated from the seminal fluid and come into contact with the elements in the female genital tract to become capacitated. Spermatozoa can then move forward through the system toward the ampulla. Since 1978, when Edwards and Steptoe performed the first successful human IVF, this technique has gained increased importance worldwide. The IVF technique has eliminated the requirement for sperm cells to ascend the female reproductive tract, but capacitation, acrosome reaction, and oocyte penetration, which require vigorous flagellar movement, are still definite requirements for a successful IVF procedure. Some of these requirements necessitate tremendous energy expenditure.

When sperm and oocyte meet, penetration of the cumulus begins, and the outer acrosomal membrane and plasma membrane of the sperm fuse. Acrosomal contents (i.e. hyaluronidase, acrosin) are released and assist in the passage of sperm through the oocyte investments. Several factors such as pH, the egg factors, ATPases of sperm plasma membrane (i.e. Ca-ATPase) and possibly vigorous flagellar movements (hyperactivation) are involved in the acrosome reaction.

The following observations indicate that sperm energy metabolism is responsible for several events during

fertilization: a) When sperm cells are in a state of low flux (low metabolic activity, low flagellar activity), lactate, an end product of glycolysis, is not processed further by mitochondrial oxidation. It is therefore released into the medium surrounding the sperm. Lactate release may reduce activity of certain acrosomal enzymes (i.e. acrosin) which require higher pH for their optimal activity. Therefore, optimal energy production by spermatozoa and continuous use of energy via vigorous flagellar beating (high flux) prevents such a lactate release and possibly inhibits some of the acrosomal enzymes due to this release. b) Some sugars, especially fructose, are inhibitors of acrosin, which is an acrosomal proteinase implicated in sperm penetration through the zona pellucida (55). When sperm is in a state of low flux (low motility or low energy metabolism), either normally or abnormally, substrate cycling occurs. Exogenous glucose (or fructose if glucose concentration is low) is phosphorylated via glycolysis; the products of phosphorylation, glucose-6-phosphate and fructose-6-phosphate, are dephosphorylated by various sperm phosphatases. Glucose and fructose are then released into the surrounding medium (56). Fructose, which competes poorly with glucose for subsequent transport across the membrane and rephosphorylation, is excluded from re-entry until the extracellular glucose is consumed. This extra fructose in the microenvironment of spermatozoa-oocyte may inhibit acrosin. Substrate cycling and release of fructose into the surrounding medium will not occur if spermatozoa are

in a state of high flux (56).

Sperm flagellar movement seems to be very important for passage through the investments surrounding the oocytes. When a spermatozoon enters the viscous matrix of the cumulus oophorus, a typical whiplash pattern of hyperactivated movement may be physically constrained. However, the increased bending ability of the flagellum, which is probably due to an increase in Ca^{2+} concentration (17), becomes an efficient means of generating thrust against the zona (45). A spermatozoon in the process of passing through the zona pellucida, which is a tough and highly polymerized acellular layer, shows vigorous flagellar beating (45), suggesting that the sperm spends tremendous amounts of energy while penetrating the oocyte.

The importance of vigorous flagellar beating and a continuous supply of energy becomes more apparent when one considers the average time course of penetration when sperm and oocyte meet. Studies by Blandau (57) showed that sperm cells begin their penetration 15-60 minutes after being placed with the oocytes. A sperm must attach to the zona pellucida for at least 15 minutes before it begins penetrating the zona by making a narrow slit and passing through, following an oblique path into the perivitelline space (45). The average time required for the sperm head to penetrate the zona pellucida is 20 minutes. Studies in mice have shown that shortly before spermatozoa break through the inner surface of the zona, they often move back and forth as if in a channel, then suddenly penetrate the inner zonal membrane to enter the perivitelline

space. Sperm cells then plunge into the vitelline membrane in less than one second. At this time, flagellar beats slow down and shortly thereafter stop.

There are several types of sperm dysfunction during the interaction with an oocyte. It is possible that the sperm may fail to bind to the zona pellucida or that it may bind but not penetrate or only partially penetrate the zona (57). Could any of these malfunctions be potentiated by a decline in sperm energy metabolism and energy source ?

It seems that many sperm functions require energy expenditure and that ATP is the major mediator of these activities. Using pooled semen of fertile donors, Calamera et al. (58) determined that a human sperm contains approximately 150 moles of ATP and 85 moles of ADP. These researchers also showed that the motility of sperm is positively related to their ATP content. ATP content of motile sperm was significantly higher than that of immotile sperm (12 micrograms / 100 million sperm vs. 4.6 micrograms / 100 million sperm, respectively). Several authors (58-64) have investigated the possible role of ATP in sperm fertilizing capability. Unfortunately, findings have not been consistent.

Comhaire et al. (59, 60) in several investigations have shown that ATP determination is of value in the diagnosis of male infertility. They found (59) that the concentration of ATP per milliliter (ml) of ejaculate was significantly correlated with sperm against gravity (swim-up) and in vitro potential to the SPA. They also found that the ATP

concentration was significantly lower (10×10^{-7} M) in the semen of infertile men with normal sperm count and motility compared with matched fertile donors (87×10^{-7} M). Calamera et al. (58) and Vilar et al. (61) have found similar results.

Orlanda et al. (62) compared ATP and ADP content of human ejaculated sperm in fertile donors and infertile oligozoospermic individuals. They found significant correlation among ATP, ADP, sperm count, total motile sperm in the ejaculate, and viable sperm as assessed by 0.5% eosin Y staining. However, these investigators did not find any difference between the ATP/ADP ratio of fertile donors compared with that of oligozoospermic men.

Irvine and Aitken (63) and Chan and Wang (64), on the other hand, concluded that ATP measurement has limited value in the laboratory evaluation of sperm function. They found that the content of ATP (micromolar) in semen samples of 13 cryopreserved fertile donors and 39 infertile males was positively correlated with the SPA. However, a large part of this correlation was based upon the relationship between semen ATP and sperm count. Semen samples with higher count and good motility had better SPA results.

Chan and Wang (64) also compared ATP contents (mol/ml) of semen samples from 36 proven fertile donors with those of 233 infertile male individuals. The SPA and the hypoosmotic swelling test (HOST) were used to assess sperm fertilizing potential. They attempted to correlate ATP values with the results of these tests. They found a high-positive correlation

between seminal ATP (mol/ml) and sperm count, percent of motility, total number of sperm, total number of motile sperm in the ejaculate, HOST results, and SPA rates. However, they found no significance when the combined effect of sperm count and motility on SPA and HOST results was eliminated.

We have also been investigating the possibility of using ATP measurement as a marker for the evaluation of sperm fertilizing potential. In one study (unpublished) we determined the ATP content of semen samples from 18 donors who were regularly used in the SPA. Samples obtained from these donors at various time intervals showed significant variation in ATP content, count, motility, and SPA results. Likewise, when evaluating 84 infertile males, we found no correlation between whole semen ATP or swim-up ATP concentrations with the outcome of the SPA. Irvine and Aitken (63) stated that "the subcellular biochemical economy of the sperm cell is complex and beside ATP there are many other factors influencing the fertilizing potential of the sperm". Despite this statement, the role of energy metabolism in the overall fertilizing capability of sperm cannot be disputed. Although many investigators have evaluated seminal ATP contents of both fertile and infertile groups, results cannot be critically analyzed or compared in view of the differences between semen samples in such elements as round cells, time elapsed after ejaculation, multiplicity of noncomparable data, and inadequacy of measurement techniques. Several lines of evidence indicate that studies used to evaluate ATP determinations were carried

out under less than optimal conditions.

The commonly accepted viewpoint is that human sperm cells are able to synthesize the ATP needed for motility and other energy-requiring processes by metabolism of exogenous substrates, either by glycolysis or by mitochondrial oxidative phosphorylation (30, 69). If the glycolytic cycle stops completely, respiration can maintain full motility of the sperm (30). ATP thus formed can diffuse to more distal parts of the flagellum or to the parts of the sperm where it is needed. Considering several recent reports (22-24), it was concluded that ATP alone may not be the only factor involved in the energy metabolism of spermatozoa.

Saks et al. (22), in their studies of cardiac muscle cells, reported that although ATP splitting occurs in muscular contractions, the utilization of ATP cannot be detected in contracting muscles. This finding means that ATP concentration remains basically constant even at low temperature, where ATP recovery in glycolysis or oxidative phosphorylation does not occur rapidly. To evaluate the priority of ATP breakdown in muscle contraction, they inhibited the creatine kinase (CK) reaction in muscle cells using 1-fluoro-2,4-dinitrobenzene (FDNB). CK is a dimer, composed of two units with a relative molecular mass of 50,000. Three isoenzymes are present in human tissues: CK1 (BB), present predominantly in nerve tissue but also found in the thyroid, kidney, and intestine; CK2 or MB, present in heart muscle, the diaphragm, and esophagus (smooth muscle); and CK3 or MM, the most common form, present

in all tissues, especially skeletal muscle. On electrophoresis, the CK1 isoenzyme (BB) migrates most rapidly toward the anode (+) and is found in the pre-albumin area. The slowest moving form, CK3 (MM), is found in the gamma globulin region, whereas the heart form, CK2 (MB), with an intermediate mobility, is located in the α_2-globulin zone (19). The enzyme is responsible for the reversible conversion of creatine phosphate and ADP to ATP and creatine (Fig. 11). When CK was inhibited using FDNB, the contraction cycle was accompanied by ATP utilization at a constant creatine phosphate level. The researchers concluded that in contraction, ATP was utilized, but ADP produced by ATP breakdown was immediately rephosphorylated in the CK reaction at the expense of creatine phosphate. As a result, a new amount of ATP needed for subsequent contraction was produced.

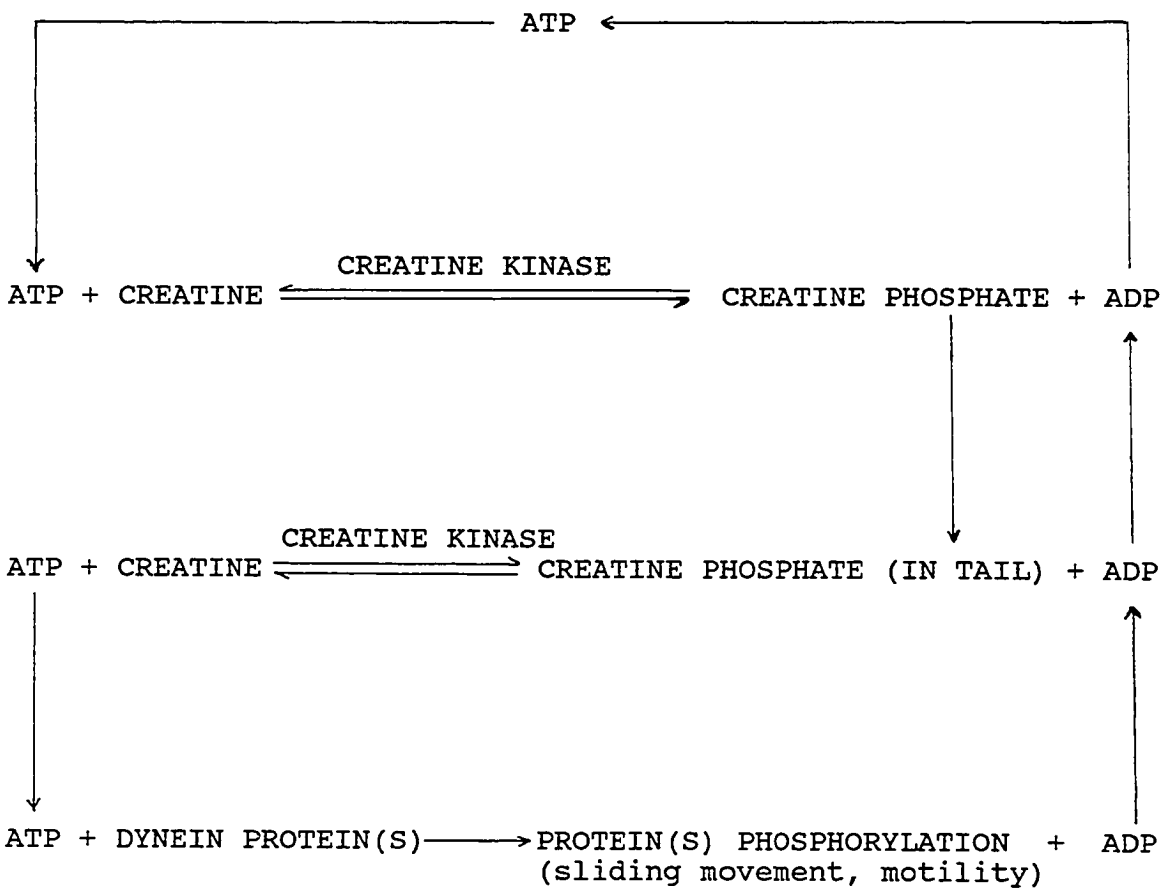
Studies of cardiac muscle cells (22) have shown that available energy production was inhibited by inducing anoxia or ischemia, or using inhibitors of glycolysis and oxidative phosphorylation. These studies have shown that under these conditions, cardiac contraction stops at high levels of intracellular ATP, with a rapid decline of creatine phosphate content. The reason is that the equilibrium constant of the CK reaction (K' = Mg-ADP + creatine phosphate/Mg-ATP + creatine) is very low and the ATP production from creatine phosphate and Mg-ADP is kinetically favorable (22). Why is creatine phosphate synthesized despite the fact that its breakdown is kinetically favored? Probably because of the different kinetic

properties of mitochondrial CK isoenzymes, may be sensitive to intramitochondrial ATP. If ATP is synthesized in the mitochondria and creatine is present, creatine phosphate is formed regardless of the concentration of extramitochondrial ATP. Why, then, does contraction stop in the cardiac muscle even though ATP concentration is high? Perhaps because much of the cellular ATP may not be directly accessible for utilization in contraction.

Studies on CK and cardiac muscle cells have shed light on the problem of intracellular energy transport. Two conclusions have been reached. The first is that ATP may be functionally compartmentalized and unable to ensure rapid transport of energy in muscle cells. The second conclusion is that the creatine phosphate pathway of energy transport involves several CK isoenzymes, each responsible for different functions and each present in different regions of the cell (i.e., MB and BB isoenzymes are myoplasmic).

In relating the above finding to mechanisms of sperm energy production and expenditure, Tombes and Shapiro (23) have studied the possible involvement of CK in the overall metabolic activity of sea urchin sperm. This sperm provides an excellent system to examine the significance of the creatine phosphate shuttle in cell regulation and energy metabolism. As with cardiac muscle (22), the utilization of energy by sea urchin sperm is strictly coupled to its production (23). ATP is produced exclusively by mitochondrial respiration and is utilized primarily for sperm motility (23). It is commonly

ATP FROM GLYCOLYSIS AND/ OR
MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION



ATP is used as an immediate source of energy for maintenance of sperm motility and osmotic balance. It is believed that creatine phosphate is used as a reserve source of energy. It is converted to ATP when it is needed.

believed that the ATP which is formed in the proximal end of the flagellum (midpiece) can reach its more distal parts by diffusion. This diffusion accounts for energy transport for sperm motility (23). However, some groups (17, 23) have demonstrated that the large pool of creatine phosphate in sperm is depleted and resynthesized when motility is induced and then inhibited by altering intracellular pH. Sperm might therefore employ a creatine phosphate shuttle to mediate phosphate energy transport from the mitochondria to the flagellum (Fig. 6). This shuttle would have the advantage of ensuring high concentrations of ADP, which is much needed in mitochondria at the time of maximal activity or vigorous flagellar activity. In this case the respiration of motile sperm cannot be increased by mitochondrial uncoupling agents (23).

Another advantage of a creatine phosphate shuttle is that it makes creatine phosphate not directly accessible to dynein ATPase molecules of the flagellum. This energy pool is converted to ATP by a specific CK isoenzyme. Two CK isoenzymes, mitochondrial and flagellar, exist for creatine phosphate synthesis and utilization, respectively (23). Inhibitors of CK (FDNB) inhibit both respiration and motility. Respiration is inhibited because of the decreased ADP availability to the mitochondria, and motility is affected, since ATP replaces creatine phosphate as the diffusing metabolite. Under these conditions, dynein ATPase of the proximal region of the flagellum preferentially uses ATP, disrupting normal production of flagellar waves. Flagellar

bending occurs only in the proximal third of the tail, whereas the distal two-thirds remain stiff. Sperm cells under this condition move less vigorously through larger arcs. This change in the sperm motility pattern indicates that, under normal conditions, creatine phosphate is the diffusing energy unit in the flagellum. Otherwise, ATP could diffuse along the entire length of the flagellum, allowing a normal motility pattern.

Creatine phosphate may well be involved in energy metabolism of the sperm cells (26). Sea urchin sperm cells treated with Triton X-100 became motile upon addition of either ATP or a combination of creatine phosphate and ADP. Addition of creatine phosphate or ADP alone did not induce motility as long as AMP was added to inhibit myokinase.

Although sea urchin sperm cells are a convenient vehicle for the demonstration of the creatine phosphate shuttle, similar systems may operate in sperm cells of other species (23). Human sperm motility and velocity can be increased significantly by addition of creatine phosphate (24).

Considering the relationship between ATP and creatine phosphate, it is believed that, under normal circumstances, the following cycle occurs in the energy metabolism of sperm:

a) ATP is synthesized via glycolysis and/or mitochondrial oxidative phosphorylation. It is converted to creatine phosphate by specific CK isoenzymes and is carried to various parts of the sperm, where it can be used as a reserve source of energy.

b) To maintain major energy-requiring sperm functions (i.e. motility, maintenance of osmotic balance, and other membrane functions), ATP is utilized as an immediate source of energy. ATP is formed from creatine phosphate by a specific type of CK isoenzyme. ATP concentration is kept at a constant level at the expense of creatine phosphate.

c) Creatine phosphate is replenished from the ATP synthesized via glycolysis and/or mitochondrial oxidative phosphorylation, and the cycle continues. Under unusual or abnormal conditions, the cycle may be interrupted. A normal level of immediate energy (ATP) concentration may not indicate that the concentration of reserve energy (creatine phosphate) is at the required level.

About 70% of spermatozoal ATP is consumed to maintain its motility at 37°C (30). Beside motility, sperm has to maintain its osmotic balance, which is also a crucial part of sperm function. This is accomplished partly via different ATPases present in the head, midpiece, and tail sperm membrane (25), which also serves as a barrier preventing energy loss (26). Having an intact and functional membrane is therefore critical for a spermatozoon. Sperm cells with intact membranes incubated in a hypoosmotic medium resist the change by absorbing excess water which causes them to swell and form coiled or thickened tails. This technique, HOST, has been used to assess the integrity of sperm membrane (27, 33).

2. Hypoosmotic swelling test (HOST) and its significance

Mammalian sperm cells emerge highly differentiated and are capable of acquiring a mature motility pattern and fertilizing ability. This process of sperm maturation involves a series of morphological and biochemical modifications which are obvious in the plasma membrane (22). The sperm membranes are also implicated in other important transformations such as motility (i.e., Ca-Mg ATPases and Na-K ATPase), controlling the effect of external and internal factors regulating sperm, capacitation, acrosome reaction, and sperm-oocyte fusion. A functionally incompetent membrane may cause severe disturbances in many sperm functions, such as abnormal motility patterns and excessive energy loss through the membrane. White et al. (26) observed that the motility of sea urchin sperm cells treated with Triton X-100 ceased a short time after the treatment, and ATP content of sperm cells declined drastically. They concluded that membrane damage stops motility of sperm primarily because of a loss of endogenous ATP. Loss of creatine phosphate is also a possibility, which they did not investigate.

To develop a simple technique for assessing functional integrity of the sperm membranes, Drevius and Ericksson (72) demonstrated that exposing bovine sperm to hypoosmotic conditions resulted in an influx of water across the sperm membrane which, in turn, caused an increase in sperm volume and bulging or swelling of the plasma membrane. The sperm tail appeared to be particularly susceptible to such a change in

osmolarity of the medium and exhibited morphological features such as coiling or thickening (Fig. 12). This test, HOST, is based on the assumption that the ability of the sperm tail to swell in the presence of a hypoosmotic solution is a sign that the membrane is functionally active and intact.

More recently, Jeyendran et al. (27) evaluated the functional integrity of sperm membranes in 23 semen samples of a mixed population of fertile and infertile individuals. They observed a good positive correlation ($r=0.90$) between the percentage of sperm in a semen sample which were capable of undergoing swelling and the percentage of hamster zona-free oocyte sperm penetration (SPA). Evaluating the HOST results from 83 IVF patients, Van der Ven et al. (33) found that under the conditions of their studies the HOST was a more accurate predictor of a successful IVF outcome than the conventional semen parameters. Semen samples with > 60% of the sperm cells capable of undergoing swelling exhibited higher fertilization capability. Neither group investigated ATP and CK content of the semen samples. Evaluating 271 infertile and fertile individuals, Chan et al. (71) did not find a high correlation between the HOST and the outcome of the SPA. They did, however, find that the semen samples with abnormal semen parameters (i.e. motility and morphology) had lower percents of swelling than those with normal semen parameters. A weak positive correlation was also observed between the percentages of swelling and sperm motility ($r=0.22$, $p<0.05$).

The importance of sperm energy metabolism in fertilizing

ability cannot be disputed. Many causes of infertility may be related to defects in sperm energy production, transfer, and utilization. It is essential to assess the energy availability of the sperm in terms of immediate energy level (ATP), reserved energy source (CK), and membrane integrity, as measured by the HOST.

3. Sperm morphology and its significance

Besides spermatozoal energy levels and the functional integrity of the plasma membrane, there are other factors which may alter the fertilizing potential of spermatozoa. Sperm morphology is one of these factors. Evaluation of sperm morphology can be a sensitive index of the germinal epithelium. Unfortunately, for years emphasis has been put on assessment of the types and percentages of abnormal sperm forms, rather than normal forms. Although normal spermatozoa have been morphologically defined, the percent of normal forms has usually been determined indirectly by evaluating the percentage of abnormal sperm forms. Reports of abnormal forms varying from 14 to 40 types are indicative of that trend (28). Kruger (73) recently introduced his new strict method of assessing normal sperm morphology. In his study of Norfolk IVF patients, he showed a good correlation between IVF outcome and his method of assessing sperm normal morphology. Since then, I have employed his technique and have introduced some modifications. The results have substantiated the value of this technique for evaluating male partners of infertile couples applying for IVF.

It is believed that determination of ATP, CK, HOST, and the percentage of normal sperm morphology for male fertility potential correlate well with IVF outcome.

CHAPTER III

STATEMENT OF THE RESEARCH

The design of the study is to satisfy the following specific aims:

A. Specific Aim 1

Since about 20% of infertile males show no apparent abnormal semen parameters (1), I will attempt to identify new factors which may influence infertility. My preliminary investigation showed that in some of these individuals, ATP content of spermatozoa was significantly lower than in those who had produced a pregnancy in Norfolk IVF program ($p<0.002$). These preliminary results indicated that the modified method of ATP determination might be of value in evaluating such patients further. The study will also include seminal CK activity and HOST determinations of the samples from these individuals. In addition, our computer-assisted technique of evaluating basic semen parameters will be enormously valuable in determining these parameters more accurately than by manual methods. Semen samples from at least 30 individuals with idiopathic infertility will be chosen on a random basis. The following questions will be investigated:

1. What range of values exists for seminal ATP in males with idiopathic infertility? Do these values show any relationship

with the outcome of IVF when the evaluated semen sample is used to inseminate the oocyte? In a study of IVF outcome, factors such as fertilization rate/oocyte, cleavage rate, number of embryos transferred, number of cells/embryo, clinical pregnancy rate/cycle and per transfer, miscarriage rate, and term pregnancy will be evaluated.

2. Is there any association between the HOST and IVF outcome? Is the association (if any) similar to that of ATP and/or CK activity? At least 25 semen samples from IVF patients whose CK activity and ATP have been determined will be evaluated by the HOST.

3. Is there any association between sperm morphology (percent of normal sperm) and IVF outcome?

4. Is there any relationship between the basic semen parameters of men in the IVF study and the range of values in their seminal ATP, CK activity, and HOST?

5. Do all men with low or high seminal ATP show similar seminal CK activity and HOST trends? Are these trends related to IVF success or failure?

B. Specific Aim 2

In an IVF trial, semen samples are processed for a swim-

up recovery, and the most motile spermatozoa recovered are used to inseminate the oocyte. Our preliminary results indicated that, in predicting the outcome of IVF, swim-up determinations of ATP are of more value than whole semen determinations. Moreover, I found that the ATP content of spermatozoa drops drastically in some, but not all, men whose semen samples have been processed for a swim-up. To further elucidate these findings, swim-up ATP of at least 100 men with normal semen parameters participating in Norfolk IVF program will be determined. These samples will be tested by on the protocol outlined above. The following questions will be investigated:

1. What range of values exist for swim-up ATP in males with no apparent cause of infertility? Do these values show any relationship with the outcome of IVF when the same sample is used to inseminate the oocyte? At least 100 swim-up samples will be evaluated.

2. Is there any association between swim-up CK activity and the outcome of IVF? Does this association (if any) correlate with that of ATP and IVF outcome? At least 25 swim-up samples will be evaluated.

3. What is the relationship of swim-up HOST and IVF outcome? Is the association (if any) similar to that of ATP and/or CK activity determined from the sample? At least 25 swim-up samples will be evaluated.

4. What is the relationship between whole semen ATP and swim-up ATP? Do all samples with a drastic drop in ATP following swim-up perform poorly in IVF? How does this drastic drop (if so determined) correlate with the CK and HOST values obtained from the same semen and swim-up samples?

5. Are the results of swim-up ATP, CK activity, and HOST predictive of IVF results?

6. What is the relationship between basic swim-up parameters determined by computer assisted analysis and IVF outcome? Is there any association between basic swim-up parameters and swim-up ATP, CK activity, or HOST? How does this association (if any) correlate with IVF outcome?

C. Specific Aim 3

Forty to fifty percent of infertility is due to semen abnormalities, namely, poor sperm count, motility, and morphology (59). In most cases, however, no identifiable cause(s) for the poor semen quality can be found.

It is possible that spermatozoal energy imbalance in the form of ATP deficiency and/or abnormal CK activity or membrane defects may be shown by the HOST to have a role in the abnormalities observed in spermatozoa. To reduce multiple factors in the study, semen and swim-up samples from at least 100 individuals with only abnormal sperm morphology will be

evaluated to determine whether, besides their known morphology problem, there are other (metabolic) abnormalities observed. Can that problem be related to the results obtained from their seminal ATP, CK activity, and HOST? The following questions will be investigated:

1. What is the association between abnormal semen or swim-up morphology and the IVF outcome? Do all individuals with abnormal semen and/or swim-up morphology fail to fertilize oocytes or produce pregnancies? Do patients with the poorest morphology pattern perform the worst in an IVF attempt?

2. What range of values exists for seminal and swim-up ATP in men with morphology problems? Do these values show any association with abnormal semen parameters? What is the relationship between seminal and /or swim-up ATP and IVF outcome? Do men with morphology problem but successful IVF results (if any) show different ATP values than those of the same group but with failed IVF?

3. Is there any association between seminal or swim-up CK activity and the outcome of IVF in these patients? Does this association (if any) correlate with ATP or other basic semen parameters?

4. Do these patients, when compared with non-male factor patients, show more pronounced drops in ATP content of

spermatozoa when their semen samples are processed for swim-up? How does this decline (if any) correlate with IVF outcome?

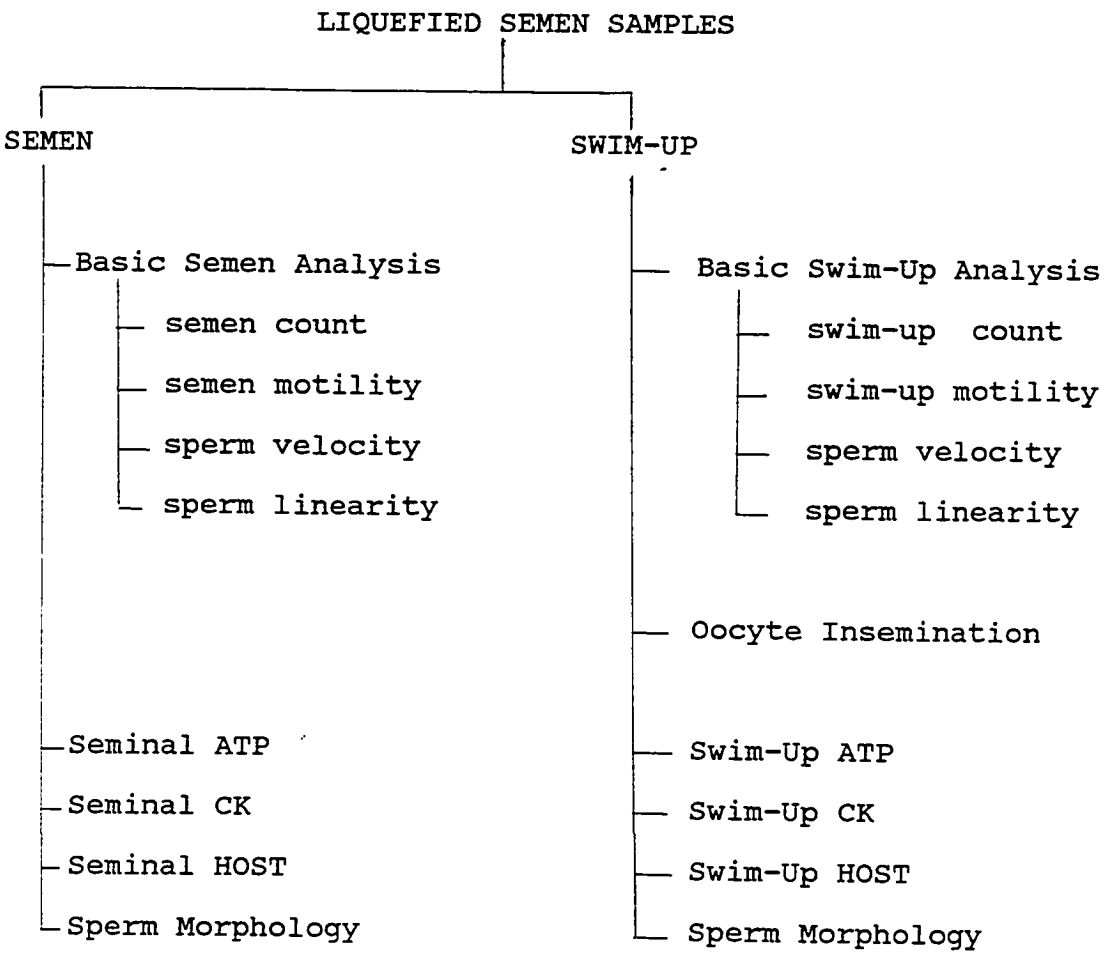
D. Specific Aim 4

The last goal is to devise a formula or formulae to predict the outcome of IVF based on the values obtained from these tests. It is equally important to evaluate whether these values can be repeated when patients are screened before their acceptance in to the program. An important reason for predicting IVF outcome is to assess the chance of an individual for success before, and not at the time of, the procedure.

CHAPTER IV

MATERIALS AND METHODS

A. Outline of the Study Protocol



B. Experimental Techniques

1. Automated analysis of human semen

Various methods for the objective determination of human semen parameters have been developed. These methods fall into two categories. The first are those methods which provide an overall measurement based on the displacement of a large number of spermatozoa; the second are those which provide individual measurements of trajectories from a smaller number of spermatozoa. The first consists essentially of physical methods (92). These include a) the measurement of changes in impedance, b) the measurement of the intensity of the diffused light, c) microphotometry, d) the measurement of optical density, e) measurement of ultraviolet absorption, and f) laser light scattering.

Such methods of measurement are rapid but provide little information on the kinetic heterogeneity of a semen sample. They provide a quantitative, generic description of sperm motion. We must be able to assess this heterogeneity, since a semen sample of poor motility may contain a sufficient number of spermatozoa with normal motion characteristics which these methods cannot measure (92).

The other methods are based on image analysis, the basic principles of which are 1) recording the images of moving spermatozoa in a microscopic field of observation, 2) reconstructing the trajectory for each spermatozoon by the analysis of sequential images from the recording, and (3)

describing the trajectories by appropriate parameters (velocity, lateral head displacement, beat cross frequency, etc.). Several types of recording techniques such as cinematography, still camera multiple exposure track analysis, videographic analysis, and computerized videomicrography are currently being used for sperm motion analysis.

Time-exposure photography (e.g., cinematography, still camera tracking) is time-consuming. Although fairly precise, this direct micrographic method, which analyzes individual spermatozoa, necessitates many measurements. Even when computerized, this method requires as much as 10 to 20 minutes per sample, making it unsuitable for routine use (93). A videomicrography system eliminates the necessity of developing 35-mm negatives; however, it does not eliminate the subjectivity introduced by the human evaluation of videotapes.

In our laboratory, semen samples are analyzed by an automated system analyzer (Cellsoft Cryo Resources, Ltd., New York, N.Y.) equipped with an updated software version capable of performing a sophisticated evaluation of sperm motion characteristics (Fig. 7). The software in the system can extract information on sperm location in successive video frames and further process it to provide comprehensive data on sperm motion.

The Cellsoft System includes an IBM AT computer (Turnkey System, International Business Machines Corp., Boca Raton FL.) with a monochrome monitor, Okidata dot matrix printer (Okidata, Tokyo, Japan), Olympus BH-2 phase-contrast microscope (Olympus

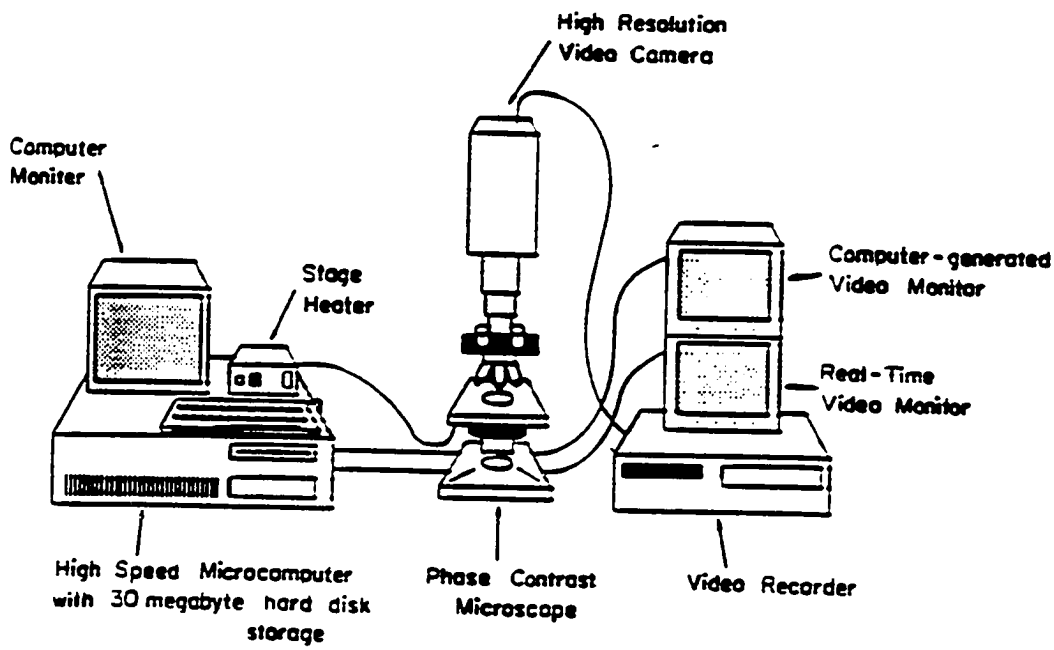
Optical Co., Ltd., Tokyo, Japan) equipped with a model MP-100M stage warmer and with 10x and 20x negative high-phase contrast objectives and 6.7x projection ocular, high-resolution video camera, two cathode ray tubes as video monitors (GBC, Taiwan), video recorder (Panasonic, Denver, CO.), Makler counting chamber (Sefi Medical Instruments, Israel) and the Cellsoft software for automated motion analysis (Fig. 7).

To prepare samples for analysis, all fresh samples are obtained by masturbation after 2-5 days of abstinence and are evaluated for basic semen analysis within 30-45 minutes after ejaculation. For the analysis, each sample is mixed well, and 5 microliters (µl) are put into a Makler chamber and placed on the stage warmer of the Cellsoft microscope. At least 200 cells and/or 4 different fields on the Makler chamber are analyzed. Attention is paid to having a uniform distribution of cells in the chamber and not choosing the fields with the highest number of spermatozoa.

When the chamber containing semen is placed on the microscope stage of the system and is focused, an image of the field is transferred to the video monitor through the video camera. The video images are digitized by the system for calculation of sperm motion characteristics and final data output. The computer processor stores sequential images (frames) of each spermatozoon and electronically superimposes it to form the video equivalent of a multiple-exposure photograph. The system therefore relies on image size, shape,

Figure 7.

Major components of an automated system used for computerized evaluation of sperm concentration and motion characteristics



and/or threshold grey level (luminescence, the negative image in the second video monitor of the objects in the first video monitor), and the thresholds setting to distinguish putative spermatozoa and differentiate them from semen debris (germ cells, white blood cells, etc.) that may be present in the field of view (Table 3). The computer acquires the video data at 30 frames/second and converts the images into a digitized one with a resolution of 512 x 512 pixels (pixel=0.688 μm). Objects analyzed are those within the central portion of this image consisting of a 320 x 400-pixel matrix representing an area of 215 x 265 μm. Due to the light effect, the sperm head, appearing luminescent, is observed on the first monitor. A threshold grey level is arbitrarily set for each field of view to erode the sperm image on the second monitor until the middle piece and flagellum are no longer visible. This setting also filters out other debris found in semen, which differ markedly in size and luminosity from spermatozoa.

The computer evaluates the digitized image of any object in the central field from pixel area setting (4 pixels for low setting and 25 for high setting) and luminosity; the computer determines whether the object is a spermatozoon or debris. If the object is a spermatozoon, the centroid is calculated and its X- and Y- coordinates are stored. Uncertainty in the location of the centroid is <0.5 pixel or 0.05 μm at the magnification used. The image is tracked for about 30 frames ; only spermatozoa present in the first through "n" frames are analyzed. Minimum duration of tracking, <n, is set for

TABLE 3.

GENERAL PARAMETERS (SETTINGS) USED FOR COMPUTERIZED SEMEN ANALYSIS

PARAMETER	VALUE
Sampling frequency (frames/second)	30
Number of frames to analyze	15
Maximum velocity (micron/second)	200
Minimum velocity	10
Minimum number of frames for motility analysis	2
Minimum number of frames for linearity analysis	8
Minimum number of frames for velocity analysis	8
Pixel size (micron)	0.688
Minimum size to exclude an object from analysis (pixels)	4
Maximum size to exclude an object from analysis (pixels)	25
Minimum number of frames for calculation of lateral head displacement (μm)	7
Minimum velocity for the calculation of lateral head displacement	16
Minimum linearity for the calculation of lateral head displacement	3

calculation of each parameter (Table 3). Based on the coordinates of each centroid, a circular area of possible location in the next frame is calculated for each spermatozoon. This circle has a radius equal in pixels to 1/30 of the setting for maximum velocity. If a spermatozoon is detected within this area in the next frame, it is assumed to be the same cell. If a second centroid is in the same area of possible location, the computer considers the paths of the two spermatozoa. Should this occur before a specified number of frames for a given parameter are digitized, analysis of such paths is terminated. The thresholds shown on Table 3 are chosen to best fit the characteristics of both freshly ejaculated and swim-up preparations of human spermatozoa. To determine these optimal settings, spermatozoa from several different samples can be analyzed different times by the system and be looked at on the video monitor. Settings can be altered in a systematic fashion until the number of motile and immotile spermatozoa detected by the computer approximate the number the observed. A limit of 15 frames was chosen for the analysis of semen parameters, because our study using 5 to 20 frames/field indicated that the variation about the mean reached a minimum when at least 12 frames/field were analyzed. A minimum velocity threshold of 10 μm/second was chosen to exclude movements due to cell collisions or drifts in the crowded field of analysis. Apparent movement of centroid because of variations in the image (hollow around unfocused images) is also excluded by this minimum setting. A minimum velocity of 20 μm/second was not

chosen in order to include most moving spermatozoa which may have lower velocities. A setting of 200 um/second was chosen because most spermatozoa rarely move more rapidly, and a higher cutoff would increase the area of possible location of a centroid in successive frames and needlessly increase the number of colliding spermatozoa and intersecting paths. Setting eight successive frames as the criterion for determination of linear velocity and linearity maximizes the number of spermatozoa included in these calculations without severely increasing the variation about the mean of these parameters. A requirement for greater than eight successive frames would markedly decrease the number of spermatozoa analyzed for velocity and linearity determinations. A requirement for less than eight successive frames might increase the number of spermatozoa included in the calculations, but, according to my findings, it would significantly increase the variation about the mean for these parameters. Accuracy of linear measurements was verified using the projected image of a hemocytometer to calibrate the video screen and compare the straight line path of a spermatozoon with that derived from straight line velocity. No significant difference was observed using these settings.

The computer output for each field analyzed includes the following:

a) Number of putative spermatozoa identified in the first frame represents the concentration of spermatozoa and is reported as million sperm/ml semen.

b) Total number and percentage of spermatozoa (percent of motility) tracked in two frames of analysis with a linear velocity > 10 µm/second.

c) Mean linear velocity (µm/second) of all motile spermatozoa tracked for at least eight successive frames and the cumulative frequency distribution of these cells at 10 um velocity increments. Linear velocity is calculated by adding the distances of the linear path (an approximation of the actual path) between the position of a sperm centroid in all successive frames and dividing the interval during which the cell was tracked (Fig. 8A). Straight-line velocity is calculated from the straight line path between the first and last positions of a centroid (sperm head). Curvilinear velocity is an equivalent term for linear velocity (Fig. 8A).

d) Mean linearity of all motile spermatozoa tracked for eight successive frames and their cumulative frequency distribution at one-unit intervals. The linearity is calculated by dividing the length of the straight line distance of cell track (straight line velocity) by the linear velocity, multiplied by 10. A value of 10 indicates that the sperm swam in a perfectly linear manner. Small values are indicative of spermatozoa that swam in a nonlinear manner.

e) The motility index is calculated as the mean linear velocity multiplied by percentage of motile sperm (percent of motility).

f) Mean amplitude of lateral head displacement for motile spermatozoa. Amplitude of lateral head displacement (um) is twice the lateral head displacement or distance the centroid (head) deviates from the average path and is an estimate of the shift or yaw of a sperm head (Fig. 8B). The lateral displacement of the centroid in each frame is multiplied by 2 to transform the data from a half to a full cycle, and the values are averaged for each spermatozoon.

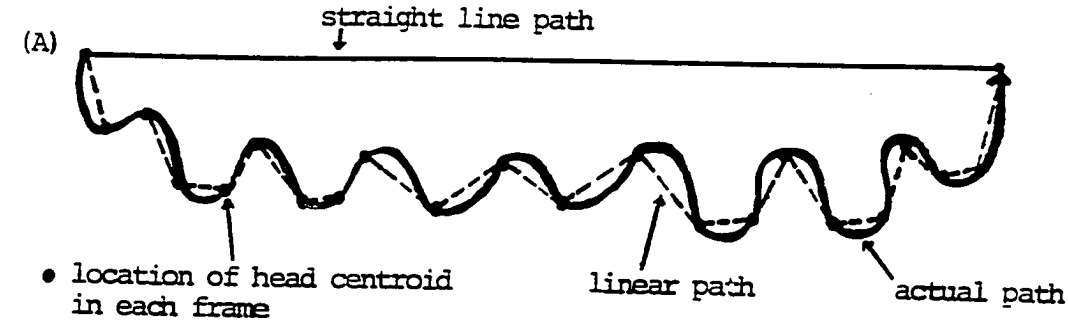
g) Mean beat cross frequency (1/second) is the frequency with which the linear path of the centroid crosses the average path (Fig. 8B).

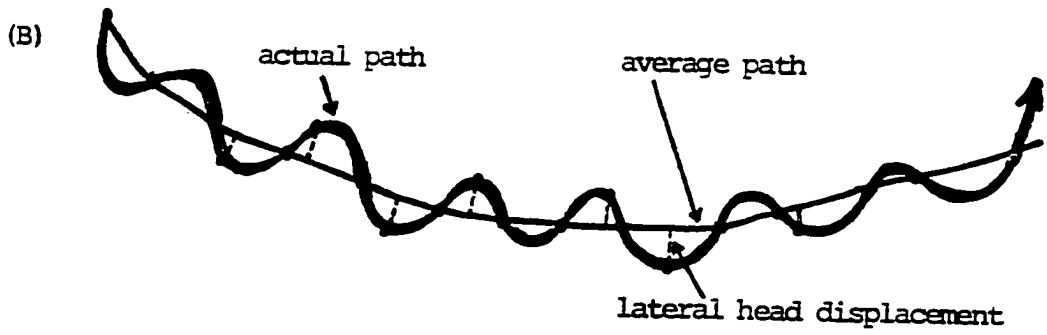
h) Percentage of motile spermatozoa with circular motion. A spermatozoon is considered to have a circular path (motion) if all tangents from the average path are on the same side of the path (Fig. 8D) and the radius of the average path (circular) is less than a designated value for the radius. Spermatozoa with an average path radius of <80 um are considered to have circular motion (Fig. 8D). In contrast, spermatozoa with more linear motion (Fig. 8C) have tangents from their average paths on both sides of the path.

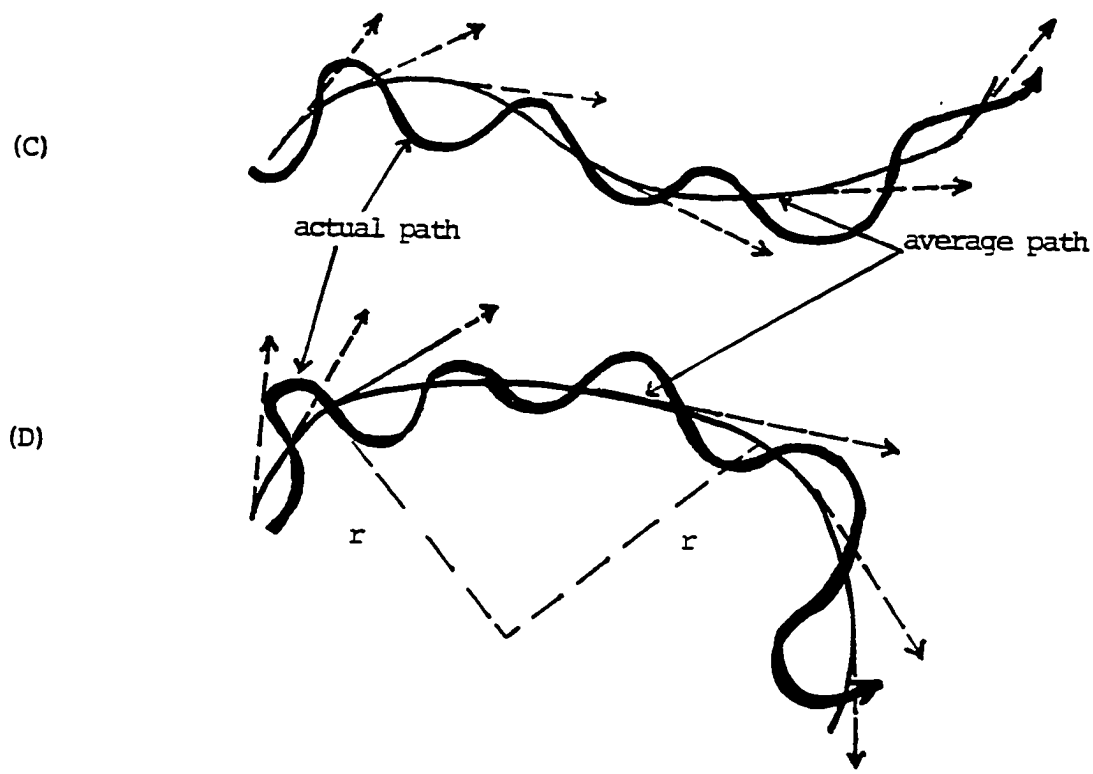
i) Percentage of progressively motile spermatozoa can also be calculated, but without the aid of the system, by subtracting the percentage of cells with circular motion from the percentage of motile spermatozoa determined by the system.

Figure 8.

Path of the centroid of a sperm head and parameters measured by an automated semen analysis system







2. ATP Determination

a. Preparation of semen specimens: Semen samples were obtained from individuals for human IVF as well as for ATP, CK, HOST, and preparation of two slides for morphology evaluations. All specimens were collected in special containers by masturbation, allowed to liquefy for 30 minutes, and evaluated for sperm concentration, motility, and other basic semen parameters via computer-assisted image analysis (Cellsoft). Only samples with normal count (≥20 million/ml), motility (≥40%), and number of round cells (≤1 million) were included in the study. Samples were immediately used for whole semen ATP, CK, HOST, and morphology evaluations or were processed for swim-up in Ham's F10 supplemented with 7.5% fetal cord serum (FCS). Swim-up preparations were then used to inseminate the oocyte and at the same time were processed for swim-up ATP, CK, morphology, and HOST determinations. Approximately 10 µl of semen were used to prepare duplicate slides for semen morphology assessment.

b. Swim-up preparation: After liquefaction for 30 minutes, 1-2 ml of semen was aliquoted into 4-6 centrifuge tubes and combined with an equal volume of Ham's F10 supplemented with 7.5% human fetal cord serum (H-FCS). Tubes then were centrifuged for 10 minutes at 270 x g, and supernatants were discarded. The same amount of H-FCS was added, pellets resuspended, and centrifugation was repeated once, followed by discarding the supernatants. Fresh H-FCS was gently layered over the pellets, and the specimen tubes were

incubated for 1 hour at 37° C in 5% CO_2 in air. Finally, the supernatants, containing the motile sperm fraction, were collected, pooled, and used for IVF as well as for CK, HOST, and ATP measurements, and preparation of two swim-up morphology slides.

c. Chemiluminescence methodology for ATP determination: Of the various methods - spectrophotometry, fluorometry, radioimmunometry, and enzyme immunometry - available for detecting, monitoring, or analyzing biological compounds, luminescence is one of the safest, simplest, and most sensitive methods for many applications. The technique, which was extensively studied in the 19th century with the pioneering work of Dubois, has not been widely received until recently because of the lack of adequate instrumentation, the limited availability of reagents, and the lack of methodology for many applications. Luminescence methodologies have been developed for a wide variety of applications. Determination of cellular ATP and creatine phosphate content is one of them (75).

Chemiluminescence may be defined as the chemical production of light, often confused with fluorescence. The difference between them is the source of energy that produces molecules in an excited state. In fluorescence, an incident light source is needed to excite the molecules to the singlet state, after which they return to the ground state with the emission of light of a higher wavelength. This process is temperature-independent and has a very short life. By contrast, in chemiluminescence the creation of an excited state

is through an enzyme-catalyzed reaction (a chemical reaction), and the decay from the excited state back to the ground state is accompanied by emission of light which is temperature-dependent, long-lasting, and measurable by a special photometer.

There are numerous terrestrial and marine organisms that emit light. Some of these systems have been applied in analysis of cell metabolism. Firefly luciferin and luciferase have been prepared in a highly purified crystalline state from Photinus pyralis. A luciferin-luciferase mixture, in the presence of molecular oxygen, reacts with ATP and produces light. In the presence of luciferin and Mg-ATP, luciferase catalyzes a reaction in which adenylate is transformed from ATP to the carboxyl group of luciferin. The enzyme-bound luciferyladenylate interacts with molecular oxygen through a series of steps leading to the decarboxylation of luciferin, leaving the enzyme-bound product (oxyluciferin) in the excited state (Figs. 9 and 10).

Figure 9. Reactions in the ATP assay

$$COOH\text{-}LH + Mg\text{-}ATP + E \longrightarrow E.LH + AMP + ppi$$

$$E.LH + AMP + O_2 \longrightarrow E.P^* + CO_2 + AMP$$

$$E\text{-}P^* \longrightarrow E + P + light\ (560\ nm)$$

KEY:

LH: Luciferin

E: Luciferase

*: Excited State

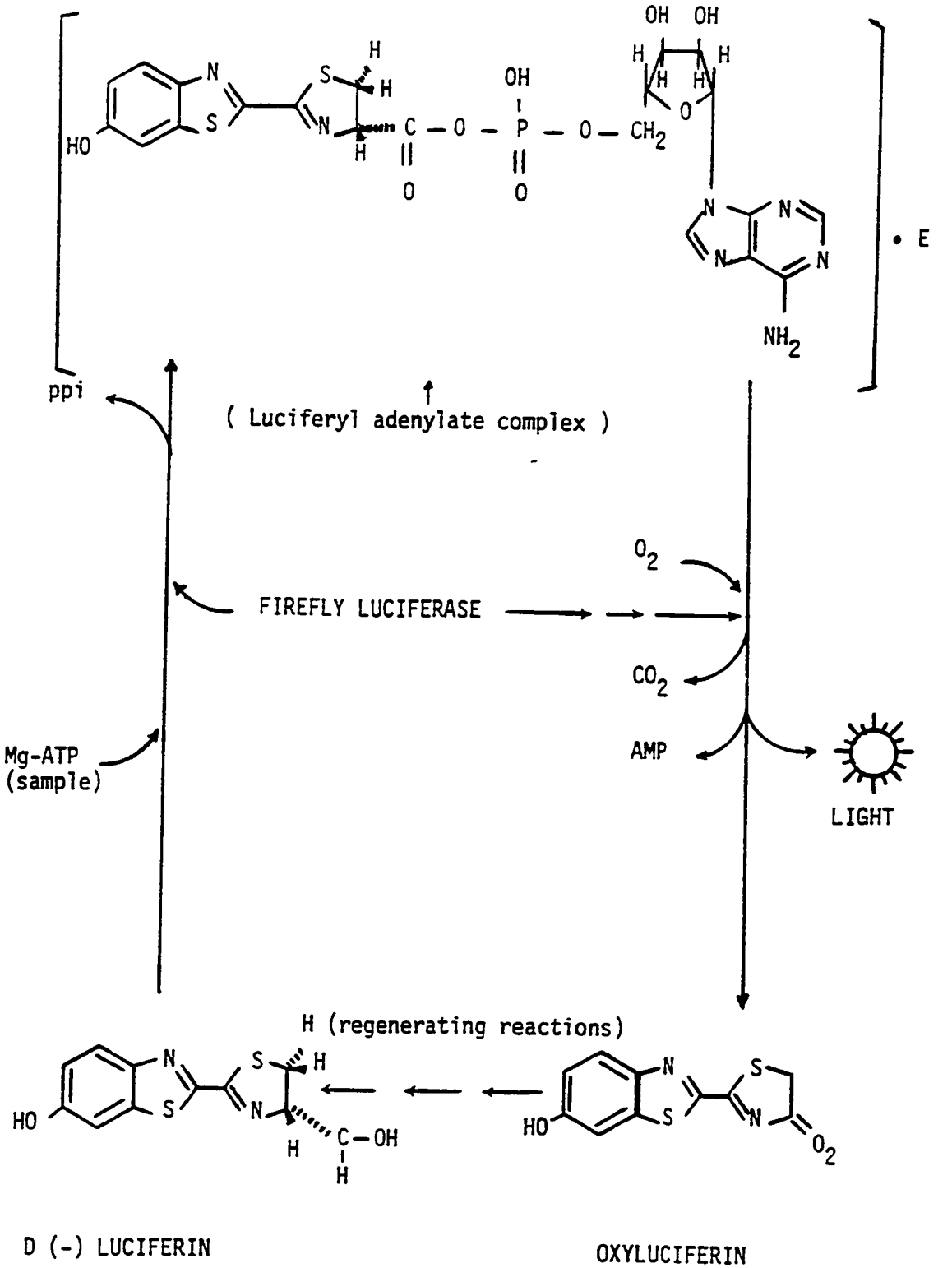
P: Product

All components of the assay except the compound to be measured must be in excess. If LH2, E, and molecular oxygen are in excess, the light intensity is directly proportional to the ATP concentration. For this reason the firefly system is a very sensitive reagent for determining ATP over a wide range of concentrations. The reaction is best carried out at 25° C in a buffer (e.g., 0.5M Tris), pH 7.75 (76), with a magnesium ion for rapid initiation. Although cytidine and inosine triphosphates can also stimulate light emission, their presence in significant amounts has not been reported. Certain ions such as SCN^-, I^-, $NO3^-$, Br^-, and Cl^- can inhibit the reaction (75).

d. Reagents, equipment, methodology and calculation of results: Reagents for the assay included 0.5 M Tris acetate-0.02 M EDTA buffer, pH 7.75; luciferin-luciferase (LKB

Figure 10.

Reactions in ATP determination via chemiluminescence methodology.



D (-) LUCIFERIN

OXYLUCIFERIN

REACTIONS IN THE CHEMILUMINESCENCE DETERMINATION OF ATP

Instruments, Gaithersburg, MD), ATP standards (LKB Instruments) and ultrapure water, which was used in all reagent preparations. Equipment for the assay included ATP luminometer (Integrating Photometer Model 3000, Biospherical Co., San Diego, CA), pH Meter (Corning Model 5), and boiling water bath.

ATP in semen or swim-up samples was extracted by boiling in Tris-EDTA buffer, followed by measurement with a luciferin-luciferase mixture. For a final volume of 750 µl, 730 or 740 µl of boiling Tris-EDTA buffer were mixed with 20 or 10 µl, respectively, of whole semen or swim-up sample in cryotubes and were immediately placed in boiling water for 15 minutes. The sample was added to the boiling Tris. A combination of boiling and chelation of Mg^{2+} rendered ATPase inactive, preventing ATP catalysis by the enzyme. Extracts were then stored at -20° C for a maximum period of 24 hours or were assayed immediately. When assayed, an additional volume of Tris-EDTA buffer, followed by 150 µl of luciferin-luciferase, was added to the extracts and the light output (B) was quantified for one minute in an integrated ATP photometer using disposable scintillation vials. One hundred ul of 50×10^{-7} M dilution of ATP standard were then added, and the light output (C) was quantified again for one minute. Addition of an internal standard compensated for sample turbidity and sample matrix effects. Proper standards and controls were used to check for the possible effect of different media (Ham's F10/FCS) on the assay results. To eliminate the contribution of sperm count to variability in test results, all results were reported as picomoles of ATP/1

million sperm (pmol/10^6 sperm).

Since 10 or 20 µl of each sample were used to quantitate one minute of light output (B), and 100 µl of internal standard were used, sample light output was multiplied by 10 or 5, respectively, to equilibrate the volumes of sample and internal standard. Light output for ATP over the range of 10^{-1} to 10^{-11} M was linear. ATP in the semen or swim-up was reported as Y x 10^{-7} M ATP where Y was the amount of ATP calculated from formula below:

$$\frac{(B)}{(C) - (B)} \times 50 \times 10^{-7} \text{ M}$$

B was a one-minute reading of the sample alone, and C was the standard mixed with the sample reading. To convert the results into pmol ATP/10^6 sperm, the results obtained from the above formula were divided by semen or swim-up count (in millions) and adjusted to pmol ATP/10^6 sperm.

3. Creatine kinase determination

a. Preparation of semen samples, as noted in ATP methodology.

b. Sample manipulations: A specific amount of semen or swim-up samples containing at least 5 million sperm was washed at 3° C to remove the seminal fluid or swim-up medium by diluting 50-fold into the cold saline and centrifuging at 1000 x g for 10 minutes. Pellets were then frozen at -70° C for

further evaluation. To perform the assay, sperm creatine phosphate was extracted by mixing the pellet with a buffer medium containing 15 millimolar (mM) Tris, 15 mM HEPES (pH=8.0), 5m M $MgCl_2$, 150 mM KCl, 1mM dithiothreitol, and 0.04% Triton X-100 as a permeabilizing agent.

c. CK activity determination: Spectrophotometric assays were performed at 22° C. CK activity in the reverse direction was measured by following the creatine phosphate-dependent production of ATP from ADP through the hexokinase and glucose-6-phosphate dehydrogenase-coupled assay (74). CK (creatine-N-phosphotransferase) catalyzes the reversible phosphorylation of ADP or creatine to ATP or creatine phosphate. The CK activity measurement in the reverse direction is based on a three-step reaction (Fig. 11). In the first step, CK catalyzes the synthesis of ATP from creatine phosphate and ADP. In the second step, the ATP is used for glucose-6-phosphate synthesis in the presence of hexokinase. In the third step, the glucose-6-phosphate is oxidized to 6-phosphogluconate with reduction of NADP to NADPH with an increase in absorbance measurable at 340 nm. Measurement is carried out in an assay volume of 0.56 ml containing 45 mM of creatine phosphate, 2 mM of ADP, 20 mM of N-acetyl cytosine, 5 mM of AMP, 20 mM of diadenosine pentaphosphate, and 125 mM of imidazole-acetate buffer, pH 6.5. A CK test kit can be purchased from the Sigma Chemical CO. (St. Louis, MO). The CK activity is expressed in units/100 million sperm (74).

Figure 11. Chemical determination of creatine kinase

$$\text{creatine phosphate} \xrightleftharpoons[\text{pH 6.5}]{\text{CK}} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{D-glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{D-glucose-6-phosphate}$$

$$\text{D-glucose-6-phosphate} + \text{NADP} \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^{+}$$

Following the initial lag phase of less that 90 seconds, the steady state reaction rate is determined by the increase in absorbance at 340 nanometers (nm) and at 22° C (74).

4. Hypoosmotic swelling test (HOST)

a. Rationale: Sperm cells with functional (biochemically active) membranes increase in volume when exposed to a hypoosmotic medium. This medium should exert an osmotic stress great enough to cause an influx of water but small enough to prevent lysis of the membrane. The increase in volume causes an expansion of the sperm membranes which is readily detectable in the tail area by the curling of the tail fibers. Thus, a

sperm that swells in the HOST, as shown by a curling tail, has an intact membrane.

b. Preparation of hypoosmotic medium: To prepare the HOST solution, 2.7 gm of fructose (M.W= 180.16) and 1.47 gm of sodium citrate dihydrate (M.W.=294.1) were dissolved in 200 ml of deionized distilled water. This mixture had an osmolarity of 160-165 mOsmol compared with the osmolarity of 300-350 mOsmol for the seminal fluid under normal condition. One ml aliquots of the HOST solution were pipetted into separate test tubes, capped, and frozen (-22° C) until use.

c. Procedure for the HOST: The test tube containing the HOST solution was thawed by incubation at 37° C for 10 minutes, and 0.1 ml of a well-mixed fully liquefied semen or swim-up sample was added to the solution, mixed, and incubated for 1 hour at 37° C. After incubation, a drop of well-mixed sample was placed on a glass slide with a cover glass and observed under phase contrast (x400) for coiling or swelling of the sperm tail (Fig. 12). At least 200 sperm/sample were counted, and the percent of sperm with coiled or thickened tails was calculated. Since some semen samples had spermatozoa with coiled tails before exposure to the HOST, each sample was observed before exposure to the HOST solution. The percent of sperm with coiled tails in the untreated sample was subtracted from the percent obtained after treatment to determine the actual percent of sperm that coiled in the HOST. Normal range was established from the values obtained from samples which produced pregnancies. Only samples with normal acrosin and

Figure 12.

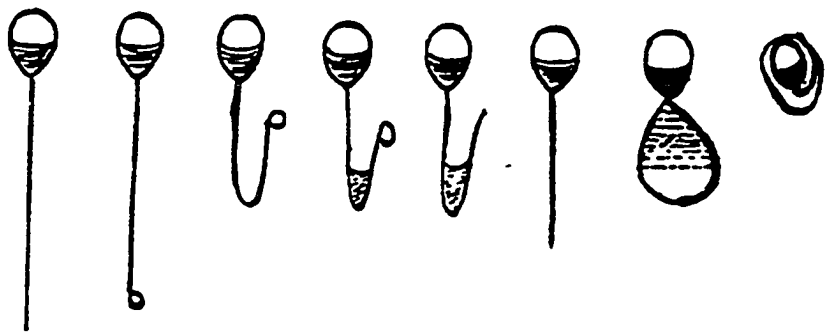
Schematic drawings of typical changes of human spermatozoa incubated in a hypoosmotic medium (hypoosmotic swelling test, HOST):

a: No change, indicative of a dead or abnormal sperm

b-e: Various types of coiling seen in spermatozoa with "functionally intact" membranes

f: Thickening of the tail, also seen in spermatozoa with intact membranes

g-h: Complete coiling similar to other types of coiling (b-e)



basic semen parameters were included in the study.

5. Determination of the percent of normal sperm

a. Preparation of morphology slides: Two morphology slides were prepared for each liquefied semen and swim-up sample used in IVF. The same samples were used for determination of ATP, CK, and HOST. A thin smear of 5 µl semen or swim-up was made on a slide precleaned thoroughly with 70% ethanol. The slides were air-dried at room temperature, and one of the duplicate slides for each sample preparation was stained for morphology analysis.

b. Staining technique for sperm morphology: Air-dried slides were fixed for 15 seconds (or 12 dips) with fixative composed of 1.8 mg/ml of triarylmethane methanol (Diff-Quick). Slides were then dipped into solution 1 (1 gm/l xanthene in sodium azide-preserved buffer) for 15 seconds (12 dips), then into solution 2 (0.625 gm/l azure A and 0.625 gm/l methylene blue in buffer) for 10 seconds (7 times). Between the fixing step and each of the staining steps, the excess solutions were drained from the slides by blotting the slide edges on an absorbent paper. The slides were read on the day of staining.

c. Methods of assessment of sperm morphology: Spermatozoa were considered normal when the head had a smooth oval configuration with a well-defined acrosome comprising 30% to 70% of the sperm head. The length of a normal sperm head was 5 to 6 µm with a diameter of 2.5 to 3.5 µm (Fig. 13). By

this method of assessment, normal sperm should not show any neck, midpiece, or tail defects. A micrometer in the eyepiece of the microscope was used for routine measurements, which were carried out under an oil immersion objective. At least 200 sperm were evaluated on each slide. Sperm with characteristics deviating from the above values were considered abnormal and were placed collectively into one abnormal grouping.

Based on the percent of normal sperm, semen samples were categorized into 3 groups:

Normal group (N): $\geq$ 14% normal

Good group (G): $\geq$ 4% to < 14% normal

Poor group (P): < 4% normal

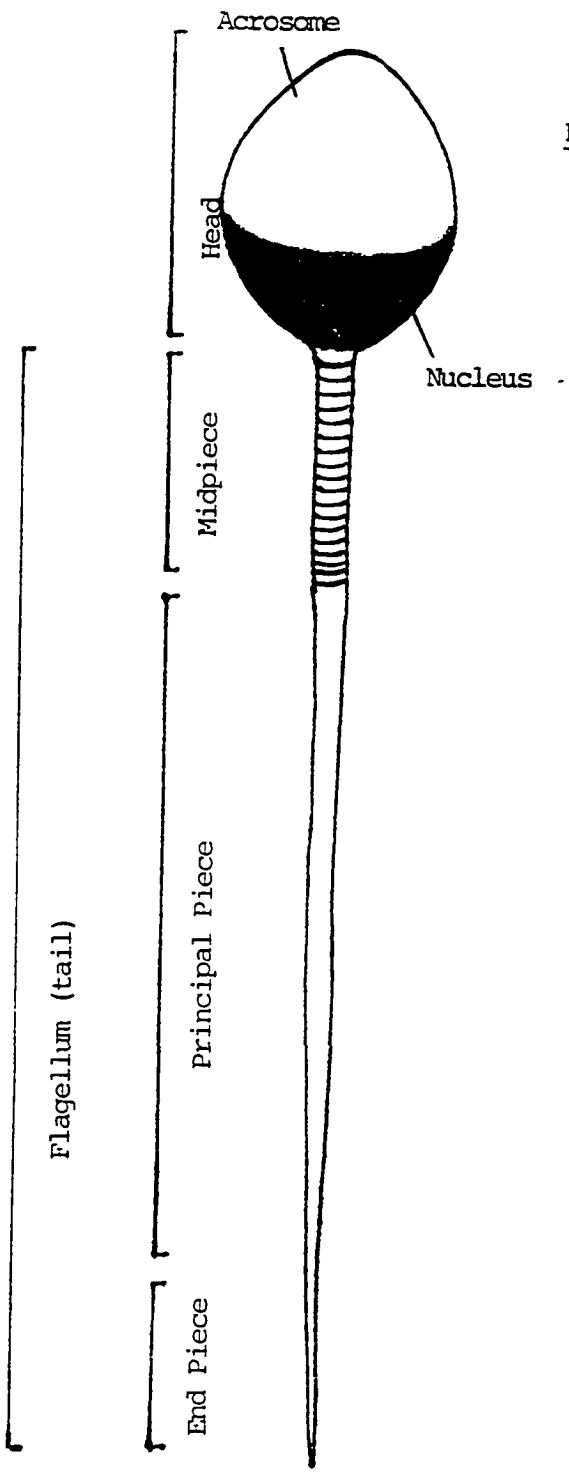
Classification of abnormal sperm forms was not attempted because in my previous study of 81 patients in series 26; no correlation had been found between the percentage of these abnormal forms and the IVF outcome (unpublished observation).

C. Statistical analysis

The relationships between basic semen and swim-up parameters, ATP, CK, HOST, the percentage of normal sperm, and IVF outcome were examined by Statistical Analysis System (SAS) software. Statistical parameters were determined by analysis of variance (ANOVA) and Spearman rank correlation coefficients. For a given r value, $p < 0.05$ was taken as a significant correlation between two parameters. The relationships between the sperm parameters and the fertilization and pregnancy rates were examined by multiple regression analysis in the SAS

Figure 13.

Schematic drawing of a normal spermatozoon according to new strict criteria of assessing sperm morphology



HEAD

length: 5-6 μm

width : 2.5-3.5 μm

smooth configuration

30-70% acrosome

general linear model (GLM) procedure. The SAS GLM procedure allows examination of all submodels of the complete multiple regression model. Multiple regression analysis was also used to obtain the coefficients used in discriminant function analysis. Multiple regression is a powerful set of statistical techniques that allow one to analyze the relationship between a dependent variable (e.g., fertilization or pregnancy rate) and a set of independent variables (e.g., basic seminal parameters, ATP, CK, HOST). These techniques are especially useful because they do not require that the independent variables be uncorrelated with one another. Through multiple regression analysis we could obtain the best prediction equation (regardless of the meaning of the equation) that indicates how scores on the independent variables could be weighted and summed to obtain the best possible prediction of fertilization or pregnancy rate (in our study) for the samples which were evaluated. We would also obtain statistics that indicate how accurate the prediction equation is and how much of the variation in fertilization or pregnancy rate is accounted for by the joint linear influences of all or a selected number of independent variables. Using this method, we could also evaluate the relationship between the dependent variable and a particular independent variable. In this case a simple regression analysis will not provide an appropriate answer because fertilization or pregnancy rate is confounded with other independent variables.

In simple regression analysis, values of the dependent

variable are predicted from a linear function of the form $Y = A + BX$, where Y is the predicted value on the dependent variable Y, X is the independent variable, B is the weight (a constant) assigned to the independent variable X, and A is a constant representing the Y intercept. The difference between the actual (Y) and the predicted value of a dependent variable (Y) for each case is called the residual or the error (deviation) in prediction, $Y - \bar{Y}$. The regression strategy involves the selection of A and B so that the sum of the squared residuals, $(Y-\bar{Y})$, is smaller than any possible alternative values. Optimum values for B and A are obtained from these formulae:

$$B = \frac{\leq(X-\bar{X})(Y-\bar{Y})}{\leq(X-\bar{X})^2}, \qquad A = \bar{Y} - BX$$

Multiple regression is an extension of simple regression analysis in which several independent variables, instead of just one, are used simultaneously to predict the outcome for each subject on some dependent variable (e.g., fertilization or pregnancy rate). The regression solution takes the form of the general linear model (GLM):

$Y = A + B1X1 + B2X2 + ... + BkXk$

where B's are partial coefficients for each independent variable calculated in a way that brings the Y values predicted from the equation as close as possible to the Y values obtained from measurement.

The statistical GLM was also used with the fertilization

rate or the number of pregnancies as the dependent variable to determine a threshold of ATP concentration to indicate where the chances of fertilization or pregnancy were significantly impaired ($p<0.05$).

Discriminant function analysis was used to devise formulae for predicting the pregnancy outcome of IVF. The major purpose of discriminant function analysis is to find out the best combination of independent variables to maximize differences among groups (e.g., pregnant versus non-pregnant). It is similar to multivariate analysis of variance (MANOVA) turned around. In MANOVA we ask whether grouping procedure produces significant differences on a combination of independent variables (e.g., basic seminal parameters, ATP, CK, HOST). If the answer is yes, then that combination of independent variables can be used to discriminate among groups. In MANOVA, independent variables are the predictors, and dependent variables are the multivariables. In discriminant function analysis these dependent variables become predictors, which are traditionally called independent variables.

The analytic model for discriminant function analysis follows that of MANOVA. A column matrix of scores on predictor (dependent) variables is formed within each group (e.g., pregnant versus non-pregnant), one for each case. By subtracting an appropriate mean from each score, matrices of difference scores are formed. These matrices are squared, by multiplying each matrix by its transpose, and summed. In this way, cross-products matrices (S matrices) are formed, analogous

to sums of squares in analysis of variance. Determinants (a measure of generalized variance of a matrix) of the various matrices are found; ratios between them provide tests of hypotheses about the ability of the dependent variables to differentiate among groups.

With discriminant function analysis based on two sets of conditions, two different formulae were devised for predicting the pregnancy outcome of IVF. One formula was based on the mean pregnancy rate (%) and was calculated with all patients in the study combined as one group. The second formula was constructed on the mean pregnancy rate of patients with no apparent semen abnormality.

The following IVF variables were calculated and considered as factors involved in IVF outcome:

1. Fertilization rate/oocyte for each group was calculated by dividing the number of fertilized oocytes by the total number of inseminated preovulatory oocytes.

2. Pregnancy rate/cycle was calculated by dividing the total number of pregnancies by the number of patients who underwent laparoscopy. Only clinical pregnancies were considered for IVF outcome analysis.

3. Pregnancy rate/transfer was calculated by dividing the number of pregnancies by the number of patients who reached the transfer stage.

4. Ongoing pregnancy rate was calculated by dividing the number of ongoing pregnancies by the number of total clinical pregnancies.

5. Spontaneous abortion rate was calculated by dividing the number of spontaneous abortions by the number of total clinical pregnancies.

6. Average number of preovulatory oocytes/patient was calculated by dividing the number of preovs recovered at aspiration by the total number of patients who underwent laparoscopy.

7. Average of embryos transferred/patient was calculated by dividing the number of embryos transferred by the number of patients who reached the transfer stage.

8. Cleavage rate was calculated by dividing the number of embryos that reached at least a 2-cell stage by the number of fertilized oocytes.

9. Number of cells/embryo was calculated by dividing the number of cell(s) which developed after insemination by the number of fertilized oocytes.

CHAPTER V

RESULTS

A. Preliminary Studies

Our modified technique of ATP determination yielded excellent within-run and between-run precisions (Table 4), especially for swim-up preparations. Four within run coefficients of variation (CV) for low (n=5) and high (n=8) swim-up ATP were 5% and 7%, respectively. Grouping of samples into low (≤ 40 picomol/million sperm) and high swim-up ATP (> 40 picomol/million sperm) was based on our preliminary findings (using statistical GLM of SAS) that all patients with swim-up ATP of <40 picomol/million sperm showed no pregnancy in the series 25 IVF program. No such threshold for seminal ATP could be found, although no pregnancy was achieved from semen samples with seminal ATP <64 picomol/million sperm.

Compared with swim-up samples, semen samples showed higher CVs (Table 4) but still within the limit of the assay methodology. Between-run precision studies were conducted using 10 replicate readings of a single sample with low ATP and another sample with high ATP over a two-week period. Calculated CVs were also excellent, especially for swim-up preparations for both samples with low and high ATP levels (Table 4).

Effects of storage (freezing) on ATP concentration was also determined using two semen and two swim-up samples with

TABLE 4

PRECISION STUDIES FOR SEMEN AND SWIM-UP ATP

	Semen		Swim-up	
	Mean (SEM)	CV (%)	Mean (SEM)	CV (%)
WITHIN-RUN				
Low ATP(n=5)[a] ≤ 40 pmol/10^6 sperm	40 (2.3)	10	36 (1.2)	5
High ATP(n=8)[a] > 40 pmol/10^6 sperm	82 (3.4)	12	76 (1.9)	7
BETWEEN-RUN				
Low ATP (n=1)[b] ≤ 40 pmol/10^6 sperm	42 (3.1)	11	39 (1.6)	7
High ATP (n=1)[b] > 40 pmol/10^6 sperm	90 (3.0)	9	87 (2.1)	6

a: Within-run (4 runs for each sample) mean ATP concentration and CV for 5 samples with low ATP (≤40 pmol/10^6 sperm) and 8 samples with high ATP (>40 pmol/10^6 sperm) were determined.

b: Between-run mean ATP concentration and CV were determined using 10 replicate readings of a single sample with low ATP (38 pmol/10^6 sperm) and another sample with high ATP (87 pmol/10^6 sperm).

low ATP and four with high ATP. No drastic change in ATP concentration was observed up to day 6 of storage (< 8% change). After day 6, however, the change was higher (> 10%). No sample used in our studies was stored for more than 24 hours.

To determine whether whole semen and swim-up ATP determinations could be of value in predicting the outcome of an IVF program, we studied 37 patients from Norfolk series 25. Results from whole semen samples showed a wide range of seminal ATP concentration (43 to 1028 pmol/million sperm). Statistical analysis showed no correlation between seminal ATP and IVF variables. Based on the results from the swim-up measurements in conjunction with fertilization rates and pregnancies produced by these samples, four groups could be identified:

Group 1 (n=12): Samples with low ATP,
< 100% fertilization,
and no pregnancy
(mean ± SEM swim-up ATP= 38 ± 4
pmol/million sperm)

Group 2 (n=4): Samples with high ATP,
< 100% fertilization,
and resulting pregnancy
(mean ± SEM swim-up ATP= 63 ± 5
pmol/million sperm)

Group 3 (n=8): Samples with high ATP, 100% fertilization rate, and resulting pregnancy (mean ± SEM swim-up ATP= 72 ± 4.5 pmol/million sperm)

Group 4 (n=13): Samples with high ATP, 100% fertilization rate, and no pregnancy (mean ± SEM swim-up ATP= 67 ± 4.1 pmol/million sperm)

Using ANOVA, the Wilcoxon 2-Sample test, and Kruskal-Wallis test (chi-square approximation), it was found that the swim-up mean ATP concentration in group 1 was significantly different ($p < 0.001$) from those of the other groups. Using the same methods of analysis, no significant difference between the count and percent of motility of group 1 and to the other groups was found.

Based on these preliminary studies, it was decided to continue the study to obtain more data and to include the new strict method of evaluation of sperm morphology.

B. Studies on Norfolk IVF Series 27

1. ATP determinations and results

Seventy-eight patients in this series were studied (blind study). All samples obtained from these patients had a

normal count (≥ 20 million/ml) and percent of motility (≥ 40%). No sample with primary male factor infertility other than morphology was included . With the exception of samples with abnormal count and motility, selection of patients for the study was random. Seminal and swim-up ATP, as well as morphology, were determined for these patients. ATP results were similar to those of the preliminary studies, and statistical analyses indicated no specific threshold for seminal ATP where the chances of fertilization or pregnancy could be impaired, although no pregnancy resulted from samples with seminal ATP <65 pmol/ million sperm. Based on the ATP results from the swim-up samples, two groups were identified: patients with low swim-up ATP and those with high swim-up ATP (Table 5). When these results were compared with the IVF outcome, all patients with low swim-up ATP had a significantly lower fertilization rate/oocyte (FR/O) and a lower pregnancy rate (PR). Both groups had similar swim-up count and percent of motility. The average number of preovulatory oocytes retrieved from each wife was even higher in patients receiving semen samples with low ATP concentrations. These results may indicate that FR/O and PR differences between the two groups are not due to differences in count, percent of motility, or the number of preovs recovered. There were no differences in the average number of embryos transferred/person and the cleavage rate between the two groups. Although not statistically significant ($p < 0.06$), the number of cells/embryo was lower in the group with low swim-up ATP (Table 5). Seminal

TABLE 5

CORRELATION BETWEEN SWIM-UP ATP AND HUMAN IVF RESULTS

(Series 27)

	ATP≤40 (n= 13)	ATP>40 (n=65)	SIG.*
Mean Swim-Up ATP/ Million Sperm	35.3	101.1	$p < 0.01$
Mean Swim-Up Count/ % Motility	19/86	27/87	NS
Fertilization Rate/ Oocyte	54.6	93.8	$p < 0.04$
Pregnancy Rate/Cycle	0.0	30.8	$p < 0.001$
Pregnancy Rate/ Transfer	0.0	31.3	$p < 0.001$
Ongoing Pregnancy Rate	0.0	24.6	$p < 0.008$
Abortion Rate	-	20.0	$p < 0.001$
Average Preovulatory Oocyte/ Patient	4.5	3.3	$p < 0.05$
Average Embryo Transferred/Patient	2.2	3.3	NS
Cleavage Rate	96.3	94.8	NS
Number of Cells/ Embryo	3.8	4.2	NS

ATP concentration in picomol/ million sperm.
No patient with primary male factor infertility other than morphology was included.
SIG.*: significance
NS: no significance

ATP determinations showed no significant correlation with the IVF outcome.

2. Morphology Assessments

Ninety-two patients in Norfolk series 27 were chosen for sperm morphology evaluations. These patients had no other primary male factor infertility and were chosen on a random basis. Some of the same patients (n= 78) had ATP determined on their swim-up preparations. Based on the percent of normal sperm, patients were grouped into three groups (Table 6):

P group (n=10): normal sperm < 4%

G group (n=65): normal sperm ≥ 4% to ≤ 14%

N group (n=17): normal sperm ≥ 14

These three groups showed significant differences in their mean rate of normal sperm. FR/O was significantly lower in the P group than in the with G or N group. FR/O, however, could not be used to differentiate the N and G groups. The pregnancy rate (PR) was significantly different in all groups (Table 6). There was no difference among the groups with regard to the average number of preovulatory oocytes recovered/patient, average number of embryo(s) transferred/patient, and the cleavage rate or number of cells/embryo. The number of cells/embryo in the group with poor sperm morphology was lower, though not statistically significant, than in the G or N group. Grouping the patients from this series into pregnant and non-pregnant revealed that the average number of cells/embryo in the pregnant group was slightly higher

TABLE 6

CORRELATION BETWEEN SPERM MORPHOLOGY AND HUMAN IVF RESULTS

(Series 27)

	P (n=10)	G (n=65)	N (n=17)
Mean Normal Sperm (%)	2.0[a]	7.9[b]	19.1[c]
Fertilization Rate/Oocyte	27.5[a]	83.5[c]	96.3[c]
Pregnancy Rate/ Cycle	0.0c	24.7[a]	41.2[b]
Pregnancy Rate/ Transfer	0.0[c]	25.4[a]	43.8[b]
Ongoing Pregnancy	0.0[c]	15.4[a]	35.3[b]
Abortion Rate	-	37.5[c]	14.3[a]
Average Preov/ Patient	4.0[a]	3.3[a]	3.2[a]
Average Embryos Transferred/Patient	2.0[a]	2.7[a]	2.8[a]
Cleavage Rate	89.5[a]	95.2[a]	97.9[a]
Number Cells/ Embryo	3.7[a]	3.8[a]	4.2[a]

P: < 4% normal forms
G: ≥4 to < 14% normal forms
N: ≥ 14% normal forms
No patient with primary male factor infertility other than morphology was included.

Numbers with identical superscripts are not significantly different. Those with unidentical superscripts are significantly different as follow:
a from b ($p<0.003$), a from c ($p<0.001$) and b from c $p<0.001$)

($p<0.054$) than in the non-pregnant group (mean 4.2 vs. 3.6, respectively). In a further study of the relationship between sperm morphology and seminal or swim-up ATP, we found that 70% of patients with the P pattern had low (≤40 pmol/million sperm) swim-up ATP but slightly higher whole semen (seminal) ATP compared to the other two groups. In contrast to the semen samples with the P pattern, only 33% of semen samples with the G pattern showed low swim-up ATP. Seminal ATP in this group was similar to those with normal morphology. Only 6% of semen samples with normal morphology had low swim-up ATP (Table 7).

3. Hypoosmotic swelling test in IVF series 27

Seventy-eight patients who were studied for ATP were also evaluated for the assessment of their sperm membrane integrity based on the HOST results. No significant correlations between any of IVF variables and the HOST results in semen or swim-up samples were observed (Tables 11 and 12).

C. Studies on Norfolk IVF Series 30

To obtain more data and ascertain whether the significant results in series 27 could be repeated, we decided to randomly choose some patients from Norfolk IVF series 30. Semen and swim-up CK were determined to evaluate their usefulness in predicting IVF outcome.

1. ATP results, series 30

ATP results from semen evaluations showed no

TABLE 7

PERCENTAGE OF SAMPLES WITH LOW OR HIGH SWIM-UP ATP IN THREE MORPHOLOGY GROUPS OF PATIENTS FROM SERIES 27 (S-27) AND SERIES 30 (S-30)

	P PATTERN		G PATTERN		N PATTERN	
	S-27	S-30	S-27	S-30	S-27	S-30
LOW ATP	7/10 (70%)	3/5 (60%)	15/65 (33%)	18/45 (40%)	1/17 (6%)	1/20 (5%)
HIGH ATP	3/10 (30%)	2/5 (40%)	50/65 (77%)	27/45 (60%)	16/17 (94%)	19/20 (95%)

LOW ATP: swim-up ATP ≤ 40pmol/million sperm
HIGH ATP: swim-up ATP > 40 pmol/million sperm

significant correlation with series 30 IVF outcome. Swim-up results, however, showed a similar trend and level of significance as in series 27 (Table 8). Although FR/O was higher in patients with low ATP, pregnancy results remained low as in series 27. No pregnancy was achieved in the group with low ATP. As in series 27, the significant difference in pregnancy rate between two groups may not be related to differences in swim-up count, motility, or the average number of embryos transferred/person. The number of cells/embryo transferred was lower (3.8 and 3.6) for low ATP groups of series 27 and 30, respectively, compared to groups with high ATP (4.2 and 4.4). The difference, however, was not significant ($p < 0.06$).

2. Morphology results, series 30

Eighty-two IVF patients with no semen abnormalities other than morphology were studied to evaluate the role of sperm morphology in determining IVF outcome. Stained morphology slides prepared from swim-up preparations could not be read because of precipitation of fetal cord serum proteins around the sperm heads during the fixation step. Seminal morphology was evaluated and showed an excellent correlation with the pregnancy rate (Table 9). Fertilization rate, however, showed no differences among the three groups, contrary to series 27. The reason for the improvement was that before series 27, all oocytes were inseminated with 50,000 spermatozoa/oocyte/ml of medium. In some patients of series 27

TABLE 8

CORRELATION BETWEEN SWIM-UP ATP AND HUMAN IVF RESULTS

(Series 30)

	ATP≤40 (n=5)	ATP>40 (n=50)	SIG.*
Mean Swim-Up ATP/ Million Sperm	31.2	108.3	$p < 0.001$
Mean Swim-Up Count/ % Motility	16.3/88	19.4/92	NS
Fertilization Rate/ Oocyte	100.0	74.0	$p < 0.05$
Pregnancy Rate/Cycle	0.0	30.0	$p < 0.005$
Pregnancy Rate/ Transfer	0.0	30.6	$p < 0.006$
Ongoing Pregnancy	0.0	30.0	$p < 0.006$
Abortion Rate	-	0.0	$p < 0.001$
Average Preovulatory Oocyte/ Patient	4.4	1.0	$p < 0.03$
Average Embryos Transferred/Patient	3.0	3.0	NS
Cleavage Rate	92.9	93.8	NS
Number of Cells/ Embryo	3.6	4.4	NS

ATP concentration in picomol/ million sperm
No patient with primary male factor infertility other than morphology was included.
SIG.*: significance
NS: no significance

TABLE 9

CORRELATION BETWEEN SPERM MORPHOLOGY AND HUMAN IVF RESULTS

(Series 30)

	P (n=17)	G (n=45)	N (n=20)
Mean Normal Sperm (%)	1.9[a]	7.8[b]	16.6[c]
Fertilization Rate/ Oocyte	91.9[a]	93.2[a]	96.4[a]
Pregnancy Rate/ Cycle	5.9[a]	24.4[b]	40.0[c]
Pregnancy Rate/Transfer	6.3[a]	25.0[b]	40.0[c]
Ongoing Pregnancy	0.0[a]	15.6[b]	30.0[c]
Abortion Rate	100.0[a]	36.4[c]	25.0[b]
Average Preov / Patient	4.8[a]	4.2[a]	4.2[a]
Average Embryos Transferred/Patient	2.9[a]	3.0[a]	2.8[a]
Cleavage Rate	91.3[a]	95.7[a]	92.9[a]
Number of Cells/ Embryo	3.3[a]	4.4[a]	3.5[a]

P: < 4% normal forms
G: ≥ 4% to < 14% normal forms
N: ≥14% normal forms
No patient with other primary male factor infertility was included.

Numbers with identical superscripts are not significantly different. Those with unidentical superscripts are significantly different as follow:
a from b ($p<0.01$), a from c ($p<0.001$) and b from c ($p<0.02$)

and all patients of any series thereafter, the number of spermatozoa/oocyte/ml of medium was increased to 100,000 or more (depending on severity of the abnormality) if the morphology evaluations of the husband's semen showed the P or G pattern. Three groups of patients in IVF series 30 had similar results in other IVF variables. Similar relationships between sperm morphology and the seminal and swim-up ATP were observed (Table 7). All patients with normal sperm morphology had normal seminal and swim-up ATP. Forty percent of the G pattern samples, 60% of the P pattern samples, and 5% of samples with normal morphology had low swim-up ATP. Seminal ATP in the G group was similar to that of the normal group. Semen samples with the P pattern had slightly higher seminal ATP than the other two groups.

3. CK evaluations, series 30

Swim-up samples prepared for CK studies gave a wide range of results. Since CK results are based on CK activity/100 million sperm, and since upon centrifugation no thick pellet is formed by a swim-up sample, these variations may have caused the erratic results (Table 13).

Compared with IVF variables, seminal CK showed no significant correlation in normozoospermic semen samples (Table 13). Creatine Kinase values also showed no significant correlation with the ATP or total semen count (Figs. 14 and 15). To evaluate whether CK isoenzymes can indicate any difference, semen samples from 27 patients of series 30 were

analyzed.

Analysis of CK isoenzymes has an advantage over the reverse enzymatic method of determining CK activity because sperm count is not used in calculating the final results. During sample (pellet) preparations for CK measurement, sperm count may change, introducing error in to the study. The CK isoenzyme ratio of MM+MB/MB+BB, however, revealed no significant difference between samples which produced pregnancy and those which did not (Table 10). The semen CK MM+MB/MB+BB ratio also showed no significant correlation with seminal ATP or sperm count (Figs 14 and 15).

4. CK MM+MB/MB+BB ratio in oligozoospermic samples

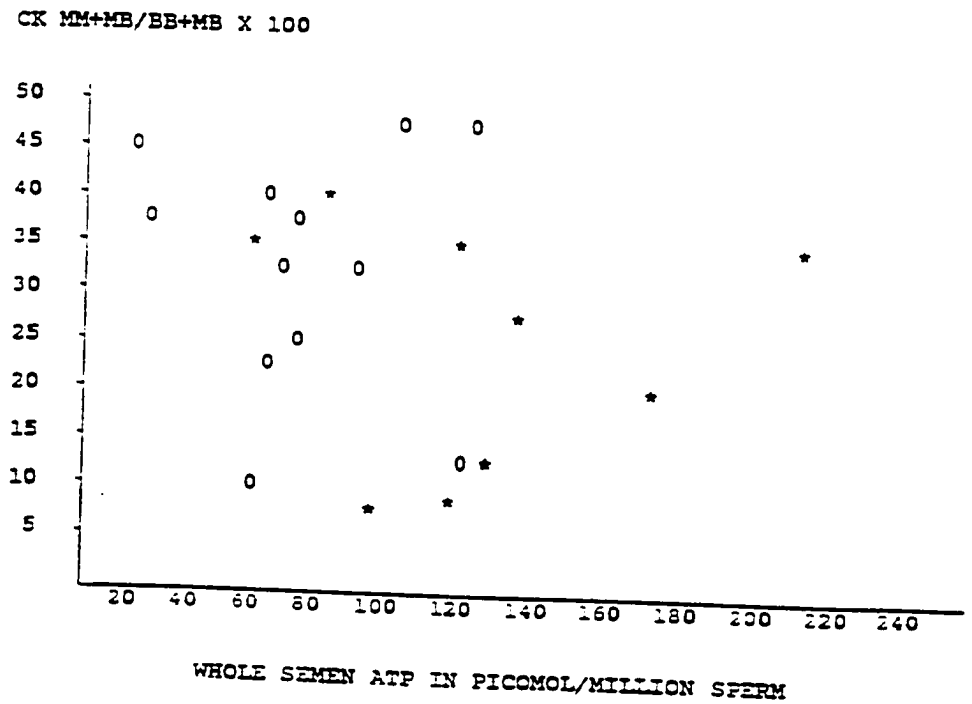
In a separate study, CK MM+MB/MB+BB ratios of 10 oligozoospermic samples from patients not participating in the IVF program were determined. The mean ratio was significantly lower than in normozoospermic samples. Creatine Kinase MM+MB/MB+BB ratios in these samples, however, showed no significant correlation with ATP (Table 10).

D. Combined Results From IVF Series 27 and 30

1. ATP results in combined series: When patients of IVF series 27 and 30 were combined and grouped by their swim-up ATP concentrations (low ATP vs. high ATP), some interesting results were observed (Table 11). In addition to pregnancy rates which were significantly different between the two groups ($p<0.001$), other variables exhibited significant differences. Semen and

Figure 14.

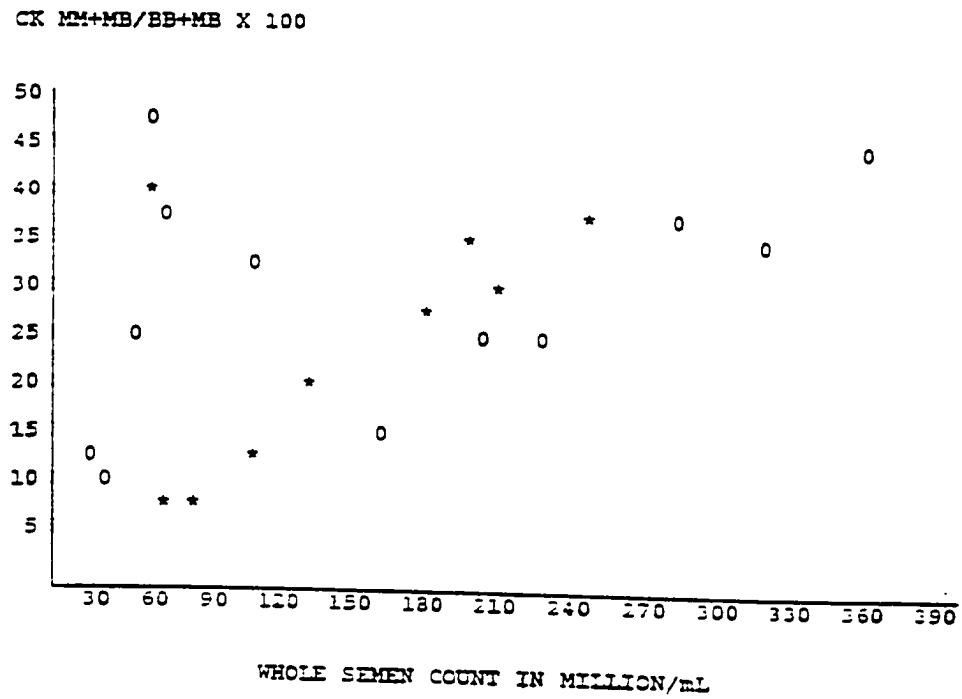
The relationship between whole semen CK MM+MB/BB+MB and ATP and the resulting pregnancy (*) and non-pregnancy (0), series 30; n=21.



No patient with primary male factor infertilty other than morphology included

Figure 15.

The relationship between whole semen CK MM+MB/BB+MB and sperm count and the resulting pregnancy (*) and non-pregnancy (0), series 30, n=21.



No patient with primary male factor infertility other than morphology included

TABLE 10

MEAN CK ISOENZYME RATIO[a] IN ORIGINAL SEMEN FOR SERIES 30

GROUP	CK RATIO[a] (SEM)
PREGNANCY (n=9)	23.4[b] (4.4)
NO PREGNANCY (n=22)	27.2[b] (3.1)
OLIGOZOOSPERMIC (n=11)	13.1[c] (3.3)

a: MM+MB/BB+MB ratio

b: not significantly different

c: significantly ($p < 0.05$) different from b

SEM: standard error of the mean

swim-up counts (not percents of motility) of these two groups were significantly different ($p < 0.05$), and the percent of normal sperm was different ($p < 0.05$). There was a significant difference ($p < 0.05$) between swim-up linearity of the low ATP group and the high ATP group. Spermatozoa in swim-up samples with low swim-up ATP were less linear (5.2) than those with high swim-up ATP (7.3). Sperm mean linear velocity was also slightly lower in the group with low swim-up ATP. However, sperm velocity in semen or swim-up samples of these two groups was not different.

2. Morphology results in combined IVF series 27 and 30: When patients in IVF series 27 and 30 were combined and classified by their percentage of normal sperm, the same trend as in series 27 or 30 was observed. The pregnancy rate and swim-up ATP were significantly different in the three groups ($p < 0.01$). Swim-up count and the percentage of motility in the P group were significantly different from the G or N groups ($p < 0.05$ and 0.008, respectively) (Table 12). Swim-up samples of semen with normal morphology exhibited higher sperm mean linear velocity and lower sperm mean linearity than those of the P or G pattern (Table 12).

Analyzing the results of different series as well as the combined series has shown that pregnancy outcome is a very sensitive indicator of IVF outcome in our studies (Table 13). The relationships between sperm parameters, the assays performed, and pregnancy rates were examined by multiple

TABLE 11

COMPARISON OF SEMINAL AND SWIM-UP VARIABLES IN PATIENTS WITH LOW AND HIGH SWIM-UP ATP (SERIES 27 AND 30 COMBINED)

	LOW ATP			HIGH ATP			
VARIABLE	MEAN	(SEM)	(n)	MEAN	(SEM)	(n)	SIG.
Count 1	93.5	(13.6)	(18)	134.3	(10.5)	(106)	p<0.05
Mot 1	51.8	(5.6)	(18)	67.1	(1.7)	(106)	NS
Normal 1	4.8	(0.8)	(21)	10.2	(0.6)	(118)	p<0.05
Velo 1	39.6	(1.9)	(16)	41.1	(0.7)	(114)	NS
Line 1	5.8	(0.3)	(16)	5.8	(0.1)	(114)	NS
ATP 1	111.6	(20.7)	(20)	124.5	(6.5)	(105)	NS
Count 2	16.5	(3.7)	(13)	27.5	(2.0)	(97)	p<0.05
Mot 2	89.2	(3.0)	(13)	98.0	(7.3)	(97)	NS
Velo 2	59.2	(2.2)	(11)	63.9	(1.1)	(82)	NS
Line 2	5.2	(0.3)	(11)	7.3	(1.0)	(82)	p<0.05
Hos 1	80.0	(4.9)	(18)	86.3	(3.0)	(106)	NS
Hos 2	89.0	(3.0)	(13)	93.1	(2.1)	(97)	NS
ATP 2	30.2	(1.7)	(21)	89.6	(3.5)	(119)	p<0.001
Preg	0.0	(0.0)	(21)	30.3	(4.2)	(119)	p<0.001

LOW ATP: swim-up ATP ≤ 40 pmol/million sperm
HIGH ATP: swim-up ATP > 40 pmol/million sperm

No patient with primary male factor infertility (other than morphology) was included.

Number 1 after each variable denotes "seminal," and 2 denotes "swim-up".

Normal: percent of normal forms
Mot: percent of motility
Velo: mean velocity in micrometers/second
Line: mean linearity
Count: sperm count in million/ml semen or swim-up
Hos: percent (mean) of coiled or thickened sperm tail in semen or swim-up samples
Preg: pregnancy rate

TABLE 12

COMPARISON OF SEMINAL AND SWIM-UP VARIABLES IN PATIENTS GROUPED BASED ON THEIR PERCENT NORMAL SPERM MORPHOLOGY (SERIES 27 AND 30 COMBINED)

	NORMAL (N)			GOOD (G)			POOR (P)		
VARIABLE	MEAN	(SEM)	(n)	MEAN	(SEM)	(n)	MEAN	(SEM)	(n)
Count 1	141.3	(17.8)	(28)	121.3	(7.5)	(82)	122.2	(54.1)	(16)
Mot 1	72.4	(2.3)	(28)	64.7	(2.1)	(82)	54.4	(6.0)	(16)
Normal 1	18.2	(1.1)	(32)	7.9	(0.3)	(97)	2.0	(0.3)	(19)
Velo 1	41.3	(1.3)	(30)	41.0	(0.9)	(80)	39.7	(1.7)	(14)
Line 1	5.3	(0.2)	(26)	5.9	(0.1)	(80)	6.0	(0.2)	(14)
ATP 1	124.9	(16.7)	(25)	114.5	(6.5)	(83)	178.4	(26.0)	(18)
Count 2	31.6	(4.7)	(27)	25.8	(2.1)	(67)	17.2	(5.7)	(12)
Mot 2	95.8	(2.9)	(27)	93.1	(0.7)	(67)	76.2	(7.3)	(12)
Velo 2	68.9	(2.5)	(25)	63.7	(1.2)	(55)	58.4	(1.8)	(9)
Line 2	5.5	(0.2)	(25)	6.9	(1.0)	(55)	7.1	(6.2)	(9)
Hos 1	85.2	(6.5)	(28)	81.6	(6.1)	(82)	80.1	(8.6)	(16)
Hos 2	91.0	(3.5)	(27)	89.3	(3.9)	(67)	82.6	(3.6)	(12)
ATP 2	106.3	(9.2)	(30)	73.0	(3.3)	(89)	70.9	(13.5)	(17)
Preg	40.6	(8.8)	(32)	25.8	(4.5)	(97)	0.0	(0.0)	(19)

Normal (N): Percent of normal sperm of $\geq$ 14
Good (G) : Percent of normal sperm $\geq$ 4 but < 14
Poor (P) : Percent of normal sperm < 4

No patient with primary male factor infertility (other than morphology) was included.
Number 1 after each variable denotes "seminal," and 2 denotes "swim-up".
Mot: percent of motility; Velo: velocity in um/second; Line: linearity; Preg: pregnancy rate
ATP: in pmol/ million sperm
Count: in million sperm/ ml semen or swim-up

ONLY THE FOLLOWING WERE SIGNIFICANTLY DIFFERENT FROM EACH OTHER:

1. Percent of motility in the P pattern ($p<0.008$) compared with that of the G or the N
2. Percent of normal forms: from G ($p<0.05$), P from N ($p<0.01$), G from N ($p<0.04$)
3. Linearity 1 was lower ($p< 0.006$) in the N pattern compared with the G or the P pattern
4. ATP 1 in P group was higher ($p<0.006$) than in the G or the N
5. Pregnancy in N group was higher compared with the G ($p<0.02$) and the P ($p<0.01$). Pregnancy rates in the G and P groups were also different ($p<0.02$).

regression analysis in the SAS general linear model procedure. The multiple regression analysis examines the contribution of all the independent variables to the variation in pregnancy, which is the dependent variable.

E. Formulae to Predict the Pregnancy Outcome of IVF

A combination of semen ATP and sperm velocity in semen, the percentage of normal sperm, swim-up ATP, motility, and linearity gave the highest contribution to the pregnancy outcome. By discriminant function analysis, two formulae were devised from which the chance of achieving pregnancy could be calculated. Formula 1 was constructed from the pregnancy rate (25%) obtained by combining all patients in both series into one group and determining the overall pregnancy rate. Variables which were significant contributors to the pregnancy outcome and were used to devise formula 1 were as follows:

1. Percentage of normal sperm in semen (Normal)
2. Sperm velocity in semen (Velo 1)
3. Seminal ATP concentration in pmol/million sperm (ATP 1)
4. Sperm ATP concentration in swim-up samples (ATP 2)
5. Sperm linearity in swim-up samples (Line 2)
6. Sperm percent of motility in swim-up samples (Mot 2)

To use the formula, values for each variable are plugged into the "Yes" formula for assuming that the patient will become pregnant and into the "No" formula for assuming that the patient will not become pregnant. The numerical values for each "Yes" and "No" formula are then compared. The formula

TABLE 13

COMPARISON OF SEMINAL AND SWIM-UP VARIABLES IN PREGNANT AND NON-PREGNANT PATIENTS OF SERIES 27 AND 30 COMBINED

	PREGNANT			NON-PREGNANT			
VARIABLE	MEAN	(SEM)	(n)	MEAN	(SEM)	(n)	SIG.
Count 1	125.9	(13.3)	(29)	125.1	(10.9)	(101)	NS
Mot 1	64.1	(3.6)	(29)	64.6	(1.9)	(101)	NS
Normal 1	10.5	(0.6)	(39)	6.9	(0.6)	(112)	$p<0.05$
Velo 1	38.2	(1.5)	(28)	41.7	(0.7)	(96)	NS
Line 1	5.7	(0.2)	(28)	5.8	(0.1)	(96)	NS
ATP 1	138.6	(12.5)	(30)	121.6	(7.7)	(100)	$p<0.056$
Count 2	26.3	(3.4)	(26)	26.2	(2.2)	(84)	NS
Mot 2	91.6	(1.6)	(26)	98.6	(8.5)	(84)	NS
Velo 2	63.9	(1.9)	(22)	61.7	(1.2)	(71)	NS
Line 2	5.7	(0.2)	(22)	7.4	(1.1)	(71)	$p<0.05$
CK 1	0.1	(0.1)	(21)	0.2	(0.1)	(28)	NS
ATP 2	97.2	(7.4)	(36)	75.0	(3.8)	(104)	$p<0.03$

No patient with primary male factor infertility (other than morphology) was included.

Number 1 after each variable denotes "seminal," and 2 denotes "swim-up".

Mot: percent of motility
Normal: mean percent of normal sperm
Velo: velocity in micrometers/second
Line: mean sperm linearity
CK: creatine kinase in units/100 million sperm
ATP: in pmol/million sperm
SIG.: significance

with the highest numerical value determines the category into which the patient falls.

Formula 1:

"Yes" = -21.21 + (0.26)(Normal 1) + (0.65)(Velo 1) + (0.05)(ATP 1) + (0.03)(ATP 2) + (.02)(Line 2) + (-0.001)(Mot 2)

"No" = -18.88 + (0.21)(Normal 1) + (0.71)(Velo 1) + (0.04)(ATP 1) + (0.02)(ATP 2) + (0.04)(Line 2) + (-0.001)(Mot 2)

Using this formula and assuming that at least one mature and healthy oocyte (metaphase II) is recovered, we found an 87.8% correlation between pregnancy prognosis and outcome (Table 14).

Formula 2 for predicting pregnancy was constructed on the pregnancy rate of patients with normal morphology in combined series 27 & 30. The "Yes" and "No" groupings were as follows:

"Yes" = -19.80 + (0.27)(Normal 1) + (0.65)(Velo 1) + (0.05)(ATP 1) + (0.04)(ATP 2) + (0.02)(Line 2) + (-0.001)(Mot 2)

"No" = -18.6 + (0.20)(Normal 1) + (0.71)(Velo 1) + (0.04)(ATP 1) + (0.01)(ATP 2) + (0.04)(Line 2) + (-0.001)(Mot 2)

TABLE 14

DISCRIMINANT FUNCTION ANALYSIS FOR PREDICTING PREGNANCY OUTCOME BY SEMINAL AND SWIM-UP VARIABLES SERIES 27 & 30 COMBINED

FROM PREG	NO	YES	TOTAL
NO	59	3	62
	95.2%	4.8%	100.0%
YES	13	7	20
	65.0%	35.0%	100.0%
TOTAL PERCENT	87.8%	12.2%	100.0%

BASED ON THE MEAN PREGNANCY RATE OF 25% OBSERVED FOR ALL PATIENTS IN SERIES 27 AND 30 COMBINED REGARDLESS OF THE TYPE OF THEIR SEMEN ABNORMALITIES (morphology or ATP).

By this formula a 70% correlation between pregnancy prognosis and outcome was found (Table 15).

TABLE 15

DISCRIMINANT FUNCTION ANALYSIS FOR PREDICTING PREGNANCY OUTCOME BY SEMINAL AND SWIM-UP VARIABLES SERIES 27 & 30 COMBINED

FROM PREG	NO	YES	TOTAL
NO	44	18	62
	71.0%	29.0%	100.0%
YES	6	14	20
	30.0%	70.0%	100.0%
TOTAL			
PERCENT	61.0%	39.0%	100.0%

BASED ON THE PREGNANCY RATE OF NEARLY 45 % OBSERVED IN PATIENTS INSEMINATED WITH SEMEN SAMPLES OF NORMAL (N) MORPHOLOGY WHICH ALSO SHOWED NO OTHER APPARENT SEMEN ABNORMALITIES.

CHAPTER VI

DISCUSSION AND CONCLUSION

During the past 25 years, major steps have been taken toward the understanding and treatment of infertility. The amount of information generated by basic and clinical research has increased so tremendously that it has become extremely difficult to manage. And yet, not all the information has been useful in guiding us toward a meaningful diagnosis. Some of the past studies have been plagued with numerous difficulties in data collection and interpretation. In many cases these difficulties were the direct consequences of the complex nature of the problem of infertility.

Some problems with identifying and evaluating an infertile population are obvious; others are less clear. The first problem is that of obtaining meaningful data. There are reasons for infertility, representing the biological variations in human populations. To these basic problems might be added the occurrence of undetected variations, such as chemical exposure, which may add to the variation and complicate the study of infertility. In considering factors that may be responsible for infertility, one must also consider the manner in which the results are correlated and interpreted.

Many tests have been advocated to evaluate the function of

spermatozoa and to estimate the capacity of these cells to fertilize. The characteristics which determine the fertilizing potential of spermatozoa in vivo (after intercourse or artificial insemination) may be different from those in vitro.

For in vivo fertilization, sperm motility may be of crucial importance, since spermatozoa must swim into and/or through the female reproductive tract, primarily by their own motility. A combination of tests which directly and/or indirectly measure sperm parameters (percent of motility, amplitude of lateral sperm head displacement,velocity, and linearity) may reflect sperm function.

However, investigations of the influence of sperm factors (concentration, percentage of motile sperm, and/or morphology) on IVF success are confusing. Since pregnancies have been achieved with semen samples showing abnormal or even pathological characteristics, it has been assumed that IVF could be used as a treatment for male infertility, even though no controlled studies have been done. The reports of Edwards et al. (80) and Cohen et al. (81) that a majority of men with abnormal semen were able to fertilize human oocytes in vitro have strengthened this opinion. However, explanations are rarely given for the observed failures of IVF and the efficacy and the accuracy of the classical criteria of evaluating sperm characteristics. In a study by Jeulin et al. (82), the percentage of motile spermatozoa in the patient's semen samples was used as a selection criterion for IVF candidates, and no difference was found between the low (< 33%) and high (> 66%)

cleavage rate with respect to this characteristic. Our studies indicate that, with the critical evaluation of several sperm functional and anatomical characteristics, one may answer questions about the IVF failures. In two recent cases of IVF in Norfolk, pregnancies were achieved from semen samples with characteristics below normal according to classical criteria but normal by sperm function tests such as ATP (nonpublished observations).

Besides evaluating sperm characteristics more critically than by the classical method, assessing sperm fertilizing competency from a metabolic point of view has gained interest among several investigators. Since some of the most important sperm activities are energy-dependent, and since ATP is the major source of immediate energy, its determination as a marker of fertilizing potential of sperm has been suggested. Unfortunately, findings have not been consistent. There are several lines of evidence indicating major sources of error in evaluating the role of ATP in infertility.

Semen samples from fertile donors have been used to investigate male infertility. Results from these donors may(not) represent a fertile group at the time of the experiment. Moreover, use of tests such as SPA to evaluate these samples introduces errors to the studies. The results of the SPA are not always consistent, mainly because none of the studies have been similar. Since a combination of several factors may contribute to infertility, the results of any semen evaluation will be more meaningful if samples obtained from

infertile individuals are compared. This will help the assessment of the possible contribution of each factor to overall infertility. IVF, rather than SPA, is the best method of evaluating a seminal parameter or seminal constituents and their role in fertilization and pregnancy outcome. Human cervical mucus is a barrier for seminal fluid and seminal elements other than spermatozoa. Cervical mucus, in fact, is a swim-up medium for motile spermatozoa and sets up its own rise condition. Infertility studies of only whole semen samples are not complete studies. Swim-up preparations of these samples must always be analyzed. Moreover, ATP determinations in swim-up samples must be carried out at the time of oocyte insemination. In ATP studies, for example, round cells (germ cells and/or white blood cells) present in most semen samples introduce errors into the studies. We therefore may assume that a semen sample containing a proportionally high number of round cells may have an ATP concentration that does not represent the functional capacity of the sperm. Thus, a better correlation may be expected if spermatozoa are separated from other components before ATP is analyzed. Irvine et al. (63) studied of the role of ATP and concluded that the ATP assay has no value in male infertility studies. They used washed spermatozoa and SPA for their evaluations. Aside from containing many immotile spermatozoa, washed semen samples may contain a large amount of other cellular elements such as exfoliated germ cells, genital tract cells, cellular debris, and amorphous materials, some of which are not readily visible

by regular light microscopy. Moreover, lack of full capacitation and extreme variation in the percentage of motility among washed samples make this technique unsuitable for the study. A high number of immotile spermatozoa with the digestive enzymes in proximity with the oocyte may not be beneficial, and results may not be correlated with the ATP values. Most swim-up preparations of normal semen samples have motilities within the 80%-95% range. Use of swim-up samples reduces sample variability in sperm motility characteristics and gives a purer and more uniform sample for other andrological evaluations. Russell et al. (83), in their study of semen samples from 63 infertile men in an IVF program, used a swim-up technique to obtain "the best spermatozoa available" for the insemination of oocytes. According to their findings, the overall motility was significantly enhanced from 51.8% in the unprocessed samples to 89.1% in the swim-up samples. Normal morphology assessed by both light and electron microscopy was significantly improved in their swim-up technique. When the swim-up sample was quantitatively inadequate, use of the non-swim-up sample to fertilize the human oocyte was unsuccessful (83). Sperm concentration improvement (e.g., washed concentrated semen) has never been shown to improve the IVF results. Gerris et al. (84) found that sperm densities in the original ejaculate of more than 10 million/ml did not significantly improve IVF. In contrast, they found that sperm motility played an important role.

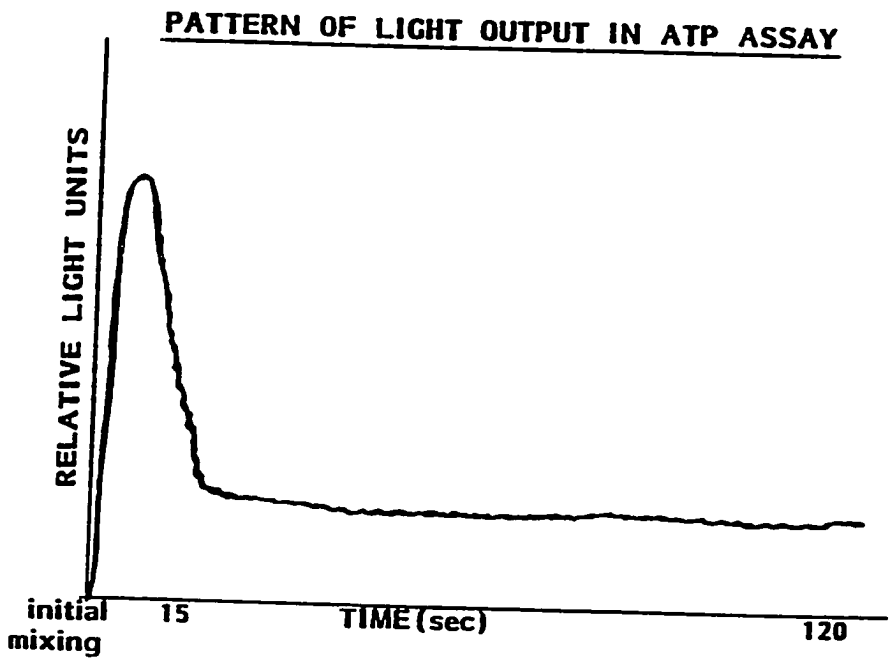
Adenosine triphosphate content of spermatozoa declines with time (66-68). We have found that the decline is more significant in some samples than in others (nonpublished observation) and that there is a possibility of a significant ATP decline only one hour after ejaculation. Since, under normal circumstances, spermatozoa separate from the seminal plasma and move (or are moved) forward toward the upper genital tract and reach the Fallopian tubes within 30 minutes, ATP determinations must be made out within 30-45 minutes after ejaculation. In some studies, semen samples have been processed at various times after ejaculation (even after two hours) for ATP determinations.

Many other errors have been noted in ATP studies. Considering the dilution factor, the volume of luciferin-luciferase mixture has not been high enough to keep ATP as a limiting reagent at all ATP concentrations. Another error is the improper use of instrumentation. The first portion of the light output in an ATP assay (Fig. 16) is very unstable and sensitive to many assay conditions. This portion of light must be eliminated, but not every instrument used in ATP studies has a delay switch for this purpose.

It is essential to correct assay and instrumentation problems and to evaluate semen and swim-up preparations for study of infertile males. The ultimate means of validating andrological assays and their usefulness in the evaluation of male infertility would be to correlate the assay values from semen samples of infertile men with human oocyte fertilization,

Figure 16.

The pattern of light output in a typical chemiluminescence assay.



cleavage, and pregnancy outcome initiated by these samples. Therefore, human IVF instead of SPA or other assays is more relevant.

In evaluating the results from our IVF patients in series 27 (Table 4), we noticed that those with low swim-up ATP had significantly lower fertilization and pregnancy rates than those with high swim-up ATP. In series 30 (Table 6) there was no difference in fertilization rate for the two groups. This change can be attributed to our decision to increase the number of spermatozoa used to inseminate oocytes from a standard 50,000 spermatozoa/oocyte/ml of medium to amounts that depended on the severity of the problem. This decision, initiated after series 27, improved the fertilization rate but not the pregnancy rate. We believe that increasing the number of sperm/occyte increases the percentage of sperm with fertilizing capacity.

It also seems that fertilization and post-fertilization may not be totally related events. Increasing the number of sperm/oocyte in the insemination dish may increase the chance of polyploidy in samples which otherwise may have the same fertilization rate with lower numbers of spermatozoa. Since about 65% of patients with the G pattern had normal swim-up ATP, and since about 43% of these produced pregnancies, it is important that ATP evaluations also be carried out to identify this subgroup within the G group. Although many factors may influence the pregnancy outcome of IVF, the inability to achieve pregnancy in groups with low swim-up ATP in both series

points to the value of this assay in the evaluation of IVF patients. The mechanism by which low ATP may influence the pregnancy outcome is unknown.

Another finding is the lack of correlation between whole semen ATP and IVF pregnancy outcome, although when data from both series were pooled, whole semen ATP values became significant ($p < 0.05$) (Table 9). This significance was in contrast to our preliminary findings, possibly because in our study of series 27 and 30 we included only semen samples with < 1 million round cells/ ml of semen. Since round cells contain high concentrations of ATP, elimination of samples with a high number of round cells may have reduced the error introduced into the study of the role of whole semen ATP. The observation that no pregnancy was achieved from semen samples with seminal ATP of < 65 pmol/million sperm and the fact that the contribution of seminal ATP to IVF outcome became significant when data from series 27 and 30 were combined, make it important to look at seminal ATP results more critically. Any semen sample with an ATP < 65 pmol/million sperm should be considered for a second seminal ATP and a swim-up evaluation to assess its potential for IVF. Swim-up ATP remained a highly significant indicator ($p < 0.001$) of IVF pregnancy outcome when data from both series were combined. In series 27 and 30, grouping patients by the percent of normal sperm showed a similar trend for pregnancy results as that when patients were grouped by their swim-up ATP. However, determination of swim-up ATP in patients with the G pattern could help in subgrouping

these patients into two classes: normal (>40 pmol/million sperm) swim-up ATP and low (≤40 pmol/million sperm) swim-up ATP. G pattern patients with normal swim-up ATP may be inseminated with 50,000 spermatozoa/oocyte/ml of medium. This recommendation must be evaluated separately. Semen samples with the P pattern in combined series 27 and 30 also resulted in no pregnancy. This may imply that the determination of sperm morphology and other basic semen parameters in P pattern patients may be enough to assess the quality of the semen samples. However, it may not be necessary to inseminate oocytes with more than 50,000 sperm/oocyte/ml of medium in P pattern patients with normal swim-up ATP (Table 5).

Higher linear velocity along with significantly lower mean linearity in patients with normal sperm morphology (Table 12) may be significant. Since mean linearity is the ratio of straight line velocity over linear velocity, it may indicate that spermatozoa in swim-up samples prepared from semen with normal morphology (N pattern) were more hyperactivated than in samples from the G or P group. Spermatozoa in the swim-up samples with low swim-up ATP were also less linear than those with high swim-up ATP (Table 11). If almost none of the samples with normal sperm morphology had low swim-up ATP, then why did these samples have a lower mean linearity similar to that of the group with low swim-up ATP? The answer possibly lies in the differences in their mean linear velocities. Samples with low swim-up ATP had a lower mean linear velocity and a lower mean linearity. This means that spermatozoa with

low swim-up ATP were not only swimming more slowly but also more erratically (in tight circles), indicating lack of hyperactivation. Contrary to this group, samples with high velocity and low linearity (N pattern) may be in a state of hyperactivation where an increase in lateral head amplitude (less linearity) is accompanied with a higher (but not a lower) velocity.

We evaluated the contribution of sperm morphology and swim-up ATP to the IVF pregnancy using multiple regression analyses of data from series 27 and 30. Swim-up ATP (ATP 2) and sperm morphology (Normal 1) together explained 31.2% of the variance. This percentage increased to 68% when sperm velocity in semen (Velo 1), seminal ATP (ATP 1), sperm linearity in swim-up samples (Line 2) and the percentage of motility of swim-up samples (MOT 2) were included.

The formulae constructed to predict IVF pregnancy outcome were based on this finding. Based on the number of patients evaluated in the combined series, these formulae can well predict the true potential of pregnancy in IVF. These formulae can also be used to screen potential IVF patients. However, reproducibility studies are also needed. The validity of these formulae in predicting IVF outcome must be examined by the assay results from patients before IVF and their pregnancy results after IVF.

Determination of semen and swim-up CK did not result in any new or meaningful finding. First, some semen samples may contain elements other than spermatozoa, with a potentially

significant concentration of CK. Although CK could not be found in white blood cells (which may be present in semen), its presence in germ cells which are metabolically active cannot be disputed. Technical errors in sample preparation and manipulation, and assay methodology may also be responsible for variable and erratic results. When semen samples are processed for CK, a reducing agent such as dithiothreitol (DTT) must be added to prevent CK oxidation. An inaccurate determination of sperm count will also affect the CK results, since the CK activity results are expressed as a function of the number of spermatozoa. Care must be taken when the supernate is discarded; a major portion of sperm cells may thus lost. This step becomes critical when swim-up samples are being processed. These samples will not form a thick and uniform pellet. Centrifugation at a higher force to form a good pellet is not advisable because this may damage some fragile sperm cells and cause the enzyme to leak. To overcome these problems, one may add DTT to the samples being prepared for CK determination and determine the count after centrifugation and discarding of the supernatant or one may measure CK isoenzymes rather than total CK activity. Determination of CK isoenzymes is not affected by the contribution of sperm count. One may be able to identify different types of CK isoenzymes at varying concentrations. Implementing these steps may identify a subgroup with high ATP and with no resulting pregnancies. Can some samples with high ATP and no pregnancies have low reserve energy in the form of CK? Can these samples contain a CK isoenzyme with low

catalytic activity?

HOST results showed no correlation with IVF outcome. Sperm membrane integrity may not be determined by this assay, as indicated also by Chan et al. (64).

Sperm morphology determination of swim-up samples is also plagued with problems. Since swim-up medium contained 7.5% (V/V) fetal cord serum, the serum precipitated on and around the spermatozoa when the slides were fixed for staining. This made it difficult to evaluate the sperm morphology. The medium can be diluted and washed from spermatozoa by centrifugation, although this may change sperm morphology; the effect must be studied.

Treatment of infertility in recent years has greatly increased the number of options available for infertile couples. The technical advances accompanying fertility treatment generally have placed more emphasis on exteriorization of the reproductive process. For IVF the reproduction process has been largely bypassed, since gametes are obtained outside the normal means for conception. Egg quality and egg quantity, as obtained by assisted stimulation and laparoscopy, have improved. Washing and storage of spermatozoa in media has eliminated the detrimental properties of seminal plasma on sperm fertilizing ability and have allowed capacitation. Further improvements in sperm quality have involved a technique that takes advantage of sperm swimming abilities. This technique, variously referred to as the rise or swim-up procedure, is frequently used in IVF and, to a

lesser extent, in intrauterine insemination.

With the advent of IVF and other clinical techniques, it is important to use the best spermatozoa available from a semen sample. Many reports indicate an increase in the percentage of motile forms as well as percentage of sperm with normal morphology. Our automated method of assessing the percent of sperm percent motility, mean velocity, mean linearity, and sperm morphology according to strict criteria and using swim-up samples makes our results more valuable. Our method of assessing sperm with normal morphology is less subjective and has a higher reproducibility. Our method determines the percent of normal sperm in semen rather than the percent of abnormal forms. Various studies associating the type and percent of abnormal sperm with infertility conditions have been inconclusive (28). With these results, one can more accurately evaluate the male contribution to infertility. However, other sperm functions must also be considered. For example, a spermatozoon swimming from point A to point B may use a significantly different amount of energy than another spermatozoon, while both may contribute equally to the percent of motility and mean velocity. Since many important sperm functions are active processes, determination of immediate ATP and reserve (CK and its isoenzymes) energy levels is of great importance. But, this determination is valuable only if all major technical and procedural errors are eliminated. Our method of measuring ATP is more accurate and reproducible. We have modified the procedure and eliminated all known factors

which might affect the results. We have found that swim-up ATP is of more value than seminal ATP in correlation with IVF pregnancy outcome. The value of swim-up ATP is even greater in patients with G and P patterns of sperm morphology. Semen samples of G or P pattern but with normal swim-up ATP may be used to inseminate oocytes at 50,000 spermatozoa/oocyte/ml of medium, a finding that must be further verified.

IVF pregnancy outcome can be predicted by the formulae we devised. These may be used in screening patients for IVF. However, our data may not be reproducible in other centers for several reasons. First, there is no fixed and standard procedure for sperm capacitation and the swim-up technique. Temperature, pH, osmolarity, duration of preinsemination capacitation, the time interval between ejaculation and processing, the exact volume inseminated, and the exact number of capacitated spermatozoa in the insemination dish vary from one team to another, making comparison of results difficult. Second, in many studies one knows little about the correlation between IVF results and the characteristics of the ejaculate used. A study of such correlation (like the one we conducted) may help to determine whether our definition of the lower limits beyond which successful IVF is unlikely can be repeated.

Of 11 patients evaluated for both semen and swim-up ATP before IVF, three showed low swim-up ATP. No IVF pregnancy resulted from the samples of these patients, although data are too small to make a definite conclusion. This points to the need for more reproducibility studies to confirm our

preliminary findings in patients before and after IVF.

In conjunction with the parameters determined in our study, investigations must also be made to determine the contribution of oocytes to IVF outcome with a particular stimulation protocol.

The contribution of both seminal and swim-up ATP, CK, HOST, and sperm morphology to IVF outcome using samples with abnormal count, motility, and the number of round cells must also be evaluated. Why an increase in the concentration of sperm cells/oocyte in an insemination dish increases the fertilization rate but not the pregnancy rate must also be investigated. This is a very important study because recent interest in micromanipulation techniques (microinjection of sperm nuclear material into an oocyte).

CHAPTER VII

SUMMARY

With the advent of IVF and other clinical techniques, it is important to use the best sperm available from a semen sample. To achieve this goal, many centers use swim-up preparations. Many reports indicate an increase in the percentages of motile forms and spermatozoa with normal morphology. However, other sperm functions and characteristics must be considered concurrently. In implementing multivariable analysis and assessing the contribution of different seminal factors to IVF outcome, we analyzed major semen parameters such as sperm concentration, percent of motility, mean linearity, mean velocity, and sperm morphology according to the new strict criteria, in the same ejaculate used for IVF. Since many important sperm functions are active processes, determination of immediate (ATP) and reserve (CK and its isoenzymes) energy levels in spermatozoa was also included in the study. Our findings indicate the value of multivariable analysis in infertility investigations. For example, we showed that information about the percent of sperm motility or its mean linearity may not be sufficient for assessing the quality of semen samples used for IVF. A sperm swimming from point A to

point B may use an amount of energy significantly different from that of another sperm, although both may contribute equally to the measured percent of motility or the mean linearity. It was showed that spermatozoa in swim-up samples with almost equal percents of motility can have significantly different energy sources in the form of ATP. It was also demonstrated that sperm mean linearity per se is of no value if it is not evaluated along with other sperm (or semen) parameters. Spermatozoa in swim-up samples which did not produce a pregnancy had similar (low) mean linearity as samples with the highest pregnancy rate (40% in samples with normal morphology and with normal swim-up ATP). The value of mean linearity became apparent when it was evaluated along with sperm mean velocity in association with sperm morphology and its ATP content. Swim-up samples with no pregnancies (low swim-up ATP and often poor sperm morphology) had low mean linearity and low velocity, whereas samples which produced the highest number of pregnancies had a low mean linearity but high mean velocity. This may be indicative of sperm hyperactivation in samples which produced the pregnancies and erratic sperm movement in the samples with no resulting pregnancies.

In this study, the Comhaire ATP procedure was modified and all factors which were shown to affect the results were eliminated. It was found that swim-up ATP is more valuable than seminal ATP in correlation with IVF pregnancy outcome. It was also demonstrated that the swim-up samples with ATP <40 pmol/million sperm and/or with poor sperm morphology produced

the lowest number of pregnancies (0% to 5.6%). In contrast, samples with normal sperm morphology or with normal swim-up ATP produced the highest number of pregnancies (25% to 40%). The value of swim-up ATP was even greater in the evaluation of samples with a G or P pattern of sperm morphology. About 30% of samples with the P pattern and 60% of those with the G pattern exhibited normal swim-up ATP. Currently, in samples with a P or G pattern, a higher number of spermatozoa are used to inseminate the oocytes. To prevent polyploidy, it is necessary to investigate the possibility of inseminating oocytes at 50,000 sperm/oocyte/ml of medium in samples with normal swim-up ATP but with a P or G pattern.

The pregnancy outcome of IVF can be predicted through the formulae we devised. Our criteria for the selection of samples must be kept in mind: normal count and motility, with a normal number of round cells in the semen. The contribution of oocytes to IVF outcome was not determined, since only patients with at least one recovered, healthy, metaphase II oocyte were studied.

Of 11 patients evaluated for swim-up ATP before IVF, no pregnancy resulted from three samples with low swim-up ATP. More reproducibility studies are needed to confirm findings in patients before and after IVF.

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